

HANDBOOK OF BACTERIOLOGY

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MACKIE & McCARTNEY'S
**HANDBOOK OF
BACTERIOLOGY**

A Guide to
The Laboratory Diagnosis and
Control of Infection

Edited by

ROBERT CRUICKSHANK,
M.D., F.R.C.P., D.P.H., F.R.S.E.

*Professor of Bacteriology, University of Edinburgh.
Adviser in Bacteriology S.E. Regional Hospital Board (Scotland)
and Department of Health for Scotland.
Senior Consultant in Bacteriology, Royal Infirmary, Edinburgh.*

AND

Members of the Staff of the Bacteriology Department,
University of Edinburgh

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LIST OF CONTRIBUTORS

COGHLAN, Joyce, D., B.Sc., Ph.D.

Bacteriology of Water, Milk, Foods; Leptospira.

COLLEE, J. G., M.B., Ch.B.

Clostridia.

CRUICKSHANK, R., M.D., F.R.C.P., D.P.H., F.R.S.E.

Immunity; Neisseriae; Corynebacteria; Mycobacteria; Brucella;
Haemophilus; Bordetella.

DUGUID, J. P., M.D., B.Sc.

Morphology, Physiology and Genetics of Bacteria; Infection;
Staining and Cultivation; Sterilisation; Pathogenic Fungi.

GILLIES, R. R., M.D., D.P.H.

Streptococci; Pneumococci; ⁷The Intestinal Bacteria; Proteus;
Pyocyanine; Lactobacilli; Pasteurella.

GOULD, J. C., M.D., B.Sc.

Antimicrobial Agents; Staphylococci; The Anthrax Bacillus;
Phage Typing; Protozoa.

SWAIN, R. H. A., M.A., M.D., F.R.S.E.

Serological Methods; Animal Inoculation; The Cultivation of
Viruses; Spirochaetes; Rickettsiae; Bartonella; Actinomycetes;
Psittacosis, Pox, Respiratory, Enteric, Arbor and Miscellaneous
Viruses.

WILKINSON, J. F., M.A., Ph.D.

Physical and Chemical Methods; Bacteriophage.

PREFACE

IT is 35 years since the first edition of this textbook was published. That "Mackie and McCartney" quickly became a most popular handbook of practical bacteriology was shown by the rapid succession of new editions and reprints, the latest of which appeared in 1956. This fruitful partnership was ended by the untimely death of Professor Mackie in 1955 and the recent retirement of Dr. McCartney from active laboratory work. As Professor Mackie's successor, I accepted, after careful consideration, an invitation to assume the editorship of new editions of this textbook, with the proviso that the task of revision would be shared with staff members of the Bacteriology Department of Edinburgh University. The names of the team of collaborators and the sections of this tenth edition for which they are mainly responsible are given in the front of the book.

Inevitably, with the many advances in microbiology, extensive revision and considerable expansions were necessary and this edition contains some 230 pages more than its immediate predecessor, in spite of the uniform use of smaller print for the technical methods in Part II. The number of chapters has been increased from 24 to 46, partly because of new material and partly by subdivision of composite chapters, particularly in Part III dealing with systematic microbiology. Thus, there are now eight chapters on viruses in place of one; the intestinal pathogens are considered in four separate chapters; and the space devoted to ~~microbial biology~~, infection and immunity has been nearly doubled. Throughout Part II the practical aspects of the laboratory diagnosis of infection in the individual and of the epidemiology of specific infectious diseases in the community has been given fuller consideration in the hope that this textbook will become even more widely used by medical students and doctors in many lands as a laboratory guide to the rational treatment and prevention of infective disease. Fuller use has also been made of illustrative figures and diagrams than in earlier editions.

This textbook is used by students, both graduate and undergraduate, of veterinary medicine and we are indebted to Mr. G. Fraser, Ph.D., M.R.C.V.S., of the Bacteriology Department, The Royal (Dick) Veterinary College, for revision of the sections on animal infections.

The burden of preparing the index has fallen most heavily on Dr. R. R. Gillies, and we have had help in the proof reading from Drs. G. P. B. Boissard, J. G. Collee, and F. L. Constable of this Department. We have had much helpful advice from numerous other colleagues both in Edinburgh and further afield, and to all of them we would express our grateful thanks. We are also greatly indebted to Mr. C. Macmillan and Mr. J. Parker of E. & S. Livingstone, Ltd., for their patient helpfulness in the preparation of this edition.

1960

ROBERT CRUICKSHANK

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PART I

MICROBIAL BIOLOGY:

Infection and Immunity

CHAPTER I

MORPHOLOGY OF BACTERIA

BACTERIOLOGY or Microbiology, as applied to medicine, embraces the study of the micro-organisms that are parasitic on man. Microbes are designated *parasites* when they obtain their nourishment from the body of a living host. Some parasites have the power to produce disease and these are described as *pathogenic*, while others are normally harmless and are described as *commensal*. The latter term (lit. "table companion") properly applies to the numerous micro-organisms which live harmlessly in the mouth, throat and intestine, sharing the food eaten by their host. Its meaning, however, has been usefully widened to include harmless parasites living elsewhere in the body, e.g. in the nose and on the skin. The distinction between commensal and pathogenic varieties is not absolute; some normally commensal species are potential pathogens, able in certain circumstances to cause disease, while some pathogenic species occasionally assume a commensal role, e.g. in the so-called *healthy carriers* of infection.

In veterinary science, bacteriology is specially concerned with the micro-organisms responsible for disease in domesticated animals. As many infective diseases are common to man and animals, medical and veterinary bacteriology are closely related branches of the general subject. Pathogenic organisms show great diversity in their degree of adaptation to different host species. Some are infective for the human species only, others for certain animals but not man, and yet others for man as well as one or more animal species. The host range of the causative microbe is an important factor determining the epidemiology of a disease, since wild or domesticated animals of different species may act as the source of infection. (The term "source" of infection applies to the normal growth habitats of the microbe, e.g. the human or animal host; objects contaminated with live non-growing microbes are termed "vehicles" or "reservoirs" of infection.)

Many microbial species are *non-parasitic*, or *free-living*. These abound in soil, water, mud and other such natural habitats, and with rare exceptions are harmless to man and animals. They nourish themselves by a variety of mechanisms. The protozoa capture, ingest and digest internally solid particles of organic foodstuff such as bacteria and plant debris (holozoic nutrition).

The algae and photosynthetic bacteria absorb carbon dioxide and soluble inorganic substances, and gain their energy by photosynthesis from sunlight (holophytic nutrition). Certain bacteria, the *autotrophs*, also utilise carbon dioxide as the sole source of carbon, but gain their energy by oxidation of an inorganic substance. Finally, there are numerous species of bacteria and fungi, described as *saprophytic*, which utilise organic nutrient substances obtained from dead plant or animal matter; they absorb soluble nutrients through their cell surfaces, and when utilising solid material, must first decompose this externally into soluble products by the action of extracellular enzymes. Saprophytes are responsible for the decomposition and eventual mineralization of all organic waste, e.g. animal corpses and excreta, dead and fallen vegetation, and sewage. They are also responsible for the natural spoilage of food and fodder. A few species of saprophytic bacteria and fungi are able in special circumstances to infect the human or animal body, and cause disease (e.g. producing tetanus or gas gangrene when the tissue defence mechanisms are disordered by wounding). The term "saprophyte" is sometimes also applied to those commensal parasites which feed on dead organic matter within the body of a living host, for instance in the intestinal contents.

Micro-organisms may be defined as living creatures which are microscopical in size and relatively simple, often unicellular in structure. The diameter of the smallest body that can be resolved and seen clearly by the naked eye is about $100\ \mu$ (1 micron, or $\mu=0.001$ millimetres). All but a few of the micro-organisms are smaller than this and a microscope is therefore necessary for their observation. The light microscope under optimal conditions can resolve bodies down to $0.2\ \mu$ in diameter, and this includes all microbes except the viruses, most of which are still smaller. The electron microscope has a limit of resolution approaching $0.001\ \mu$ (i.e. $1\ m\mu$ or millimicron), and can resolve even the smallest viruses ($0.01\ \mu$ diam.). It should be noted that when bacteria or fungi are allowed to grow undisturbed on a solid or semi-solid substrate, their numerous progeny accumulate to form "colonies" which are readily visible to the naked eye.

Living material, or protoplasm, is organised in units known as "cells". Each cell consists of a body of protoplasm, the *protoplast*, enclosed by a thin semi-permeable *cell membrane* or *cytoplasmic membrane*, and also, in most cases, by an outer, relatively rigid *cell wall*. The protoplast is differentiated into a major part, the *cytoplasm*, and an inner body, the *nucleus*, which contains the hereditary determinants of character, the *genes*, borne on thread-

like *chromosomes*. The bodies of higher plants and animals are multicellular, with interdependence and specialisation of function among the cells, the different kinds of cells being segregated in separate tissues. Many micro-organisms, on the other hand, are unicellular, existing as single self-sufficient cells, unattached to their fellows. Other micro-organisms grow as aggregates of cells joined together by their cell walls in clusters, chains, rods, filaments or mycelia (*i.e.* meshworks of branching filaments), and some grow as a plasmodium, or multinucleate mass of cytoplasm. Generally, these morphologically multicellular microbes are physiologically unicellular, each cell being self-sufficient and, if isolated artificially, able to nourish itself, grow and reproduce the species. Some specialisation of cell function, approaching that of true multicellular organisms, is encountered in colonies of moulds and higher bacteria; thus, certain cells comprising an aerial mycelium are specialised for the formation and dissemination of spores, and are dependent for their nutrition on the activities of other cells comprising a vegetative mycelium.

The majority of micro-organisms may be classified in the following large biological groups: (1) Algae, (2) Protozoa, (3) Slime moulds, (4) Fungi proper, or *Eumycetes*, including the moulds and the yeasts, (5) Bacteria or *Schizomycetes* ("fission fungi"), (6) *Rickettsiales* and (7) Viruses or *Virales*. Since the algae and slime moulds contain no species of medical or veterinary importance, they will not be dealt with in this book. The main differential characters of the other groups are as follows:

Protozoa.—Non-photosynthetic unicellular organisms (a few are colonial) with protoplasm clearly differentiated into nucleus and cytoplasm; holozoic, saprophytic or parasitic; regarded as the lowest forms of animal life, though certain flagellate protozoa are very closely related to photosynthetic flagellate algae in the plant kingdom; reproduce asexually by binary fission or multiple fission (schizogony), and in some cases also by a sexual mechanism. Some exhibit a definite life cycle with both sexual and asexual phases, and some form round, thick-walled resting cells, or "cysts".

Fungi.—Non-photosynthetic micro-organisms possessing relatively rigid cell walls; saprophytic or parasitic. **Mould** forms.—Grow as branching filaments (hyphae) which interlace to form a meshwork (mycelium); the hyphae are coenocytic (*i.e.* have a continuous multinucleate protoplasm), being non-septate or septate with a central pore in each cross-wall; reproduce by the formation of various kinds of sexual and asexual spores developed from the vegetative (feeding) mycelium or from an

aerial mycelium which effects their air-borne dissemination. *Yeast* forms.—Ovoid or spherical cells which reproduce asexually by budding and also, in many cases, sexually with the formation of sexual spores; do not form a mycelium, although the intermediate *yeast-like fungi* may form a "pseudomycelium" of elongated cells.

Bacteria.—A varied group of small micro-organisms with primitive cellular organisation; generally unicellular, but the cells may grow attached to one another in clusters, chains, rods, filaments or, as in the "higher bacteria" (*Actinomycetales*), a mycelium. Their cells are smaller (mostly 0·4–1·5 μ in short diameter) than those of protozoa and fungi, in most cases have relatively rigid cell walls, are spherical (coccus), rod-shaped (bacillus), comma-shaped (vibrio), spiral (spirillum and spirochaete) or filamentous, and show little structural differentiation when examined by ordinary microscopical methods. Special staining methods show that they possess a central nuclear body which contains desoxyribonucleic acid and divides by simple fission without evidence of mitosis or chromosomes. They reproduce mainly by simple transverse fission. Certain species form endospores as a resting phase and some (actinomycetes) reproduce by formation of conidia (exogenously formed asexual spores). Many species are motile by means of flagella and some by active flexion of the cell body. Most are saprophytic or parasitic, and a few autotrophic or photosynthetic.

Rickettsiales.—Simple unicellular organisms which are rod-shaped, spherical or pleomorphic; smaller than most bacteria, though still resolvable by the light microscope (*i.e.* over 0·2 μ diam.) and mostly not filterable through bacteria-stopping filters; strict parasites which can grow only in the living tissues of a suitable animal host, usually intracellularly. A few exceptional species can grow in cell-free nutrient media containing body fluids.

Viruses.—The smallest and simplest of the micro-organisms; range between 0·3 μ and 0·01 μ in diameter, mostly being ultra-microscopic (*i.e.* smaller than resolvable by the light microscope) and filterable through bacteria-stopping filters; strict intracellular parasites capable of growth only within the living cells of an appropriate animal, plant or bacterial host, and never on inanimate nutrient medium. The viruses which infect and parasitise bacteria are named *bacteriophages* or *phages*.

The bacteria and viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and sub-tropical countries, while the common fungal infections are mainly superficial (*e.g.* skin infections) and of minor severity.

The remainder of this chapter will be devoted to the general biology of the bacteria. That of the other groups of micro-organisms will be dealt with in Chapters XXXV to XLV.

MORPHOLOGICAL STUDY OF THE BACTERIA

Microscopical examination is usually the first step taken for the identification of an unknown bacterium. The bacterium may be allocated to one or other of the major groups when its *morphology* and *staining reactions* have been observed. The morphological features of importance are the size, shape and grouping of the cells, and their possession of any distinctive structures such as endospores, flagella (or motility), capsules and intracellular granules. Staining reactions are observed after treatment by special procedures such as the Gram and Ziehl-Neelsen stains, the different kinds of bacteria being shown in separate colours due to their different permeability to certain decolorising agents. A preparation stained by one of these methods usually suffices for observation of the general morphology of the bacterium, but some morphological features can be demonstrated only by the application of further special stains.

Unstained Preparations of Living Organisms.—The morphology of bacteria can be studied in the first place by examining them microscopically in the unstained condition, suspended in a thin film of fluid between a glass slide and cover-slip (*i.e.* in an "unstained wet film"). In this way their general shape can be seen and their motility determined (pp. 18, 15). Certain very slender bacteria, however, such as the spirochaetes, are so feebly refractile that they can not be seen by the ordinary microscopic methods, and *dark-ground illumination* (p. 96) or *phase-contrast microscopy* (p. 103) is necessary for their demonstration.

Electron microscopy (p. 106) is now applied to the morphological study of bacteria. Although rarely available for routine diagnostic work, it is important in enabling demonstration of certain cell structures, *e.g.* fimbriae (p. 16), which cannot be observed by the light microscopes.

For the study of the development of individual organisms and the growth of bacteria in colonies (p. 30), the "agar-block" method of Ørskov, and the microscope-incubator may be used (p. 265). These methods enable living bacteria to be observed at intervals during their actual growth on a suitable substrate, and present a more natural picture than other procedures involving manipulations which may sometimes create artificial appearances.

Stained Preparations.—The microscopical examination of

fixed and stained preparations is usually an essential routine procedure. ! The bacteria are more readily discovered and studied when immobilised by fixation and darkly stained in contrast with the bright background. ! *Simple staining* is effected by the application of a watery solution of a single basic dye, e.g. methylene blue, methyl violet or basic fuchsin, or sometimes along with a mordant, e.g. dilute carbol fuchsin (p. 111). ! The coloured, positively charged cation of the basic dye combines firmly with negatively charged groups in the bacterial protoplasm, especially with the abundant nucleic acids. ! This staining is retained through subsequent washing with water to remove excess dye from the slide. Acidic dyes, having coloured anions, do not stain bacteria except at very acid pH values, and thus can be used for "negative staining" (see below). Cells or structures which stain with basic dyes at normal pH values are described as *basophilic* and those which stain with acidic dyes as *acidophilic*.

Prior to staining, the film or smear of bacteria must be fixed on the slide. *Fixation* is usually effected by heat; the slide is first thoroughly dried in air and then heated gently in a flame. Vegetative bacteria are thereby killed, rendered permeable to the stain, stuck to the surface of the slide and preserved from undergoing autolytic changes. Chemical fixatives are used for sections of infected tissue and films of infected blood, since they cause less damage to the tissue cells; they include formalin, mercuric chloride, methyl alcohol and osmic acid (p. 142 *et seq.*).

It should be noted that the bacterial cell wall (see below) is not stained by ordinary methods and the coloured body seen corresponds to the cell protoplasm only. This is usually much shrunken as a result of drying. Chains of stained bacteria thus show the coloured bodies separated by gaps which are the sites of unstained connecting cell walls.

Beaded and *Bipolar Staining*.—Certain bacteria do not colour evenly with simple stains. Thus, the diphtheria bacillus shows a "beaded" appearance, with alternate dark and light bars. The plague bacillus shows "bipolar staining", the ends being more deeply coloured than the centre. The uneven staining may be due to the manner in which the protoplasm shrinks when the cell is dried and fixed.

"*Negative*" or *Background Staining* is of value as a rapid method for the simple morphological study of bacteria. The bacteria are mixed with a substance such as India ink, or nigrosin (pp. 111, 122), which, after spreading as a film, yields a dark background in which the bacteria stand out as bright, unstained objects.

Silver Impregnation methods (p. 136) are utilised for the staining

of spirochaetes, especially for demonstrating these organisms in tissues. The slender cells are thickened by a dark surface coating of silver deposit.

Impression Preparations (p. 127) are used for cytological studies when it is desired to avoid the distortion of cell structure and colonial arrangement inevitable in the normal procedure of preparing a smear, drying it and fixing it by heat. Bacteria newly spread on the surface of an agar medium, or grown on agar to form small colonies, are fixed *in situ*, without drying, to a slide or cover-slip. This is done by allowing a chemical fixative to diffuse through the agar from below. The fixed bacteria adhere to the glass when the agar is removed and can then be stained.

Staining Reactions.—The staining reactions of bacteria are of the greatest importance in their differentiation and identification. (*Gram's staining reaction* (p. 112) has the widest application, dividing all bacteria into two categories named "Gram-positive" and "Gram-negative", according to whether or not they resist decolorisation by acetone, alcohol or aniline oil after staining with a pararosaniline (triphenylmethane) dye, e.g. crystal or methyl violet, and subsequent treatment with iodine.) The Gram-positive bacteria resist decolorisation and remain stained a dark purple colour. The Gram-negative bacteria are decolorised and then counter-stained light pink by the subsequent application of basic fuchsin, safranine, neutral red or dilute carbol fuchsin.) In routine diagnostic work, a Gram-stained smear is often the only preparation examined microscopically, since it shows clearly the general morphology of the bacteria as well as revealing their Gram-reaction. It should be noted that species which are characteristically Gram-positive, may appear Gram-negative under certain conditions of growth; thus, some show an increasing proportion of partly or wholly Gram-negative cells in ageing cultures on nutrient agar. Gram-reactivity appears to reflect a fundamental aspect of cell structure and is correlated with many other biological properties. Thus, the different species of a single genus generally show the same reaction. Gram-positive bacteria are more susceptible than Gram-negative bacteria to the antibacterial action of penicillin, acids, iodine, basic dyes, detergents and lysozyme, and less susceptible to alkalies, azide, tellurite, proteolytic enzymes, lysis by antibody and complement, and plasmolysis in solutes of high osmotic pressure.

\ The mechanism of the Gram stain is not fully understood. (Gram-positive organisms are able to retain basic dyes at a higher hydrogen-ion concentration than the Gram-negative species, showing an isoelectric point of pH 2-3 as compared with pH 4-5. The more acidic character

of their protoplasm, which is enhanced by treatment with iodine, may partly explain their stronger retention of basic dyes. It has also been suggested that the difference in Gram reaction depends upon a difference in the permeability of the cell wall or cytoplasmic membrane. After staining with methyl violet and treatment with iodine, a dye-iodine complex, or "lake", is formed within the cell, which is insoluble in water but moderately soluble and dissociable in the acetone or alcohol used as the decoloriser. Under the action of the decoloriser, the dye and iodine diffuse freely out of the Gram-negative cell, but not from the Gram-positive cell, presumably because the latter's surface is less permeable to the decoloriser or its iodine solute. Whatever the complete mechanism of the reaction may be, Gram-positivity appears to depend upon the integrity of the cellular structure and the presence in the cell of a specific magnesium ribonucleate-protein complex (and maybe also of other specific compounds). Thus, Gram-positive bacteria become Gram-negative if they are ruptured mechanically, or if their magnesium ribonucleate is removed by autolysis or by treatment with bile salt or the enzyme ribonuclease. From cytological studies it seems that Gram-positive staining colours the whole cell, including the cell wall, but chemical analysis of isolated cell walls has not shown them to contain magnesium ribonucleate.

The *acid-fast staining reaction*, as revealed by the Ziehl-Neelsen method (p. 116), is of value in distinguishing a few bacterial species, e.g. the tubercle bacillus (p. 537), from all others. These "acid-fast" bacteria are relatively impermeable and resistant to simple stains, but when stained with a strong reagent (basic fuchsin in aqueous 5 per cent. phenol, applied with heat), subsequently resist decolorisation by strong acids, e.g. 20 per cent. sulphuric acid. Any decolorised non-acid-fast organisms are counter-stained in a contrasting colour with methylene blue or malachite green. /

The acid-fast bacteria have an exceptionally rich and varied content of lipids, fatty acids and higher alcohols, and their acid-fastness has been attributed to this. When the lipids, including those firmly bound in the protoplasm, are removed by treatment with suitable solvents, the cells are no longer acid-fast. One of the lipids peculiar to acid-fast bacteria exhibits the property of acid-fastness in the free state; this is *mycolic acid*, a high molecular weight hydroxy acid containing carboxyl groups. The mere presence of such a substance in the cell is not by itself sufficient to explain acid-fastness, since the character is lost when the cell is ruptured by mechanical means or autolysis. Acid-fastness therefore depends on the structural integrity of the cell, its content of lipids and, possibly, a special anatomical disposition of the lipids.

Bacterial Protoplasm and Nuclear Bodies.—The protoplasm of the bacterial cell is a viscous watery solution, or soft gel,

containing a variety of organic and inorganic solutes, and numerous small granules ($10\text{--}30\text{ }\mu$ diam.) consisting of protein and ribonucleic acid. When unstained bacteria are examined, or bacteria stained by the usual methods, the protoplast shows no differentiation into nucleus and cytoplasm. However, by a special method it is possible to demonstrate the presence of bodies which correspond to nuclei, although differing morphologically from the organised nuclei of animal or plant cells. After suitable fixation, the bacteria are treated with hydrochloric acid to reduce the staining affinity of the cytoplasm by removing its ribonucleic acid content; on subsequent staining the nuclear bodies become deeply coloured and the cytoplasm but slightly (p. 128). The nuclear bodies are oval or elongated transversely in the cell. They react positively to the Feulgen test for desoxyribonucleic acid, an essential constituent of the nuclei of higher organisms. Unlike the intracellular storage granules described below, they are constantly present in all cells and under all conditions of culture. The nuclear bodies appear to increase by growth and simple fission, and not by mitosis. Only a single body is present in some cells, while in others, as a result of nuclear division preceding cell division, two, four or even more nuclear bodies may be present.

Cell Wall and Cytoplasmic Membrane.—The bacterial protoplast is limited externally by a very thin, elastic cytoplasmic membrane, and outside this covered by a relatively rigid cell wall. The *cytoplasmic membrane* is about $5\text{--}10\text{ }\mu$ thick and consists mainly of lipo-protein. It constitutes an osmotic barrier impermeable to many small molecular solutes, but is capable of selectively transporting certain nutrient compounds and waste products into or out of the cell. It has little mechanical strength and is supported by the enclosing cell wall to which it adheres. The *cell wall* is $10\text{--}25\text{ }\mu$ thick, relatively rigid but with some elasticity, and responsible for maintaining the characteristic shape of the bacterium. It is freely permeable to solute molecules under 10,000 in molecular weight and $1\text{ }\mu$ in diameter. The chemical nature of the cell wall appears to differ considerably among the bacterial species, though it generally contains carbohydrate and protein or peptide components. By the usual staining methods, the cell wall remains invisible, but it is revealed by staining after treatment with tannic acid and, without staining, by use of the electron microscope.

Intracellular or Inclusion Granules.—In many species of bacteria, round granules are observed in the protoplasm. These are not permanent or essential structures, and may be absent under certain conditions of growth. They appear to be lifeless aggregates

of substances concerned in cell metabolism, e.g. an excess metabolite stored as a nutrient reserve. They consist of volutin, lipid, glycogen- or starch-like polysaccharide, or sulphur, the first two being of common occurrence in parasitic and saprophytic bacteria. Their demonstration may assist in the identification of certain organisms; thus, the diphtheria bacillus may be recognised by its content of volutin.

Volutin Granules (syn. *metachromatic* or *Babes-Ernst* granules) have an intense affinity for basic dyes. With toluidine blue or methylene blue, they stain *metachromatically* a red-violet colour, contrasting with the blue staining of the bacterial protoplasm. By special methods (e.g. Neisser's and Albert's, pp. 120, 119) employing also iodine and/or a counter-stain, the granules can be demonstrated with even greater colour contrast; their staining, unlike that of the protoplasm generally, is not bleached by the iodine or displaced by the counter-stain. The metachromatic staining of volutin granules is thought to be due to their content of polymerised inorganic metaphosphate (polymetaphosphate). This is an energy-rich compound which may act as a reserve of energy and phosphate for cell metabolism. Volutin granules are slightly acid-fast, resisting decolorisation by 1 per cent. sulphuric acid; they are more refractile than the protoplasm and are sometimes distinguishable in unstained wet films. By electron-microscopy they appear as very opaque, clearly demarcated bodies.

Lipid granules are recognised by their affinity for fat-soluble dyes such as Sudan black; thus, by Burdon's method (p. 125) they are coloured black in contrast to the remaining protoplasm which is counter-stained pink with basic fuchsin. The granules are spherical, of varying size and highly refractile, being easily seen in unstained preparations. They are slightly acid-fast and may be stained by the modified Ziehl-Neelsen method used for spores (p. 120). They resist staining by basic dyes and appear as unstained spaces in bacteria treated by simple stains or by Gram's method. In the bacteria so far subjected to chemical analysis, the granules appear to consist mainly of polymerised β -hydroxybutyric acid (or poly- β -hydroxybutyrate). The manner in which the lipid content of bacteria varies with the conditions of culture suggests that this substance may act as a carbon and energy storage product.

Bacterial Spores.—Some species, particularly those of the genera *Bacillus* and *Clostridium* (pp. 559, 674), develop a highly resistant resting-phase, or *endospore*, whereby the organism can survive in a dormant state through a long period of starvation or other adverse environmental conditions. The process does not

involve multiplication: in "sporulation", each vegetative cell forms only one spore, and in subsequent "germination" each spore gives rise to a single vegetative cell. Although it has been suggested that spores are formed spontaneously as an intermediate stage in a bacterial life cycle, it seems more likely that sporulation occurs as a response to starvation. It does not take place as long as conditions continue to favour maximal vegetative growth, but occurs when growth is being arrested, as in the later stages of artificial culture. In certain species, sporulation can be induced by depletion of the supply of one of the nutrients necessary for vegetative growth, e.g. the carbon and energy source, the nitrogen source, sulphate, phosphate or iron salt; at the same time, the process requires a continued supply of other minerals (K, Mg, Mn and Ca salts) and favourable conditions of moisture, temperature, pH, oxygen tension, etc. The spore is formed inside the parent vegetative cell (hence the name "endospore"). It develops from a portion of protoplasm near one end of the cell (the "forespore"), incorporates part of the nuclear material of the cell and acquires a thick covering layer, the "cortex", and a thin, but tough, outer "spore coat". The appearance of the mature spore varies according to the species, being spherical, ovoid or elongated, occupying a terminal, subterminal or central position, and being narrower than the cell, or broader and bulging it. Finally, the remainder of the parent cell disintegrates and the spore is freed.

Spores are much more resistant than the vegetative forms to all injurious chemical and physical influences, including exposure to disinfectants, drying and heating. Thus, their killing requires application of moist heat at 100°–120° C. for a period (e.g. 10 min.) in which heating at 60° C. would suffice to kill the vegetative cells. Spores may remain viable for many years, either when in the dry state or in moist conditions unfavourable to growth, as in absence of nutrients sufficient to maintain the minimal metabolism of the vegetative form. The high resistance of spores has been attributed to several factors in which they differ from vegetative cells: the impermeability of their cortex and outer coat, their high content of calcium and dipicolinic acid, their low content of water (maybe 5–20 per cent.), and their very low metabolic and enzymatic activity.

Germination of the spore occurs when the external conditions become favourable to growth by access of moisture and nutrients, in particular of trigger nutrients such as L-alanine, inosine or glucose in certain *Bacillus* species. Spores which have survived exposure to severe adverse influences such as heat are found to be

much more exacting than normal in their requirements for germination. For this reason, specially enriched culture media are used when testing the sterility of materials which have been exposed to disinfecting procedures (p. 234). In the process of germination, the spore swells, its cortex disintegrates, its coat is broken open and a single vegetative cell emerges.

In unstained preparations the spore is recognised within the parent cell by its higher refractivity. It is larger than lipid inclusion granules and often ovoid, in contrast to the spherical shape of the latter. When mature it resists coloration by simple stains and Gram's stain, appearing as a clear space within the stained cell protoplasm. Spores are slightly acid-fast and may be stained differentially by a modification of the Ziehl-Neelsen method (p. 120).

Some of the mycelial bacteria (*Actinomycetales*) form *conidia*, resting spores of a kind different from endospores. The conidia are borne externally by abstraction from the ends of the parent cells (conidiophores), often in chains, and are disseminated by the air or other means to fresh habitats. They are not specially resistant to heat and disinfectants.

Bacterial Capsules and Loose Slime.—Certain bacteria form a relatively thick gelatinous covering layer, or *capsule*, outside their cell wall, and are described as "capsulate". In many species the substance of the watery capsular gel is a complex polysaccharide; in some, polypeptides or proteins are the main constituent. The development of the capsules may be dependent on certain favourable environmental conditions; thus their size may vary with the amount of carbohydrate available for nutrition of the bacteria, and they may even disappear in the later stages of growth on an artificial culture medium. Capsules play an important part in determining the immunological specificity of bacteria and in conferring virulence, as by rendering them less susceptible to phagocytosis (*vide pneumococcus*, p. 494).

Capsules have little affinity for basic dyes and are usually invisible by ordinary staining methods. Such stains, moreover, may sometimes produce misleading capsule-like haloes, clear or coloured, around non-capsulate bacteria. The most reliable method of demonstration is by "negative staining" with India ink, this being used in wet films to avoid the great shrinkage which capsules undergo when dried; other special methods of differential staining are also available (p. 122). Some bacteria form a capsular layer which is too thin (under $0.2\ \mu$) to be resolved by the light microscope, but whose presence outside the cell wall is inferred from immunological and chemical examinations; such envelopes

have been termed *microcapsules*. The M-protein layer in non-capsulate *Streptococcus pyogenes* (p. 480) and the O antigens of Gram-negative bacilli (p. 66) are examples. It has been calculated that the O antigen may account for 5–10 per cent. of the dry weight of the cell and cover the cell wall as a layer 0·01–0·02 μ in thickness.

Loose or free slime is produced extracellularly by many capsulate organisms and some non-capsulate species. It is a viscid colloidal material similar in chemical nature to the capsules and secreted outside them. It readily disperses into solution in a liquid medium, but in growths on solid medium it remains as a matrix in which the bacteria are embedded, conferring on the growths a sticky or "mucoid" character. Slime can be demonstrated in wet-film India ink preparations of these surface cultures.

Flagella and Motility.—Motile strains of bacteria possess filamentous appendages known as *flagella*, which effect propulsive movements and act as organs of locomotion. The flagellum is a long, thin filament which is twisted spirally in an open, regular wave-form. It is commonly about 0·02 μ thick and several times the length of the bacterial cell. It originates in the bacterial protoplasm and is extruded through the cell wall. According to the species, there may be one or several flagella per cell, and in rod-shaped bacteria they may be "peritrichous", originating from the sides of the cell, or "polar", originating from one or both ends. Where several occur on a cell, they may function coiled together as a single "tail". Flagella consist largely or entirely of a protein, "flagellin", belonging to the same chemical group as myosin, the contractile protein of muscle. They are clearly demonstrated by the electron microscope, particularly in metal-shadowed preparations and usually appear as simple fibrils without internal differentiation. They are invisible in ordinary preparations by the light microscope, but may be shown by the use of special staining methods which involve mordanting and deposition of stain (p. 124), and in special circumstances by dark-ground illumination (p. 96). Because of the difficulties of these methods, the presence of flagella is commonly inferred from the observation of motility.

(*Motility* may be observed either microscopically (p. 95) or by noting the occurrence of spreading growth in semi-solid agar medium (p. 198). On microscopic observation of wet films, motile bacteria are seen swimming in different directions across the field, with a darting, wriggling or tumbling movement. True motility must be distinguished from a drifting of the bacteria in a single direction due to a current in the liquid, and also from Brownian

movement, which is a rapid oscillation of each bacterium within a very limited area due to bombardment by the water molecules.

Among the spirochaetes, motility appears to be a function of the cell body, since flagella do not occur. The most characteristic movement is a fast spiral rotation on the long axis with slow progression in the axial line; movements of flexion and lashing movements may be observed. Some spirochaetes possess an axial filament and others a band of fibrils wound around their surface from pole to pole. It has been suggested that these structures may contribute to motility, either through being themselves contractile or by acting as stiffeners for recoil against the contractile protoplast.

The function of motility in pathogenic bacteria is not known. It is generally regarded as assisting spread through the body fluids and tissues, but non-motile pathogens are not obviously less capable in this respect. Motility may also assist uptake of nutrients by continuously changing the body fluids in contact with the bacterial cell surface.

Fimbriae.—Certain pathogenic and non-pathogenic Gram-negative bacilli possess filamentous appendages of a kind different from the flagella. These "fimbriae" are unrelated to motility and may occur in non-motile as well as motile strains. They are shorter than flagella, about half as thick, without the wavy shape and usually much more numerous, up to several hundred being borne peritrichously by each cell. They cannot be seen by the light microscope, but are clearly demonstrated by the electron microscope in metal-shadowed preparations. The functions of fimbriae are not known with certainty. They may act as organs of adhesion for attaching the bacilli to surfaces of various kinds. Thus, fimbriate bacilli adhere strongly to many kinds of cells and when mixed with red blood cells, bind them together so as to cause haemagglutination. They are also enabled to grow as a skin or "pellicle" on the surface of stagnant liquid media. Most potentially fimbriate strains readily undergo a reversible variation between a fimbriate phase and a non-fimbriate phase, the former generally predominating in growths in liquid media and the latter in growths on solid media.

Pleomorphism and Involution.—In the course of growth, bacteria of a single strain may show considerable variation in size and shape, forming a proportion of cells which differ grossly from the normal, e.g. swollen spherical and pear-shaped forms, elongated filaments and filaments with localised swellings. This pleomorphism occurs most readily in certain species (e.g. *Streptobacillus moniliformis*, *Pasteurella pestis*), in ageing cultures on

artificial medium and, especially, in the presence of antagonistic substances such as penicillin, glycine, lithium chloride, sodium chloride in high concentrations and organic acids at low pH. The abnormal cells are generally regarded as degeneration or "involution" forms; many are non-viable, while some may grow and revert to the normal form when transferred to a suitable environment. In certain cases the abnormal shape seems to be the result of defective cell-wall synthesis; the growing protoplasm expands the weakened wall to produce a grotesquely swollen cell which later bursts and lyses. There is evidence that penicillin exerts its bactericidal influence by interfering with cell-wall synthesis.

So-called *L-forms* have been derived from many kinds of bacteria, particularly from cultures grown under conditions inducing pleomorphism, as in the presence of penicillin. These are fragile, plastic, often spherical cells which lack a normal rigid cell wall, but are capable of continued growth through repeated subcultivations in special osmotically protective media. Under certain circumstances they may revert to the normal bacterial form. *L-forms* are resistant to penicillin although their parent forms are sensitive.

Bacterial Reproduction.—Among the "lower" or true bacteria, multiplication takes place by *simple binary fission*. The cell grows in size, usually elongating to twice its original length, and the protoplast becomes divided into two approximately equal parts by the ingrowth of a transverse septum from the cell wall. In some species, this cell-wall septum splits in two and the daughter cells separate almost immediately. In others, the cell walls of the daughter cells remain continuous for a while and the organisms grow adhering in pairs, clusters, chains or filaments. Under favourable conditions, growth and division are repeated with great rapidity, *e.g.* every half-hour, so that one individual may reproduce thousands of millions of new organisms in less than a day. Among the spirochaetes transverse fission occurs as in other bacteria.

In the "higher" or mycelial bacteria, growth takes place by extension of the vegetative filaments, and multiplication by transverse division of these into shorter forms, or by the liberation of numerous conidia (*vide supra*) which later germinate and give rise to fresh mycelia.

Some observers have described more complex processes of reproduction among bacteria and postulated life cycles comprising different morphological phases. In many cases, however, the forms presumed to be "intermediate phases" have in fact been degenerate involution cells, and the evidence at present available

does not warrant acceptance of such views. Sexual reproduction does not occur commonly in bacteria, but genetical evidence has been obtained that, in a few varieties, the conjugation of two individual cells and genetic recombination may rarely occur (p. 38).

MORPHOLOGICAL CLASSIFICATION OF BACTERIA

The main groups of bacteria are distinguished by microscopical observation of their morphology and staining reactions, initially in a Gram-stained preparation. They can be classified simply as follows:

I. Lower Bacteria.—Simple, generally unicellular structures, never in the form of a mycelium or sheathed filaments.

1. Coccii—spherical or nearly spherical cells.
2. Bacilli—relatively straight rod-shaped (cylindrical) cells.
3. Vibrios—curved rod-shaped cells ("comma-shaped").
4. Spirilla—spirally twisted, non-flexuous rods.
5. Spirochaetes—thin, spirally twisted, flexuous filaments.

II. Higher Bacteria.—Filamentous organisms, some being sheathed and some growing with branching to form a mycelium. May have certain cells specialised for reproduction; e.g.

Actinomycetes—simple branching filaments forming a mycelium; may form conidia; non-motile.

Coccii

The main groups of coccii are distinguished according to their predominant mode of cell grouping and their reaction to the Gram stain. The following groups correspond with biological genera:

(1) *Diplococcus*.—Cells mainly adherent in pairs and slightly elongated in axis of pair; Gram-positive (e.g. *D. pneumoniae*, p. 494).

(2) *Streptococcus*.—Cells mainly adherent in chains, due to successive cell divisions occurring in the same axis; Gram-positive (e.g. *Strept. pyogenes*, p. 479).

(3) *Staphylococcus*.—Cells mainly adherent in irregular clusters, due to successive divisions occurring irregularly in different planes; Gram-positive (e.g. *Staph. aureus*, p. 466).

The genus *Micrococcus*, comprising only non-pathogenic species, is similar to *Staphylococcus*, though some of its species differ in size, in being motile or in being only weakly Gram-positive.

(4) *Gaffkya*.—Cells mainly adherent in plates of four (tetrads), or multiples thereof, due to division occurring successively in two planes at right angles; Gram-positive (e.g. *Gaff. tetragena*, p. 478).

(5) *Sarcina*.—Cells mainly adherent in cubical packets of eight, or multiples thereof, due to division occurring successively in three planes at right angles; Gram-positive (e.g. *Sarc. lutea*, p. 478).

(6) *Neisseria*.—Cells mainly adherent in pairs and slightly elongated at right angles to axis of pair; Gram-negative (e.g. *N. meningitidis*, p. 502).

(7) *Veillonella*.—Generally very small cocci arranged mainly in clusters and pairs; Gram-negative (e.g. *Veill. parvula*, p. 513).

The different cocci are relatively uniform in size, about 1 μ being the average diameter. Some species are capsulate and a very few are motile. It should be noted that a pure growth will usually show, in addition to the predominant cell grouping (e.g. clusters or long chains), a number of single cocci, pairs and very short chains.

Bacilli

The primary subdivision of the rod-shaped bacteria is made according to their staining reactions by the Gram and Ziehl-Neelsen methods, and whether or not endospores are formed. Some of the groups thus distinguished include several biological genera, and these can be recognised only by study of their physiological characters in artificial cultures.

(1) The *Acid-fast Bacilli*.—In giving an acid-fast staining reaction by the Ziehl-Neelsen method, members of the genus *Mycobacterium*, including the tubercle bacillus (p. 537), are distinguished from all other bacilli.

(2) The *Gram-positive Spore-forming Bacilli*.—Apart from some rare saprophytic varieties, the only bacteria to form endospores are those of the genera *Bacillus* (aerobic, p. 559) and *Clostridium* (anaerobic, p. 674). They are primarily Gram-positive, but very liable to become Gram-negative in ageing cultures. The size, shape and position of the spore may assist recognition of the species; e.g. the tetanus bacillus is characterised by its bulging, spherical terminal spore ("drum-stick" form).

(3) The *Gram-positive Non-sporing Bacilli*.—These include several genera. *Corynebacterium* (p. 514) is distinguished by a tendency to slight curving and club-shaped or ovoid swelling of the bacilli, and their arrangement in parallel and angular clusters due to the snapping mode of cell division. *Erysipelothrix* (p. 533) and *Lactobacillus* (p. 623) are distinguished by the tendency to

grow in chains and filaments, and *Listeria* (p. 533) by the occurrence of motility and flagellation.

(4) The *Gram-negative Bacilli*.—These include numerous genera belonging to the families *Pseudomonadaceae*, *Achromobacteraceae*, *Enterobacteriaceae*, *Brucellaceae* and *Bacteroidaceae*. *Pseudomonas* is distinguished by its polar flagellation, while motile members of the other families are peritrichously flagellate.

Vibrios and Spirilla

Vibrios are recognised as short, non-flexuous curved rods (e.g. *V. cholerae*, p. 626) and spirilla as non-flexuous spiral filaments (e.g. *Sp. minus*, p. 730). They are Gram-negative and mostly motile, having polar flagella and showing very active, "darting" motility.

Spirochaetes

The spirochaetes are slender, flexuous spiral filaments, and their staining reaction, when demonstrable, is Gram-negative. They are distinguished from the spirilla in being capable of active flexion of the cell body and in being motile without possession of flagella. The different varieties are recognised by their size, shape, wave form and refractivity, observed in the natural state in unstained wet films by dark-ground microscopy. The pathogenic species are classified in three genera:

(1) *Borrelia*.—Larger and more refractile than the other pathogenic spirochaetes, and more readily stained by ordinary methods; coils large and open, with a wave-length of 2–3 μ ; a leash of 8–12 fibrils, each about 0·02 μ thick, is seen twisted round the whole length of the protoplast, by electron microscopy (e.g. *Borr. recurrentis*, p. 726).

(2) *Treponema*.—Thinner filaments in coils of shorter wavelength (e.g. 1·0–1·5 μ), typically presenting a regular "corkscrew" form; feebly refractile and difficult to stain except by silver impregnation methods; by electron microscopy, a leash of 3–4 fibrils is seen wound round the protoplast within the cell wall (e.g. *Trep. pallidum*, p. 708).

(3) *Leptospira*.—The coils are so fine and close (wavelength about 0·5 μ) that they are barely discernible by dark-ground microscopy, though clearly seen winding round a single axial filament by electron microscopy. One or both extremities of the spirochaete are "hooked" or recurved, so that it may take the shape of a walking-stick, an S or a C (e.g. *L. icterohaemorrhagiae*, p. 716).

Actinomycetes

These mycelial bacteria, or *Actinomycetales*, include three main genera:

(1) *Actinomyces*.—Gram-positive, non-acid-fast, tending to fragment into short coccoid and bacillary forms, and not forming conidia; anaerobic (e.g. *Actinomyces israelii*, p. 660).

(2) *Nocardia*.—Similar to *Actinomyces*, but aerobic and mostly acid-fast (e.g. *Noc. farcinica*, p. 664).

(3) *Streptomyces*.—Vegetative mycelium not fragmenting into short forms; conidia formed in chains from aerial hyphae (e.g. *Streptomyces griseus*, p. 659).

CHAPTER II

PHYSIOLOGY OF BACTERIA

CLASSIFICATION AND IDENTIFICATION

The physiology and biochemistry of bacteria are studied by observations made with "cultures" grown in the laboratory on artificial nutrient medium.

Bacteria are subject, as regards their growth and viability (*i.e.* survival in the living state), to various external influences, *e.g.* nutrient materials, atmosphere, temperature, moisture, hydrogen-ion concentration, light and a wide variety of chemical substances which are inhibitory or lethal (*e.g.* chemotherapeutic agents and disinfectants, pp. 394, 166).

Bacterial Nutrition.—The growth of bacteria is dependent on an adequate supply of suitable food materials, the specific nutrient requirements varying in the different species according to their natural environmental adaptations. Some species are able to grow under a wide range of conditions, while others, especially the more strictly parasitic (*e.g.* gonococcus, p. 507), are very exacting and restricted in their requirements with regard not only to food but also to temperature and other factors. While it is hardly possible to reproduce exactly the natural environmental conditions of pathogenic bacteria, suitable artificial culture media have been devised for the great majority.

The chemical constitution of all bacteria is essentially similar, the principal components being water, proteins, nucleic acids, polysaccharides, lipids, mineral salts and small amounts of certain essential metabolites. The molecular sub-units, such as the amino acids, nucleotides and fatty acids, are mostly the same as those occurring in higher organisms. The growth of bacteria involves the synthesis of these mainly complex organic substances from the simpler components of the nutrient medium, and the differing nutritional requirements of bacteria reflect their differing synthetic abilities.

The main general nutrient requirements are as follows: (1) a *source of energy*, required for the endergonic synthetic reactions of growth and repair, maintenance of membrane equilibria, motility, etc.; (2) a *source of carbon*; (3) a *source of nitrogen*, required mainly for the synthesis of proteins and nucleic acids; (4) a supply of *inorganic salts*, particularly phosphate and sulphate among the

anions, and potassium, sodium, magnesium, calcium, iron, manganese and cobalt among the cations; and (5) in certain bacteria, a supply of various "accessory growth factors", or "bacterial vitamins", required in small amounts for the synthesis of essential metabolites.

Some non-parasitic bacteria are able, like the plants, to utilise carbon dioxide as the sole source of carbon and are called *lithotrophs* (or autotrophs). Energy is obtained by the oxidation of inorganic compounds (chemolithotrophs) or from sunlight (photolithotrophs). The majority of bacteria, however, including all the parasitic species, require organic nutrients such as carbohydrates, amino acids, peptides or lipids to serve as the sources of carbon and energy. These organisms are called *organotrophs* (or heterotrophs). The various species show considerable differences in their requirements for nitrogen sources and growth factors. Some, especially among the non-parasitic species, have comprehensive synthetic abilities and are therefore *non-exacting* nutritionally; they are able to synthesise all their amino acids, requiring only an inorganic nitrogen source (*e.g.* a nitrate or an ammonium salt), and also all their essential metabolites, thus not requiring any preformed growth factors. For example, *Escherichia coli* (p. 608) can grow on a "simple synthetic medium" which contains only glucose (carbon and energy source), ammonium sulphate (nitrogen and sulphur source), phosphate buffer, potassium chloride, magnesium chloride and traces of other inorganic salts; alternatively, an amino acid such as alanine might serve as the combined source of carbon, energy and nitrogen.

Other bacteria, in the course of evolution towards a strictly parasitic mode of life, have increasingly obtained their amino acids and growth factors from the tissues of their host, and have lost the power of themselves synthesising these compounds. Many species are thus nutritionally *exacting* (*e.g.* *Streptococcus pyogenes*) and can grow in a synthetic medium only if it contains a wide range of different amino acids and growth factors. The *amino acids*, *e.g.* tryptophane, leucine, methionine, cystine and histidine, are required as components for the formation of the cell proteins, including the enzymes. Among the *growth factors*, purines and pyrimidines are required as components for the synthesis of nucleic acids, and others are required in minute amounts as precursors for the formation of coenzymes. The latter are often identical with mammalian vitamins, and most of the B vitamin group, for instance, are required in some or other bacterial species (*e.g.* thiamine, riboflavin, nicotinic acid, pyridoxine, *p*-aminobenzoic acid, folic acid, biotin, vitamin B₁₂, etc.).

Under appropriate conditions in synthetic media, the amount of bacterial growth is linearly proportional to the concentration of a growth factor or essential amino acid whose supply is deficient in relation to the other nutrients. This is the principle of *micro-biological assay*, wherein the amount of a growth factor or amino acid is measured according to the amount of the growth that it supports (e.g. assay of vitamin B₁₂ using *Lactobacillus leichmannii*). The method has the advantage of specificity and high sensitivity. In the case of vitamins it is possible to determine as little as 0.001 µg. per ml., and in some cases (biotin, B₁₂) considerably less.

The transformation of simple substances into the complex constituents of bacteria involves the formation in stages of various intermediate substances of increasing chemical complexity, each stage in the synthesis being catalysed by an appropriate enzyme. Any interference with an intermediate metabolite or with its associated enzyme will inhibit growth by preventing the metabolite from being utilised for further synthesis. It is by such interference that many antiseptics, chemotherapeutic drugs and antibiotics bring about the inhibition of growth or killing of the organism (p. 394). Furthermore, an enzyme may be lost as a result of gene mutation (p. 38) and the variant strain thus become nutritionally exacting in respect of a metabolite which the parent strain can synthesise for itself.

Nutrient Broth.—Although synthetic culture media have been described for many kinds of bacteria, they are laborious and expensive to prepare, and of limited application. In routine practice, media are made from a few natural products which contain a wide range of nutrient factors and so can support growth of all but the most exacting species. The most generally useful liquid medium is *nutrient broth* (p. 187). This is a watery solution of two commercial products, "peptone" and "meat extract", and enough sodium chloride to give isotonicity with body fluids. "Peptone" is a crude soluble product obtained by digesting a protein material such as meat with proteolytic enzymes such as trypsin, pepsin and papain. It is mainly a mixture of polypeptides and amino acids, which serve as sources of nitrogen, carbon and energy, and also inorganic salts and small amounts of some growth factors. By itself, as in the form of "peptone water" (p. 199), it can support the growth of many kinds of bacteria, but the addition of meat extract supplements its deficiencies and makes a more widely useful medium. "Meat extract" consists of the water-soluble components of meat, excepting proteins which are removed in its preparation. It contains a wide range of growth factors and mineral salts, a small amount of sugar and some amino

acids. Peptone and meat extract give a clear solution and, being free from coagulable protein, are suitable for incorporation in media which have to undergo sterilisation by heat. Certain growth factors, however, are liable to destruction by heat and adsorption by filters, so that excessive heating or filtration of media must be avoided if they are to be retained. In place of peptone, an acid or tryptic hydrolysate of the milk protein casein may be used, and in place of meat extract, an autolysate of brewer's yeast, *i.e.* "yeast extract". While commercial products are usually employed, a meat extract may be freshly prepared from lean meat in the laboratory ("infusion broth", p. 190); and, instead of adding peptone, meat may be digested with trypsin, as in the preparation of "digest media" (*e.g.* Hartley's broth, p. 192).

Other substances may be added to enrich nutrient broth and solid media based on peptone and meat extract (*e.g.* nutrient agar, p. 195). Thus, for the highly exacting haemophilic bacteria (p. 649), blood is added as a source of haem (X factor) and coenzyme I or II (V factor), deficient in the basic medium. In "selective" or "enrichment" media, substances are added to inhibit the growth of unwanted bacteria or enhance that of desired kinds. In "indicator media", substances are added which yield a visible change with particular kinds of bacteria, as an aid to identification.

Influence of Oxygen and Redox Potential.—The majority of bacteria are described as *facultative anaerobes* because they are able to grow either aerobically, *i.e.* in the presence of air and free oxygen, or anaerobically, in its absence; with most such species, growth is more abundant aerobically. Certain other species will grow only in the presence of air or free oxygen, and are described as *strict* or *obligatory aerobes*. Still others will grow only in the absence of free oxygen and are usually killed in its presence; these are known as *strict anaerobes*. In the latter case, the ultimate determining factor is the state of oxidation of the environment, this being best described in terms of the oxidation-reduction, or "redox" potential (p. 286 *et seq.*). A sufficiently low redox potential for the growth of strict anaerobes is usually provided by placing the culture medium in an atmosphere of hydrogen, with the complete exclusion of free oxygen (*e.g.* in a McIntosh and Fildes anaerobic jar, p. 256). It has been suggested that in the presence of oxygen a strict anaerobe is liable to produce toxic peroxides which it cannot destroy owing to lack of catalase, an enzyme present in most aerobes and facultative anaerobes. Finally, there is a group of organisms which grow best in the presence of a trace only of free oxygen and often prefer an increased concentration of carbon dioxide; these are called *microaerophilic*.

The natural environment of a bacterium is determined by its oxygen and redox potential requirements. Thus, a strict aerobe like the tubercle bacillus will grow best in a well-aerated environment such as the animal lung, and a strict anaerobe like *Clostridium welchii* requires an anaerobic environment such as in the contents of the intestine or in dead tissue in a lacerated wound.

Bacterial Respiration and Oxidation Reactions.—The aerobic or anaerobic nature of a micro-organism is related to the nature of its respiratory metabolism. Thus, aerobes obtain their energy by a series of coupled oxidation-reduction reactions in which the ultimate hydrogen acceptor is atmospheric oxygen; in this *aerobic respiration*, their carbon and energy source may be oxidised completely to carbon dioxide and water. Anaerobes, on the other hand, oxidise compounds at the expense of some hydrogen acceptor other than oxygen. Usually their carbon and energy source acts both as hydrogen acceptor and donator in a series of oxido-reductions. This process is known as *fermentation* and leads to the formation of a variety of waste products such as ethanol in yeasts and organic acids in bacteria, e.g. lactic acid in lactobacilli and streptococci, and a mixture of lactic, acetic and formic acids in the enterobacteriaceae. The nature of the fermentation products is of significance in the classification of bacteria. Each of the series of oxidation-reduction reactions involves transfer of hydrogen or equivalent electrons, and is catalysed by an enzyme specific for the substrate acting together with a coenzyme. The energy from each oxidation step is produced mainly as chemical-bond energy in certain energy-carrier substances, particularly adenosine triphosphate, available for subsequent endergonic synthetic reactions, etc.

Influence of Carbon Dioxide.—It is now recognised that all bacteria require the presence of a small amount of carbon dioxide for growth, an amount normally provided by the atmosphere or by oxidation and fermentation reactions within the cell itself. Some bacteria, however, require a much higher concentration of carbon dioxide (5–10 per cent.), which must be provided in the environment of the culture medium (e.g. *Brucella abortus*, when first isolated from the body, p. 643).

Influence of Temperature.—(a) *On Growth.*—For each species there is a definite temperature range within which growth takes place. The limits are the “maximum” and “minimum” temperatures, and an intermediate “optimum” temperature can usually be recognised at which growth is most rapid. In the laboratory, bacteria are grown at this optimum temperature in a thermostatically controlled *incubator*. The optimum temperature

of a bacterium is approximately that of its natural habitat, e.g. about 37° C. in the case of organisms which are parasitic on man and warm-blooded animals. These, and many saprophytes of soil and water, which grow best at between 25° and 40° C., are termed *mesophilic*. Some mesophiles have a wide growth temperature range (e.g. 5°–43° C. for *Pseudomonas pyocyannea*, p. 622), while others are more restricted (e.g. 30°–39° C. for *Neisseria gonorrhoeae*, p. 507). None of them grow appreciably at temperatures below 5° C., as in a domestic refrigerator (3°–5° C.), and few at more than 45° C.

A group of soil and water bacteria, the *psychrophiles*, grow best at temperatures below 20° C., usually quite well at 0° C. and in some cases, slowly, down to about –7° C. on unfrozen media. Their importance lies in their ability to cause spoilage of refrigerated and frozen food, though none are pathogenic. Another group of non-parasitic bacteria, the *thermophiles*, grow best at high temperatures between 55° and 80° C., and have minimum growth temperatures ranging from 20° to 40° C. (facultative thermophiles), or even above 40° C. (strict thermophiles). These organisms are important as a cause of spoilage in under-processed canned foods, since many form spores of exceptionally high heat-resistance.

(b) *On Viability*.—Heat is an important agent in the artificial destruction of micro-organisms, the effect depending under moist conditions on the coagulation and denaturation of cell proteins, and under dry conditions, on oxidation and charring. Among the bacteria which are parasites of mammalian animals, non-sporing forms in the presence of water generally cannot withstand temperatures above 45° C. for any length of time. The time of exposure to heat which is necessary for killing is shorter the higher the temperature, and various other factors influence the exact amount of heating required. Thus, bacteria are more susceptible to "moist heat", e.g. in hot water or saturated steam (p. 151), than to "dry heat", e.g. in a hot-air oven (p. 150). They are rendered more susceptible by the presence of acid, alkali or any chemical disinfectant, and less susceptible by the presence of organic substances such as proteins, sugars and fats, and also by their own occurrence in large numbers. The "thermal death point" of a particular organism may be defined as the lowest temperature which kills it under standard conditions within a given time, e.g. ten minutes. Under moist conditions, it lies between 50° and 65° C. for most non-sporing mesophilic bacteria, and between 100° and 120° for the spores of most sporing species (e.g. about 105° C. for *Cl. tetani* and 115° C. for *Cl. botulinum*). The extreme limit of resistance to moist heat is shown by the

spores of a non-pathogenic, strictly thermophilic bacillus, *B. stearothermophilus*, whose killing requires exposure to 121° C. for 10–35 minutes. With dry heat, the 10-minute thermal death points of the different sporing bacteria are mostly between 140° and 180° C.

At low temperatures some species die rapidly, but the majority survive well. Cultures of the latter may be preserved for long periods at between 3° and 5° C. in a domestic-type refrigerator, or in the frozen state at between –20° and –70° C. in a "deep freeze" cabinet (p. 267). The process of freezing kills a proportion of the bacterial cells present, and this is least if freezing is effected rapidly (e.g. by use of solid carbon dioxide).

Influence of Moisture and of Desiccation.—Four-fifths by weight of the bacterial cell consists of water and, as in the case of other organisms, moisture is absolutely necessary for growth. Drying in air is injurious to many microbes, and the different species vary widely in their ability to survive when dried under natural conditions, as in infected exudate smeared on clothing or furniture, and converted to dust. Thus, the gonococcus, *Treponema pallidum* and the common cold virus appear to die almost at once, while the tubercle bacillus, *Staph. aureus* and the smallpox virus may survive for several months. Bacterial endospores survive drying especially well; for instance, those of *Bacillus anthracis*, when dried on threads, have survived for over sixty years.

Even delicate, non-sporing organisms may survive drying for a period of years if they are desiccated rapidly and completely, preferably while frozen, and thereafter maintained in a high vacuum (0.01 mm. Hg., or less) in a sealed glass ampoule which is stored at room temperature in the dark. This is the basis of the *lyophilisation* or "freeze-drying" process of preserving bacterial cultures in the laboratory (p. 268).

Influence of Hydrogen-ion Concentration.—This is an essential factor in bacterial metabolism and growth. The majority of commensal and pathogenic bacteria grow best at a neutral or very slightly alkaline reaction (pH 7.2 to 7.6). Some bacteria, however, flourish in the presence of a considerable degree of acidity and are termed *acidophilic*, e.g. *Lactobacillus* (p. 623). Others are very sensitive to acid, but tolerant of alkali, e.g. *Vibrio cholerae* (p. 626). Strong acid or alkali solutions, e.g. 5 per cent. hydrochloric acid or sodium hydroxide, are rapidly lethal to most bacteria, the mycobacteria (e.g. tubercle bacillus) being exceptional in resisting them.

Influence of Light and other Radiations.—Darkness is a favourable condition for growth and viability. Ultra-violet rays

are rapidly bactericidal, *e.g.* direct sunlight or radiation from a mercury vapour lamp. Even diffuse daylight, as it enters a room through window glass, significantly shortens the survival of micro-organisms and may be of hygienic importance. Bacteria are also killed by cathode and Röntgen rays, and by radium emanations.

Osmotic Effects.—As a result of the presence of a semi-permeable cytoplasmic membrane, bacteria resemble other cells in being subject to osmotic phenomena. Relatively, however, they are very tolerant of changes in the osmotic pressure of their environment and can grow in media with widely varying contents of salt, sugar and other such solutes. For most species the upper limit of sodium chloride concentration permitting growth lies between 5 and 15 per cent., though *halophilic* (or osmophilic) species occur which can grow at higher concentrations up to saturation. The latter are saprophytes whose importance lies in their ability to cause spoilage of food preserved with salt or sugar. Sudden exposure of bacteria to solutions of high concentration (*e.g.* 2–25 per cent. sodium chloride) may cause *plasmolysis*, i.e. temporary shrinkage of the protoplast and its retraction from the cell wall due to the osmotic withdrawal of water; this occurs much more readily in Gram-negative than in Gram-positive bacteria. Sudden transfer from a concentrated to a weak solution, or to distilled water, may cause *plasmoptysis*, i.e. swelling and bursting of the cell through excessive osmotic imbibition of water.

Influence of Mechanical and Sonic Stresses.—Although their cell walls have considerable strength and some elasticity, it is possible to rupture and kill bacteria by exposure to mechanical stresses. A bacterial suspension may be largely disintegrated by subjection to very vigorous shaking with fine glass beads, or to supersonic or ultrasonic vibration (9,000–200,000, and over 200,000 cycles per second, respectively). These measures are used in isolating the large molecular components of the cell (p. 308).

Isolation of Pure Cultures.—Most studies and tests of the physiological, immunological and other characters of bacteria are valid only when made on a *pure culture*, i.e. an isolated growth of a single strain free from mixture and contamination with other bacteria. For this reason, in the diagnostic examination of mixed infective material, an essential preliminary is the isolation of the pertinent organism in a pure culture. This is normally achieved by the method of “plating out” on a solid culture medium, although other methods are applicable in certain cases (p. 252 *et seq.*). The solid medium most used is *nutrient agar*, a preparation

of nutrient broth jellified by addition of the polysaccharide "agar-agar", which is derived from certain seaweeds; it is usually dispensed as a flat layer in a shallow "Petri" dish. The infected material is inoculated on the surface of this and spread out very thinly. Where the bacteria are deposited singly at sufficient distance from each other (*e.g.* 1 cm.), the whole progeny of each accumulates locally during growth to form a discrete mass, or *colony*, which is readily visible to the naked eye (*e.g.* 0.5–5 mm. diam.). Each colony constitutes a pure culture, since it consists exclusively of the descendants of a single cell; it may be "picked" with a sterile inoculating wire to prepare a pure subculture in a fresh medium. Occasionally a mixed colony is formed from two bacteria that have been inoculated close together on the agar, and these must be avoided. The maintenance of pure cultures necessitates the use of properly sterilised media, containers and instruments, and continuous covering against the deposition of dust-borne bacteria from the air; during inoculation, the culture medium should be uncovered only for a few seconds.

The Morphology of Bacterial Growths on Culture Medium.—The appearance to the naked eye of a pure growth on solid or liquid medium may be a valuable guide to the identity of the species. The morphology of colonies is especially important in assisting the decision as to which out of a mixture should be subcultivated for further study. The colonies of different species may differ in such characters as size, shape, smoothness of surface, consistency, transparency, colour and their effect on the underlying medium. The growths of most bacteria are colourless, but some species are *chromogenic* and produce characteristic pigments, *e.g.* yellow, red or green; the pigment may be non-diffusible, remaining in the bacterial cells and colonies, or it may be diffusible and pass out into the surrounding medium. Some features of colonial morphology are best observed by means of a hand lens or low-power microscope (p. 254).

Growth Phases.—When bacteria are introduced into a sterile culture medium, their growth proceeds as follows. There is first a period of "lag" (*e.g.* two hours) during which there is no multiplication though there is increase in cell size accompanied by intense metabolic activity. This is followed by a phase of rapid multiplication, the "logarithmic" phase, which may occupy six to eight hours at optimum temperature; the number of cells increases in geometric progression, 1–2–4–8–16–, doubling in each successive interval of time (*e.g.* each 30 minutes), so that the logarithmic curve of growth is a straight line. Then follows a "stationary" phase lasting from a few hours to several days, in which the number of new cells reproduced tends to be equalled

by those dying. Cessation of growth may be due to exhaustion of the supply of an essential nutrient, as in dilute media, or to the accumulation of toxic waste products, as in concentrated media. Finally there is a phase of "decline", or diminution of viable individuals, the organisms progressively dying until, after several days to months, no living bacteria remain.

Diagnostic Biochemical Reactions of Cultures.—Among the pathogenic bacteria, fermentative properties are important features in the identification of certain species. Thus, various bacteria decompose particular carbohydrates (*e.g.* sugars, hexahydric alcohols, polysaccharides) with the formation of acids (*e.g.* formic, acetic and lactic acids) and, in many cases, also gases (*e.g.* carbon dioxide and hydrogen). Fermentative activity for a given compound is tested by growing the bacterium in a liquid medium containing a large amount of the compound; the formation of acids is shown by the reaction of a pH indicator dye and the formation of gas by the collection of a bubble in a small inverted "Durham" tube immersed in the medium.

Tests are also made for other enzymatic activities, including the ability to digest proteins, as in gelatin or coagulated serum, to form indole or hydrogen sulphide from peptone, to form ammonia from urea, to break down and utilise various organic acids, and to decompose hydrogen peroxide (catalase activity).

CLASSIFICATION AND NOMENCLATURE

At present no standard classification is universally accepted and applied. The older systems were based mainly on morphological characters, and while these serve a useful purpose in any broad subdivision of the bacteria as shown in the earlier parts of this chapter, they are quite inadequate for detailed biological classification. Thus, the term *Bacillus* was used in the past as a generic name for all rod-shaped bacteria, *e.g.* *Bacillus anthracis*, *B. tuberculosis* and *B. coli*, but the bacillary organisms clearly require subdivision into many separate genera in view of their heterogeneity. A system of classification and nomenclature of the bacteria was introduced some years ago by the Society of American Bacteriologists following the accepted rules of biological classification, and has since been elaborated by American systematists. A brief outline of this system is given, *but only those orders, families and genera which are of special importance in medical and veterinary bacteriology are dealt with*. Generic characters are not detailed, but these are indicated by the characters of the various type-species quoted in later chapters. For full details, the seventh edition of Bergey's *Manual of Determinative Bacteriology* should be consulted.

Much of this classification and many of the names given below have

come into general use, though older designations are sometimes still applied in medical literature. Thus, in the new system the generic term *Bacillus* applies only to those rod-shaped bacteria that are spore-forming and aerobic, but its older use for a diversity of rod-shaped organisms is still current. It is, of course, quite correct to use the term "bacillus" as the common or vernacular name for any rod-shaped bacterium, and the common names such as "anthrax bacillus," "tubercle bacillus" and "colon bacillus" in place of the international scientific names (viz. *Bacillus anthracis*, *Mycobacterium tuberculosis* and *Escherichia coli*). In Part III of this book the new names of the American system are in general given priority, but the older conventional designations are indicated and also certain biological names used commonly by British writers. Abbreviations of generic names, e.g. "B.", "Myco.", etc., used throughout the text of the book are those adopted in the following summary.

ORDERS OF BACTERIA (CLASS SCHIZOMYCETES)

PSEUDOMONADALES.—Cells rigid, in form of straight, curved or spiral rods; rarely coccoid. Usually occur singly, though rarely in pairs or chains. Usually motile, possessing polar flagella. Gram-negative.

EUBACTERIALES.—Cells rigid, and spherical or straight rod-shaped in form. Occur singly, or in pairs, clusters, chains or filaments. Motile with peritrichous flagella or non-motile. Endospores formed in one family (*Bacillaceae*). Gram-positive or Gram-negative. Not acid-fast.

ACTINOMYCETALES.—Cells rigid, and rod-shaped or filamentous, with a tendency to branching; may grow as a mycelium which may develop aerial conidia, oidiospores or sporangiospores, and so give mould-like colonies. Mostly non-motile. Usually Gram-positive and some species acid-fast.

SPIROCHAETALES.—Cells flexuous, slender and spiral-shaped. Motile by flexion of the cell body. Do not possess flagella. Gram-negative.

MYCOPLASMATALES (Pleuropneumonia-like organisms).—Cells soft, fragile and highly pleomorphic. Reproduce by fission of filaments and large bodies into coccoid elementary bodies which are filterable. Non-motile. Gram-negative. Grow on agar media and so differ from the filterable viruses.

PSEUDOMONADALES

FAMILIES

Pseudomonadaceae.—Cells are straight rods, occasionally coccoid. May form a water-soluble pigment (e.g. green or brown) or a water-insoluble pigment (e.g. yellow or red).

Spirillaceae.—Cells are rigid, curved or spiral rods.

Further families in this order include most of the photosynthetic and autotrophic species of bacteria.

GENERA

Pseudomonadaceae

Pseudomonas.—e.g. *Ps. aeruginosa* (p. 622).

Spirillaceae

Vibrio.—e.g. *V. comma* (p. 626).

Spirillum.—e.g. *Sp. minus* (p. 730).

EUBACTERIALES

FAMILIES

Achromobacteraceae.—Cells rod-shaped and Gram-negative. Non-pigmented or forming yellow, orange or brown pigments.

Grow well on ordinary peptone media. Few species can ferment sugars to give acid; glucose is usually attacked oxidatively if at all.

Enterobacteriaceae.—Cells rod-shaped and Gram-negative. Grow well on ordinary peptone media. Actively ferment glucose, and in many cases lactose and other sugars, producing acid, or acid and visible gas (CO_2 and H_2).

Brucellaceae.—Cells small, coccoid to rod-shaped, and Gram-negative. Obligate animal parasites. Many fail to grow on ordinary peptone media, requiring addition of body fluids. Many lack power to ferment sugars.

Bacteroidaceae.—Cells rod-shaped or filamentous, and Gram-negative. Most are strict anaerobes and many require enriched culture media for growth. Parasites of mammals, especially of the alimentary canal.

Micrococcaceae.—Cells spherical and Gram-positive; occur singly and in pairs, tetrads, cubical packets and irregular clusters. Many form a non-water-soluble yellow, orange or red pigment. Mostly non-motile. (*Sarcina ureae* alone forms endospores.)

Neisseriaceae.—Cells spherical to elliptical, and Gram-negative; occur mainly in pairs, with long axes parallel, or in clusters. Non-motile. Parasites of mammals.

Lactobacillaceae.—Cells are cocci or rods occurring singly, in pairs and in chains. Gram-positive. Mostly non-motile. Facultatively or strictly anaerobic. Actively ferment sugars with lactic acid as main product.

Corynebacteriaceae.—Cells rod-shaped or club-shaped; Gram-positive. Mostly non-motile.

Bacillaceae.—Cells rod-shaped and usually Gram-positive. Form endospores. Many are motile.

GENERA

Achromobacteraceae

Alcaligenes.—e.g. *Alc. faecalis*.

Enterobacteriaceae

Escherichia.—e.g. *Esch. coli* (p. 608).

Aerobacter.—e.g. *Aero. aerogenes* (p. 617), *Aero. cloacae* (p. 618).

Klebsiella.—e.g. *Kl. pneumoniae* (p. 616).

Paracolobactrum.—e.g. *Par. coliforme*.

Serratia.—e.g. *Serr. marcescens* (*B. prodigiosus*).

Proteus.—e.g. *Pr. vulgaris* (p. 619).

Salmonella.—e.g. *S. typhosa* (p. 574) and *S. enteritidis* (p. 587).

Shigella.—e.g. *Sh. dysenteriae* (p. 595).

(Note.—In Part III of this book the above classification of the Enterobacteriaceae has been modified so that the genera conform more closely with the groups proposed by the Enterobacteriaceae sub-committee of the International Committee on Bacterial Nomenclature and Taxonomy. The genera *Aerobacter* and *Paracolobactrum* are omitted and the following genera included:

Citrobacter.—e.g. *Cit. freundii* (p. 615).

Cloaca.—e.g. *Cloaca cloacae* (p. 618).

Hafnia.—e.g. *Hafnia alvei* (p. 618).

Thus, *Aerobacter aerogenes* is included in the genus *Klebsiella*, and *Aerobacter cloacae* in the genus *Cloaca*. The *Paracolobactrum* organisms are regarded as lactose non-fermenting variants in the genera *Escherichia*, *Citrobacter*, *Klebsiella*, *Cloaca* and *Hafnia*.)

Brucellaceae

Pasteurella.—e.g. *P. pestis* (p. 634).

Bordetella.—e.g. *Bord. pertussis* (p. 655).

Brucella.—e.g. *Br. melitensis* (p. 642).

Haemophilus.—e.g. *H. influenzae* (p. 650).

Actinobacillus.—e.g. *Actinobacillus mallei* (p. 570).

Moraxella.—e.g. *Morax. lacunata* (p. 653).

Bacteroidaceae

Bacteroides.—e.g. *Bacteroides fragilis* (p. 672).

Fusobacterium.—e.g. *F. fusiforme* (p. 669).

Sphaerophorus (or *Necrobacterium*).—e.g. *Sph. necrophorus* (p. 670).

Streptobacillus.—e.g. *Streptobacillus moniliformis* (p. 666).

Micrococcaceae

Micrococcus.—e.g. *M. ureae* (p. 478).

Staphylococcus.—e.g. *Staph. aureus* (p. 466).

Gaffky.—e.g. *Gaff. tetragena* (p. 478).

Sarcina.—e.g. *Sarc. lutea* (p. 478).

Neisseriaceae

Neisseria.—e.g. *N. meningitidis*, *N. gonorrhoeae* (p. 502).
Veillonella.—e.g. *Veill. parvula* (p. 513).

Lactobacillaceae

Diplococcus.—e.g. *D. pneumoniae* (p. 494).
Streptococcus.—e.g. *Strept. pyogenes* (p. 479).
Peptostreptococcus.—e.g. *Peptostrept. putridus* (p. 493).
Lactobacillus.—e.g. *Lacto. acidophilus* (p. 623).

Corynebacteriaceae

Corynebacterium.—e.g. *C. diphtheriae* (p. 514).
Listeria.—e.g. *List. monocytogenes* (p. 535).
Erysipelothrix.—e.g. *Ery. insidiosa* (p. 533).

Bacillaceae

Bacillus.—e.g. *B. anthracis* (p. 559).
Clostridium.—e.g. *Cl. tetani* (p. 676).

ACTINOMYCETALES**FAMILIES**

Mycobacteriaceae.—Cells rod-shaped, but rarely filamentous or branching. Do not form conidia or other kinds of spores.

Actinomycetaceae.—Cells filamentous and branching. Grow as a mycelium which may fragment into short rod or coccoid forms, and reproduce by budding or by spores formed through fragmentation of the mycelium and in some cases from aerial hyphae.

Streptomycetaceae.—Cells filamentous and branching. Grow as a mycelium which does not fragment. Conidia are borne on sporophores, in many cases aerial.

GENERA**Mycobacteriaceae**

Mycobacterium.—e.g. *Myco. tuberculosis* (p. 537).

Actinomycetaceae

Actinomyces.—e.g. *Actinomyces israelii* (p. 660).
Nocardia.—e.g. *Noc. madurae* (p. 665).

Streptomycetaceae

Streptomyces.—e.g. *Streptomyces griseus* (p. 659).

SPIROCHAETALES

GENERA OF THE FAMILY TREPONEMATACEAE (THE SMALL | SPIROCHAETES)

Borrelia.—*Borr. recurrentis* (p. 726).

Treponema.—e.g. *Tr. pallidum* (p. 708).

Leptospira.—e.g. *L. icterohaemorrhagiae* (p. 716).

MYCOPLASMATALES

GENERA

Mycoplasma.—e.g. *Mycoplasma mycoides* (p. 667).

CLASS MICROTATOBIOTES

This class contains the smallest of living organisms, all obligate parasites and capable of growth only in the living tissues of an appropriate host, usually intracellularly. It includes two orders:

RICKETTSIALES.—Individual organisms are over $0.2\ \mu$ in diameter, resolvable by the light microscope and not filterable through bacteria-stopping filters, except for a few species with filterable phases. Gram-negative. Only a few species can grow on cell-free nutrient media.

VIRALES.—Individual organisms are mostly less than $0.2\ \mu$ in diameter, ultramicroscopic and filterable. All are obligate intracellular parasites, and none grow on inanimate or cell-free nutrient media.

There is no generally accepted classification of the viruses into genera and species (but see p. 745).

RICKETTSIALES

GENERA

Rickettsia.—e.g. *R. prowazekii* (p. 733).

Coxiella.—e.g. *Cox. burnetii* (p. 740).

Ehrlichia.—e.g. *Ehrl. ovina*.

Cowdria.—e.g. *Cowdria ruminantium* (p. 739).

Chlamydia.—e.g. *Chl. trachomatis* (the trachoma virus, p. 767).

Miyagawanella.—e.g. *Miy. psittaci* (the psittacosis virus, p. 763).

Bartonella.—e.g. *Bart. bacilliformis* (p. 742).

Haemobartonella.—e.g. *Haemobartonella muris* (p. 742).

Anaplasma.—e.g. *An. marginale* (p. 915).

SYSTEM OF IDENTIFICATION OF BACTERIA

(1) **The Morphology and Staining Reactions of Individual Organisms** generally serves as a preliminary criterion, particularly for placing an unknown species in its appropriate biological

group (see key given on pp. 18-21). In medical bacteriology the microscopic characters of certain organisms in pathological specimens may be sufficient for diagnostic identification, e.g. tubercle bacilli in sputum. Morphology among the bacteria usually fails, however, to differentiate allied organisms, e.g. the meningococcus, gonococcus and *Neisseria catarrhalis*.

(2) **Cultural Characters**, including the growth requirements and the appearance of cultures to the naked eye (p. 30), are further criteria assisting identification, but may also be insufficient to differentiate species; e.g. different species of *Salmonella* produce indistinguishable colonies.

(3) **Biochemical Reactions**, e.g. the fermentation of various carbohydrates. Species which can not be distinguished by morphology and cultural characters may exhibit distinct differences in their biochemical reactions, e.g. typhoid and paratyphoid bacilli. Different species or types may, however, resemble one another in fermentative properties, e.g. certain types of the *Salmonella* group.

(4) **Antigenic Characters**.—In bacteriology, species and types can often be identified by specific "antibody reactions". These depend on the fact that the serum of an animal immunised against a micro-organism contains specific antibodies (for the homologous species or type) which react in a characteristic manner with the particular micro-organism (p. 57). Such antisera, for example, may agglutinate or clump the homologous organism in test-tube experiment, and this effect can be observed easily with the naked eye. An unknown bacterium may thus be identified by demonstrating its reaction with one out of a number of standard known antisera.

It should be noted here that the serum of a person or animal suffering from a bacterial infection may also exhibit specific antibody reactions. The nature of the infection may thus be diagnosed by demonstrating that the patient's serum agglutinates one out of a number of known laboratory cultures; e.g. the Widal reaction in enteric fever.

(5) **Animal Pathogenicity**.—In the case of pathogenic organisms, e.g. the tubercle bacillus, which are virulent to, and produce characteristic lesions in, laboratory animals, the inoculation test provides a reliable method of identification.

CHAPTER III

BACTERIAL GENETICS AND VARIATION

LIKE other organisms, bacteria in general breed true and maintain their characters constant from generation to generation, yet at the same time show variations of particular characters in a small proportion of their progeny. Because of the great rapidity of bacterial reproduction, such variations are frequently encountered in the laboratory, even during relatively short periods of cultivation.

The characters which a bacterium exhibits at a given time (*i.e.* its phenotype) are determined both by its genetic constitution (genotype) and by environmental conditions which influence the contemporary expression of the genetic potentialities. The observed characters of a pure growth are thus liable to two kinds of variation: (1) *heritable variation* due to change in genetic constitution, *e.g.* by mutation, transduction, lysogenic conversion or recombination through cell conjugation, and (2) *non-heritable variation* due to change in the environmental conditions, *i.e.* environmentally impressed modifications and enzymatic inductions ("adaptations").

Bacteria reproduce by asexual binary fission, normally maintaining their genetic constitution unaltered through successive generations. The hereditary cell components which determine the inherited characters, are called "genes"; they are thought to correspond to specific deoxyribonucleic acid (DNA) molecules. The bacterial cell contains a large number of different genes, probably over 1000. Each gene is capable of ensuring its own replication and also of directing the formation of a particular substance or enzyme which is responsible for some of the inherited characters. Duplication of the genes must precede cell division, and there must be a mechanism for ensuring that each daughter cell receives a complete and identical set. In the higher organisms, the genes are borne in fixed linear order along the thread-like chromosomes in the nucleus, and during mitotic cell division the latter are split longitudinally and segregated in two equal sets. Bacteria must have a comparable mechanism and this may involve a single chromosome located in their DNA-containing nuclear bodies (p. 10).

Mutation.—Replication of the cell's set of genes is usually an

exact process. However, in a small proportion of cell divisions a "mutation" may occur, a gene accidentally being lost, altered in nature or changed in position. The mutant cell and its progeny then continue to reproduce with the new genetic constitution; correspondingly, they exhibit a sudden change of character which is heritable and, apart from rare reverse mutation, permanent. Bacteria are thought to be "haploid", i.e. possessing only a single set of genes; the effect of a mutated gene is thus not masked by that of an unmutated partner, as may occur in diploid organisms.

Spontaneous Mutations of unknown cause seem to be responsible for most of the heritable variations observed in bacteria. Any gene is liable to undergo spontaneous mutation. The mutation rate of a given kind of gene is fairly constant, but different genes show different rates, mostly between once per 10^4 and once per 10^{10} cells per generation. A large bacterial colony of about 10^9 cells, the product of a similar number of cell divisions, is thus liable to contain thousands of mutant cells of different kinds, including many cells changed in the more readily mutating genes and a few changed in the less readily mutating.

Commonly observed mutations include: (1) loss of capacity to form capsules, usually with associated loss of virulence, antigenic type specificity and mucoid colony character (e.g. in *D. pneumoniae*, p. 495); (2) loss of capacity to form the O somatic antigen, with associated loss of virulence, type specificity and smooth colony texture (e.g. smooth-rough, or S-R variation in *Salmonella*, p. 67); (3) loss of flagellation and motility (e.g. in *Salmonella*, p. 66); (4) exaltation or attenuation of virulence (e.g. the origin of attenuated living vaccines, pp. 60, 845, and 778); (5) variation in nutritional requirements by loss or gain of ability to utilise a given nutrient or to synthesise an essential amino acid or vitamin (e.g. in the so-called "adaptation" or "training" of an exacting organism to grow in subculture on a simpler medium than required for its primary culture; see *Myco. paratuberculosis*, p. 557); (6) variation in fermentative ability involving loss or gain of the power to ferment a given substrate (e.g. the "late" fermentation of lactose by *B. coli mutabilis*, and by *Sh. sonnei*, p. 595); (7) drug resistance variation, i.e. acquisition of increased resistance to the action of sulphonamide, penicillin, streptomycin or other antibacterial agent (pp. 400, 551); (8) loss of sensitivity to bacteriophage (p. 874); and (9) acquisition of resistance to toxic waste products accumulating in artificial cultures (e.g. variation of *Br. abortus* to R-type cells having an increased resistance to the inhibitory metabolite D-alanine which accumulates in cultures with DL-asparagine as nitrogen source). Many character changes result in an associated change of colonial morphology, as in size, pigmentation and texture. Mutants arising in a growing colony may thus form visibly distinctive sectors or papillae.

Phase Variation.—Some heritable variations are readily reversible and occur with relatively high frequency in either direction (*e.g.* once per 1000 cell divisions). The variation of certain Gram-negative bacilli between a fimbriate and a non-fimbriate phase (p. 604), and the phase variation of flagellar antigens in *Salmonella* (p. 591) are of this kind. It has been suggested that their mechanism differs from that of true gene mutation.

Induced Mutations and Mutagenic Agents.—The rate of almost any mutation may be increased greatly, *e.g.* 10-100,000 fold, by exposure of the resting or dividing cells to X-rays, ultraviolet radiation, mustard gas, peroxides, formaldehyde, acriflavine and certain other substances. The induced mutations are undirected, the nature of the new character not being determined by the nature of the mutagenic agent.

Environmental Selection of Mutants.—The rate of most mutations is so low that the small proportion of mutant cells originating in a culture usually remains undetected and does not alter the gross characters of the culture. Indeed, most kinds of mutation are harmful or lethal, and these cells die out. Only those mutants are detected whose altered genetic constitution fits them better than the parent strain for growth and survival under the concurrent cultural conditions. Such mutants increase in number relative to the parent-type cells until they predominate in the culture and so make the new character manifest. In nature, mutation and environmental selection have probably been the main means effecting the evolution and differentiation of bacterial varieties specially adapted to life in the different habitats.

Many conditions may be selective for a mutant. (1) A given culture medium favours those mutants which can utilise its constituents more rapidly or completely than the parent-type cells. When they originate in a growing colony, such mutants may form distinctive "secondary colonies" or "papillae", due to their greater growth (*e.g.* the lactose-fermenting mutants of *B. coli mutabilis* and *Sh. sonnei* on a lactose medium). A mutant which has gained the ability to synthesise for itself an essential growth factor is selected absolutely by cultivation in a synthetic medium which lacks that factor and so cannot support growth of the parent-type cells. (2) A lethal or growth-inhibiting condition is highly selective for mutants with increased resistance to that condition, *e.g.* the presence of an antibiotic, bacteriophage or toxic waste product of metabolism. Thus, cultivation on nutrient agar containing streptomycin will give a growth of streptomycin-resistant mutants without growth of the sensitive parent-type cells. (3) Repeated passage of a pathogenic bacterium through animals of a given species is selective for mutants having increased ability to survive and multiply in this species, and thus of exalted virulence. Repeated passage in

another species, or on artificial culture medium, is selective of mutants better fitted for survival and growth in these different conditions, and thus often attenuated in virulence for the former host species (p. 862).

Since environmental selection is normally essential for the detection of mutants, it is sometimes suggested that the pertinent environmental influence is required to induce the variation or direct its character. However, it can be shown that these mutations are undirected and occur spontaneously in the absence of the environmental condition which is subsequently selective; *e.g.* streptomycin-resistant mutants originate as readily in the absence of streptomycin as in its presence, although they only emerge as dominant in the latter case.

Other Mechanisms of Heritable Variation.—Less common mechanisms are transformation by free DNA, transduction by bacteriophage, lysogenic conversion and recombination by cell conjugation. In these cases, a *directed* change is produced in the genotype of a susceptible strain through receipt of genetic material derived from a donor strain which possesses the character to be acquired.

Transformation by free DNA.—Free soluble deoxyribonucleic acid derived from a suitable donor strain has been found capable of transforming the nature of the capsular antigen, the degree of drug-sensitivity and other characters in strains of *D. pneumoniae*, *H. influenzae* and other species. The DNA acts as a gene, or collection of genes, entering the recipient bacterium, becoming incorporated in its nuclear apparatus and thereafter reproducing in step with cell multiplication. In the type transformation of *D. pneumoniae*, cells of one antigenic type, *e.g.* type 2, are changed into another antigenic type, *e.g.* type 3 or 6. The capsulate, "S"-form cells of type 2 are first allowed to undergo spontaneous mutation to a non-capsulate "R"-form which lacks type specificity and which does not revert spontaneously to the "S"-form. A small proportion of the "R" cells are then transformed to "S" cells of a chosen type (*e.g.* type 3 or 6) by mixture with the transducing substance derived from donor "S" cells of that type. The latter is supplied as heat-killed "S" cells, cell-free extracts of the "S" culture, or purified DNA obtained from the "S" cells. The few cells transformed to encapsulation are detected by selective culture in broth with anti-R antibody.

Phage-mediated Transduction.—This is a heritable change effected through the agency of a bacterial virus, or bacteriophage (p. 878). The transducing gene is derived from a donor bacterial culture which is lysogenic (*i.e.* carrying phage) or has been infected

with a phage; it leaves the donor bacterium in a liberated phage particle which has accidentally incorporated it, and enters the recipient bacterium when this is infected by the phage. The recipient cell may exchange the transduced gene for its own corresponding gene, eliminating the latter. Single antigenic, nutritional, fermentative or drug-resistance properties may thus be transduced between *Salmonella* strains (e.g. *S. typhi* may acquire the flagellar antigen of *S. typhimurium*). Usually only a very small proportion (e.g. 1 in 10^6) of the phage-treated cells acquire any particular character by transduction.

Lysogenic Conversion.—A temperate bacteriophage may confer certain properties on its bacterial host for as long as it remains present in it. These properties are acquired by other susceptible bacterial strains when they are parasitised by the phage. Thus, an avirulent strain of *C. diphtheriae* (p. 514) may acquire toxicity by infection with the β phage derived from a virulent strain. In contrast to phage-mediated transduction, the character concerned is manifested by every phage-treated bacterium which acquires stable heritable phage infection, i.e. becomes lysogenic.

Recombination by Cell Conjugation.—A form of gene recombination resembling to some extent that of sexual reproduction has been found to occur rarely in certain strains of some bacterial species, e.g. *Esch. coli*. It requires contact and conjugation between living parent cells of suitable mating types, at least one of which possesses a fertility factor, F or Hfr. It can not be induced in one parent by cell-free extracts of the other. Apparently, the donor cell (commonly F+) and the recipient cell (commonly F-) become attached to one another, and part of the gene content or chromosome of the former is transferred into the latter. Chromosomal cross-over may then occur in the diploid or partly diploid zygote and a process of segregation finally yields a haploid daughter cell with a genetic constitution derived partly from each parent. Two characters are transferred by conjugation especially readily, and often when no other genes are transferred; these are the fertility character, F+, and the ability to produce colicines (p. 600).

Non-Heritable Variation.—The influence of environmental conditions may directly modify morphologic, metabolic and other properties without involving a change of genetic constitution. Such modifications are temporary and non-heritable. All the exposed cells of identical genotype and responsive physiological state are affected simultaneously. They maintain their altered character as long as they are exposed to the inducing condition, but on withdrawal of this influence rapidly revert to their original

state. Examples of such *environmentally impressed modifications* include: (1) the suppression of flagellation and motility in *Salmonella* during cultivation on agar containing 0·1 per cent. phenol; (2) the development of pleomorphic involution cells during growth in unfavourable conditions, e.g. in *P. pestis* cultures on medium containing 3 per cent. of sodium chloride (p. 634); and (3) the increased formation of capsular polysaccharide during growth on media with a relative excess of utilisable carbohydrate (e.g. in *Klebsiella*).

Enzymatic inductions (or *adaptations*) are a special form of non-heritable variation, and a common and important mechanism enabling bacteria to exploit fully the different nutrients which they successively encounter. Many enzymes, termed "inducible" or "adaptive", are formed in significant amount only when growth takes place in the presence of their specific substrate. The enzymatic activity is not exhibited by a washed suspension of non-growing bacteria taken from a medium lacking this substrate. When such unadapted cells are grown in the presence of the substrate, they produce the enzyme after a brief interval (e.g. half an hour). Examples of inducible enzymes include those fermenting various carbohydrates in *Esch. coli* and other species, and penicillinase in *B. cereus*.

CHAPTER IV

INFECTION AND RESISTANCE TO INFECTION

INFECTION is the invasion of the tissues of the body by micro-organisms. The animal body is continually exposed to contact with numerous micro-organisms of many different species, including commensals which grow on its skin and mucous membranes, and saprophytes which grow in the soil and elsewhere. The blood and tissue fluids contain nutrients sufficient to sustain profuse growth of many of these species. However, the normal (non-immunised) body possesses a series of defence mechanisms which enable it generally to resist microbial invasion of its tissues and give it a "natural immunity" or "innate resistance" towards most micro-organisms. These mechanisms are non-specific in that each is operative against a variety of microbe species. They include mechanical barriers to the entry and spread of microbes, the antibiotic action of the normal commensal flora, microbicidal substances present in the body fluids and secretions, microbicidal substances liberated from tissue cells damaged by the infective process, and phagocytosis by macrophages (polymorphonuclear leucocytes) and macrophages (*e.g.* fixed reticulo-endothelial cells and wandering monocytes).

Pathogenic microbes are specially adapted and endowed with mechanisms for overcoming the normal body defences in one or more animal species, and can thus invade the germ-free tissues and fluids. Some pathogens invade only the surface epithelium, skin or mucous membrane, but many invade more deeply, spreading through the tissues and/or disseminating by the lymphatic and blood streams. In some cases a pathogenic microbe can infect an entirely healthy individual, while in other cases infection occurs only if the body defence mechanisms are deranged by some locally or systemically debilitating condition, *e.g.* wounding, intoxication, chilling, fatigue, malnutrition, administration of cortisone, adrenalin, etc., and various predisposing diseases and functional disorders. If the defences are sufficiently weakened, then even a saprophytic or commensal microbe, *e.g.* *Cl. welchii* or *Esch. coli*, may be capable of acting as an opportunist pathogen and causing an infection. When an organism succeeds in overcoming the normal body defences, the infection usually progresses until specific immunity mechanisms become operative, for

example that involving the formation of humoral antibodies specific for the invading microbe or its poisonous products (p. 57).

NORMAL NON-SPECIFIC DEFENCE MECHANISMS

(1) The skin and mucous membranes are important mechanical barriers to the entry of microbes into the tissues. The skin is the more resistant barrier because its outer horny layer hinders access of the microbes and their toxins to the living epithelial cells. Microbes are removed mechanically from the mucous membranes of the respiratory, alimentary and urinary tracts; they are trapped in the sticky mucous secretion covering the membrane and this is regularly removed by ciliary action or by peristalsis and evacuation. Infection often follows a disorder of this function, as in the obstructed urinary tract or in a bronchus blocked by excessive mucus. Within the body, the spread of microbes may be limited by dense cellular or fibrous tissue, or by the gelatinous inter-cellular cement substance (hyaluronic acid) or by a meshwork of fibrin deposited in the infected tissues as part of their inflammatory reaction.

(2) The antibiotic activity of the normal commensal flora of the mucous membranes may be important in denying access of potentially pathogenic organisms. Thus, the salivary streptococci produce substances (e.g. hydrogen peroxide) which are lethal to the diphtheria bacillus and the meningococcus. During the child-bearing period, the commensal *Lactobacillus acidophilus* (Döderlein's bacillus) ferments glycogen derived from the vaginal epithelium and produces a highly acid vaginal secretion which is inhibitory to many kinds of bacteria, including streptococci and staphylococci. When the normal commensal flora of the intestine is eliminated by oral administration of a broad-spectrum antibiotic drug, a serious infection may follow due to a drug-resistant *Staph. aureus* or *Candida albicans* previously excluded by the commensals.

(3) Microbicidal substances are normally present in the body secretions, tissue fluids and blood serum. *Long-chain fatty acids*, e.g. oleic acid, which are present in the slightly acid (pH 5–6) secretion of the skin, are lethal to many bacteria within an hour or two of their contact with the skin, e.g. *Strept. pyogenes* and *C. diphtheriae*; bacteria normally resident on the skin, e.g. *Staph. aureus* and *Staph. albus*, are better adapted to withstand the action of these acids. *Lactenin* is a protein present in milk (e.g. human, cow) which is selectively bactericidal for *Strept. pyogenes* and may thus protect against puerperal mastitis and neonatal

- throat infection. The *hydrochloric acid* and low pH (e.g. 1-2) of the gastric juice is lethal to a majority of ingested microbes and serves as an important protection for the intestinal canal.

Lysozyme is a small-molecular basic protein which causes lysis (disintegration) of susceptible bacteria by enzymatically decomposing a structural mucopolysaccharide component of their cell walls. It occurs in most mammalian tissues and fluids, e.g. conjunctival fluid and the mucous secretions of the nose, mouth, throat, bronchi, intestine and urinary tract, but does not occur in blood serum except when liberated from damaged polymorph leucocytes which are rich in it; a very high concentration is present in hen egg white, the source of a commercial preparation. Lysozyme acts most strongly on certain saprophytic Gram-positive bacteria, e.g. *Micrococcus lysodeikticus* and *B. megatherium*, though in certain conditions it may affect pathogens such as staphylococci and anthrax bacilli, and it accelerates the lysis of Gram-negative pathogenic bacteria under the action of antibody and complement (p. 68). When their cell walls are dissolved by lysozyme in a special osmotically protective medium, bacteria may be converted into free, spherical protoplasts which for a time remain metabolically active. The mucous secretions probably contain several other microbicidal substances besides lysozyme.

Normal ("non-immune") blood serum contains a variety of microbicidal substances, including the alpha and beta lysins. *Alpha lysin* (the alexin or complement system) is destructive to a wide range of Gram-negative bacteria and certain viruses. The lytic effect is dependent on the combined action of an antibody (p. 68), the normal serum constituent "complement" (p. 68) and magnesium ions. The main antibody is a relatively non-specific *normal antibody*, a euglobulin called *properdin*, which is thermolabile¹ at 57°-60° C. in half an hour; this antibody has a combining affinity for the cell walls of various Gram-negative and Gram-positive bacteria, certain viruses, red cells, yeast cell-wall carbohydrates (zymosan, glucan) and other biological materials. Lytic effects may also be due to the presence of trace amounts of specific immune antibodies (thermolabile at 65°-70° in half an hour) present in the supposedly non-immune animal as a result of heterogenetic stimuli (p. 61). *Beta lysin* is one of a group of substances, possibly proteins, which occur in the serum of man, rat and other animals, are thermostable² at 56° for half

¹ "Thermolabile" indicates that a particular property is lost on exposure to a certain degree of heat for a stated time.

² "Thermostable" denotes the ability to withstand a certain degree of heat for a stated time.

an hour, and are bactericidal for a variety of Gram-positive bacteria including the anthrax bacillus and some staphylococci.

(4) Microbicidal substances are liberated from tissue and blood cells damaged in the course of infection and inflammation. Those liberated from tissue cells include *lysozyme*, *long-chain fatty acids* (active on streptococci, tubercle bacilli, certain viruses, etc.), the polyamines *spermine* and *spermidine* (active on tubercle bacilli), *protamines*, *histones* and *linear basic polypeptides* (e.g. a lysine-rich peptide active on certain bacteria and viruses, and an arginine-rich peptide active on tubercle bacilli). Neutrophil polymorph leucocytes may liberate lysozyme, leukins and phagocytins. *Leukins* are thermostable substances (at 60°–100°), probably basic protamines or histones, which are active mainly against Gram-positive bacteria. *Phagocytins* are thermostable (65°) euglobulins which are bactericidal for various Gram-negative bacteria. *Plakins*, which are extractable from blood platelets, have similar properties to leukins. While the basic polypeptides, leukins and plakins have little direct action on Gram-negative bacteria, they may accelerate bacteriolysis by antibody and complement. *Haematin* and *mesohaematin* derived from red blood cells are inhibitory for various Gram-positive saprophytes and pathogens.

The metabolism of the leucocytes and other cells in inflamed tissue can ultimately cause a local accumulation of lactic acid and carbon dioxide, and a depletion of oxygen, conditions which are unfavourable for the growth of bacteria such as the tubercle bacillus and *Staph. aureus*.

(5) *Phagocytosis*.—The phagocytes are body cells which are specialised for the pursuit, capture, ingestion and intracellular destruction of invading microbes (phagocytosis). They include the polymorphonuclear and large mononuclear leucocytes of the blood and the fixed macrophages of the reticulo-endothelial system (e.g. sinusoidal cells of spleen, liver; bone marrow and lymph nodes, adventitial cells of blood vessels and histiocytes of connective tissue). Polymorph leucocytes and monocytes migrate from the blood vessels in inflamed tissues and accumulate to attack the bacteria causing a local infection, while the fixed macrophages of the reticulo-endothelial system are mainly responsible for eliminating bacteria from the blood and lymph. Phagocytes can most readily entrap bacteria when they are supported on a cellular or fibrous surface ("surface phagocytosis"); the deposition of a fibrin meshwork in inflamed tissue assists phagocytosis by providing supporting surfaces. When the phagocytes are freely suspended in fluid, e.g. oedema fluid, the opsonic activity of antibody and complement is generally required

to make phagocytosis possible (p. 68). Susceptible bacteria die and disintegrate within an hour or two after their ingestion, partly due to the secretion of acid (pH 3–5) in the vacuole surrounding them and possibly also due to the action of lysozyme and other digestive enzymes. On the other hand, many pathogenic bacteria are able to survive and multiply inside the phagocyte, thus causing its death and lysis, and eventually escape from it (e.g. tubercle bacillus, brucellae and salmonellae).

THE INVASIVE MECHANISMS OF PATHOGENIC BACTERIA

Bacteria which infect the animal body must have means of overcoming their host's defence mechanisms, of protecting themselves against the bactericidal substances in the body fluids and of avoiding ingestion or intracellular destruction by phagocytes. Such protection is generally afforded by the special nature of their outermost covering, i.e. capsule, microcapsule or somatic antigens. If this covering is lost by gene mutation or enzymatic digestion, or becomes coated with antibody and complement, the bacterium is rendered non-virulent. Thus, virulent pneumococci isolated from infected animals possess polysaccharide capsules which render them relatively resistant to capture and ingestion by phagocytes; non-capsulate mutant strains derived by artificial culture in the laboratory are susceptible to phagocytosis and thus are non-virulent. Some pathogens, e.g. *Strept. pyogenes* and *Staph. aureus*, secrete *leucocidins*, i.e. phagocyte-destroying toxins, and some, e.g. *S. typhi*, produce substances which inhibit the migration of leucocytes. The factors which enable certain pathogens such as the tubercle bacillus to survive and grow within phagocytes have not yet been defined.

While some pathogens normally enter the body through a wound, others may require mechanisms for breaching the epithelial barriers and for assisting spread through the tissues. Thus, some species produce toxins which destroy the epithelial cells (e.g. *C. diphtheriae*); some produce a "spreading factor" such as hyaluronidase which dissolves the hyaluronic-acid cement substance of tissues (e.g. *Strept. pyogenes*), and some produce a fibrinolysin which induces solution of the fibrin clots deposited as barriers to the spread of infection (e.g. *Strept. pyogenes*). Many pathogenic bacteria, especially when growing in the tissues, liberate diffusible substances (termed "aggressins") which, though non-toxic by themselves, increase the aggressiveness of the

bacteria. In some cases they may act by interfering with phagocytosis, opsonisation (p. 70) or bacteriolysis by normal or immune serum (p. 68). Various non-pathogenic bacteria, however, may possess similar properties, and the existence of a specialised substance of this type is doubtful.

BACTERIAL TOXINS

Toxins are defined as the products of bacteria which are injurious to the tissues and in virtue of which disease processes result from bacterial infection. They are classified broadly as:

- (1) Extracellular toxins, or *exotoxins*, which diffuse readily from the living bacteria into the surrounding medium.
- (2) Intracellular toxins, or *endotoxins*, which are retained within the bacterial cells until they die and disintegrate.

The majority of the pathogens produce endotoxins only, and these are significantly potent in the Gram-negative bacteria. Some, mainly Gram-positive species, develop powerful exotoxins, e.g. the tetanus bacillus, the diphtheria bacillus and certain staphylococci and streptococci. Particular pathogenic bacteria may fail to produce toxins in ordinary culture media, but do so when growing in the animal tissues, e.g. the anthrax bacillus.

Preparations of *exotoxins* may be obtained by growing the bacteria in a liquid medium and then removing them by centrifugation or by filtration through a bacteria-stopping filter (p. 170); the toxin remains in the supernatant liquid or filtrate. The exotoxins that have so far been isolated in a purified state, e.g. tetanus and diphtheria toxins, have been identified as simple proteins. Exotoxins are generally unstable substances, their toxic effect being readily annulled by chemicals (e.g. by formaldehyde, as in conversion to "toxoid", p. 517), by free oxygen (e.g. oxygen-labile haemolysins, pp. 481, 677) and by heat (e.g. diphtheria toxin at 65° C. and *Clostridium welchii* alpha toxin at 100° C.). A few are more resistant to heat, e.g. botulinus toxin and staphylococcal enterotoxin. Some are exceedingly potent, the tetanus and botulinus toxins being the most powerful poisons known and having a minimum lethal dose for man in the order of 0.0001 mg. Some are toxic to tissue cells generally and rapid in action, while others have a specialised action on particular tissues (e.g. tetanus and botulinus toxins on certain kinds of nerve cells) and produce symptoms of poisoning only after several hours or days following their introduction into the body. They may be designated according to certain of their effects, e.g. *haemolysin* (causing lysis of red blood cells), *leucocidin* (destructive to leucocytes), *necrotoxin*

(causing necrosis of tissue). By immunising animals with a preparation of exotoxin, a specific neutralising antibody (antitoxin) is developed which can be demonstrated in the blood serum of the immune animals (p. 520). The mechanisms of action of the exotoxins is with few exceptions unknown. The alpha toxin of *Cl. welchii* has been defined as a lecithinase, or lecithin-splitting enzyme, which apparently acts on the lecithin components in cell membranes, e.g. of red blood cells (p. 687). The toxin of the diphtheria bacillus appears to be the isolated protein moiety of the bacterial cytochrome-*b* enzyme and may act by interfering with synthesis of cytochrome *b* by the host tissues.

Endotoxins are structural components of the bacterial cell and are liberated only on death and disintegration of the cells. In the infected animal this occurs through phagocytosis and bacteriolysis by antimicrobial substances in the body fluids. Endotoxins are not found in bacteria-free supernatants of cultures, unless these have undergone autolysis through ageing. In several species (e.g. of *Salmonella*, *Shigella*, *Brucella* and *Neisseria*), the endotoxin has been extracted with solvents (e.g. diethylene glycol) and identified as a polymolecular lipid-phospholipid-polysaccharide-protein complex; this may comprise 5–10 per cent. of the dry weight of the cells and corresponds to the O somatic antigen of the virulent "smooth-type" organisms (p. 67). The toxic action may be demonstrated by injecting animals with such a culture extract or with killed whole cells. The endotoxins of different species all produce more or less similar symptoms, particularly pyrexia, diarrhoea, prostration and visceral haemorrhages. Immunisation with endotoxins leads to the formation of antibodies which exert important antibacterial effects (e.g. bacteriolysis, p. 68), but are incapable of neutralising completely the toxic action. Endotoxins are more stable than exotoxins and can withstand a temperature of 100° C.

In the course of an infective disease the tissues of the body at large may become hypersensitive to specific products of the infecting organism. Such *hypersensitivity*, or *allergy*, may contribute to the toxic manifestations of the disease, since microbial products which are normally non-toxic may thus become highly toxic and irritant to the tissues (p. 72).

VIRULENCE OF MICRO-ORGANISMS

Virulence is an important property of micro-organisms in relation to their pathogenic action, and is defined as the capacity to invade the tissues, multiply and produce toxic effects. It is

estimated by the *minimum lethal dose* (M.L.D.), *i.e.* the smallest dose of the organism (number or weight of cells from a culture) which will kill a particular species of animal. As a result of the varying susceptibility of individual animals to bacteria and their toxins, it is often impossible to state the exact minimum dose, and it is customary to refer to the *average lethal dose* for a number of individual animals (*i.e.* the LD 50, or dose giving a 50 per cent. mortality). It must be noted that virulence depends on two factors which may be largely independent of one another: the invasive power or aggressiveness, and the toxigenic or toxin-producing property of the organism. Thus, the tetanus bacillus is highly toxigenic but only weakly aggressive; in contrast, the pneumococcus is markedly aggressive and its toxicity is minimal. The virulence of bacterial strains cannot be fully explained in terms of known toxins and invasive mechanisms; strains which possess the same known components may still vary greatly in virulence.

The virulence of an organism can be either "exalted" or "attenuated" artificially. *Exaltation* of virulence may be produced by passing the strain through a series of individual animals of the same species, inoculating the animals one from another in succession, *i.e.* "passage". In this way a mutant strain is selected which has increased virulence for that particular species. *Attenuation* usually results when organisms are cultivated artificially for some time; thus, stock laboratory cultures are usually of low virulence, as compared with recently isolated strains. Similarly, passage through animals of a different species may select mutants having reduced virulence for the original host species (p. 845).

In many species a loss of virulence is correlated with a change of colony texture from smooth to rough and a corresponding loss of the O somatic antigens. This is the so-called S→R mutation.

SOURCES OF INFECTION FOR MAN

The epidemiology of an infective disease depends to an important extent on the nature and distribution of the sources of infection. For human diseases, the sources may be as follows:

(1) PATIENTS.—Infections due to some microbial species are acquired mainly or exclusively from ill humans with active or manifest infection, *e.g.* pulmonary tuberculosis (human-type bacilli), leprosy, whooping-cough, syphilis, gonorrhoea, measles, smallpox, mumps and influenza. Some cases of the infection may be mild or atypical, and thus not recognised as a source of danger.

(2) HEALTHY CARRIERS.—Many pathogenic species are able to

produce in certain individuals a limited or subclinical infection which is insufficient to cause the signs and symptoms of illness. Persons having such an inapparent infection are commonly capable of disseminating the causative microbes to other persons; they are then termed "carriers" and constitute an unsuspected and thus especially dangerous source of infection. Some infectious diseases are contracted from carriers as frequently or much more frequently than from patients, e.g. streptococcal, staphylococcal, pneumococcal and meningococcal infections, diphtheria, typhoid fever, bacillary dysentery and poliomyelitis. *Convalescent carriers* are persons in whom a limited, localised infection continues for a period of weeks, months or occasionally years following clinical recovery from a manifest infection. Other carriers suffer no more than subclinical infection from the time of first acquiring the pathogen; those who acquire it from a patient are termed *contact carriers* and those who acquire it from another carrier, *paradoxical carriers*. If carriage persists for more than an arbitrary period of time, e.g. one year in the case of typhoid infection, the person is called a *chronic carrier*.

(3) INFECTED ANIMALS.—Some pathogens which are primarily parasites of a different animal species may in appropriate circumstances spread from the infected animal to man and so cause human disease. Infections normally acquired from animals include bovine-type tuberculosis, salmonella food poisoning, bubonic plague, anthrax, brucellosis, leptospiral jaundice, rabies and psittacosis. In most such infections there is rarely any secondary spread from the patient to other humans; an exception is the man-to-man spread of pneumonic plague which may supervene on bubonic plague contracted by men from rodents.

(4) SOIL.—A few infective diseases of man are caused by saprophytic microbes derived from the soil, vegetation and similar habitats, e.g. tetanus, gas gangrene, maduromycosis and sporotrichosis.

In the above examples, the source of infection is described as *exogenous*, i.e. outwith the body of the person becoming infected; *endogenous* infection may occur in carriers of potentially pathogenic organisms when these previously harmless bacteria invade other surfaces or tissues in the carrier, e.g. *Esch. coli* derived from the bowel causing infection in the urinary tract.

MODES OF SPREAD OF INFECTION

Some infections, e.g. respiratory and intestinal, can spread from host to host by a variety of mechanisms, while others

e.g. venereal and arthropod-borne blood infections, are normally transmitted only by a single mechanism for which the parasite is specially adapted.

(1) **Respiratory Infections.**—The causative microbes are mainly disseminated into the environment in masses of infected secretion, *e.g.* secretion removed from the nose or mouth on fingers, handkerchief, cups, spoons, etc., or expelled in spitting or blowing the nose; they are also discharged to a lesser extent in the droplet-spray produced by sneezing, coughing and speaking, but hardly at all in normal breathing.

Hands, handkerchiefs, clothing, bedding, floors, furniture and household articles (fomites) become contaminated with the secretion and may act as vehicles or reservoirs of infection. The secretion dries and becomes pulverised into dust. When dried in dust, most kinds of respiratory microbes can remain alive for several days, and some even for several months if shielded from direct daylight, *e.g.* tubercle and diphtheria bacilli, streptococci, staphylococci and smallpox virus. The infection may then be passed to the recipient by *contact*, either (a) direct contact, *i.e.* touching of bodies as in handshaking, kissing and contact of clothing, or (b) indirect, or mediate contact, involving an inanimate vehicle of infection, *e.g.* eating utensils, door handles, towels, etc.; the recipient may finally transfer the microbes with his fingers into his nose or mouth.

Infection may be *dust-borne* and occur through inhalation of air-borne infected dust particles. Very large numbers of such particles are liberated into the air from the skin and clothing during normal body movements, from the dried parts of an infected handkerchief during its use, from bedclothes in bed-making, from the floor when walked on or brushed, and from furniture during dusting. The larger infected particles settle within a few minutes on the floor and other exposed surfaces, *e.g.* skin, clothing, wounds, surgical and medical supplies, but the smaller infected particles remain air-borne for up to one or two hours and can be inhaled into the recipient's nose, throat, bronchi or lung alveoli. Air-borne infection is mainly a danger within the room of its origin; spread to other rooms in the same building is usually slight, though it can occur if the ventilating or convectional air currents move in the appropriate direction.

Droplet-spray constitutes a third means of spread of respiratory infection. It is probably the least important, except perhaps in the case of the pathogens which are rapidly killed by drying, *e.g.* the meningococcus, whooping cough bacillus and common

cold virus. Sneezing, coughing, speaking and other forceful expiratory activities expel a spray of droplets derived almost exclusively from the saliva of the anterior mouth; this may be infected with small numbers of pathogenic microbes from the nose, throat or lungs. Very many droplets are expelled, but only a few are infected. The *large droplets* (over 0·1 mm. diam.) fly forwards and downwards from the mouth to the distance of a few feet; they reach the floor within a few seconds, or bespatter the surfaces of intervening persons and objects, but cannot be inhaled directly. The *small droplets* (under 0·1 mm.) evaporate immediately to become minute solid residues, or "*droplet-nuclei*" (mainly 1–10 μ diam.), which remain air-borne like infected dust particles and may be inhaled into the nose, throat or lungs. It is thought that very few of the droplet-nuclei are likely to be infected with pathogenic microbes, but it is possible that certain virus infections are commonly spread by droplet-nuclei, e.g. measles, chickenpox.

(2) **Skin, Wound and Burn Infections.**—These superficial infections may be acquired through contact with infected hands, clothing or other articles, through exposure to sedimentation of infected air-borne dust and through contamination by droplet-spray. Pathogenic streptococci and staphylococci derived from the respiratory tract are important causes of wound and burn infections.

(3) **Venereal Infections.**—The venereal diseases are so-called because they are transmitted almost exclusively by sexual contact. An important reason for this limitation is that the causative organisms, e.g. *Trep. pallidum* and *N. gonorrhoeae*, are extremely susceptible to the lethal effects of drying and the other conditions encountered on potential vehicles whereby infection might be transmitted less directly.

(4) **Alimentary Tract Infections.**—Here, pathogenic microbes are discharged in the faeces of infected persons and are transmitted in various ways leading to their ingestion by the recipient. Most intestinal pathogens are poorly resistant to drying; they tend to die within a period of hours, though rarely may survive on cloth or in dust for several days. They are more likely to be spread by moist vehicles such as water or moist foods, in which they may survive for up to several weeks (e.g. typhoid, dysentery and cholera organisms.) (a) *Water-borne infection* occurs through excreta contaminating a supply of water, e.g. a river or well, which is used without purification for drinking or culinary purposes. Purification of water is effected on a large scale by storage, filtration and chlorination. Small amounts may

be treated by boiling or by addition of hypochlorite tablets.

(b) *Hand Infection*.—A carrier tends to contaminate his hands with minute traces of faeces and with bacteria that pass through toilet paper; nurses may infect their hands in attending patients and touching bed-pans. Such persons may handle and contaminate foodstuffs, eating utensils and other vehicles of infection. A recipient may pick up the microbes on his fingers and thus transfer them into his mouth. (c) *Food-borne infection* may occur through a carrier handling the food, through preparation of the food in utensils infected by handling, or washing in infected water, and through flies alighting on the food after feeding on exposed infected faeces. Conditions enabling growth of the bacteria and/or production of enterotoxins in the contaminated food, are prerequisites for "bacterial food-poisoning".

(5) **Arthropod-borne Blood Infections**.—In several systemic infections the causative microbes are abundantly present in the blood and are spread to other individuals by blood-sucking arthropods such as the mosquito (malaria, yellow fever), flea (plague), louse (epidemic typhus fever, European relapsing fever), tick (Rocky Mountain spotted fever, West African relapsing fever), mite (scrub typhus) and tsetse fly (trypanosomiasis). The parasite is adapted to spread by its particular arthropod vector and is rarely transmitted by other means.

(6) **Laboratory Infection**.—Laboratory workers occasionally become infected from artificial cultures and from infected diagnostic and necropsy materials from patients and experimental animals. Some organisms are especially liable to cause laboratory infections, e.g. the brucellae, rickettsiae and *Past. tularensis*, while many others require special care, e.g. tubercle bacillus, anthrax bacillus, freshly isolated typhoid and dysentery bacilli, pathogenic leptospirae and borreliae, and the psittacosis virus. Especial danger attaches to the pipetting of infected liquids by mouth, leading to their accidental ingestion; a rubber teat or a mouth-piece with two cotton-wool filters should always be used for pipetting cultures and exudates. Accidental self-inoculation with a syringe sometimes occurs, and spraying of the conjunctiva when the needle becomes loosened from the syringe during an injection. Many laboratory procedures atomise liquids and so can contaminate the air with infected droplet-nuclei, e.g. the expulsion of liquid from a pipette or syringe, the shaking of liquid in an open vessel, the centrifugation of tubes bearing traces of liquid on their rim, and the breaking of liquid films as when a wetted stopper or a screw-cap is removed from a bottle or a drop is separated from

an inoculating loop. When working with dangerous pathogens such as the tubercle bacillus, it is recommended that all such procedures be carried out within a specially ventilated "protective cabinet" or "inoculation hood".¹

¹ A description of a suitable cabinet is given by Williams, R. E. O., & Lidwell, O.M. (1957), *J. clin. Path.*, **10**, 400.

CHAPTER V

ACQUIRED IMMUNITY

IN contrast to natural immunity or non-specific resistance discussed in Chapter IV, acquired immunity is specific in the sense that it protects against one particular pathogen or its toxic products. Acquired immunity may depend on the presence and amount of specific globulin molecules or *antibody* in the blood elicited in response to the stimulus of a foreign protein or other large molecular substance which is called the *antigen*; the antibody to diphtheria toxin is a good example and this is known as *humoral* immunity. Or it may result from an enhanced capacity of particular cells or tissues to counteract infection, and this is called *cellular* immunity. Thus in typhoid fever and brucellosis the fever may go on for weeks or months despite a high content of humoral antibodies, and it is presumably the cellular response which eventually overcomes the clinical infection. This latter form of immunity is often associated with the state of *microbial hypersensitivity* (p. 74) as in tuberculosis. Immunity may be acquired *naturally* or *artificially*; and both forms of immunity may be acquired either *actively*, i.e. the antibodies are produced by the host's tissues, or *passively*, i.e. the antibodies are supplied to the host ready made. Thus, an attack of measles gives immunity to further attacks and this is naturally acquired active immunity. Most infants are immune to measles for the first 4–6 months of life, due to the placental transfer of antibodies from mother to foetus; this is an example of a naturally acquired passive immunity. Troops on active service are given two or more injections of tetanus toxoid (see p. 683) to protect them against the risk of tetanus; this is artificially acquired active immunity. Civilians are given a dose of tetanus antitoxin following injury, and this is an example of artificially acquired passive immunity. Passive immunity is rapid in onset and immediate if the antiserum is given intravenously, and requiring only a few hours to reach adequate levels if given intramuscularly or subcutaneously. It has a short duration of a few weeks since, being a foreign protein, it is eliminated by the body. Active immunity takes time to develop but persists for months or years and, once acquired, is usually capable of restoration when it has dropped to a low level.

ANTIGENS AND ANTIBODIES

The appearance of specific antibody in the blood about the time of clinical recovery as in lobar pneumonia, the correlation between demonstrable antibody and clinical protection as in diphtheria, and the experimental demonstration that antibodies can specifically neutralise the infecting pathogen as in smallpox, are all findings that support the importance of humoral immunity. It should be made clear, however, that certain specific antibodies which appear after a clinical infection may have no protective value. Most foreign proteins of sufficient molecular weight (10,000 or over) and certain other large molecular substances, e.g. complex polysaccharides, act as antigens and elicit a specific antibody response when injected parenterally into man or other animals. The whole bacterial cell, which is commonly used in vaccines, usually contains a variety of antigens of which only one or two may be important in protection against infection. For example, the plague bacillus is now known to contain some 20 different antigens, of which only 3 seem to be concerned with infection and immunity, while the antibody to the flagellar antigen of the typhoid bacillus probably plays no part in protection. Substances which cannot stimulate the production of antibodies but which react specifically *in vivo* or *in vitro* with the antibody once it has been formed are called partial antigens or *haptens*. In most cases the union *in vitro* between antigen and antibody can be demonstrated by some physicochemical reaction (p. 63 *et seq.*), but in a few instances it can only be shown indirectly as an interference phenomenon. Some substances such as egg albumin and bacterial toxins are good antigens in the sense of provoking the production of antibody; others like haemoglobin and the nucleo-proteins are poor antigens. Animal species vary in their antibody responses. The horse, rabbit and hen are good producers of demonstrable antibodies; the guinea-pig and rat are not. The guinea-pig, on the other hand, is readily made hypersensitive to foreign protein and is used to demonstrate the characteristic features of anaphylaxis.

Active Immunity.—Granted the variations in antigenic potency and animal responses, the amount and spacing of antigen injected determines within limits the extent of the antibody response. The time of appearance and amount of demonstrable antibody is best measured by following the response to injections of bacterial *toxoids*, which are toxins detoxicated by treatment with weak formalin without losing their antigenic potency. The first injection of toxoid (or primary stimulus) elicits only a small

amount of antitoxin after a latent period of about two weeks. A second injection (secondary stimulus) some weeks later elicits a much greater increase in antibody which reaches its peak about 10 days after the injection and gradually falls over a period of months or years. Thus, two or sometimes three injections of the antigen constitute the primary course, which if properly performed with good antigens confers substantial immunity. When over a period of time immunity wanes as illustrated by a low level or absence of demonstrable antitoxin, a single *booster* or reinforcing dose will stimulate a rapid outpouring of antibody which reaches a high level and persists, as a rule, for several years (see Fig. 1).

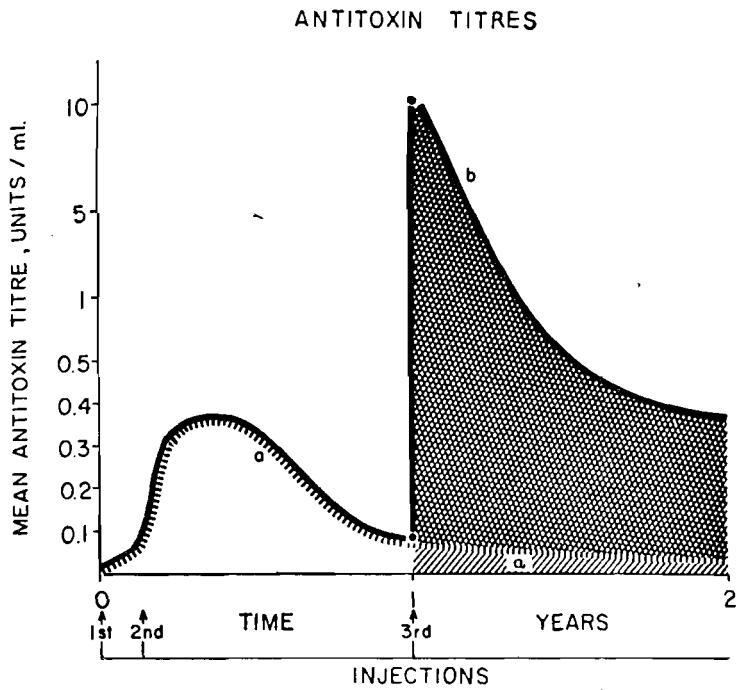


FIG. 1

Antitoxin titres in the serum of subjects receiving either (a) two doses of toxoid or (b) two doses plus a third dose about one year after first injection.

In bacterial and viral diseases where the infection is not due predominantly to powerful exotoxins, killed suspensions of the micro-organism or living attenuated cultures may be used to produce artificial active immunisation and these prophylactic

agents are called *vaccines*. In the case of killed vaccines, suspensions of the organism in its virulent phase are inactivated by heat at a temperature around 55°–60° C. or by antiseptics such as formalin, phenol, alcohol, etc., care being taken not to destroy or denature any possibly important antigens by overheating or by too strong concentrations of the antiseptic. Two or more injections of the inactivated vaccine are given, preferably at 4-week or longer intervals; examples are the vaccines against typhoid fever, whooping-cough and poliomyelitis. Sometimes, a purified bacterial fraction, e.g. pneumococcus polysaccharide derived from the capsule, can be used as the immunising agent. Living attenuated bacterial and viral vaccines have been used principally when killed vaccines have proved ineffective. A modified form of the smallpox virus has been used for many years to produce a localised infection, vaccinia, which immunises against virulent smallpox. A living non-virulent culture of the tubercle bacillus, B.C.G. vaccine, is used to increase protection against tuberculosis.

When an enhanced response to an antigen is desired in the process of active immunisation, the antigen may be mixed with an *adjuvant*. Thus, bacterial toxoids may be precipitated by or adsorbed on to alum (aluminium hydroxide or phosphate) and this mixture is a much more potent antigen than toxoid alone. Some bacterial suspensions, e.g. typhoid vaccine and some oil emulsions also act as adjuvants when mixed with toxoids or viruses. Alum adjuvants help to create a depot from which the antigen is released over a prolonged period of time and in this way produce a more persistent stimulus for the production of antibody.

Passive Immunity.—In passive immunity, the host plays no part in the production of antibodies. They may be ready made and transferred from mother to foetus via the placenta as in measles and diphtheria; or in some animal species (cow, sheep) they may be transferred from mother to offspring via the colostrum. Gamma globulin which contains most of the natural antibody in human serum may be separated from the other constituents and injected as a prophylactic measure in children exposed to measles, infective hepatitis, etc. Antibodies to certain bacteria or their toxins may also be produced artificially in animals such as the horse. Repeated injections are given until a high level or *titre* of antibody is reached when the animal is bled and the antibody is concentrated by getting rid of the albumin and non-antibody containing globulin. These concentrated antisera may be used both prophylactically and therapeutically, as in tetanus and diphtheria.

The practical aspects of artificial immunisation are discussed under individual infections and in Chapter XLVI.

Specificity is one of the pronounced characters of acquired immunity and of antibodies, and though it may be a true species-specificity it is often restricted to "types" within the species, such as the serological types of the pneumococcus. Thus, after an attack of lobar pneumonia due to pneumococcus type 1, antibodies specific to that type develop in the patient's blood and give him resistance against further attacks by type 1 pneumococci but not against other pneumococcal types. This type-specific immunity is also seen in streptococcal infections, in influenza, and presumably in other infectious diseases where there are different antigenic types within the species.

The specificity of antibody depends on the specificity of the corresponding antigen, e.g. in the case of the pneumococcus, the chemical composition of the capsular substance determines the type specificity. An organism usually contains more than one antigenic constituent, and for each of these a separate antibody is produced. When related bacterial species have certain antigenic constituents in common, as may be the case, an antiserum for one of these species exhibits to a greater or less degree *group action* towards the others.

In some instances, the occurrence of antibodies may have no aetiological significance. In typhus fever a serum-antibody is demonstrable which is specific for a particular type of *Proteus*, though this organism has no aetiological relationship to the disease (p. 735). Antibodies like that present in typhus fever, which appear to be specific for an antigen having no biological relationship to that constituting the immunising stimulus, are designated "heterophile". As originally shown by Forssman, guinea-pig tissues, and also those of certain other species, e.g. horse, when injected into a rabbit bring about the production of a heterophile antibody which is haemolytic for sheep red corpuscles. This antibody is generally designated "Forssman antibody".

THE NATURE AND PRODUCTION OF ANTIBODIES

Antibodies are protein macromolecules or polypeptides, shaped like a cigar, with molecular weights around 160,000. Because of their size and solubilities they belong to the globulins, and their other properties place them in the *gamma globulin* fraction. Indeed, gamma globulin may consist almost entirely of antibodies, of which there may be many million molecules in every ml. of blood. They may be classified as functional proteins like enzymes

and hormones, in contrast with structural or storage proteins like keratin and collagen and egg-white. These functional proteins must be synthesised by some production mechanism, and must act by adsorption on to their targets or substrates. Enzymes are good examples of functional proteins, and the production and mode of action of antibodies may have analogies with adaptive enzymes. In bacteria, for example, these enzymes appear or are stimulated into activity by a particular substrate which is then specifically attacked.

The current view on the production of polypeptides is that they are built up from long chains of amino acids, linked together by enzyme action, and held in a monomolecular layer against a film of ribonucleic acid (RNA) which is specially concerned with protein synthesis. There is presumably a template of RNA plus a protein organiser on which the new protein is built up, rather like the formation of crystals. Only when the polypeptide chain is shed off from the template does it become folded up to assume the characteristic form of globulin or albumen particles. Globulin macromolecules have a half-life of about twenty days, so fresh globulin must be produced continuously.

The production of specific antibodies may depend on the existence of a reaction in which enzymes bring about the exchange of amino acids in the middle of the peptide chain. Thus, an antigen, by contact with a developing polypeptide, could induce a particular configuration to give a pattern that would fit the inducing substrate or antigen. Once the stimulus to antibody production has been given, there is a logarithmic increase in the amount of antibody in the blood, which must mean that the capacity to manufacture antibodies is a function, not only of the cells originally stimulated, but also of their progeny. The production of antibody, say against the yellow fever virus, may go on for years after the original stimulus. This function could be accomplished by the production and genetic transfer of induced enzymes in a manner analogous to the phenomenon that occurs in bacteria.

An alternative explanation for antibody production, which has been elaborated by Haurowitz and others, is that the determinant group in the antigen persists in the tissues of the host for a long time after the introduction of the antigen, and repeatedly makes its imprint on the polypeptide chain so that antibody is produced in the process of folding up of the macromolecule. It is true that some antigens may persist in tissues for many months after inoculation, but most of them seem to disappear fairly quickly, so, unless they remain as undetectable determinant fractions, this hypothesis would not fit the facts.

The most recent theory on antibody production is based on *clonal selection*, where the unit is not a single cell but the clone—a family of cells which have multiplied asexually and so have identical genetic constitution (Burnet).¹ Clonal selection, which is accepted for bacterial cells, is now applied by Burnet to mammalian cells to explain the facts of acquired immunity and other immunological phenomena (such as immune tolerance, delayed hypersensitivity and the somatic mutation theory of cancer). As regards antibody production, the basic contention is that the capacity to produce specific globulins is a genetic attribute of the cell and that the effect of an antigen is simply to stimulate proliferation of the particular cells concerned with the production of the specific antibody (one of many thousands) which fits that particular antigen.

Another unexplained phenomenon is that the foreign antigen is taken up by the reticulo-endothelial cells, but the antibodies are manufactured mainly by the primitive plasma cells of the lymphoid system. This means that the stimulus to produce the antibodies must be transferred from one species of cell to another, unless the phagocytic cells can change to antibody-producing cells. It may be noted that although many different antibodies can be produced at the same time, any one cell usually produces only one specific kind of antibody.

ANTIGEN-ANTIBODY REACTIONS

There is a variety of methods by which we can demonstrate the presence of a specific antibody to a particular antigen, whether that be toxin, bacterium or some extract of it or virus. Most of these tests are demonstrated *in vitro*, i.e. in test-tubes or other containers; but some are demonstrable only *in vivo*, i.e. in living animals or in tissue cultures. The tests most commonly used are *neutralisation* (of toxin or living microbe), *precipitation* or *flocculation*, *agglutination* and *complement fixation*; antibodies may also specifically assist in bacteriolysis (lysin) and in phagocytosis (opsonin). The antigen is called a precipitinogen, agglutininogen, etc., and the antibody, precipitin, agglutinin, etc. Since the reactions involve the use of serum, we speak of "serology" and serological tests or reactions; similarly, bacterial strains with different surface antigens within a species are identified as serological types. Antigen-antibody tests can be used quantitatively to measure the amount of antibody present in a given volume of serum by making a series of increasing dilutions of the serum or

¹ Burnet, F. M. (1959), *The Clonal Selection Theory of Acquired Immunity*. Cambridge, University Press.

antigen or in other ways. The use of different methods for demonstrating antibody does not necessarily mean that several different antibodies are being identified; in most instances it is one antibody that is being demonstrated in different ways.

Protection Tests

If a microbe or its toxin is highly lethal for an experimental animal like the mouse or guinea-pig or, in the case of viruses, for living tissues (chick embryo or tissue culture), the average lethal dose or LD 50 (*i.e.* the dose which kills 50 per cent. of the susceptible animals or tissues) is first assayed. Some multiple of this dose, say 10 LD 50, is then mixed with different dilutions of the antibody-containing serum and after standing for 30–60 minutes, the mixtures are injected into groups of animals or eggs or tissue-cultures. Where animals are used, they should be homogeneous in regard to breed, weight, sex, etc. If a microbe or, more often, a toxin produces characteristic and measurable lesions in the skin of a susceptible animal, a series of intradermal tests with different dilutions of serum can be carried out on one animal. Diphtheria antitoxin can be measured in this way.

In *passive protection* tests the serum in different doses is first injected into the susceptible animal, and after an interval of some hours the animals are "challenged" with a fixed dose, say 10 LD 50, of the microbe or toxin. *Active protection* tests are designed to measure the immunising or protective potency of a particular antigen (vaccine or toxoid). The antigen is injected in several dilutions (or sometimes in two or more spaced doses) into groups of animals and, after an interval of 1–3 weeks, the animals are challenged with a fully virulent strain of the organism or of the toxin under test. When assays of a prophylactic agent are first carried out on experimental animals, it is usually essential to compare the laboratory findings with the protective efficiency of the prophylactic in controlled trials on human volunteers. For example, a mouse protection test of whooping-cough vaccine was found to show good correlation with its protection of young children in field trials and has therefore been adopted as the method of assay for standardising the potency of whooping-cough vaccines.

Precipitation and Flocculation Tests

When an antigen in solution, *e.g.* a toxin or bacterial extract, is layered on top of serum in a test-tube, a ring of white precipitate will form if the serum contains the specific antibody or precipitin. A quantitative assay can be made by adding increasing dilutions of

the antigen to tubes containing a constant amount of antibody until no precipitate forms. If a series of precipitin tests, using a fixed concentration of antibody and varying dilutions of the antigen, are set up in test-tubes, precipitation (sometimes called flocculation) will occur first and in greatest amount in one tube of the series. This is the optimal proportions test of Dean and Webb or equivalence zone and indicates the ratio of antigen to antibody where neither is in excess. This phenomenon is used as a method for assay of diphtheria antitoxin (Ramon flocculation test, p. 520). Precipitation tests are also used for the detection (by extraction) of group-specific and type-specific bacterial antigens as in the classification of the haemolytic streptococcus (p. 487) and in the identification of blood stains in forensic medicine.

In recent years, the occurrence of precipitation reactions in gels of agar or gelatin has been used for assay purposes and also to demonstrate the presence of more than one diffusible antigen in a microbe or toxin. There are several variations in technique for demonstrating precipitation reactions in gels, based on the method of Oudin who layered antigen solutions on top of a column of antiserum-containing agar or gelatin with the subsequent development of a band or bands of precipitation and on the Elek-Ouchterlony methods of allowing antigen and antibody to diffuse towards each other from wells or impregnated filter paper in a plate of agar gel. In this double diffusion technique several hypothetical antigens may be placed in cups around a central well of antiserum and the number of precipitin lines where antigen and antibody are in equivalence will indicate the number of antigens in each solution. Gel diffusion precipitation can be used, not only for quantitative and qualitative assays of antigens and antibodies but also to detect the presence of diffusible toxins in bacterial cultures, e.g. in the diphtheria bacillus (p. 514) and the staphylococcus (p. 465).

Agglutination

Agglutination is the most easily observed effect of an anti-bacterial serum. If the serum is added *in vitro* to a uniform suspension of the particular organism and the mixture is incubated, the bacteria become aggregated in clumps and the suspension appears flocculent or granular, the clumps or floccules being easily visible to the naked eye. The phenomenon is attributed to a specific antibody designated *agglutinin*. The agglutinin does not affect the viability of the bacteria. It also clumps dead bacteria in the same way as the living organisms. It is relatively thermo-

stable, e.g. at 56° C. as compared with complement. An electrolyte, such as sodium chloride, is necessary for its action, and agglutination tests are usually carried out in a medium of physiological saline. The physical mechanism of bacterial agglutination has not yet been fully elucidated, but depends apparently on a disturbance in the balance between the cohesive force of surface tension tending to aggregate the bacterial cells and the mutually repellent influence of the similar electrical charges carried by the cells. The electrolyte present, as well as the agglutinin, contributes to the physical changes involved in the process of agglutination. Agglutination tests are applied in diagnostic work, and for the identification of species and types of bacteria (p. 313).

Normal serum may contain natural antibodies, including agglutinins for various bacteria. In general, normal agglutination is quantitatively weak.

Agglutination Reactions in relation to Antigenic Structure and Antigenic Variation.—Among motile bacterial species (e.g. typhoid bacillus) two different kinds of "agglutinogen" (*i.e.* the antigen which stimulates agglutinin production) can be recognised; *flagellar* (contained in the flagella) and *somatic* (in the body of the organism). The flagellar antigen is usually designated by the symbol H and the somatic by O. For these different types of antigen separate agglutinins are likewise produced, also designated H and O, and the agglutination which results from the interaction of these antigens and agglutinins is described by the same symbols. H agglutination is of a "large-flake" type, *i.e.* large easily visible clumps, whereas O agglutination is of the "small-flake" or finely granular type. Differential testing of H and O agglutinins can be carried out by varying the condition of the bacterial suspension. For H agglutination, a flagellate strain of the particular organism must, of course, be used, and if the suspension is treated with formalin an almost pure H reaction is obtained, since formalin-fixed flagella interfere with O agglutination. Treatment of the suspension with alcohol, on the other hand, removes and inactivates the H antigen and an alcoholised suspension is therefore a suitable reagent for testing O agglutination (p. 318). Another method is to use a non-motile variant of the organism. The H and O antigens differ in thermostability: thus the H antigen is labile at 80°-100° C., whereas the O antigen withstands 100° C. A bacterial suspension which has been kept at 100° C. for about twenty minutes yields an almost pure O agglutination.

Some motile bacteria are *diphasic* as regards the constitution of their H antigens, *e.g.* various *Salmonella* types, and occur in

two different forms, one in which the H antigen may be relatively specific, the other in which this antigen may show characters common to allied species or types. These phases may be represented in a culture by different colonies so that if one colony is subcultured the "specific" phase may be obtained, if another, the "non-specific" phase, but such colonies do not show any morphological differences. However, the difference between the two phases may not be in respect of the specificity or non-specificity of the antigenic components (p. 590). Other species are *mono-phasic* as regards their H antigen, occurring only in the specific or in the non-specific form. It must be recognised that even a particular kind of antigen is frequently composite and consists of multiple components for each of which a separate agglutinin is produced on immunisation.

All these aspects of antigenic structure are well illustrated by the *Salmonella* group of bacteria, as is shown in the table on p. 590 which should be referred to.

S→R Variation.—In laboratory cultures of the *Salmonella* and dysentery groups two types of colony may be observed: (1) the normal smooth, round and transparent form—S (*smooth*) type, and (2) a rough, irregular and opaque variant—R (*rough*) type. The S type when suspended in 0·85 per cent. saline forms a stable suspension; the R type tends to auto-agglutinate, though it may remain stable in weaker saline solutions (e.g. 0·2 per cent.). A similar variation with the development of R colonies is seen in other bacterial groups. These types are antigenically different; thus, an antiserum for S may not agglutinate R, and an antiserum for R may not agglutinate S.

The variation from S to R is associated with a change in the somatic antigen, the variant antigen being designated by the symbol Ø (or R); the H antigen is usually unaltered. Among pathogenic bacteria the S→R transformation is frequently associated with loss of virulence. Moreover, while the antigen characteristic of the S type is often highly specific, the R antigen may exhibit characters common to other, though related, species.

It has been shown that the typhoid bacillus and certain related organisms when freshly isolated possess an additional somatic antigen which is associated with virulence (as judged by injection of cultures in mice). When the organism is continuously cultivated artificially and loses virulence this antigen is no longer present. It has been designated the Vi (virulence) antigen and can be detected by agglutination tests with an appropriate antiserum. It seems likely that various organisms possess analogous "virulence" antigens, and the capsular hapten of the pneumococcus has

a somewhat similar role. The coliform group of organisms also carry surface or K antigens which are subdivided into L, A and B antigens (p. 604).

Haemagglutination.—Soluble antigens may be adsorbed on to larger particles, e.g. collodion, which then behave as the specific antigen and are agglutinated by the corresponding antibody. Red blood cells, particularly sheep cells and group O human cells, are frequently used for this form of haemagglutination, which is designated as indirect or conditioned haemagglutination to distinguish it from direct bacterial and viral haemagglutination which does not involve antibody (pp. 16, 756). Polysaccharides are rapidly adsorbed on to red blood cells; proteins rather more slowly. In the latter case, adsorption is facilitated by preliminary treatment of the red cells with dilute tannic acid (1: 20,000). Any antibody to the red cells which may be present in the serum being tested must, of course, be first removed by absorption with "untreated" red blood cells. This method of agglutination has been used to detect antibodies to the tubercle bacillus, Vi and O typhoid antibodies, virus antibodies, etc.; it usually gives a much higher titre than is obtained by direct bacterial agglutination.

Another form of indirect agglutination is the antiglobulin reaction or Coombs test, used in blood grouping to detect weak or incomplete antibody.

Complement-Fixation; Lysin; Opsonin

The bactericidal action of an immune serum is due to a specific thermostable antibody (*bacteriolysin* or *bactericidin*) acting along with a normal non-specific constituent of serum (*complement*) which is thermolabile (at 55° C.). The antibody apparently combines firmly with the bacterial antigen and the complement then unites with the combined antigen and antibody. The antibody and complement have no independent combining affinity. It has thus been supposed that the antibody acts by "sensitising" the bacteria to the action of complement, and that the latter is the essential lytic agent.

Thus:

$$\text{Bacteria} + \left\{ \begin{array}{l} \text{Specific} \\ \text{antibody} \end{array} \right\} + \text{Complement} = \text{Bactericidal effect.}$$

$$\text{Bacteria} + \left\{ \begin{array}{l} \text{Specific} \\ \text{antibody} \end{array} \right\} = \text{No effect.}$$

$$\text{Bacteria} + \text{Complement} = \text{No effect.}$$

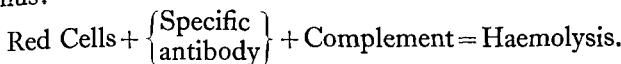
It may be noted here that complement is an exceedingly

unstable substance and becomes quickly inactivated when serum is kept at room temperature.

In vivo bacteriolysis can be demonstrated readily by *Pfeiffer's* reaction: a suspension of cholera vibrios is injected intraperitoneally in a guinea-pig along with an anticholera serum which is devoid of complement as a result of heating (*e.g.* at 55° C. for $\frac{1}{2}$ hr.), and if peritoneal fluid be drawn off with a hypodermic syringe at intervals within an hour, it is seen that the vibrios undergo progressive lysis and disappear from the fluid. In this case the complement of the blood plasma of the animal acts along with the antibody of the immune serum.

Haemolysis by a Haemolytic Antiserum is analogous to bacteriolysis—*i.e.* it is due to a specific thermostable antibody acting along with the normal complement. Haemolytic antiserum may be produced by injecting blood cell suspensions of one species of animal, *e.g.* sheep, into another species, *e.g.* rabbit.

Thus:

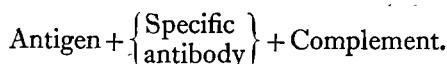


The phenomenon of haemolysis by serum can be demonstrated *in vitro* with blood suspensions and is easily visible with the naked eye, the blood becoming laked and transparent.

A suspension of red blood corpuscles in isotonic salt solution plus the antiserum which has been heated at 55° C. to destroy complement (*i.e.* red cells + specific antibody *only*) serves as an indicator for the presence or absence of complement—*e.g.* in complement-fixation tests—and is spoken of as a haemolytic system (p. 339). Serum haemolysin must not be confused with the haemolysins produced by various bacteria, *e.g.* streptococcal haemolysin, which are “toxins” or enzymes secreted by the bacteria and act directly on the red blood cell.

Even in the absence of bactericidal action, an immune serum may contain antibodies which, along with antigen, fix or absorb complement; and a *complement-fixing antibody* is therefore spoken of.

Thus:



To test for this effect, the haemolytic system is used as an

indicator: if complement has been fixed, then, on adding the haemolytic system, no haemolysis will occur.

Complement-fixation tests are employed in diagnosis, and occasionally for the identification of species in the same way as the agglutination reaction.

The *opsonic action of normal serum* is dependent on a non-specific thermolabile principle, the *normal opsonin*, which in some respects resembles complement (*vide supra*). It seems likely, however, that natural antibodies such as properdin also play a part in this effect as in the bactericidal action of the alpha lysin of normal serum (p. 46).

The increased *opsonic action of an antibacterial serum* is due to a specific thermostable antibody (*immune opsonin*) which can function independently of complement, though the latter may enhance the effect of the opsonin.

The opsonins combine with the bacteria, rendering them in some way susceptible to phagocytosis but without directly affecting their viability. Thus, if a serum is allowed to interact with organisms, which are then separated from it by centrifuging and washing with salt solution, they are still susceptible to phagocytosis by leucocytes though the serum has been removed, and the leucocytes have also been freed from serum. The opsonin is apparently "absorbed" from the serum by the organisms, and bound by them.

The *opsonic index* is a numerical expression of the opsonic power of the serum of a person for a given organism as compared with normal, and has been regarded as significant of the degree of resistance possessed by the person to the particular infection. The index can be estimated according to the following system: a preparation of leucocytes, the bacteria in question and the patient's serum are mixed, and, after a period of incubation, film preparations are made from the mixture and suitably stained; by counting under the microscope the number of bacteria phagocytosed by fifty, or preferably a larger number of, leucocytes, the average for one leucocyte can be calculated—the *phagocytic index*. A similar experiment is carried out, substituting the pooled serum of two or three normal persons, and the phagocytic index again estimated. The opsonic index is then calculated by dividing the phagocytic index of the serum in question by that of the normal serum. Thus unity is normal, and the opsonic power of the serum is greater or less than normal according as the index is greater or less than unity.¹

¹ Wright, A. E., & Colebrook, L. (1921), *Technique of the Teat and Capillary Glass Tube*. London, Constable.

It has been shown that the results of counting bacteria in a small number of leucocytes, *e.g.* 50, may not be a statistically accurate representation of the actual number phagocytosed.

Antiviral Sera.—The serum of a person or animal immunised against a filterable virus may exhibit properties which are analogous to those of an antibacterial serum. Thus, the virus is specifically inactivated or neutralised by the serum, and this has sometimes been spoken of as due to a viricidal antibody, the term implying the killing of the virus. The actual effect of this antibody on the virus has not been fully determined, but is apparently independent of complement. Specific precipitating and complement-fixing reactions have also been observed with antiviral sera. Where *elementary* or virus bodies can be separated and concentrated to form a suitable suspension, their specific agglutination by an antiserum can be demonstrated, as is exemplified by the elementary bodies of variola.

CELLULAR IMMUNITY

Although the development of specific humoral antibodies can be demonstrated following a clinical infection with most bacterial and viral pathogens, these antibodies may play an insignificant part in the patient's recovery from the infection. For example, despite the high level of specific antibodies present in the blood of patients with brucellosis in the second or third week of the illness, the clinical infection may continue for 6–12 weeks or longer; and if in this infection or in typhoid fever the illness is terminated early by chemotherapy, relapses of infection are more likely to occur. The lack of correlation between humoral antibodies and immunity is best illustrated from studies in experimental tuberculosis. Immunity to tuberculosis in a susceptible animal like the guinea-pig follows inoculation with living attenuated cultures *e.g.* B.C.G. vaccine, and, less surely, with killed bacillary suspensions; humoral antibodies similar to those in the immunised animal can be produced by injections of protein-lipid fractions of the tubercle bacillus without inducing any immunity. Serum from immune guinea-pigs transferred to susceptible animals gives no protection against tuberculous infection. Again, tubercle bacilli in collodion capsules permeable to antibody-globulins and planted intraperitoneally in immunised guinea-pigs grow at the same rate as similar cultures in susceptible animals. Thus, all the available evidence indicates that humoral antibodies play little or no part in immunity to tuberculosis.

The evidence regarding a specific cellular immunity is still

rather contradictory. Lurie showed that monocytic cells from an immunised guinea-pig when injected into the eye chamber of a rabbit along with living tubercle bacilli prevented the growth of the organisms, whereas normal monocytes failed to do so. Some workers have described similar results with mixtures of "immune" and normal monocytic cells and tubercle bacilli *in vitro*, but their findings need corroboration. Recent observations on patients with agammaglobulinaemia have demonstrated the selective importance of humoral and cellular immunity in different infections. Agammaglobulinaemia is a sex-linked genetic anomaly seen only in males in which there is an absence of gamma globulin and humoral antibodies, including the blood iso-agglutinins. Children with this anomaly fail to produce antibody in response to antigenic stimulation and are highly susceptible to acute bacterial and toxic infections, *e.g.* pneumonia, streptococcal infections and diphtheria. On the other hand, the common virus infections including measles, chickenpox and mumps run a normal course and are followed by clinical immunity. The child can also be successfully vaccinated against smallpox and develops hypersensitivity to tuberculin following B.C.G. vaccination (*q.v.*, p. 551). Agammaglobulinaemic children therefore seem to develop a cellular immunity to infections by intracellular pathogens such as viruses and the tubercle bacillus, and in such infections, humoral antibody is not essential for protection or clinical cure.

ANAPHYLAXIS; ALLERGY; MICROBIAL HYPERSENSITIVITY

Under certain conditions the body tissues, or a particular tissue or organ, develop hypersensitivity to foreign antigens which may be protein or protein-linked substances or certain chemicals. Three main categories of hypersensitivity are recognised: *anaphylaxis*, which is an artificially acquired hypersensitivity to foreign protein and is mainly an experimental phenomenon; *allergy*, which is naturally acquired and results from exposure to a variety of antigens such as pollen, dandruff, certain foods, etc.; and *microbial hypersensitivity*, which develops after certain infections, *e.g.* tuberculosis. In all three categories the tissue reaction manifests itself by a contraction of smooth muscle, *e.g.* of the bronchioles in asthma, and by increased capillary permeability leading to oedema, urticaria, rhinorrhoea, etc. Sometimes the reaction is so violent and generalised as to cause death, or, if localised, tissue necrosis. These phenomena seem to be associated with the release of histamine, serotonin or allied substances,

which occurs when there is union between antigen and antibody in certain relative proportions at the cell surface or intra-cellularly. Tissue hypersensitivity is undoubtedly related to immunity, but sometimes, instead of being protective, it may contribute to specific disease, as in acute rheumatism, or to tissue damage as in tuberculosis.

Anaphylaxis and allergy have certain points in common. The tissue reaction is immediate (within a few minutes) and the hypersensitivity can be passively transferred in the serum. Allergy, however, is of long duration and has a strong hereditary predisposition, whereas anaphylaxis is limited in time and is probably not genetically linked. Microbial hypersensitivity is characterised by delayed reactions (1-4 days) and by the failure to transfer the hypersensitivity in serum, although in some instances it may be transferable by cell suspensions.

Anaphylaxis.—If a guinea-pig is injected subcutaneously with a very small dose of horse serum (*e.g.* 0.001-0.01 ml.), and after an interval of ten days receives a larger second dose of the same serum (*e.g.* 0.2 ml. intravenously or 5 ml. subcutaneously), it will develop within a few minutes a sudden illness or *anaphylactic shock* in which the chief manifestation is spasmodic contraction of unstriped muscle, particularly that of the small bronchi. The serum is non-toxic *per se* when given to an unsensitised animal even in large doses.

Anaphylactic shock is more liable to occur and is more marked when the injection is intravenous, and when a large dose is given, than when the injection is subcutaneous or a small quantity is introduced. Thus, a dose which would produce shock if given at once, when introduced in repeated small fractions may not lead to an anaphylactic shock, and the animal is desensitised in this way. If, after the sensitising injection but before hypersensitivity has developed, a second dose of the substance is given, the animal is protected for a time against a subsequent injection.

Haptens may produce anaphylactic shock in animals specifically sensitised with the complete antigen of which the hapten is a constituent.

Serum anaphylaxis is of practical importance in medicine in relation to serum therapy—for example, when it is necessary to give a second dose of a prophylactic or therapeutic serum after an interval of 10 days or more, and especially by intravenous injection; the risk of anaphylactic shock may, in such cases, be obviated by desensitisation with very small doses of serum. Fortunately, the human subject is liable to anaphylaxis much less

than are certain animals. Tests for hypersensitivity and the hazards of repeat injections are discussed under tetanus (p. 683).

Serum Sickness or Serum Disease.—It should be noted that after a *single* dose of foreign serum (*e.g.* a therapeutic antiserum from the horse) given for the first time, in a considerable proportion of normal persons toxic effects may ensue. These occur after an interval of eight to twelve days, and may consist of fever, an erythematous or urticarial eruption, swelling of lymph glands and joints, albuminuria, etc., and there may be an inflammatory reaction at the site of the injection. Such symptoms may be related to anaphylaxis; thus, at the time when symptoms appear, the antigen (the serum-protein injected) may still be present in the system, and antibody is beginning to appear in the blood and tissues so that an antigen-antibody reaction may occur *in vivo* as in anaphylactic shock.

A single injection of foreign serum may, in certain persons, particularly those with a personal or family history of allergy, produce immediate symptoms of shock. In the administration of antisera such severe reactions can be avoided by a preliminary test dose (see tetanus, p. 683).

Allergy.—In certain persons, probably as a result of genetic factors, hypersensitivity may occur towards a considerable variety of substances of protein or non-protein nature, so that when the person is exposed to contact with the substance to which he is sensitive, toxic effects result, *e.g.* coryza, asthma, urticaria, gastrointestinal disturbance, etc. This form of hypersensitivity has been designated either as *atopy*, where there is a marked hereditary predisposition, *e.g.* hay fever, asthma, eczema; or *allergy*, where the genetic factor is much less important and there is almost always a history of contact, *e.g.* contact dermatitis, drug allergy and certain food allergies. Allergy can be tested for by cutaneous reactions with preparations of the particular allergen, *e.g.* pollen extracts. Also, when the serum of an allergic person is injected into the skin of a non-sensitive person, and after an interval the allergen is injected at the same site, an urticarial wheal results (Prausnitz-Küstner reaction). These localised responses appear quickly and are known as *immediate* hypersensitivity reactions.

Microbial Hypersensitivity.—Sensitisation to microbes or their products occurs in a variety of bacterial, viral or fungal infections. This microbial hypersensitivity develops in the course of a naturally acquired or experimentally produced infection and is not usually demonstrable until 1–2 weeks, or sometimes longer, after the onset of infection. It is found in chronic bacterial

infections characterised by intracellular parasitism, e.g. tuberculosis, leprosy, brucellosis; in certain virus infections, e.g. psittacosis, lymphogranuloma venerum; and in both superficial and systemic fungal infections, e.g. ringworm, histoplasmosis. This hypersensitivity of infection has been studied particularly in experimental and clinical tuberculosis. The finding, as demonstrated by Koch, that a guinea-pig infected with virulent tubercle bacilli would after an interval of 10 days or more react to the intradermal injection of living or killed tubercle bacilli by a delayed local inflammatory reaction is known as the *Koch phenomenon* and has become the basis of the tuberculin test (p. 543) which is used as evidence of antecedent tuberculous infection. In addition to the local reaction at the site of injection, tuberculin may also elicit focal inflammatory reactions around existing tuberculous lesions and may, in fact, activate quiescent "healed" foci. These local and focal reactions do not appear until 12-24 hours or longer after the injection of tuberculin and this is known as the *delayed hypersensitivity reaction*. Microbial hypersensitivity, although it cannot be passively transferred in serum as in the Prausnitz-Küstner phenomenon, is transferable in the cells of an induced peritoneal exudate from a sensitised animal, which suggests that in this form of hypersensitivity the antibody is closely bound to certain cells.

From this summary it must be obvious that the various categories of tissue hypersensitivity have many similarities associated apparently with the reactions that follow union of antigen and antibody on or in the sensitised cells.

Shwartzman Phenomenon.—This reaction merits consideration in relation to the general subject of hypersensitiveness. It was observed by Shwartzman that, when a culture-filtrate of the typhoid bacillus had been injected into the skin of a rabbit (preparatory inoculation) and after 24 hours (but not later than 32 hours) the same filtrate was injected intravenously (provocative inoculation), an intense reaction occurred at the site of the intradermal injection, viz. an area of haemorrhagic inflammation with subsequent necrosis. A general reaction can be induced by giving both inoculations intravenously and is characterised by bilateral cortical kidney necrosis. The reaction is not specific: thus, after the intradermal injection of typhoid bacillus filtrate, or other endotoxin-containing organisms, a coliform bacillus filtrate injected intravenously may excite the reaction. The precise significance of this phenomenon is still somewhat obscure, but it may contribute to the toxæmia of Gram-negative bacterial infections.

Arthus Phenomenon.—This is a local hypersensitivity reaction characterised by an acute inflammatory response going on to tissue necrosis following repeated injections of an antigen. It may sometimes be encountered in man, *e.g.* after frequent injections of rabies vaccine. |

PART II

TECHNICAL METHODS

The draw-tube should be adjusted to the *tube length* (p. 82) for which the objective is corrected; for the majority of microscopes this is usually 160 mm., but as the revolving nose-piece has a length of 18 mm. the draw-tube should be extended only to the 142-mm. mark. Leitz objectives, however, are corrected for a tube length of 170 mm. In modern microscopes the scale on the draw-tube now includes the length of the nose-piece, so the engraved figures indicate the actual tube length. Many manufacturers also engrave a line round the draw-tube at the correct tube length, while in the cheaper models the draw-tube is rigidly fixed at this length and cannot be altered. It is essential that an objective should be used at its proper tube length, particularly the apochromatic objectives and oil-immersion achromatic objectives (*vide infra*), if the maximum resolution is to be obtained.

The fixed *stage* is fitted with two spring clips. A *mechanical stage* is of great advantage, and is really necessary when a large area of a microscopic preparation has to be searched, as in the examination of films of sputum for tubercle bacilli, or of blood for malaria parasites. An attachable mechanical stage can be obtained for almost any model, but the "built-in" mechanical stage is more satisfactory and is recommended for routine bacteriological work.

The *substage* is an important part of the microscope and one to which frequently little attention is paid. The mechanism for raising the substage should be rigid and free from lateral movement. Where critical work has to be done with highly corrected objectives and condenser, centring screws should be fitted. The *condenser*, which is used for focussing light on the object to be examined, is usually of the two-lens Abbe type; but if apochromatic or semi-apochromatic objectives are used, a condenser of similar optical quality must be employed. In fact, it is recommended that the ordinary Abbe condenser should never be used for bacteriological work owing to its poor optical correction, but that an aplanatic condenser (which gives a solid cone of light) or an achromatic condenser which is not only aplanatic but corrected for chromatic aberration should always be employed. The modern oil-immersion objectives are so well corrected and admit such a high incident cone of light that their performance is much impaired if an ordinary Abbe condenser is used. The *iris diaphragm* is an important part of the substage, as it controls the angle of light which passes into the condenser. For example, if the diaphragm is partially closed and a high numerical aperture objective is used, the definition will be much impaired.

Binocular Microscopes.—Where much microscopic work has to be done and for routine examinations we recommend that the microscope should have a binocular body, as, by using both eyes, a considerable amount of eye strain and fatigue is avoided. In the binocular body the rays of light from the objective are divided by a half-silvered surface inclined at an angle of 45 degrees which permits one half of the light to pass vertically, while the remainder is reflected horizontally. Each half of the rays is directed into its appropriate eye-piece by means of prisms.

The eye-piece sockets can be adjusted to the interocular distance of the observer, while one of the ocular tubes is adjustable to correct individual differences between the two eyes.

Inclined binocular microscopes are very suitable for routine use, as the eye-pieces are inclined towards the observer and it is not necessary to tilt the stand as with the straight binocular or monocular bodies. Consequently the stage is kept horizontal and this is of particular advantage when dealing with wet films or using dark-ground illumination. (Similarly an inclined eye-piece fitting for a monocular tube may be obtained.)

It should be noted that the inclined binocular body may increase the actual magnification by $1\frac{1}{2}$ times. This factor shown as $1.5 \times$ is engraved on the body. Lower-power eye-pieces only should be used; $6 \times$ is the most convenient, and $8 \times$ is the highest practicable for routine use.

Binocular microscopes have interchangeable monocular and binocular bodies, which are removable without disturbing the objectives, so that a monocular body can readily be used for photography, micrometry, etc.

OBJECTIVES AND EYE-PIECES

For general purposes, ordinary achromatic objectives are quite satisfactory, and are admirable for routine work and students' use. The quality of the present-day objective is extremely good, and for ordinary work the purchase of the more expensive types is not recommended. The most useful objectives are $\frac{2}{3}$ -in. or 16-mm., $\frac{1}{6}$ -in. or 4-mm., the $\frac{1}{12}$ -in. (thus designated, but actually $\frac{1}{4}$ -in.) or 2-mm. oil-immersion, and, for dark-ground illumination and blood work, the $\frac{1}{7}$ -in. or 3.5 mm. oil-immersion lens should also be added. With a monocular body these should be used in conjunction with a 10-magnification ($10 \times$) Huygens eye-piece. A $5 \times$ eye-piece is often supplied, and is sometimes employed for searching when a larger field is desired without altering the objective. It is not practicable to use the ordinary (Huygens) eye-piece above $12 \times$, and even this magnification gives some distortion and haziness of outline. Higher magnifications may be obtained by the use of apochromatic objectives and compensating eye-pieces. With binocular microscopes, either straight or inclined, $6 \times$ or $8 \times$ eye-pieces are sufficient, as, owing to the division of the rays, less light enters each eye-piece. With $10 \times$ eye-pieces the definition is not so sharp and the field is apt to be too dark when ordinary illuminants are used.

MAGNIFICATION

The objective works at a distance from the object a little more than its focal length. A real, inverted and enlarged image is formed in the upper part of the tube, and this real image is magnified further by the eye-piece. Thus, the total magnification is the product of the separate

magnifications of the objective and of the eye-piece, and depends on three factors:

- (1) The focal length of the objective.
- (2) The magnifying power of the eye-piece.
- (3) The distance between the lens system of the objective and the image produced—the “optical tube length”.

In actual practice, however, when calculating the magnification, the “mechanical tube length” is used; the diagram below illustrates optical and mechanical tube lengths.

The “optical tube length”—AB—is the distance between the posterior principal plane of the lens system of the objective and the plane of the image which is in the upper part of the draw-tube.

The “mechanical tube length”—CD—is the distance between the

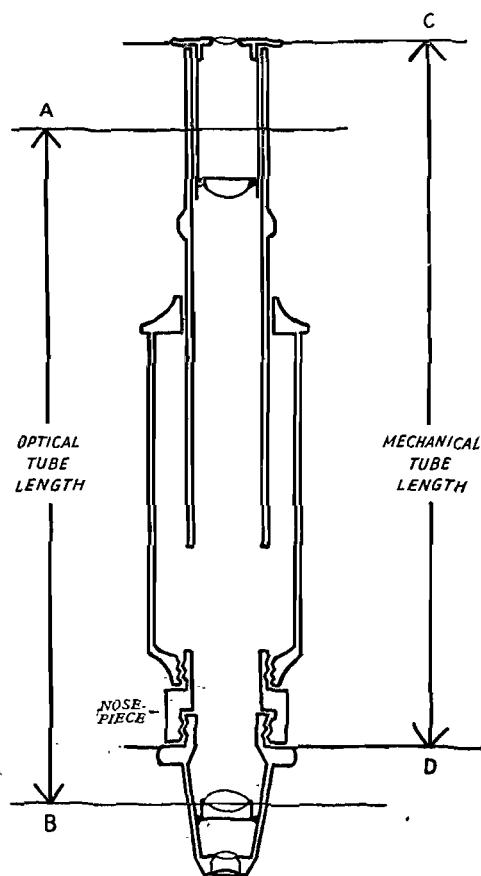


FIG. 2

eye-lens of the eye-piece and the point where the objective fits into the lower end of the body-tube or nose-piece. In adjusting the draw-tube to a given tube length, it must be remembered that the draw-tube scale may read from the foot of the body-tube and does not include the nose-piece, the length of which is 18 mm. Allowance must be made for this—*e.g.* if the objective works at 160 mm. tube length, the scale of the draw-tube is set at 142 mm., which, with the length of the nose-piece, gives the required total length of 160 mm. In modern instruments the draw-tube scale includes the length of the nose-piece and the correct tube length is often indicated on the draw-tube by an engraved ring. The tube length may easily be verified by measuring with a ruler from the bottom of the nose-piece to the upper end of the draw-tube.

When calculating the magnifying power of a given objective and eye-piece, the optical tube length may be taken for practical purposes as equal to the mechanical tube length.

It is emphasised that objectives are designed to work at a definite tube length, and any variation from this distance may seriously impair the definition obtained, particularly when apochromatic and high-power achromatic objectives are used.

The initial magnifying power of the objective is first determined, and then multiplied by the magnifying power of the eye-piece, when the total magnification is obtained. The objective acts as a convex lens, and its magnification can easily be calculated as follows:

$$\text{magnification of objective} = \frac{\text{size of image}}{\text{size of object}}$$

$$= \frac{\text{distance of image from objective}}{\text{distance of object from objective}}$$

The distance of the image from the objective is the "optical tube length", which is approximately equal to the "mechanical tube length", and this is determined from the draw-tube scale, as described above.

The distance between the object and the objective depends on the objective used, and this distance is adjusted by means of the coarse adjustment when the object is focussed. It may be taken as approximately equal to the focal length of the objective. Suppose an object is examined with a 16-mm. ($\frac{2}{3}$ -in.) objective and a $10\times$ eye-piece, the tube length being 160 mm.; the size of the image produced by the objective alone depends on the ratio of the tube length to the focal length of the objective—*i.e.* 160 mm.: 16 mm. (ten times); this real image is now magnified ten times by the $10\times$ eye-piece, making a total magnification of 100 diameters. If a 4-mm. ($\frac{1}{8}$ -in.) objective is employed the distance of the image is the same (160 mm.), but the distance between objective and object is only 4 mm., hence the initial magnification of the objective is 160: 4—*i.e.* 40. This is further magnified by the $10\times$ eye-piece to 400 diameters. Similarly a 2-mm. objective has an initial magnification of 80, and when used in con-

junction with a $10\times$ eye-piece gives a total magnification of 800 diameters.

Thus: total magnification =

$$\frac{\text{tube length}}{\text{focal length of objective}} \times \text{eye-piece magnification.}$$

It is thus seen that the magnification varies inversely as the focal length of the objective, the shorter the focal length the greater the magnification.

Makers now engrave the initial magnification on the objective mount and refer to the objective by its magnification and numerical aperture (*vide infra*); thus, the 16-mm. ($\frac{2}{3}$ -in.) is 10/0.28, the 4-mm. ($\frac{1}{8}$ -in.) is 40/0.65, and the 2-mm. ($\frac{1}{2}$ -in.) is 95/1.28 (or 1.30). As the eye-pieces are also designated by their magnifying power, the total magnification is easily and correctly determined, provided of course the proper tube length is used.

The $\frac{1}{2}$ -in. objective has, in reality, a shorter focal length than that by which it is designated, and gives a magnification of 95–100 diameters,

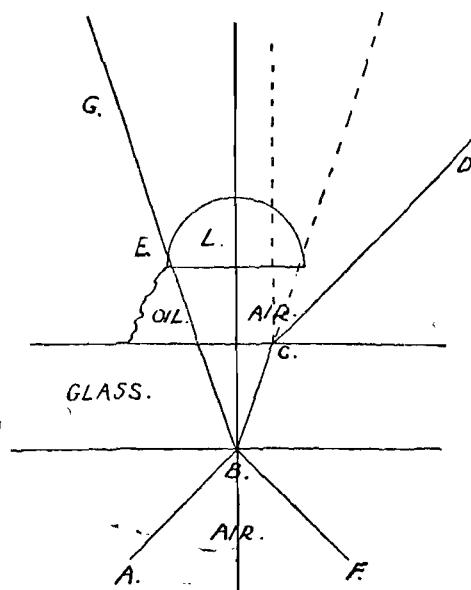


FIG. 3

Diagram showing the paths of rays through (1) a dry lens (on right), and (2) an oil-immersion lens (on left) (*after Spitta*).

Note the refraction of the oblique ray ABCD in passing from the glass slide to air, as compared with the ray FBEG. L is the front lens of the objective.

according to the make. When used in conjunction with a $10\times$ eyepiece the total magnification is 1000 diameters.
The magnification usually employed in bacteriological work is 800–1000 diameters.

OIL-IMMERSION OBJECTIVES

The $\frac{1}{2}$ -in. oil-immersion lens works very close to the cover-slip, and the intervening space between objective and cover-slip is filled with cedar-wood immersion oil. The reason for this is that when an oblique ray of light emerges from a dense medium (glass) into a rare medium (air) it is refracted outwards—*i.e.* away from the normal (see diagram—ABCD). As the brightness of the image depends upon the light entering the objective, and the resolution (*vide infra*) depends on the effective aperture, this refraction of light diminishes not only the brightness but the clearness of the image. If, however, the space between objective and object is occupied by immersion oil, which has the same refractive index as glass, the rays of light do not undergo refraction and pass into the objective (see diagram—FBEG).

The high power ($\frac{1}{6}$ -in.) is a “dry” lens and must *not* be used with immersion oil. Oil must be used only with lenses specially computed to work with this fluid. Such objectives have “oil immersion” engraved on them.

NUMERICAL APERTURE

Objectives are rated not only by their focal length but also by their *Numerical Aperture* (N.A.). The numerical aperture may be defined simply as the ratio of the diameter of the lens to the focal length.¹ It is expressed mathematically as follows:

$$\text{N.A.} = n \sin U$$

where n is the refractive index of the medium between object and objective (air, 1.0; cedar-wood immersion oil, approximately 1.5), and $2U$ the *angle of aperture*—*i.e.* the angle formed by the two extreme rays of light, which, starting from the centre point of the object, reach the eye of the observer (*vide* diagram, p. 86).

$$\begin{aligned} \text{That is, } \text{DAC} &= 2U \\ \text{BAC} &= U \end{aligned}$$

$$\sin U = \frac{EF}{EA}$$

It is thus seen that the numerical aperture, other things being equal,

¹ The numerical aperture has been expressed in this manner to simplify description, but this is true only for objectives of long focal length, where EA is approximately equal to FA (see diagram). With short-focus lenses of high numerical aperture this definition is not correct. The length EA is then much greater than the distance of the objective from the slide (FA).

depends on EF, which is half the diameter of the lens. Objectives, therefore, may have equal focal lengths, but different numerical apertures depending on the diameter of the front lens.

The theoretical limit of the angle DAC is 180° —i.e. when the objective is actually on the object—and therefore the theoretical limit of U is 90° . The greatest possible N.A. of a dry lens cannot exceed 1, since the refractive index of air ($n=1$), and $\sin 90^\circ=1$. Actually the highest practical N.A. of a dry lens is 0.95. On the other hand, the introduction of cedar-oil between the objective and object gives n a value of 1.5. The highest theoretical value, therefore, of $n \sin U$ for an oil-immersion objective is $1.5 \times \sin 90^\circ$ —i.e. 1.5. In practice, however, the highest N.A. of an oil-immersion objective (attained in an apochromat) is 1.4. The ordinary $\frac{1}{2}$ -in. objective for bacteriological purposes has a N.A. of 1.28 or 1.3.

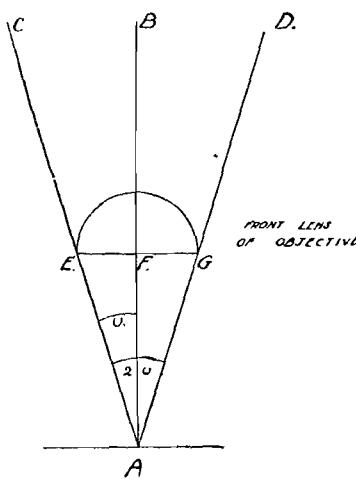


FIG. 4

Diagram to illustrate numerical aperture.

The essential qualities of an objective depend on its numerical aperture, and these are:

- (1) *brightness of image*, which, other things being equal, varies as the square of the N.A.;
- (2) *resolving power* and *defining power*, which vary directly as the N.A.

The *depth of focus*, while not dependent entirely on the N.A., varies in inverse proportion to it.

It is thus apparent why oil-immersion objectives give such good results—the N.A. being increased by the high refractive index of the oil. In general, it may be said that in the case of two lenses of equal focal length, the one with the higher N.A. is the better lens and is to

be preferred. The 16-mm. or $\frac{2}{3}$ -in. objective should have a N.A. of at least 0.28; the 4-mm. or $\frac{1}{6}$ -in., a minimum N.A. of 0.65; while the $\frac{1}{2}$ -in. oil-immersion should have a N.A. of not less than 1.28.

RESOLUTION

The *resolving power* (as apart from magnifying power) of a lens is its capacity to separate two adjacent points, and this property determines the amount of structural detail that can be observed microscopically. The limit of resolution is attained when the magnification reaches 1450 diameters. Theoretically, with axial illumination two points closer together than half the wavelength of the light used cannot be resolved. It is not possible to attain this theoretical limit under visual working conditions, and in practice the limit is reached at about 0.00025 mm. ($0.25\ \mu$).

Resolution, however, must not be confused with visibility, because it is possible to see "elementary bodies" (of virus diseases) as small as $0.074\ \mu$ with ordinary white light, and even smaller, $0.067\ \mu$, with green light (Coles). It should also be realised that the bodies observed have been stained and often treated with a mordant (*vide* Paschen's method, p. 140), so that the stained elementary body may be larger than the natural one, and thus brought within the limits of visibility. With ordinary microscopic methods, by using at the correct tube length an apochromatic objective of N.A. 1.4, and a high-power compensating eye-piece, in conjunction with an oil-immersion condenser, the whole optical system and illuminant being carefully centred, stained particles of a diameter smaller than $0.25\ \mu$ can be seen.

To illustrate the difference between visibility and resolution, if this printed page is placed a certain distance away (about 10 feet) it is possible to see that the print consists of a number of letters. It is not possible, however, to distinguish the form and shape of the actual letters as such at this distance. The letters are visible but their details cannot be resolved.

With ultra-violet light, which has a much shorter wavelength than visible light, greater resolution (2-3 times) can be obtained. With the introduction of the electron microscope (p. 106), in which the electrons have an equivalent wavelength as small as $1/100,000$ th of that of ordinary light, resolution has been increased one hundred fold as compared with the best light microscope.

DEFINITION

This is the capacity of the objective to render the outline of an object distinct, and depends on the elimination of "spherical" and "chromatic" aberration.

Spherical aberration is caused by the periphery of the lens refracting more than the central portion. The peripheral rays, therefore, focus

on the axis at a shorter distance from the lens than the central ones, with the result that the image is distorted.

Chromatic aberration is caused by the ray of white light being dispersed into its component colours as it is refracted through the lens, a spectrum being formed. The blue rays are refracted more, and come to a focus nearer the lens than the red rays. The different component colours do not come to the same focus and hence cannot blend to form white light. As a result, the image is fringed with colours and the outline is hazy.

Both chromatic and spherical aberration may be corrected by the combination of lenses of different dispersive power—e.g. convergent convex lenses of crown glass having low dispersive power, and divergent concave lenses of flint glass having high dispersive power. By this means two of the spectrum colours are combined, and the ordinary achromatic objective is constructed of lens systems made in this manner.

APOCHROMATIC OBJECTIVES

While achromatic objectives fulfil all ordinary purposes, they are not sufficiently corrected for critical work, such as photography and resolution of minute objects, for which apochromatic objectives must be employed. These represent the highest degree of optical perfection, and are, in consequence, very expensive. Apochromats show almost complete colour correction, and the essential factor in their construction is the use of the mineral *fluorite*. Fluorite possesses the following valuable optical properties:

(1) high degree of transparency; (2) low refractive index; (3) extremely small dispersion.

As a result of the use of fluorite at least three colours may be united, thus eliminating the secondary spectrum. This endows the objectives with a brilliance and "crispness" of image not attainable with ordinary lenses, and enables the maximum resolving power to be obtained.

Apochromatic objectives must be used only in conjunction with the "compensating" eye-pieces supplied by the same maker, and care must be taken to adjust the tube length carefully and to employ a highly corrected and properly centred condenser.

A series of objectives containing a certain amount of fluorite, which are intermediate in quality between the apochromatic and achromatic objectives, has been introduced by several makers. They are known as "semi-apochromatic" or "fluorite" lenses, and some of them have a performance approaching that of the apochromatic objectives. The oil-immersion lenses of this series are very useful for dark-ground illumination.

The student or beginner in microscopy is advised not to purchase apochromatic or fluorite lenses, as the present-day achromatic or ordinary objectives have been brought to such a degree of excellence that all routine examinations and much research work can be done with them.

Brief Specification of a Microscope suitable for Routine Bacteriological Work

Microscope, with coarse and fine adjustments, fitted with a removable inclined binocular body. Built-in mechanical stage with verniers, quadruple nose-piece, rackwork substage with centring screws.

Objectives

Achromatic $10\times$ (16-mm.).

$40\times$ (4-mm.).

Fluorite $45\times$ (3.5-mm.) oil-imm. (magnification and focal length may vary slightly according to make).

Achromatic $95\times$ (2-mm. or $\frac{1}{2}$ -in.) oil-imm.

Paired Eye-pieces

$6\times$ and $8\times$.

Condenser

Aplanatic or achromatic.

Note.—(a) Some makers supply a $10\times$ objective specially computed to work with compensating eye-pieces. As it is an advantage to use this type of eye-piece with the other three objectives, it is recommended that this objective and compensating eye-pieces should be specified when ordering.

(b) If micrometry or photographic work is to be done, an interchangeable monocular tube is required.

(c) For dark-ground illumination, a special concentric condenser is necessary, and also a funnel stop for the $\frac{1}{2}$ -in. objective.

(d) When more than one condenser is used it is advisable to have a substage in which the condensers can easily be changed.

ILLUMINATION

The clarity and sharpness of the microscope image depend not only on the excellence of the optical system but also on the illuminant employed. For a monocular instrument with an Abbe condenser, and particularly for lower powers, a 40- or 60-watt opal bulb in a simple lamp housing is sufficient, but for a modern instrument with an inclined binocular body, well corrected oil-immersion objectives and an aplanatic or achromatic condenser, this type of illuminant is far from satisfactory. Unless the bulb is much overrun there is not sufficient light to see small details, and the advantages of modern optical systems can be nullified by poor illumination. If the opal bulb lamp is used it is of advantage to overrun the bulb, e.g. a 200-volt bulb on a 240-volt mains supply. The life of the bulb is shortened, but that is not a serious matter compared with the illumination obtained. The opal

bulb should be in a well ventilated housing with a hood over the aperture to prevent direct light from reaching the eyes and should preferably be fitted with an iris diaphragm. The latter is very useful for centring the light in the microscope field and helps to diminish glare. The amount of light required for a good visual image depends on many factors: the intensity of the bulb, the magnification used (for example less light is required for a 16-mm. objective than a 2-mm.), the amount of light in the room (less light is required if the microscope is at the back of the room than on the bench at the window), and the time of day (much more illumination is required if there is sunshine than on a dull day or in the evening). With microscopical work, therefore, the amount of illumination required is always changing and a sliding variable resistance of 250 ohms to carry 0.75 or 1 amp. fitted with a switch is strongly recommended. This is most desirable if an overrun bulb is used, and an absolute necessity if a high-intensity projection type of bulb is employed. The resistance is placed in series with the opal bulb and in series with the primary winding of the transformer if a low-voltage projection bulb is used.

The full resistance should always be used when the lamp is switched on so that the bulb warms up comparatively slowly, and then the slide of the resistance is moved until optimum illumination is reached. With a change of objective the slide is easily adjusted to suit the intensity of light required, and this method of control is of great value when much microscopic work is done and where the objectives are changed frequently as in histological work.

With binocular microscopes the amount of light reaching each tube is only about one-third of that of a monocular instrument as light is absorbed by the glass prisms. In consequence a more intense source of light must be used. In order that the whole of the field shall be evenly illuminated, a corrected lamp condenser is necessary and this must be capable of being focussed. An iris diaphragm in front of this condenser is essential, and provision for a filter holder, preferably of the sliding type, should be made.

High-intensity lamps of this type are produced by several makers and a new improved model specially suitable for high-power microscopy has recently been described.^{1,2} This lamp uses a large "solid source" filament bulb of 12 volt 250 watts capacity, but it is actually run at 6 volts and the intensity is controlled by a sliding resistance in the primary of the transformer. The lamp is not only suitable for ordinary microscopy but is useful for dark-ground illumination and phase-contrast microscopy. Its high intensity ensures ample illumination even with dense filters. It is particularly useful for photomicrography.

It may be desirable sometimes to have a more intense beam of light than is possible with a 6-volt transformer, as when filters are required with dark-ground illumination, or when high-power photomicrography is undertaken. In these cases a 9-volt transformer to take 18 amps.

¹ McCartney, J. E. (1951), *J. clin. Path.*, **4**, 234.

² Obtainable from R. R. Beard Ltd., 10 Trafalgar Avenue, London, S.E.15.

and tapped at 6 volts should be used. The 6-volt tapping is employed for ordinary work and the higher voltage output only for special purposes.

Other sources of light may be used for special types of work, such as a mercury vapour lamp for fluorescence microscopy, or even a high-intensity D.C. arc-lamp as in cinephotomicrography.

Light Filters

A blue daylight filter such as is supplied by most manufacturers should be fitted in the substage ring underneath the microscope condenser when artificial light is used. Filters, however, are not specially required in bacteriological microscopy except for the method of detecting tubercle bacilli as detailed on p. 546. Where much microscopic work has to be carried out, particularly with unstained objects as in dark-ground illumination or phase-contrast microscopy, the use of a pale-green filter, Wratten No. 66,¹ can be recommended. This filter cuts out glare, sharpens detail and is very restful to the eyes. After a short time in use, the green colour is not noticed. It can also be recommended when searching for malaria parasites or tubercle bacilli. In the latter case the organisms appear darker and are more easily recognised when only scanty individuals are present.

CARE OF THE MICROSCOPE

The microscope is an instrument of precision, and care must be taken to preserve its accuracy. The instrument should be kept at a uniform temperature and not exposed to sunlight or any source of heat. When not in use it must be protected from dust under a transparent plastic cover or in its box. Failing these, it should be covered with a clean cloth. The microscope should be cleaned at intervals, and its working surfaces very lightly smeared with soft paraffin (vaseline). With binocular microscopes dust may collect on the surfaces of the prisms. This may be removed by passing a soft-camél-hair brush down the eye-piece tubes after removing the eye-pieces. On no account must the prism case be opened and the prisms removed, as this will completely alter the optical alignment and necessitate the return of the instrument to the maker before it can be used again.

If the microscope has to be moved, it should be lifted by the upright limb and not held by the body-tube.

The oil-immersion objective must be cleaned each day after use by wiping the front lens with a well-washed silk or cotton handkerchief. Alternatively, a fine tissue paper known as "lens paper" may be used (books of which are supplied by most manufacturers), and this is very suitable for the purpose. Oil remaining on the lens-front dries and becomes sticky; later it hardens and is then difficult to remove.

¹ Supplied by Kodak Ltd., size 2 in. square, cemented in "D" quality glass.

Canada balsam accidentally present on the lens from a mounted microscopic specimen may also dry hard in the same way. When cleaning the objective *do not use alcohol*, as the cement that unites the component lenses may be soluble in alcohol, and, in consequence, the lens systems may become disorganised and the objective spoiled. Benzol or xylol must be used to remove dried oil, and if the oil is hard, repeated applications on a soft cloth are necessary.

Dry objectives—*e.g.* $\frac{2}{3}$ -in. and $\frac{1}{6}$ -in.—are cleaned with a piece of well-washed silk or fine cotton, or lens paper. If any oil or Canada balsam is accidentally present on the front lens it must be removed with a soft cloth moistened in benzol or xylol and the lens quickly dried with a soft cloth. On no account must the component parts of an objective be unscrewed.

DIRECTIONS FOR USING THE MICROSCOPE WITH CONDENSER AND OIL-IMMERSION LENS

Before commencing to examine a specimen, special attention must be paid to the following:

- (1) The objectives and eye-piece must be clean.
- (2) The draw-tube must be adjusted to the correct length.
- (3) The plane side of the mirror must be used.
- (4) The condenser must be properly fitted into the substage, so that it can be racked up practically flush with the stage. In microscopes where the condenser is inserted from below into a sleeve fitting, the condenser is often not properly pushed into place and cannot be racked up sufficiently high for its focus to be in the same horizontal plane as the specimen.

For bacteriological work it is recommended that artificial light should always be used. The 40- or 60-watt opal bulb lamp or high-intensity lamp described on p. 93 is employed according to the type of instrument and work to be carried out. It is not advisable to place the microscope at a window, as the daylight entering the eyes renders the vision less acute. A suitable arrangement is to use the microscope on a small table at one side of the room so that the observer's back is towards the window.

When examining an object, using a simple 40- or 60-watt opal bulb lamp, the manipulations of the microscope should be carried out in the following order:

- (1) Set up the microscope, place the object on the stage, and adjust the plane side of the mirror to the illuminant so that the light is reflected into the condenser.
- (2) Focus the specimen with the low-power objective, using the coarse adjustment.
- (3) Manipulate the mirror until the image of the illuminant is seen in the centre of the field; if the lamp has an iris diaphragm this should be closed and the mirror adjusted until the aperture of the iris is

concentric with the edge of the field. Rack the condenser up or down until the edge of the iris is sharply focussed.

It is essential, particularly when examining tissues, to use the low power first, in order to locate organisms and observe the tissue changes. A suitable field having been obtained, the slide must be kept in place by means of the right-hand clip if a mechanical stage is not used.

(4) Rack up the objective a short distance and place a drop of cedar-wood immersion oil on the portion of the specimen immediately below the objective.

(5) Raise the condenser so that its upper surface is practically level with the stage. (This is not necessary if the lamp iris has been focussed as in (3).)

(6) Make sure that the iris diaphragm of the substage condenser is widely open.

(7) Rotate the nose-piece until the oil-immersion lens is in position.

(8) With the eye at the level of the stage, lower the objective by means of the coarse adjustment *until the lens is seen just to touch the oil*.

(9) Apply the eye to the microscope, and observe if the field is well illuminated. If not, adjust the mirror until maximum illumination is secured.

(10) *Carefully* focus down, *using the coarse adjustment*, and when the object has come into view, use the fine adjustment to secure sharp definition. It is often necessary at this stage to raise or lower the condenser very slightly so that the optimum illumination is secured.

When the object is sharply focussed and the condenser is so adjusted that the image of the illuminant is seen in the field, the condition is termed *critical illumination*.

In using a binocular microscope the same directions should be observed, but, in addition, the eye-pieces should be adjusted to the correct interocular distance of the observer when the specimen is focussed with the low-power objective (*vide* direction No. 2).

Method of using a High-intensity Lamp

When a high-intensity lamp incorporating a condenser is used it should be remembered that this system has an optical axis which must be adjusted so that it is continuous with the optical axis of the microscope. To do this the Köhler method of illumination is recommended in which the image of the filament of the bulb is focussed by the lamp condenser on the iris diaphragm of the substage condenser of the microscope. The front lens of the lamp condenser now acts as the source of illumination, and it is focussed by the substage condenser in the plane of the preparation to be examined, the lamp iris diaphragm being used as a guide for this purpose.

(1) Rack up fully the substage condenser of the microscope and see that the blue glass supplied with the microscope is in the ring under the condenser.

(2) Set up the microscope and lamp so that the distance of the beam

of light from the iris diaphragm of the lamp by way of the mirror to the iris diaphragm of the condenser is about 10 in.

(3) Switch on the lamp, open the lamp iris fully and decrease the resistance so that there is a bright beam of light shining on the mirror. Adjust the lamp by altering its vertical tilt and by moving the base so that the beam of light is in the centre of the mirror.

(4) Focus the lamp condenser so that an image of the filament is formed on the closed substage iris of the microscope, and then open this iris diaphragm again. Once the correct setting is found, the lamp condenser needs no further adjustment.

(5) Pull back the resistance so that the light is much dimmed, place a preparation on the microscope stage, and focus it with the low-power (16-mm. or $\frac{2}{3}$ -in.) objective.

(6) Close the lamp iris and adjust the mirror so that the image of this iris is in the centre of the field.

(7) Focus the microscope condenser up or down until the image of the lamp iris is sharp, when the object is also in focus.

(8) Open up the lamp iris fully so that the whole of the field is illuminated.

In practice, these adjustments can be made in a few seconds. To obtain the maximum definition the lamp iris should be closed and focussed in the field for each objective. This will be possible with the 4-mm. ($\frac{1}{6}$ -in.) and the 3.5-mm. ($\frac{1}{7}$ -in.) oil-immersion objectives, but not with the 2-mm. ($\frac{1}{12}$ -in.) objective. For routine work, however, this is not necessary.

It is important to note (a) that before switching on the lamp the full resistance is in and then the illumination is increased as desired, and (b) that before switching off the lamp, the bulb is dimmed to its full amount. If these precautions are taken, the life of the bulb will be very much prolonged.

CENTRATION OF THE CONDENSER

If the condenser mount of the microscope possesses centring screws, the centration of the condenser with respect to the objectives must be checked from time to time as follows. After the microscope and the illuminant have been set up as previously described, close the condenser iris diaphragm to its limit. Rack down the condenser until the image of the condenser iris appears in the field. If it is not concentric, adjust the centring screws until it is so. It will be of advantage to open up the condenser iris until its aperture is almost that of the field for the final centration. Then open the iris diaphragm fully and rack up the condenser to its normal position.

When the observer has to examine a specimen for any length of time, as when searching for bacteria, he must adopt a comfortable position. Both forearms should rest on the table, and if there is no mechanical stage the slide is moved with the left hand while the right hand manipulates the fine adjustment.

EXAMINATION OF LIVING UNSTAINED ORGANISMS

✓ In the case of bacteria, "hanging-drop" preparations are frequently used for this purpose, and a glass slide having a circular concavity in the centre is employed.

There should be no difficulty in observing a satisfactory specimen if the following procedure is adopted:

(1) By means of a match dipped in vaseline, a ring or square (according to the shape and size of the cover-slip) is outlined round the concavity.

(2) With a wire loop (p. 248) place a drop of fluid containing the organisms on a cover-slip laid on the bench.

For this purpose a fluid culture is used or the condensation fluid of a slope culture (p. 247). A further alternative is to emulsify a small amount of culture from the surface of a solid medium in a drop of broth or normal saline, taking care that the emulsion is not too dense.

(3) Invert the slide over the cover-slip, allowing the glass to adhere to the vaseline, and quickly turn round the slide so that the cover-slip is uppermost. The drop should then be "hanging" from the cover-slip in the centre of the concavity.

(4) Place the slide on the microscope, rack down the condenser slightly and partially close the iris diaphragm. (Excessive illumination renders the organisms invisible.)

(5) With the low power, focus the edge of the drop so that it appears across the centre of the field.

(6) Turn the high-power ($\frac{1}{6}$ -in. or 4-mm.) lens into position and focus the edge of the drop. Obtain the best illumination by lowering or raising the condenser, and secure sharp definition by reducing the aperture of the diaphragm.

Instead of employing a hanging-drop preparation, a film of the fluid between an ordinary slide and cover-slip may be used, but in this case the edge of the cover-slip should be sealed with vaseline to prevent evaporation of the fluid.

Motility of organisms can be detected in this way, and their shape, approximate size and general structure can be observed. *It is advisable to use the high-power dry lens* and not the oil-immersion objective. Owing to the viscosity of the oil, the cover-slip is apt to move during focussing, and currents are thus caused in the fluid, which produce an appearance of motility in the organisms. ✓

It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid (p. 15).

A *warm stage* is very convenient when examining fresh unstained preparations for amoebae and other protozoa. There are several types of warm stage available, some of which consist of a thin, flat metal box filled with hot water, or through which warm water can circulate,

and having an aperture in the centre by which the light passes to the preparation. Improved forms are electrically heated and have an automatic temperature control. The warm stage keeps the preparation at body temperature, and enables the movements of organisms to be studied, as these movements may cease if the material is kept for any length of time at room temperature.

A simple warm stage may easily be improvised from a sheet of thin copper (18-gauge) shaped like the letter "T", with the long arm 5-6 in. in length. The top of the "T" is the size of a microscope slide (3 in. x 1 in.) and in the centre is an aperture $\frac{1}{2}$ in. in diameter. The copper "T" is placed on the microscope stage with the long arm projecting forward, and the aperture over the condenser. The preparation is placed on the copper strip and secured by the stage clips. The projecting part of the "T" is warmed by means of a small Bunsen flame or spirit lamp. Care must be taken that the preparation is not overheated.

DARK-GROUND ILLUMINATION

This method renders visible delicate organisms, such as the spirochaete of syphilis, which cannot be seen in unstained preparations with an ordinary microscope.

By means of a special condenser the specimen is illuminated by oblique light only. The rays do not enter the tube of the microscope, and, in consequence, do not reach the eye of the observer unless they are "scattered" by objects (e.g. bacteria) of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated on a dark background.

Three requisites are necessary for adapting a microscope for dark-ground illumination:

- (1) A "dark-ground" condenser.
- (2) A suitable illuminant of sufficient intensity.
- (3) A stop which reduces the numerical aperture of the objective to less than 1.0, *if the ordinary oil-immersion lens is used.*

The Condenser.—A special condenser must be employed and may be of the paraboloid or of the concentric spherical reflecting type. The latter is recommended. The function of the special condenser is to focus the light on the object, the paths of the rays being such that no direct light passes into the front of the lens. The illustration shows the paths of rays through the concentric reflecting condenser. The condenser should be furnished with a centring device, and it must be emphasised here that success with dark-ground illumination depends on the accurate centring of the condenser. There must be immersion oil between the slide and condenser.

The Illuminant.—A lamp of sufficiently powerful intensity should be employed.

If *Direct Current* only is available, the small arc lamp or the "pointolite" lamp (a proprietary name applied to a lamp consisting of two

tungsten electrodes in a vacuum across which an arc is struck) should be used. The "pointolite" is more convenient to use than the arc lamp.

Alternating Current is now almost universal, and high-intensity low-voltage lamps worked through a transformer and having a condensing lens are satisfactory for this purpose (p. 93). Complete lamps are obtainable from several makers.

The Funnel Stop.—When the objectives employed for dark-ground illumination have a numerical aperture of more than 1.0 (as in the

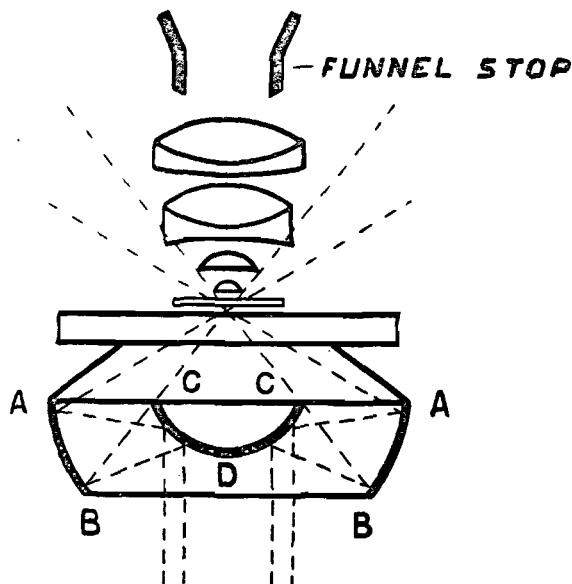


FIG. 5

Diagram showing the paths of rays through the condenser and a $\frac{1}{2}$ -in. oil-immersion lens fitted with a funnel stop. AB and CDC are reflecting surfaces. The surface at CC is opaque. (After E. Leitz.)

case of ordinary oil-immersion lenses), a special stop to reduce the N.A. to less than 1.0 must be employed. This consists of a small funnel-shaped piece of metal or vulcanite which fits into the objective behind the back lens. It is advisable to procure the stop from the maker of the lens employed. The stop is easily inserted and removed, and the objective can at once be converted for ordinary use.

Alternatively an *objective adaptor*, with a small iris diaphragm, may be used. The front part of the oil-immersion objective is removed and screwed to the adaptor, which then takes the place of the objective on the nose-piece. The numerical aperture of the objective may be reduced as desired by manipulating the iris diaphragm in the adaptor.

Some makers incorporate an iris diaphragm in the mount of the objective itself so that it can be used for bright or dark-ground illumination without further alteration.

Certain manufacturers have introduced for dark-ground illumination with bicentric condensers, special oil-immersion fluorite objectives, which are used without a funnel stop. These are $\frac{1}{2}$ -in. N.A. 1.15, and $\frac{1}{4}$ -in. N.A. 0.95. The latter lens (referred to on p. 81) can be recommended for routine dark-ground observation.

The Preparation!—The preparation should be as thin as possible in order to secure a satisfactory dark background, and so that the moving objects shall, as far as possible, be in one plane. A preparation which is too thick greatly diminishes the contrast in the dark field, and in order to obtain satisfactory contrast the objective has to be stopped down considerably, thus diminishing its resolving power. The preparation should not be too dense, otherwise there is an excessive number of particles which "scatter" the light. This causes lack of contrast even to a greater degree than a thick preparation. Some manufacturers supply special cells for dark-ground work so that when the cover-slip is placed over the cell the preparation has a definite and uniform thickness.

The thickness of the slide employed is important. The slides should be 1.0-1.1 mm. thick, and when a suitable supply has been obtained they should be used only for dark-ground work. They should be thoroughly clean and free from grease. The object to be examined must be at the focus of the condenser, the focal length of which is about 1.2 mm. If, therefore, too thick a slide is used, the focus of the condenser will be below the specimen and poor illumination will result; if the slide is too thin, the distance between the condenser and slide is such that a large amount of oil must be employed to make contact.

Method of using Dark-Ground Illumination with the Oil-Immersion Objective

The microscope with special condenser, and with the N.A. of the objective reduced by a funnel stop or iris diaphragm adaptor, is placed in front of the illuminant. It is advisable to have the microscope in the upright position and not inclined, to avoid running of the oil. The condensing lens of the lamp is adjusted so that a slightly converging beam of light is obtained. With the plane side of the mirror, direct the light into the dark-ground condenser. Using the low-power ($\frac{3}{4}$ -in. or 16 mm.) objective, focus the surface of the condenser so that the engraved concentric rings on the surface come into view. These rings show the centre of the condenser, and if the condenser is out of centre adjust the centring screws so that the rings become concentric with the edge of the field.

Should the condenser have no engraved rings the centring may be accomplished as follows.

A slide preparation is placed on the stage, and oil contact between it and the condenser established. The preparation is focussed with the $\frac{2}{3}$ -in. objective, and, if the mirror is properly adjusted, a bright ring of light is noticed in the field. Focus the condenser cautiously up or down so that the ring of light contracts to the smallest bright spot obtainable. If this spot of light is not in the centre of the field, alter the centring screws of the condenser accordingly.

The accurate centring of the condenser is of the utmost importance and the time spent in this manipulation will be amply rewarded by the brilliant illumination obtained. The preparation to be examined must be covered with a No. 1 cover-slip, and it is advisable to ring round the cover-slip with vaseline to prevent evaporation. Place a large drop of immersion oil upon the under surface of the slide and also on the upper lens of the condenser, and a similar drop on the cover-slip. Place the slide on the microscope stage, taking care that the upper surface of the condenser is well below the slide. Rack up the condenser until oil-contact is made between the whole surface of the upper lens of the condenser and the slide; then bring the oil-immersion lens into position so that it touches the oil on the cover-slip. Now carefully focus the specimen. A slight adjustment of the condenser, up or down, may be necessary, and some manipulation of the mirror may also be required. After a little practice an evenly illuminated field with an intensely dark background and brilliantly lit objects may be obtained with a minimum of trouble.

Where much dark-ground examination has to be done, it is recommended that a microscope be reserved solely for this work and kept ready with the illuminant in position, so that it is always available for immediate use. It is convenient to have the lamp and microscope fixed to a board for this purpose. The microscope, when not in use, should be covered to exclude dust.

After use, the condenser and objective should be carefully wiped free from oil.

Discarded preparations may be dropped into a covered jar of xylol (or benzol) kept for the purpose. When a sufficient quantity has been accumulated it will be found that they can easily be cleaned, as the oil and vaseline have been dissolved off by the xylol.

Dark-Ground Illumination with Low- and Medium-Power Lenses

Dark-ground illumination is easily obtained with a low-power lens whose numerical aperture does not exceed 0.3, e.g. the $\frac{2}{3}$ -in. objective, by placing a central patch or stop below the condenser. Most manufacturers supply a set of stops which fit into the ring below the iris diaphragm. Alternatively, a circle of glass with a central patch of black gummed-paper about 10–12 mm. in diameter may be used. The ordinary source of illumination is quite sufficient. Such dark-ground illumination may be used for observing slide-agglutination and

for cells, casts, etc., in urinary deposits. With the higher power dry lenses, however, it is not so easy to secure satisfactory dark-ground illumination unless special condensers are used. Some microscope manufacturers make dry dark-ground condensers to work with $\frac{1}{8}$ -in. objectives up to numerical apertures of 0.65, but these are expensive and usually require a high-intensity lamp to work satisfactorily. The results, however, are very beautiful.

Where the N.A. of the objective does not exceed 0.65, dark-ground illumination can be secured with an "achromatic" or "aplanatic" condenser (not Abbe condenser), an expanding iris or suitably large central stop being used. As such condensers are suitable for ordinary microscopy it is possible to change over from direct transmitted light to dark-ground illumination, without removing the condenser, by merely inserting the stop. A high-intensity illuminant is, however, necessary, and immersion oil is placed between the condenser and slide (*vide supra*). An intermediate objective adaptor with iris diaphragm is often of value in reducing the N.A. of the $\frac{1}{8}$ -in. objective sufficiently to obtain a uniform dark field.

MICROMETRY

In bacteriological work the unit of measurement is 0.001 mm., designated a *micron* or μ . The measurement of microscopic objects is accomplished by means of the stage micrometer in conjunction with a micrometer eye-piece. The stage micrometer consists of a 3×1 in. slide on which is a millimetre scale graduated in hundredths of a millimetre. This scale may be engraved, but is usually made by a photographic process. The micrometer eye-piece consists of a special eye-piece in which a graduated scale, mounted on the diaphragm, can be focussed by means of the movable eye-lens.

When measurements are to be made, the micrometer eye-piece is inserted into the draw-tube, the tube length is accurately noted, and the rulings on the stage micrometer focussed by the appropriate objective according to the size of the object to be measured. The number of divisions on the eye-piece scale corresponding to a definite number of divisions of the millimetre stage scale is determined. The stage micrometer is removed, and the object to be measured is next focussed. The number of divisions of the eye-piece scale which just cover the object are noted.

The millimetre value of each division of the eye-piece scale depends on the objective used and the tube length employed, and is usually determined each time a measurement is taken. Sometimes it is advisable to increase or diminish the draw-tube length so that the stage and eye-piece scales coincide or bear a geometric relation to each other—*e.g.* 1 division of the former to 10 of the latter.

Example : Using a $\frac{1}{2}$ -in. objective and a $6 \times$ micrometer eye-piece at 165 mm. tube length, it was found that 100 divisions on the eye-

piece scale exactly covered 11 divisions of the stage micrometer. Each division of the stage micrometer is $\frac{1}{100}$ mm.

$$\begin{aligned} 100 \text{ eye-piece divisions} &= 11 \text{ stage divisions} = 0.11 \text{ mm.} \\ 1 \text{ eye-piece division} &= 0.0011 \text{ mm.} \\ 1 \text{ eye-piece division, therefore, with the} \\ \text{given objective, eye-piece and tube} \\ \text{length} &= 1.1 \mu. \end{aligned}$$

The stage micrometer was removed and a stained slide of blood showing malaria crescents was substituted. The diameter of a red blood corpuscle covered 7 divisions of the eye-piece scale—*i.e.* 7.7μ . A polymorph leucocyte covered 11 divisions, while the length of a malaria crescent was equal to 10 divisions, showing the sizes of these objects to be 12.1μ and 11μ respectively.

If the draw-tube is so adjusted that 1 division of the stage micrometer equals 10 of the eye-piece scale, then each division of the latter corresponds to 1μ .

Photographic Method of Micrometry.—A more accurate method is to photograph a film of the organisms or cells under a high magnification. Without disturbing the microscope or camera, the slide is removed from the microscope stage and the stage micrometer substituted. A photograph of the stage micrometer is then taken at exactly the same magnification. By means of a pair of fine dividers the length of the organism on the print is taken, and its exact measurement found by applying this distance to the micrometer print.

FLUORESCENCE MICROSCOPY

When certain materials, *e.g.* vaseline, uranium ores or uranium glass, solutions of quinine, aesculin and various dyes, are exposed to ultra-violet (U.V.) light, they alter the wavelength of the invisible light and so become luminous and are said to fluoresce. If tissues or bacteria are treated with a fluorescent dye and examined under the microscope, ultra-violet light being used instead of ordinary visible light, they become luminous and are seen as bright objects in a dark field. Moreover, these fluorescent dyes may have a selective action on certain tissues or bacteria which are thus readily identified and recognised.

Dyes specially suited for fluorescence microscopy are auramine, coriphosphin O, morin, thioflavin S, thiazo yellow G, berberine sulphate, fuchsin, primuline, etc. They are used in very dilute solutions, 1 in 1000—1 in 10,000, and may be employed for double staining, *e.g.* fuchsin and coriphosphin.

For fluorescence microscopy the usual source of U.V. light is either a high-pressure mercury vapour lamp or direct-current carbon arc lamp suitably enclosed. As ordinary glass absorbs a considerable amount of U.V. light (particularly the shorter wavelengths) the condensing lenses of the lamp are made of quartz. Similarly, the microscope condenser

is made of quartz, while the microscope slide is of special glass which passes U.V. light. The visible rays from the lamp are cut out by a dense filter of "Wood's glass" which allows only the invisible U.V. rays to pass. As the Wood's glass also transmits very deep-red and infra-red rays, the light, before it passes the filter, goes through a solution of 4 per cent. copper sulphate to remove these rays. The rest of the microscope, *i.e.* objectives and eye-piece, are as ordinarily used since the U.V. rays, when they reach the specimen, cause it to fluoresce with visible light. A special filter is placed over the eye-piece to prevent any harmful effect of U.V. rays entering the eye.

It should be noted, however, that the absence of quartz lenses and condenser should not deter anyone from undertaking fluorescence microscopy, provided a satisfactory light source is available. Glass will transmit the long-wave U.V. rays, *i.e.* of wavelength greater than 300 m μ , and good fluorescent images may be obtained. With the quartz equipment, of course, much more U.V. light is transmitted, but for diagnostic use this equipment does not justify the extra expense, and the ordinary microscope condenser is sufficient, particularly if used in a completely darkened room.

It is important to use an adequate source of U.V. light, and a suitable mercury vapour lamp is the B.T.H. 250-watt, ME/D compact source 3-pin "MERCRA", used with a choke wired in series. The lamp is enclosed in a suitable metal box in which is incorporated a single convex lens to project the image of the mercury arc on to the mirror. Two filters are required, a deep blue in front of the lamp to cut out the visible light rays, and a yellow one in the eye-piece. These filters are complementary, the yellow absorbing completely any blue rays so that the background is black. The blue filter is two pieces of blue glass, Chance's O.B. 1 and O.B. 10, cemented together, and the yellow filter for the eye-piece consists of Wratten No. 15 and Chance's O.Y. 12. A simple but efficient blue filter consists of a solution of ammoniacal copper sulphate solution ($CuSO_4$ 7.8 gm., NH_4OH 30 ml., water 270 ml.) in a triangular Roux bottle. The yellow filter is Ilford Minus Blue (Micro 4). It is convenient to use the gelatin filter and cut a small circle to fit on the diaphragm inside the eye-piece. The mercury vapour lamp must be switched on and allowed to run for 10 minutes before commencing microscopic examination. The ordinary high-intensity lamp (p. 93) has been suggested for fluorescence microscopy, but it is too poor in U.V. light to be of practical value.

Fluorescence microscopy can be applied to the examination of the tubercle bacillus by substituting a solution of auramine for carbol fuchsin in the Ziehl-Neelsen method, *i.e.* the preparation is overstained with auramine and then decolorised with acid-alcohol. The tubercle bacilli still retain the stain when the rest of the material has lost it.

METHOD.—*Staining solution.*

Auramine "O"	0.3 g.
Phenol	3.0 g.
Distilled Water	97.0 ml.

Dissolve the phenol in water with gentle heat. Add the auramine gradually and shake vigorously until dissolved. Filter and store in a dark stoppered bottle.

Decolorising solution. 75 per cent. industrial alcohol containing 0.5 per cent. NaCl and 0.5 per cent. HCl.

Potassium Permanganate Solution. 1 in 1000.

Stain a thin smear of sputum with auramine solution for fifteen minutes. Rinse under the tap and decolorise for 5 mins, with the acid-alcohol. Wash well in tap water, apply permanganate solution for 30 secs., wash well in tap water and allow to dry. (Do not use blotting paper to dry.)

The film is examined dry with a $\frac{2}{3}$ -in. objective, or preferably with an 8-mm. objective and a high-power eye-piece. The tubercle bacilli are seen as yellow luminous organisms in a dark field. A darkened room is an advantage. It has been claimed by some workers¹ that this technique gives a higher percentage of positive results than the ordinary Ziehl-Neelsen method.

Immersion objectives can be used, but as ordinary cedar-wood immersion oil and practically all oils are fluorescent, only special non-fluorescent liquid paraffin, or sandal-wood oil, is permissible.

PHASE CONTRAST MICROSCOPY²

One of the difficulties of examining microscopically living, unstained biological specimens is that they are immersed in a fluid of almost the same refractive index as themselves. In order to see them distinctly it is necessary either (a) to close considerably the iris diaphragm, thereby reducing the numerical aperture, or (b) to use dark-ground illumination. The latter procedure is satisfactory with very small or slender objects such as spirochaetes, but its use in bacteriology is limited.

By means of phase contrast microscopy, it is possible to examine living cells with the full aperture of the objective. In consequence internal details are effectively brought out.³

Phase contrast microscopy can be used with any type of microscope, either monocular or binocular.

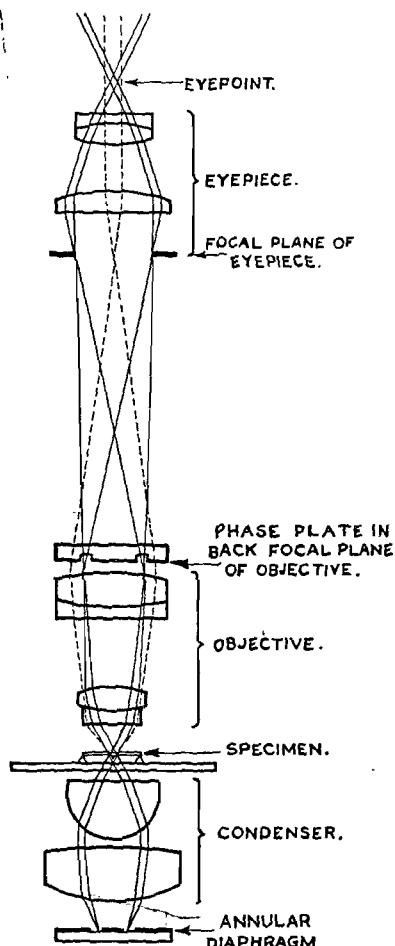
It is necessary to have:

- (1) A special condenser below which is a rotating metal disk carrying a series of annular diaphragms. These are disks of glass rendered opaque but with a narrow ring of clear glass. Each objective requires a different size of annulus according to its

¹ Richards, O. W., Kline, E. K. & Leach, R. E. (1941), *Amer. Rev. Tuberc.*, **44**, 255; Bogen, E. (1941), *ibid.*, **44**, 267; Oscarsson, P. N. (1941), *Acta med. scand.*, **108**, 240; Richards, O. W., & Miller, D. K. (1941), *Amer. J. clin. Path.*, **ii** (Technical section), 1; Lampert, H. (1944), *Lancet*, **2**, 818.

² Bennett, Jupnik, Osterberg & Richards (1951), *Phase Microscopy: Principles and Applications*, New York and London; Martin, L. C. (1951), *J. Quekett micr. Cl.*, Ser. 4, **3**, 237; Taylor, E. W. (1946), *J. roy. micr. Soc.*, **66**, 1.

numerical aperture; thus, for the 16-mm. objective the ring is narrow and about $4\frac{1}{2}$ mm. in diameter, whereas for the 2-mm. objective it is wider and about 18 mm. in diameter. The size of the annulus is such that the condenser forms an image of it in the back focal plane of the objective.



SOLID LINES REPRESENT DIRECT RAYS.
DOTTED LINES REPRESENT DIFFRACTED RAYS.

FIG. 6

Diagram illustrating the paths of light rays
in phase contrast microscopy

Reproduced by permission of American Optical Company

- (2) Special phase objectives. These are ordinary objectives at the back of which, *i.e.* in its back focal plane, is inserted a phase plate consisting of a disk of glass having a circular trough etched in it and of such a depth that the light after passing through it has a phase difference of a quarter of a wave-length compared with the rest of the plate (*vide diagram*, p. 104).

The objective is focussed on the specimen. The appropriate annulus for the objective is rotated into position under the condenser. The condenser is then focussed so that the image of the annulus is superimposed on the phase plate at the back of the objective. A special magnifier (supplied with the outfit) is inserted in place of the eye-piece and through it the back focal plane of the objective is observed. The annulus and phase ring should coincide. If not exactly coincident the centring screws under the condenser are adjusted to achieve this. The eye-piece is now re-inserted and the specimen examined.

All powers of the microscope can be used, provided that each objective has its own phase plate fitted, and there is an appropriate annulus for it below the condenser.

The principle briefly is as follows. If a diffraction grating is examined under the microscope, diffraction spectra are formed in the back focal plane of the objective. The detail observed in the image is due to interference between the direct and diffracted beams. Unstained objects such as bacteria or cells may be considered as similar to a diffraction grating; that is, the detail consists of alternate strips of material with slightly different refractive indices, through which light acquires small phase differences, and these form the image. With ordinary illumination, however, such slight differences are almost completely obscured by the intensity of the direct light beam, and hardly any detail can be observed at all.

As will be seen from the diagram, the direct light from the annular diaphragm passes only through the trough in the phase plate. The diffracted beam having a slightly different path goes through the thicker glass of the phase plate outside the trough and in consequence is retarded one-quarter of a wavelength with respect to the direct beam. When these two beams (direct and diffracted) unite they are not in phase, and these phase differences are apparent as appreciable changes in intensity. The details of the object stand out sharply and distinctly on a grey background, and being observed at full aperture, there is maximum resolution.

As there is a great difference in intensity between the direct beam and the diffracted beam, the trough of the phase plate through which the direct beam passes is covered with a light-absorbing material, usually a thin deposit of silver or other metal, so that the intensity of the direct beam is much reduced and approaches that of the diffracted beam. In consequence of this, and as the illumination is much restricted by the narrow annular diaphragm, a high-intensity lamp must be used.

Phase contrast microscopy is most valuable in general biology, but has less application in bacteriological work. It is useful, however, in examining the growth and subdivision of bacteria, flagellar movement, intestinal and other protozoa, such as amoebae, *Trichomonas*, etc., and living blood cells.

ELECTRON MICROSCOPE

Within recent years the electron microscope has been used to demonstrate details of structure of bacteria and viruses that are far beyond the power of resolution of the ordinary light microscope.¹ The instrument is, however, costly, elaborate and technically difficult to maintain.

The principles of its construction are comparatively easy to understand. The resolution, and hence the degree of magnification of a microscope, depends on the wavelength of light used (p. 87). It has been shown that electrons have an equivalent wavelength inversely proportional to their speed, and the particular wavelength used in the electron microscope is 1/100,000 that of ordinary light so that high resolution and great magnification are possible. The electron microscope consists of a source of electrons—the "electron gun"—which, in order to obtain electrons of high speed, has an accelerating or plate voltage of 50,000, stabilised to an accuracy of ± 1 volt. Higher voltages up to 100,000 are employed in the newer instruments. The electrons pass through a series of electro-magnetic fields which act as lenses and cause the necessary convergence of the electron beam. There are three such magnetic lens systems corresponding to the condenser, objective and eye-piece of the light microscope.

The material to be examined is mounted on a collodion membrane (which must not be more than 1μ thick), supported by a metal gauze screen, and placed in the path of the electrons, the whole being maintained in a high vacuum, of the order of 10^{-4} to 10^{-6} mm. of mercury, to prevent the electrons being deflected by air molecules.

The shadow image is first examined visually on a fluorescent screen, and recorded photographically as required. Owing to the high resolving power of the microscope, the photographic image may be enlarged many times. It is usual to take the negatives at 10,000–20,000 diameters and enlarge these up to 100,000 diameters. Particles one hundred times smaller than those resolvable by an ordinary microscope have been photographed, and in the latest models it has been possible to resolve particles of 10 Ångstrom units ($1 m\mu$).

¹ Cosslett, V. E. (1951), *Practical Electron Microscopy*. London, Butterworth. (This book includes the important references to literature on the subject.)

CHAPTER VII

STAINING METHODS

As bacteria consist of clear protoplasmic matter, differing but slightly in refractive index from the medium in which they are growing, it is difficult with the ordinary microscope, except when special methods of illumination are used, to see them in the unstained condition. Staining, therefore, is of primary importance for the recognition of bacteria.

The use and general principles of bacterial staining have been discussed in Chapter I.

METHODS OF MAKING FILM OR SMEAR PREPARATIONS

Before describing the various staining processes, details of the methods employed in making films must be considered.

Film preparations are made either on cover-slips or on the ordinary 3×1 in. glass slides, usually the latter. It is essential that the cover-slips or slides should be perfectly clean and free from grease, otherwise uneven films will result.

Cover-slips.—These should be $\frac{3}{4}$ or $\frac{7}{8}$ in. square, and of No. 1 thickness. (Thicker cover-slips—No. 2—may prevent the oil-immersion objective from coming near enough for the specimen to be focussed.) They are cleaned by placing them in a mixture of nitric acid, 6 parts; potassium bichromate, 6 parts; water, 100 parts. They should be dropped one by one into the fluid. The solution is contained in an evaporating dish and boiled. Alternatively, they may be cleaned in the dichromate-sulphuric acid solution (p. 293) by the method described for slides on p. 108. The cover-slips are then well washed, first in tap water and later in distilled water, and stored in a stoppered jar in 50 per cent. alcohol. Before use they are dried with a soft clean cloth, such as an old handkerchief. For routine use, the cover-slips may be sufficiently clean as supplied by the maker and require only to be wiped free of grit and dust with a clean dry cloth.

Slides.—These may be treated in a manner similar to cover-slips. A quicker and quite satisfactory method for ordinary routine use is to wipe the slide with a clean dry cotton cloth and, holding its end with forceps, roast it free of grease by passing it 6–12 times through a blue Bunsen flame. The heating should be as strong as is possible without cracking the slide. Cracking is rendered less likely by allowing the slide to cool somewhat before laying down, or by laying it on a warmed metal rack. Another method of cleaning is to moisten the finger with water, rub it on the surface of some fine sand soap such as "Bon ami", and then

smear the surface of the slide. After removing the soapy film with a clean cloth the surface is clean and free from grease. For special purposes, such as the staining of flagella, slides are cleaned with hot dichromate-sulphuric acid solution followed by flaming, as described on p. 124. If the slide is perfectly clean a drop of water can be spread over its surface in a thin even film; otherwise the water collects into small drops and a film cannot be made.

After the films have been made and examined the slides should be discarded. They should not be cleaned and used again, since it is difficult to ensure that all organisms are removed.

In the case of fluid material, e.g. broth cultures, urine, sputum, pus, etc., one loopful (or more) is taken up with the inoculating wire (p. 248) and spread thinly on the slide. A little experience will soon determine the amount required, and in spreading the films it will be found that there are both thick and thin portions, which is not disadvantageous. The slide is then held in the palm of the hand high over a Bunsen flame and dried. The film is fixed by passing the *dried* slide three times slowly through the flame, or by heating through the glass slide. In the latter method the slide is held, film upwards, in the top of the Bunsen flame for a few seconds so that the slide becomes hot. Care must be taken not to char the film, and when the slide is just too hot to be borne on the back of the hand, fixation is complete.

In making films on cover-slips and staining them, Cornet's forceps is used to hold the slip in a horizontal position, the forceps resting on the bench.

Films on cover-slips require a minimum of time for fixing owing to the thinness of the glass.

With solid material, such as cultures on agar, etc., it is necessary to place a loopful of clean water on the slide. The loop is then sterilised and a minute quantity of material, obtained by just touching the growth, is transferred to the drop, thoroughly emulsified, and the mixture is spread evenly on the slide. The resulting film is fixed and dried as above. *Beginners are very apt to take more material than necessary from the culture and thus make too thick films.*

STAINING OF FILMS

The stains are poured directly or filtered on to the slide. When staining is completed, the dye is washed off with water, and the slide is allowed to dry in the vertical position or is placed between two sheets of white fluffless blotting-paper or filter-paper. The drying of the film is completed over the Bunsen flame. Such stained films may be mounted in Canada balsam under a cover-slip, or may be examined unmounted with the oil-immersion lens, a small drop of cedar-wood oil being placed directly on the film. If it is desired to mount the preparation later, the oil can be removed with xylol.¹

¹ Benzol may be substituted for xylol.

STAINING OF TISSUE SECTIONS

The sections being embedded in paraffin (p. 144), it is necessary to remove the paraffin so that a watery stain may penetrate. The paraffin is first removed with xylol, the xylol removed with alcohol,¹ and the alcohol replaced by water. The staining process is then proceeded with. After staining, the section must be dehydrated with absolute alcohol, then cleared in xylol and finally mounted in Canada balsam under a cover-slip. The Canada balsam (which is a resin) is dissolved in xylol in order to render it of suitable consistency.

Technique.—The slide bearing the paraffin section is placed in a jar of xylol for some minutes to remove the paraffin. The section is then treated with a few drops of absolute alcohol, when it immediately becomes opaque. A few drops of 50 per cent. alcohol are poured on, and the slide is finally washed gently in water. If the tissue has been fixed in any mercuric chloride preparation, such as Zenker's fluid, the section should be treated with Gram's iodine solution for a few minutes (p. 115), then with 95 per cent. alcohol and finally water. The sections are now ready to be stained by the appropriate method. After staining and washing with water, the slide is wiped all round the section with a clean cloth to remove excess of water. The bulk of the water in the section may be removed by pressing between fluffless blotting-paper. The section is treated *immediately* with a few drops of 95 per cent. alcohol, then absolute alcohol. The slide is again wiped all round the section, a few more drops of absolute alcohol are poured on and the slide is then immersed in xylol. When cleared, the slide is removed, and excess of xylol round the section is wiped away, a drop of Canada balsam is applied and the section mounted under a No. 1 cover-slip. It is essential that the section should not be allowed to dry at any period of the process, and that dehydration with absolute alcohol should be complete in order that the section may be thoroughly cleared.

When the bacteria are readily decolorised by alcohol, aniline-xylol (aniline, 2 parts; xylol, 1 part) should be used for dehydration. After washing, when the slide has been wiped round the section, the preparation is blotted and then treated with the aniline-xylol mixture, which clears as well as dehydrates. The aniline-xylol is then replaced by xylol. This can be done conveniently by holding the slide almost vertically and dropping xylol from a drop bottle on to the slide just above the section. The xylol flows over the section and quickly removes the aniline. The preparation is mounted immediately in Canada balsam.

¹ Industrial methylated spirit (not mineralised) may be used for making up stains, decolorising, dehydration of tissues and treatment of sections instead of rectified spirit. The type known as "Toilet spirit, acetone free (66 O.P.)", is quite satisfactory. Similarly, industrial methylated spirit, absolute (74 O.P.), can be used instead of absolute alcohol for staining, dehydration and histological technique. Not only are these industrial spirits much cheaper than rectified spirit and absolute alcohol, but permits for obtaining them duty-free are more readily granted by the Customs Authorities.

D.P.X. Mounting Medium

A mounting medium which replaces Canada balsam has been devised by Kirkpatrick and Lendrum.¹ It consists of polystyrene (a synthetic resin) dissolved in xylol, with a plasticiser—dibutyl phthalate—to ensure flexibility. There is, however, much shrinkage and the mounting fluid should be applied generously. The mountant termed D.P.X. is made up as follows:

Mix dibutyl phthalate (B.D.H.)	5 ml.
with pure xylol	35 ml.
and dissolve "Distrene 80 " ²	10 g.

D.P.X. medium is water-clear, inert and does not become acid or cause fading of stained preparations. It is used in the same way as Canada balsam.

If polystyrene of a low molecular weight (about 3000) is used, much less xylol is required and no plasticiser need be added. Moreover, there is practically no shrinkage, which is a great advantage over D.P.X.

SIMPLE STAINS

These show not only the presence of organisms but also the nature of the cellular content in exudates.

METHYLENE BLUE

Of the many preparations of this dye, Löffler's methylene blue is generally the most useful:

Saturated solution of methylene blue in alcohol	30 ml.
KOH, 0·01 per cent. in water	100 ml.

Films.—Stain for three minutes, then wash with water. This preparation does not readily over-stain.

Sections.—Stain for five minutes or longer. The application of the alcohol during dehydration is sufficient for differentiation. Aniline-xylol can also be used for dehydration and clearing.

POLYCHROME METHYLENE BLUE

This is made by allowing Löffler's methylene blue to "ripen" slowly. The stain is kept in bottles, which are half filled and shaken at intervals to aerate thoroughly the contents. The slow oxidation of the methylene blue forms a violet compound which gives the stain its polychrome properties. The ripening takes twelve months or more to

¹ Kirkpatrick, J., & Lendrum, A. C. (1939); *J. Path. Bact.*, **49**, 592; (1941), *ibid.*, **53**, 441.

² Normally obtainable from Messrs. Honeywill & Stein, Ltd., 21 St. James's Square, London, S.W.1.

complete. The preparation is used in a manner similar to Löffler's methylene blue; it is employed in McFadyean's reaction (p. 560). Similar staining results are obtained with

Borax Methylene Blue (Masson)

Methylene blue	10 g.
Borax	25 g.
Water	500 ml.

Warm the water to 60° C., stir in the solids, and allow to cool slowly. This staining solution improves with age.

DILUTE CARBOL FUCHSIN

Made by diluting Ziehl-Neelsen's stain (p. 117) with ten to fifteen times its volume of water. Stain for ten to twenty-five seconds and wash well with water. Over-staining must be avoided, as this is an intense stain, and prolonged application colours the cell protoplasm in addition to nuclei and bacteria.

NEGATIVE STAINING

"Negative Staining" is exemplified by Burri's India ink method which was formerly used for the spirochaete of syphilis. A small quantity of India ink is mixed on a slide with the culture or other material containing bacteria, and then by means of another slide or loop a thin film is made, allowed to dry and examined. The bacteria or spirochaetes are seen as clear transparent objects on a dark-brown background. (See also India ink methods for capsules, p. 122.)

FLEMING'S NIGROSIN METHOD

A 10 per cent. solution of nigrosin (G. T. Gurr) is made in warm distilled water (solution is effected in about an hour) and filtered. Formalin 0.5 per cent. is added as a preservative. This keeps indefinitely. A small drop of the dye is placed on a slide, bacteria are mixed with it and a smear is made either with the loop or with another slide. (A number of preparations can be made on the same slide.) Alternatively, a film of bacteria is made on a slide in the ordinary manner and fixed by heat. A drop of nigrosin is placed at one edge of the slide and spread by means of another slide over the bacterial film. Dry and examine. If mounted in Canada balsam under a cover-slip, the preparation is permanent.

Nigrosin gives an absolutely homogeneous background, and this is the simplest method of making a preliminary examination of a culture to show shape, size and arrangement of bacteria.

Most bacteria stand out as clear objects on a dark field, but some bacilli, such as those of the coliform and haemophilic groups, show in

- their central portion a slightly dark patch somewhat resembling a nucleus. This is attributed to the fact that in drying they develop a shallow depression in which some of the nigrosin lies.

The method is of value in the preliminary examination of the spore-bearing anaerobes. In these the spores are larger than the bacilli, so that when the nigrosin film is slightly thicker than usual the spores stand out as bright clear spaces while the bacillary bodies are slightly overlaid with the nigrosin.

GRAM'S STAINING METHOD

This is one of the most important methods in bacteriology, and must be employed for the diagnostic identification of various organisms. The principle of the method has been dealt with in Chapter I.

Certain bacteria when treated with one of the para-rosaniline dyes such as methyl-violet or gentian-violet, and then with iodine, "fix" the stain so that subsequent treatment with a decolorising agent—e.g. alcohol or acetone—does not remove the colour. Other organisms, however, are decolorised by this process. If a mixture of various organisms were thus stained and subjected to the decolorising agent, it would be found that some species retain the dye, and these are termed "Gram-positive", whereas others are completely decolorised and are designated "Gram-negative". In order to render the decolorised organisms visible, and to distinguish them from those retaining the colour, a contrast or counter-stain is then applied. This contrast stain is usually red, in order that the Gram-negative organisms may easily be differentiated from the Gram-positive organisms, which retain the original violet stain. Gram's method can be carried out with the basic para-rosaniline dyes only—e.g. methyl-violet, crystal-violet, gentian-violet (which is a mixture of the two preceding dyes) and victoria blue. Methyl-violet and gentian-violet are the usual stains employed, but crystal-violet is a purer stain and can be used to advantage instead of the former. The iodine solution should not be kept too long, but used shortly after being made.

JENSEN'S MODIFICATION

This modification can be recommended for routine bacteriological work.

Solutions required:

- (1) Methyl-violet, 6 B.¹ 0.5 per cent. solution in distilled water.

(The solution should be made up in bulk and filtered. It keeps indefinitely, and does not precipitate, but should be filtered again before use.)

¹ Crystal-violet in the same proportion is recommended as an alternative.

(2) Iodine Solution (Lugol's iodine)¹:

Iodine	1 g.
Potassium iodide	:	:	:	:	:	2 g.
Distilled water	100 ml.

Note that the iodine solution is three times stronger than the original Gram's iodine.

(3) Counter-stain—Neutral Red Solution:

Neutral red	1 g.
1 per cent. acetic acid	:	:	:	:	:	2 ml.
Distilled water	1 l.

Films

These are made, dried and fixed in the usual way.

(1) Pour on methyl-violet solution and allow to act for twenty to thirty seconds.

(2) Pour off excess of stain and, holding the slide at an angle downwards, pour on the iodine solution so that it washes away the methyl-violet. Allow the iodine to act for a half to one minute.

(3) Wash off the iodine with alcohol, and treat with fresh alcohol until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.

(4) Wash with water.

(5) Apply the counter-stain for two to four minutes.

(6) Wash with water and dry between blotting-paper.

This method is very simple and gives excellent results with freedom from deposit.

Dilute carbol fuchsin (1: 15) applied for twenty to thirty seconds may be substituted with advantage as a counter-stain for routine work, but for demonstrating the gonococcus and other intracellular Gram-negative bacteria the neutral-red counter-stain should be used.

Other counter-stains are basic fuchsin 0.05–0.1 per cent., or safranine 0.5 per cent., in distilled water.

For the gonococcus and meningococcus in films, *Sandiford's counter-stain* is useful, particularly when the organisms are scanty.

Malachite green	0.05 g.
Pyronine	0.15 g.
Distilled water	to 100 ml.

¹ Iodine solution does not keep well and it is convenient, especially where stains are distributed from a central source, to have potassium iodide and iodine mixed ready for solution when required. Potassium iodide tends to be hygroscopic and must be dried, otherwise the mixture becomes sticky and lumpy. Place the potassium iodide in a thin layer in a Petri dish overnight in a desiccator over calcium chloride. Mix two parts of potassium iodide by weight with one part of iodine in a mortar. Weigh out at once amounts of 7.5 grams and place them in 1-oz. screw-capped bottles (p. 185) and screw down the caps. This is sufficient for 250 ml. of solution. The mixture keeps indefinitely and easily "pours" from the bottle. For use, place the contents of one bottle into an empty 10-oz. screw-capped bottle. Add about 50 ml. distilled water and agitate until the iodine is dissolved. Make up to 250 ml. with distilled water.

(The stain keeps for about a month only.) Apply the counter-stain for two minutes, flood off with water (but do not wash) and blot. Cells and nuclei stain bluish green. Gram-positive organisms are purple-black and gonococci red. It should be noted that not all samples of pyronine are satisfactory for this stain, so that with each new purchase of pyronine the made-up stain should be tested on a film known to contain gonococci or meningococci.

KOPELOFF AND BEERMAN'S MODIFICATION¹

The following method has been found useful for class work and is also recommended for routine purposes.

Films

- (1) Make a thin film, dry and fix in the usual way with the minimum amount of heating.

Stains:

1 per cent. aqueous solution of methyl-violet,
6 B. 30 parts
5 per cent. solution of sodium bicarbonate . . . 8 parts

Allow to remain on the slide for five minutes or more.

(The above solution is apt to precipitate within a few days; it acts quite well without the addition of the bicarbonate solution and will then keep indefinitely.)

- (3) Wash off excess of stain with iodine solution and allow to act for two minutes.

Iodine Solution:

Iodine 2 g.
Normal solution of sodium hydroxide . . 10 ml.
Distilled water 90 ml.

- (4) Drain off the excess of iodine and add acetone (100 per cent.) drop by drop until no colour is seen in the washings.

(It should be noted that decolorisation is very rapid, requiring ten seconds or less, and the time should be reduced to a minimum.)

- (5) Wash slide in water.
(6) Counter-stain for ten to thirty seconds with 0.05 per cent. aqueous solution of basic fuchsin.

- (7) Wash in water, blot and dry in the air.

Sections

- (1) Remove paraffin with xylol or benzol.
 - (2) Treat the section with alcohol and wash in water.
 - (3) Flood with the stain and allow to act for five minutes.
 - (4) Wash off excess of stain with the iodine solution and allow to act for two minutes.

¹ Kopeloff, N., & Beerman, P. (1922-1923), *Proc. Soc. exp. Biol. (N.Y.)*, 20, 71.

- (5) Decolorise with acetone (*vide supra*).
- (6) Wash slide in water.
- (7) Counter-stain for ten to thirty seconds.
- (8) Wipe carefully around the section to remove as much water as possible, dehydrate quickly in absolute alcohol, clear in xylol or benzol and mount in Canada balsam or D.P.X.

WEIGERT'S MODIFICATION

Solutions employed:

(1) Carbol Gentian-violet¹:

Saturated alcoholic solution of gentian-violet	1 part
5 per cent. solution of phenol in distilled water	10 parts

(This mixture should be made up each day, as it tends to precipitate.)

(2) Gram's Iodine²:

Iodine	1 g.
Potassium iodide	2 g.
Distilled water	300 ml.

(3) Aniline-xylol:

Aniline	2 parts
Xylol	1 part

(4) Dilute Carbol fuchsin:

Ziehl-Neelsen's carbol fuchsin (p. 117)	1 part
Distilled water	9 parts

PROCEDURE

Films

These are made, dried and fixed in the usual manner.

(1) Stain with carbol gentian-violet (two to three minutes).

(2) Pour off stain, replace with Gram's iodine solution and allow to act for one minute.

(3) Dry thoroughly by blotting.

(4) Decolorise with aniline-xylol, using several changes until the stain ceases to be removed.

Breathing on the slide after the first application of aniline hastens decolorisation.

Now examine at this stage under the low power of the microscope; the nuclei of the pus cells should be of a pale-violet colour; if the nuclei are deeply stained, then decolorisation is incomplete.

(5) Wash with several changes of xylol and dry.

(6) Counter-stain with dilute carbol fuchsin, ten to twenty-five seconds. Wash with water and dry.

¹ Alternatively, 0·5 per cent. solution of crystal-violet or methyl-violet 6 B. in distilled water may be used. This keeps well and is preferable to the original formula given above.

² See footnote on p. 113.

Sections

After removing the paraffin with xylol or benzol, treating with alcohol and washing with water, counter-stain first with carmalum¹ for ten minutes and then proceed as above. After (5) the sections will be cleared and can at once be mounted in Canada balsam or D.P.X.

Note.—If Gram's method is properly carried out, Gram-positive organisms and fibrin are stained dark violet in colour. Gram-negative organisms, the nuclei and protoplasm of pus cells and tissue cells are stained pink with the counter-stain. To obviate errors from over-decolorising, a control film of a known Gram-positive organism (e.g. a pure culture of *Staphylococcus aureus*) may be made at one side of the film to be examined. For the recognition of Gram-negative organisms such as gonococci or meningococci in pus, this control must retain the violet stain while the nuclei of the pus cells are stained only with the counter-stain.

STAINING OF TUBERCLE AND OTHER ACID-FAST BACILLI

ZIEHL-NEELSEN METHOD

The ordinary aniline dye solutions do not readily penetrate the substance of the tubercle bacillus and are therefore unsuitable for staining it. However, by the use of a powerful staining solution which contains phenol, and the application of heat, the dye can be made to penetrate the bacillus. Once stained, the tubercle bacillus will withstand the action of powerful decolorising agents for a considerable time and thus still retains the stain when everything else in the microscopic preparation has been decolorised.

The stain used consists of basic fuchsin, with phenol added. The dye is basic and its combination with a mineral acid produces a compound which is yellowish brown in colour and is readily dissolved out of all structures except acid-fast bacteria. Any strong acid can be used as a decolorising agent; but 20 per cent. sulphuric acid (by volume) is usually employed. Acid-alcohol (p. 118) may also be used.

In order to show what has been decolorised, and to form a contrast with the red-stained bacilli, the preparation is counter-stained with methylene blue.

Malachite green is also recommended as a counter-stain in the Ziehl-Neelsen method. A stock solution of 1 per cent. in distilled water is made, and for use a small quantity is diluted with distilled water in a drop-bottle so that fifteen to twenty seconds' application of the weak stain gives the background a pale green tint. Deep counter-staining must be avoided. The pale green background is pleasant for the eyes,

¹ Carmalum: carminic acid, 1 gram; potassium alum, 10 grams; distilled water, 200 ml.; dissolve with gentle heat; filter and add formalin, 1 ml., as preservative.

and is thought by some workers to make scanty organisms more easily noticed. This counter-stain is required for the method described on p. 546, where the use of a deep blue-green filter is recommended for the easy recognition of tubercle bacilli.

Ziehl-Neelsen's (strong) Carbol Fuchsin:

Basic fuchsin	1 g.
Absolute alcohol	10 ml.
Solution of phenol (5 per cent. in water)	100 ml.

Dissolve the dye in the alcohol and add to the phenol solution.

An alternative and quicker preparation is as follows:

Basic fuchsin (powder)	5 g.
Phenol (cryst.)	25 g.
Alcohol (95 per cent. or absolute)	50 ml.
Distilled water	500 ml.

Dissolve the fuchsin in the phenol by placing them in a one-litre flask over a boiling water-bath for about five minutes, shaking the contents from time to time. When there is complete solution add the alcohol and mix thoroughly. Then add the distilled water. Filter the mixture before use.

Films

These are made, dried and fixed in the usual manner:

(1) Flood the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for five minutes, heat being applied at intervals to keep the stain hot. The stain must not be allowed to evaporate and dry on the slide.

(2) Wash with water.

(3) Pour on the slide 20 per cent. sulphuric acid from a drop-bottle. The red colour of the preparation is changed to yellowish brown. After about a minute in the acid, wash the slide with water, and drop on more acid. This process is repeated several times. The object of the washing is to remove the compound of acid with stain and allow fresh acid to gain access to the preparation. The decolorisation is finished when, after washing, the film is a faint pink.

(4) Wash the slide well in water.

(5) Treat with 95 per cent. alcohol for two minutes.

(6) Wash with water.

(7) Counter-stain with Löffler's methylene blue or dilute malachite green for fifteen to twenty seconds.

(8) Wash, blot, dry and mount.

Acid-fast bacilli stain bright red, while the tissue cells and other organisms are stained blue or green according to the counter-stain used.

Note.—The practice of using staining jars in the Ziehl-Neelsen method is to be condemned, as with a positive sputum, stained tubercle bacilli may become detached and float about in the staining fluid or decolorising agent. After a number of strongly positive films have been passed through the staining jars the number of free stained tubercle

bacilli may be considerable. Negative material may, during the staining process, pick up these bacilli and so appear positive when examined microscopically. These false positives can give rise to serious errors of diagnosis. Each slide, therefore, should be stained individually by pouring on the stain from a drop-bottle, the washing done by a stream of tap water and the subsequent decolorising and staining fluid also added to the film from drop-bottles. When drying with blotting-paper, a fresh clean piece of paper is used for each slide and then discarded. The practice of using a number of large sheets for drying a succession of slides is also condemned as tubercle bacilli from a positive film may adhere to the blotting-paper and subsequently be transferred to a negative film.

Other organisms met with in diagnostic work are "acid-fast" in addition to the tubercle bacillus, e.g. the smegma bacillus which may be found in samples of urine. Treatment with alcohol in addition to acid may, however, decolorise this type of organism, whereas the tubercle bacillus is both acid- and alcohol-fast. The decolorisation with alcohol is therefore important when examining urine for the presence of the tubercle bacillus.

Instead of employing 20 per cent. sulphuric acid as a decolorising agent, 3 per cent. hydrochloric acid in 95 per cent. alcohol (industrial methylated spirit) may be used. The necessity for subsequent treatment with alcohol as in the original method is obviated. The time of decolorisation is longer with the acid-alcohol than with sulphuric acid, but the former is much less corrosive and more convenient to make up and employ, while its use definitely excludes organisms which are acid-fast but not alcohol-fast.

It should be noted that in films stained by Ziehl-Neelsen's method, red-stained organisms in the midst of hyaline material must not be regarded as tubercle bacilli, as such material may be resistant to decolorisation.

Sections

Sections are treated with xylol to remove paraffin, then with alcohol, and finally washed in water.

(1) Stain with Ziehl-Neelsen's stain as described for films, but heat gently, otherwise the section may become detached from the slide.

(2) Wash with water.

(3) Decolorise with 20 per cent. sulphuric acid or acid-alcohol as for films. The process takes longer owing to the thickness of the section, and care must be exercised in washing to retain the section on the slide.

(4) Wash well with water.

(5) Counter-stain with methylene blue or malachite green for a half to one minute.

(6) Wash with water.

(7) Wipe the slide dry all round the section, blot with filter-paper or fluffless blotting-paper, and treat with a few drops of absolute alcohol. Pour on more absolute alcohol, wipe the slide again and clear in xylol.

(8) Mount in Canada balsam or D.P.X.

Leprosy bacilli are also acid-fast, but usually to a lesser degree than the tubercle bacillus. They are stained in films or sections in the same way as the tubercle bacillus, except that 5 per cent. sulphuric acid is used for decolorisation.

STAINING OF DIPHTHERIA BACILLUS AND VOLUTIN-CONTAINING ORGANISMS

The diphtheria bacillus gives its characteristic volutin-staining reactions best in a young culture (eighteen to twenty-four hours) on a blood or serum medium.

ALBERT'S METHOD

Laybourn's modification,¹ in which malachite green is substituted for methyl green, is given here instead of the original method.

Solution 1:

Toluidine blue	0.15 g.
Malachite green	0.2 g.
Glacial acetic acid	1 ml.
Alcohol (95 per cent.)	2 ml.
Distilled water	100 ml.

Dissolve the dyes in the alcohol and add to the water and acetic acid.

Allow to stand for one day and then filter.

Solution 2:

Iodine	2 g.
Potassium iodide	3 g.
Distilled water	300 ml.

Note.—The iodine solution used in Jensen's modification of Gram's method (p. 112) works equally well.

Technique

Make film, dry and fix by heat.

Apply Solution 1 for three to five minutes.

Wash in water and blot dry.

Apply Solution 2 for one minute.

Wash and blot dry.

By this method the granules stain bluish black, the protoplasm green and other organisms mostly light green.

This method can be recommended for routine use.

¹ Laybourn, R. L. (1924), *J. Amer. med. Ass.*, 83, 121.

NEISSER'S METHOD (Modified)

The following modification of Neisser's method gives better results than the original:

Neisser's methylene blue stain:

Methylene blue	1 g.
Ethyl alcohol (95 per cent.)	50 ml.
Glacial acetic acid	50 ml.
Distilled water	1 l.

(1) Stain with Neisser's methylene blue for three minutes.

(2) Wash off with dilute iodine solution (iodine solution of Kopeloff and Beerman's modification of Gram's method, p. 114, diluted 1 in 10 with water) and leave some of this solution on the slide for one minute.

(3) Wash in water.

(4) Counter-stain with neutral red solution for three minutes, using the same solution as that employed in Jensen's modification of Gram's method (p. 112).

(5) Wash in water and dry.

By this method the bacilli exhibit deep blue granules, the remainder of the organism assuming a pink colour.

LINDEGREN'S METHOD

The volutin inclusion granules of bacteria, yeasts and other microbes stain intensely with basic dyes even under conditions so acid, e.g. pH 2.5, as to prevent staining of the other cell constituents.

Staining Solution:

Saturated aqueous toluidine blue	4.0 ml.
Glacial acetic acid	1.5 ml.
Formalin (40 per cent. formaldehyde)	20.0 ml.

Method

Place a loopful of the cells in a tube with 10 drops of the stain and stand overnight. Centrifuge and mount a loopful of the deposited cells between slide and cover-slip. Alternatively, with easily staining organisms, mount the cells directly in a drop of stain on a slide and apply a cover-slip. The granules appear purple and the rest of the cell practically colourless.

STAINING OF SPORES

If spore-bearing organisms are stained by ordinary dyes, or by Gram's stain, the body of the bacillus is deeply coloured, whereas the spore is unstained and appears as a clear area in the organism. This is the way in which spores are most commonly observed. If desired, however, it is possible by vigorous staining procedures to introduce dye

into the substance of the spore. When thus stained, the spore tends to retain the dye after treatment with decolorising agents, and in this respect behaves similarly to the tubercle bacillus (q.v.).

ACID-FAST STAIN FOR SPORES

Films, which must be thin, are made, dried and fixed in the usual manner with the minimum amount of heating.

(1) Stain with Ziehl-Neelsen's carbol fuchsin (p. 117) for three to five minutes, heating the preparation until steam rises.

(2) Wash in water.

(3) Treat with $\frac{1}{4}$ or $\frac{1}{2}$ per cent. sulphuric acid for one to several minutes, the period being determined by trial for each culture. Alternatively, excellent results are obtained by decolorising in a 2 per cent. solution of nitric acid in absolute ethyl alcohol; the slide is dipped once rapidly in the solution and immediately washed in water.

(4) Wash with water.

(5) Counter-stain with 1 per cent. aqueous methylene blue for three minutes.

(6) Wash in water, blot and dry.

The spores are stained bright red and the protoplasm of the bacilli blue.

It should be noted that the spores of some bacteria are decolorised more readily than those of others and that lipid inclusion granules may stain dark red, appearing like small spherical spores.

Nigrosin method (see also p. 111).—As an alternative to counter-staining with methylene blue in the acid-fast staining method for spores, a drop of 10 per cent. nigrosin solution may be spread thinly over the dried, decolorised film with the edge of another slide. This provides a dark background which outlines the unstained bacillary bodies.

MALACHITE GREEN STAIN FOR SPORES

(*Schaeffer and Fulton's Method*, modified by Ashby¹)

Films are dried and fixed with minimal flaming.

(1) Place the slide over a beaker of boiling water, resting it on the rim with the bacterial film uppermost.

(2) When, within several seconds, large droplets have condensed on the underside of the slide, flood it with a 5 per cent. solution of malachite green and leave to act for one minute while the water continues to boil.

(3) Wash in cold water.

(4) Treat with 0.5 per cent. safranine or 0.05 per cent. basic fuchsin for thirty seconds.

(5) Wash and dry.

This method colours the spores green and the vegetative bacilli red. Lipid granules are unstained.

¹ Ashby, G. K. (1938), *Science*, **87**, 443.

STAINING OF CAPSULES

The capsules of bacteria present in animal tissues, blood, serous fluids and pus are often clearly stained when these materials are treated by one of the common stains such as basic fuchsin, polychrome methylene blue, Leishman's stain (p. 130) and Gram's stain (which colours them with the red counter-stain). Special capsule stains may be of little advantage in such cases. On the other hand, when artificial cultures of bacteria are being examined, the capsules normally are not coloured by ordinary staining methods and special methods must be employed for their demonstration, e.g. "negative" or "relief" staining.

DEMONSTRATION OF CAPSULES IN DRY INDIA INK FILMS¹

- (1) Place a loopful of 6 per cent. glucose in water at one end of a slide. Add a small amount of bacterial culture to this and mix to form an even suspension. Add a loopful of India ink to the drop, and mix.
- (2) Spread the mixture over the slide in a thin film with the edge of a second glass slide. Dry thoroughly by waving in the air.
- (3) Fix the film by pouring over it some undiluted Leishman stain or methyl alcohol. Drain off excess at once and dry thoroughly by warming over a flame.
- (4) Drop on methyl violet solution as used in Gram's stain, and stain for one or two minutes. Wash in water. Blot and dry over a flame.
- (5) Examine directly with the oil-immersion objective.

DEMONSTRATION OF CAPSULES IN WET INDIA INK FILMS

If a permanent preparation is required for demonstration of bacterial capsules, it is necessary that a dry-film method should be employed, as described above; otherwise capsules are best observed in very thin wet films of India ink.² This latter is the simplest, most informative and most generally applicable method of capsule demonstration. The capsules do not become shrunken since they are not dried or fixed and they are clearly apparent even when very narrow.

A microscope slide is carefully wiped free of grit particles. A loopful of India ink is placed on it. A small portion of solid bacterial culture is emulsified in the drop of ink, or else a loopful of a liquid culture is mixed with the ink. A clean cover-slip is placed on the ink drop; it is pressed down firmly through a sheet of blotting-paper, so that the ink film becomes very thin and thus pale in colour. The film should be so thin that the bacterial cell with its capsule is "gripped" between the slide and cover-slip, neither being overlaid by ink nor capable of moving about.

On microscopical examination with the oil-immersion objective

¹ Butt, E. M., Bonynge, C. W., & Joyce, R. L. (1936), *J. infect. Dis.*, **58**, 5.

² Duguid, J. P. (1951), *J. Path. Bact.*, **63**, 673.

the highly refractile outline of the bacterium is seen (the bacterial cells can be seen more clearly if a phase-contrast microscope is used). Between this refractile surface-membrane and the dark background of ink particles there is a clear space which represents the bacterial capsule; the capsular zone may be from a fraction of a micron to several microns in width. Non-capsulated bacteria do not show this clear zone; the ink particles directly abut the refractile cell wall and, in consequence, these bacteria are not easily seen. When solid bacterial culture is newly mixed with the ink, any *loose slime* in it can be seen as irregular strands and masses, lighter than the ink, which gradually disperse from the bacteria and dissolve in the ink.

Note.—Sometimes a bottle of India ink becomes contaminated with a capsulated saprophytic bacterium. To avoid error from this cause, a film of the ink alone should be examined microscopically and proved free from capsulated bacteria.

DEMONSTRATION OF CAPSULES BY RELIEF STAINING WITH EOSIN¹

Staining solution:

10 per cent. water-soluble eosin, "yellowish" or "bluish", or erythrosin in distilled water	4 parts
Serum (human, rabbit, sheep or ox, heated at 56° C. for thirty minutes)	1 part
Crystal of thymol.	

Allow the mixture to stand at room temperature for several days. Centrifuge and store the supernatant fluid at room temperature; it will keep for several months.

On a slide with a 1-mm. diameter wire loop, mix one drop of exudate (or fluid culture, or a suspension in *broth* from an agar slope culture) with one drop of Ziehl-Neelsen's carbol fuchsin stain diluted 1: 5, and allow to stain for half a minute. Then add one drop of the eosin solution and leave for about one minute. Spread a film with cigarette paper (like a blood film). Allow to dry (do not heat), and examine with the oil-immersion objective.

If intense coloration of the bacterium is not essential, the preliminary staining with dilute carbol fuchsin may be omitted. Films of capsulated organisms prepared by this method show a practically homogeneous red background with an unstained capsular area prominently shown, and the bodies of the organisms stained red of about the same intensity as the background or slightly darker. The capsules are thus seen by "relief staining". If free slime is present in the culture, it is often seen as a light granular or fibrous deposit distributed throughout the red background between the bacilli.

¹ Howie, J. W., & Kirkpatrick, J. (1934), *J. Path. Bact.*, 39, 165.

DEMONSTRATION OF FLAGELLA

Because of their extreme thinness, flagella are best demonstrated by the electron microscope in metal-shadowed films. They can also be demonstrated by the light microscope, using special staining methods which require most careful attention to details of technique. To enable resolution, the flagella must be thickened at least tenfold by a superficial deposition of stain. In spite of this, their characteristic arrangement and wave form should be distinguishable.

STAINING OF FLAGELLA BY LEIFSON'S METHOD

(Modification used at Microbiology Department,
Lister Institute of Preventive Medicine)

The stain, basic fuchsin with tannic acid, is deposited on the bacteria and flagella from an evaporating alcoholic solution. The degree of staining is controlled by an exact determination of the duration of exposure. Good results depend to a large extent on the preliminary thorough cleaning and flaming of the glass slides.

(1) Clean the slides with absolute alcohol, rubbing with a fine cotton cloth. Then immerse them in concentrated sulphuric acid saturated with potassium dichromate (p. 293) for several days at room temperature or for an hour at 90° C. (In the latter case it is advisable to place the beaker of solution in a strong metal container with sand while heating.) *During all subsequent stages until staining is complete, take care not to finger the slides, even at their edges, and do not let them touch surfaces not properly cleaned and grease-free.* Using forceps, transfer the slides to cleaned Coplin jars in which they will be kept for rinsing, drying and storing; do not overcrowd. Rinse thoroughly with tap water and finally with distilled water. Allow to drain and dry in air with the jar inverted on clean blotting-paper. Store with the jar closed to prevent contamination by air-borne dust. Just before use, flame the slide for a few seconds, passing it with each face downwards about six times through a blue Bunsen flame. Place on a clean warmed metal rack and allow to cool. Mark or number the slide with a diamond while holding with forceps.

(2) Fix the broth culture, or saline suspension of an agar culture, by adding formalin to give a final formaldehyde concentration of 1-2 per cent. (w/v). Sediment the bacilli by centrifuging at 2000-3000 r.p.m., preferably in a horizontal centrifuge. Decant the supernatant liquid and gently resuspend the bacilli in distilled water by rotating the tube alternately in opposite directions, rolling it between the palms of the hand. Centrifuge again and gently resuspend in fresh distilled water so as to obtain a final suspension which is only slightly cloudy (e.g. equal to Brown's opacity tube No. 1, see p. 305). With a flamed platinum loop, place a large loopful of the suspension on the prepared slide and

gently spread over an area 1–2 cm. in diameter. Allow to dry in air at room temperature or in an incubator at 37° C. Do not fix film.

(3) The stain is prepared as follows:

Tannic acid	10 g.
Sodium chloride	:	:	:	:	:	:	5 g.
Basic fuchsin	4 g.

Thoroughly mix the powdered ingredients in these proportions and store dry in a stoppered container. Prepare the solution by adding 1.9 g of the powder mixture to 33 ml. of 95 per cent. ethyl alcohol and, when mostly dissolved (*e.g.* in ten minutes), adding distilled water to make a final volume of 100 ml. Adjust the pH to 5.0 (at least within 0.2) by addition of NaOH or HCl, using a pH meter (p. 279). Store the solution in a stoppered bottle in the refrigerator at 3°–5° C., where it may remain stable for several weeks.

Alternatively, prepare three stock solutions: (1) tannic acid, 3.0 per cent. (w/v) in water with 0.2 per cent. (w/v) phenol as preservative; (2) sodium chloride, 1.5 per cent. (w/v) in water; and (3) basic fuchsin, 1.2 per cent. (w/v) in 95 per cent. ethyl alcohol. (The basic fuchsin must have a pH of 5.0; it may be compounded thus by mixing one part of pararosaniline hydrochloride with three parts of pararosaniline acetate. Allow several hours for solution in the alcohol.) Mix the three solutions in exactly equal proportions to prepare the stain.

(4) Place the prepared slide horizontally on a carefully levelled staining rack. Pipette exactly 1 ml. stain on to the slide so that it covers the whole surface. Leave at room temperature for exactly the required time, using a stop watch. Several similar preparations should be stained for different times, *e.g.* for six, eight, ten and twelve minutes, so that the best may be chosen. The optimal duration of staining will vary with the batch of stain, the room temperature and other factors; the apparent thickness of the flagella increases with the duration of staining. Rinse off the stain gently by placing the slide under a slowly running water tap; do not pour off stain before rinsing. Counter-stain with methylene blue, *e.g.* with borax methylene blue (p. 111) for thirty minutes, to colour the bacterial protoplast. Wash with water, rinse with distilled water, drain, dry in air and examine by oil-immersion.

STAINING OF INTRACELLULAR LIPID

WITH SUDAN BLACK

BURDON'S METHOD

Sudan black stain:

Sudan black B powder	0.3 g.
70 per cent. ethyl alcohol	:	:	:	:	:	100 ml.

Shake thoroughly at intervals and stand overnight before use. Keep in a well-stoppered bottle.

Method

- (1) Make a film, dry in air and fix by flaming.
 - (2) Cover the entire slide with Sudan black stain and leave at room temperature for fifteen minutes.
 - (3) Drain off excess stain, blot and dry in air.
 - (4) Rinse thoroughly with xylol and again blot dry.
 - (5) Counter-stain lightly by covering with 0.5 per cent. aqueous safranine or dilute carbol fuchsin (p. 111) for five to ten seconds; rinse with tap water, blot and dry.
- Lipid inclusion granules are stained blue-black or blue-grey, while the bacterial cytoplasm is stained light pink.

STAINING OF CELL POLYSACCHARIDES BY THE PERIODIC ACID SCHIFF (PAS) METHOD OF HOTCHKISS

The polysaccharide constituents of bacteria and fungi are oxidised by periodate to form polyaldehydes which yield red-coloured compounds with Schiff's fuchsin-sulphite; the proteins and nucleic acids remain uncoloured. The method may be used to reveal fungal elements in sections of infected animal tissue; the fungi stain red, while the tissue materials, except glycogen and mucin, fail to take the stain.

Periodate Solution.—Dissolve 0.8 gram periodic acid in 20 ml. distilled water; add 10 ml. of 0.2 M sodium acetate and 70 ml. ethyl alcohol. The solution may be used for several days if protected from undue exposure to light.

Reducing Rinse.—Dissolve 10 grams potassium iodide and 10 grams sodium thiosulphate pentahydrate in 200 ml. distilled water. Add, with stirring, 300 ml. ethyl alcohol, and then 5 ml. of 2 N hydrochloric acid. The sulphur which slowly precipitates may be allowed to settle out.

Fuchsin-Sulphite Solution.—Dissolve 2 grams basic fuchsin in 400 ml. boiling water, cool to 50° C. and filter. To the filtrate add 10 ml. of 2 N hydrochloric acid and 4 grams potassium metabisulphite. Stopper and leave in a dark cool place overnight. Add 1 gram decolourising charcoal, mix and filter promptly. Add up to 10 ml. or more 2 N hydrochloric acid until the mixture when drying in a thin film on glass does not become pink. Preserved in the dark and well stoppered, the stain remains effective for several weeks.

Sulphite Wash.—Add 2 grams potassium metabisulphite and 5 ml. concentrated hydrochloric acid to 500 ml. distilled water. This should be freshly prepared.

Procedure

- (1) Dry films in air and fix by flaming. For sections, fix tissue with usual fixatives; bring to 70 per cent. ethyl alcohol and wash thoroughly with this.

(2) Treat with periodate solution for five minutes at room temperature. Rinse with 70 per cent. alcohol.

(3) Treat with reducing rinse for five minutes. Rinse with 70 per cent. alcohol.

(4) Stain with fuchsin-sulphite for fifteen to forty-five minutes.

(5) Wash twice or thrice with sulphite wash solution. Wash with water.

(6) Counter-stain, if desired, with dilute aqueous malachite green (e.g. 0.002 gram per 100 ml.).

(7) Wash with water. Dehydrate and mount by the usual methods.

Control sections are prepared similarly, omitting step (2).

Unless easily soluble polysaccharides such as glycogen are to be demonstrated, the method may be simplified by substituting distilled water for the alcohol in the periodate solution and tap water for rinsing in steps (1)-(3), e.g. see below.

MODIFIED PAS STAIN FOR FUNGI IN TISSUE SECTIONS

(1) Bring sections to distilled water.

(2) Treat for five minutes with a freshly prepared 1 per cent. solution of periodic acid in water.

(3) Wash in running tap water for fifteen minutes and rinse in distilled water.

(4) Stain with fuchsin-sulphite for fifteen minutes.

(5) Wash twice with sulphite wash solution for five minutes.

(6) Wash in running tap water for five minutes and rinse in distilled water.

(7) Counter-stain with dilute aqueous malachite green or with 0.1 per cent. light green in 90 per cent. alcohol for one minute.

(8) Dehydrate rapidly in absolute alcohol, clear in benzol and mount in Canada balsam.

IMPRESSION PREPARATIONS

These have been used in the morphological study of the pleuro-pneumonia group of organisms¹ and of "rough" and "smooth" colonies of various bacteria.²

The essential part of the technique is to remove a small slab about 2 mm. thick of the solid medium (e.g. serum-agar) on which the organism is growing and place it colony downwards on a cover-slip. The whole is immersed in fixative, so that the fixing fluid penetrates through the agar to reach the colony. When the bacteria are fixed, the agar is removed carefully from the cover-slip which is well washed for two hours in distilled water, suitably stained and mounted. As fixative

¹ Klieneberger, E. (1934), *J. Path. Bact.*, **39**, 409.

² Bisset, K. A. (1938), *J. Path. Bact.*, **47**, 223.

Bouin's fluid (p. 144) may be used, or Flemming's solution.¹ For staining, methylene blue or dilute carbol fuchsin may be employed, but Giemsa's stain, applied by the slow method (p. 133), is the most satisfactory for the pleuropneumonia organism. The agar slabs, after fixation, may also be embedded, and vertical sections of the colony cut with a microtome.

DEMONSTRATION OF NUCLEAR MATERIAL IN BACTERIA

ROBINOW'S METHOD²

Robinow has shown that chromatinic structures can be differentiated from the protoplasm if osmic-acid fixed cells are first treated with 1 *N* HCl at 60° C. and then stained with Giemsa's solution.

METHOD

Fixation

Cut a small square from an agar plate on which the organisms are growing in a thin layer and place it in a deep dish (well sealed with a greased glass plate) in which 5 ml. of 2 per cent. osmium tetroxide, wetting three layers of glass balls, produces a strong concentration of osmic vapour. Expose the agar for two to three minutes in the vapour.

Place the square face downwards on a clean cover-slip, remove the agar, dry the film of fixed bacteria deposited on the cover-slip and fix in warm alcohol-mercuric-chloride (Schaudinn's fluid, p. 144) for five minutes. Wash in water and store in 70 per cent. alcohol.

(Note.—If osmium tetroxide is not available, a simpler method of fixation is to immerse the agar square, bacteria-carrying side uppermost, in a shallow layer of methyl alcohol for five minutes. The agar block is dried in air before pressing on a slide or cover-slip for transfer of the bacteria. Secondary fixation in Schaudinn's fluid may be omitted.)

Staining

Transfer films from 70 per cent. alcohol to 1 *N* HCl at 60° C. for ten minutes to "hydrolyse". Rinse in tap water and twice in distilled water and float on a staining solution made with 2-3 drops of Giemsa stain³ per ml. of phosphate buffer (p. 130). Stain for thirty minutes at 37° C., rinse and mount in water, and examine at once. This method shows the chromatinic structures quite clearly.

¹ Osmic acid, 0.1 per cent.; chromic acid, 0.2 per cent.; glacial acetic acid, 0.1 per cent. The osmic and chromic acids, when mixed, will keep only for three to four weeks, while the acetic acid should only be added immediately before use.

² Robinow, C. F. (1944), *J. Hyg. (Lond.)*, **43**, 413; Dubos, R. J. (1949), *The Bacterial Cell*. Cambridge, Mass.; Harvard Univ. Press. Addendum.

³ G. T. Gurr's R66 Giemsa stain.

If sealed with wax, water mounted preparations will keep their colour contrast for a few days.

If the film is to be mounted in Canada balsam or similar mountant, stain in Giemsa solution for several hours, wash, dehydrate for a few seconds in acetone 14 parts, xylol 6 parts; then acetone 6 parts, xylol 14 parts, then 3 changes of ten minutes each in xylol, and mount in Canada balsam.

Feulgen staining of desoxyribonucleic acid in the nuclear bodies may be effected by staining with Schiff's fuchsin-sulphite (p. 126) for one hour at 15–20° C. instead of staining with the Giemsa stain in the above method.

To demonstrate the *cell wall*, make impression preparations fixed in Bouin's fluid, as described on p. 127. Mordant for twenty to thirty minutes with 5–10 per cent. tannic acid and stain with 0.02 per cent. crystal violet in water for about one minute. Mount in water.

To demonstrate the *cytoplasm*, make impression preparations fixed in Bouin's fluid and stain briefly in 0.01 per cent. thionine, dilute Giemsa's stain or 0.1 per cent. methylene blue.

THE ROMANOWSKY STAINS

The original Romanowsky stain was made by dissolving in methyl alcohol the compound formed by the interaction of watery solutions of eosin and zinc-free methylene blue. The original stain has now been replaced by various modifications which are easier to use and give better results; these are: Leishman's, Wright's, Jenner's and Giemsa's stains. The peculiar property of the Romanowsky stains is that they impart a reddish-purple colour to the chromatin of malaria and other parasites. This colour is due to a substance which forms when methylene blue is "ripened", either by age, as in polychrome methylene blue, or by heating with sodium carbonate. The latter method is employed in the manufacture of Leishman's and Wright's stains. The ripened methylene blue is mixed with a solution of water-soluble eosin, when a precipitate, due to the combination of these dyes, is formed. The precipitate is washed with distilled water, dried and dissolved in pure methyl alcohol.¹ Each modification of the Romanowsky stain varies according to the "ripening" and the relative proportions of methylene blue and eosin.

According to the nature of the microscopic preparation, different stains are employed. Thus, for the cytological examination of blood, Jenner's stain may be used, but Leishman's stain is now generally employed; for the malaria parasite and trypanosomes, Leishman's and Wright's modifications give the best results, while the pathogenic spirochaetes (particularly the *Treponema pallidum* of syphilis) and certain protozoa can be demonstrated best by Giemsa's stain.

¹ The methyl alcohol must be "pure, for analysis", and have a pH of 6.5. If too acid, the reaction must be adjusted (with the usual indicators) by the addition of 0.01 N NaOH.

The Romanowsky stains are usually diluted for staining purposes with distilled water, when a precipitate is formed which is removed by subsequent washing.

LEISHMAN'S STAIN

This stain may be purchased ready for use or made by dissolving 0.15 gram of Leishman's powder in 100 ml. pure methyl alcohol. The powder is ground in a mortar with a little methyl alcohol, the residue of undissolved stain allowed to settle and the fluid decanted into a bottle. The residue in the mortar is treated with more methyl alcohol, and the process is repeated until all the stain goes into solution. The remainder of the methyl alcohol is now added. The stain can be used within an hour or two of making.

Films

Dry unfixed films are used. The stain is first used undiluted, and the methyl alcohol fixes the film. The stain is then diluted with distilled water, and the staining proper carried out.

(1) Pour the undiluted stain on the unfixed film and allow it to act for one minute.

(2) By means of a pipette and rubber teat add double the volume of distilled water to the slide, mixing the fluids by alternately sucking them up in the pipette and expelling them. Allow the diluted stain to act for twelve minutes.

(3) Flood the slide gently with distilled water, allowing the preparation to differentiate in the distilled water until the film appears bright pink in colour—usually about half a minute.

(4) Remove the excess of water with blotting-paper and dry in the air.

It is important that the reaction of the distilled water be neither acid nor alkaline. Any slight variations from neutrality may alter considerably the colour of granules in white blood corpuscles, etc., and give rise to supposed "pathological" appearances in cells which are really normal. A simple method of ensuring a suitable reaction of the distilled water is to keep large bottles of it—e.g. aspirator bottles—specially for these stains. Add 2 or 3 drops of 1 per cent. aqueous neutral-red solution. The usual reaction of distilled water is slightly acid, and a few drops of 1 per cent. sodium carbonate solution should be added until the solution shows the faintest possible suggestion of pink colour.

Much trouble will be eliminated if a buffer solution is used instead of distilled water for diluting the stain and washing the slide. It is made as follows:

Na ₂ HPO ₄ (anhydrous)	5.447 g.
KH ₂ PO ₄	4.752 g.

Mix together in a mortar and keep as such. The buffer mixture is quite stable.

Add 1 gram of buffer mixture to 2 litres of distilled water and this gives a pH of 7.0, which is suitable for most work.
Some samples of stain may require a slightly more acid solution, of pH 6.8. For this mix

Na_2HPO_4 (anhydrous)	4.539 g.
KH_2PO_4	5.940 g.

Add 1 gram of the mixture to 2 litres of distilled water.

Note.—When staining is excessively bluish, as in old films, good differentiation is obtained by brief washing with 1 per cent. NaH_2PO_4 .

Shute¹ maintains that fifteen seconds' fixation with the undiluted stain is sufficient and that only four drops of stain are necessary. The slide is rocked for twelve to fifteen seconds and then eight to twelve drops of water are added and thoroughly mixed. Staining proceeds for fifteen minutes and the diluted stain is flooded off in two to three seconds only. If washed for longer, Schüffner's dots will not be seen. Shute advocates a pH of 7.2 for the diluting fluid.

For demonstrating Schüffner's dots in Benign Tertian Malaria the use of Giemsa's stain following Leishman's stain has been recommended as follows²:

Fix thin blood film with Leishman's stain for fifteen to sixty seconds. Dilute with twice the volume of buffer solution at pH 7.0 and stain for fifteen minutes.

Wash off with dilute Giemsa's stain (*e.g.* G. T. Gurr's R66)—1 drop of stain to 1 ml. buffer solution at pH 7.0—and stain with this for a further thirty minutes.

Wash with buffer solution.

Blot and dry.

Sections

(1) Treat the section with xylol to remove the paraffin, then with alcohol and finally distilled water.

(2) Drain off the excess of water and stain for five to ten minutes with a mixture of 1 part Leishman's stain and 2 parts of distilled water or buffer solution.

(3) Wash with distilled water.

(4) Differentiate with a weak solution of acetic acid (1: 1500), controlling the differentiation under the low power of the microscope until the protoplasm of the cells is pink and only the nuclei are blue.

(5) Wash with distilled water or buffer solution.

(6) Blot, dehydrate with a few drops of absolute alcohol, clear in xylol and mount in Canada balsam or preferably D.P.X. mounting medium (p. 110).

Note.—If the eosin tint is too pronounced, it can be lightened by the use of very dilute caustic soda solution (1: 7000) which is washed off whenever the desired colour has been obtained.

¹ Shute, P. G. (1950), *Trans. roy. Soc. trop. Med. Hyg.*, 43, 364.

² Dinscombe, G. (1945), *Brit. med. J.*, 1, 298.

J. H. WRIGHT'S STAIN

This is similar to Leishman's stain and is used more in America than in this country. It should be purchased ready for use. The method of staining is, for all practical purposes, the same as for Leishman's stain.

GIEMSA'S STAIN

This consists of a number of compounds made by mixing different proportions of methylene blue and eosin. These have been designated Azur I, Azur II and Azur II-eosin. The preparation can be purchased made up, but batches may vary considerably.

We can recommend the following method of preparation devised by Lillie,¹ which gives consistent and reliable results. It is excellent for staining blood films for malaria parasites, and also mouse or rat blood for trypanosomes.

(1) *Azure B Eosinate*.—Dissolve 10 grams methylene blue in 600 ml. distilled water. Add 6.0 ml. concentrated sulphuric acid. Bring to the boil and add 2.5 grams potassium bichromate dissolved in 25 ml. distilled water. Boil for twenty minutes. Cool to 10° C. or lower (place in refrigerator overnight). When cold add 21 grams dry sodium bicarbonate slowly with frequent shaking. Then add a 5 per cent. solution of eosin (yellowish) and shake constantly until the margin of the fluid appears pale blue or bluish-pink. About 205 ml. will be required, and 150 ml. of this can be added at once. Filter immediately, preferably on a vacuum funnel with hard paper. When the fluid has been drawn through and the surface begins to crack, add 50 ml. distilled water. Allow to drain, and wash again with a second 50 ml. distilled water. Now wash with 40 ml. alcohol (95 per cent.) and repeat with a second 40 ml. alcohol. Dry the precipitate at room temperature or 37° C. (not higher). This constitutes Azure B eosinate.

(2) *Azure A Eosinate*.—Proceed exactly as above, but use 5.0 grams potassium bichromate (in place of 2.5 grams) and dissolve it in 50 ml. distilled water.

(3) *Methylene Blue Eosinate*.—Dissolve 10 grams methylene blue in 600 ml. cold distilled water and precipitate as before with 5 per cent. eosin solution, filtering and drying as above.

To make the finished stain, grind the three eosinates separately into fine powder in separate clean mortars. Then weigh out 500 mg. azure B eosinate, 100 mg. azure A eosinate, 400 mg. methylene blue eosinate, and 200 mg. finely ground methylene blue. Decant the mixed powder on to the surface of 200 ml. solvent, allowing it to settle in gradually. Then shake frequently for two or three days, keeping the bottle between 50° and 60° C. between shakings. The solvent consists of equal volumes of methyl alcohol (A.R.) and glycerol

¹ Lillie, R. D. (1943), *U.S. publ. hth Rep.*, 58, 449.

(A.R.). The proportion of stains given above should yield a satisfactory staining picture. The diluting fluid is buffer solution pH 7.0 (p. 130).

This stain may be used in a manner somewhat similar to Leishman's preparation (the "rapid method"), or prolonged staining may be carried out, as, for example, in staining spirochaetes (the "slow method"). In both cases the preparation must be fixed prior to staining, either by methyl alcohol for three minutes, or by absolute alcohol for fifteen minutes.

RAPID METHOD

- (1) Fix films in methyl alcohol—three minutes.
- (2) Stain in a mixture of 1 part stain and 10 parts buffer solution pH 7.0 for one hour.
- (3) Wash with buffer solution, allowing the preparation to differentiate for about half a minute.

(4) Blot and allow to dry in the air.

This method of staining gives excellent results with thin blood films for malaria parasites, Schüffner's dots being well defined. Trypanosomes are also well demonstrated.

A rapid method with the application of heat is useful for demonstrating spirochaetes.

Fix preparations with absolute alcohol (fifteen minutes) or by drawing three times through a flame. Prepare a fresh solution of 10 drops of Giemsa's solution with 10 ml. of buffer solution of pH 7.0 (p. 130), shake gently and cover the fixed film with the diluted stain. Warm till steam rises, allow to cool for fifteen seconds, then pour off and replace by fresh stain and heat again. Repeat the procedure four or five times, wash in distilled water, dry and mount.

SLOW METHOD

This is a specially valuable method for demonstrating objects difficult to stain in the ordinary way, e.g. certain pathogenic spirochaetes. The principle is to allow the diluted stain to act for a considerable period. As the mixture of stain and water causes a fine precipitate, care has to be taken that this does not deposit on the film.

Cover-slips.—The film is fixed in methyl alcohol for three minutes. A mixture is made in a Petri dish in the proportion of 1 ml. of stain to 20 ml. of buffer solution, pH 7.0. The cover-slip, when fixed and still wet with the alcohol, is placed carefully, film downwards, on the surface of the mixture. When properly done, the cover-slip remains floating. The lid is carefully placed on the Petri dish and the stain allowed to act overnight. The cover-slip is then washed in a stream of buffer solution, allowed to dry in the air and mounted. There should be no deposit of precipitated stain on the preparation.

Slides.—The film is fixed in methyl alcohol for three minutes as with cover-slips. The mixture of stain and buffer solution is made in a

large (6-in.) Petri dish if there are several slides to stain. A piece of thin glass rod is placed in the Petri dish, and the slides, after fixing, are laid film downwards in the fluid with one end of the slide resting on the glass rod so that there is sufficient staining fluid between the film and the bottom of the dish.

A sheet of glass slightly curved is also convenient; the slide, with the film downward, lies across the concavity, the space between containing diluted stain. The curved plate and slides should be placed in a box with a close-fitting lid to avoid evaporation.

After sixteen to twenty-four hours' staining, the slides are washed and dried as in the case of cover-slips.

Adachi's Modification.—This method has been utilised for staining the flagella of *Spirillum minus* (p. 730) and can also be applied in the staining of delicate spirochaetes. Fix the preparation for thirty to sixty seconds by osmotic acid vapour over the following solution: osmotic acid, 1 gram; distilled water, 100 ml.; 10 drops of 5 per cent. mercuric chloride; and then stain overnight in dilute Giemsa's solution (*vide supra*) to each 10 ml. of which 0.6 ml. of 1 per cent. potassium carbonate has been added.

FIELD'S RAPID METHOD OF STAINING THICK BLOOD FILMS FOR MALARIA PARASITES¹

This method can be recommended for routine use.

In preparing the blood films it is important to ensure that they are not too thick. Drying may be assisted by placing the film in the incubator. After the film is quite dry it may be passed very rapidly two or three times through a Bunsen or spirit flame, each passage occupying two to three seconds. When cool the film is ready for staining.

Field's Stain²

Solution A (methylene blue):

Methylene blue	1.3 g.
Na ₂ HPO ₄ (anhydrous)	5.0 g.
(If Na ₂ HPO ₄ , 12 H ₂ O, is used, 12.6 g.)	

Dissolve in 50 ml. distilled water; bring to the boil and evaporate almost to dryness in a water-bath, then add KH₂PO₄ (anhydrous) 6.25 grams. Add 500 ml. of freshly boiled and still warm distilled water, stir until the stain is completely dissolved and set aside for twenty-four hours. Filter before use. If a scum forms during use, filter again.

Alternatively, if Azur I is available there is no need to carry out

¹ Field, J. W. (1941), *Trans. roy. Soc. trop. Med. Hyg.*, 35, 35.

² This stain in tablet form can be obtained from G. T. Gurr, London.

the polychroming of the methylene blue as outlined above, and *Solution A* can be made as follows:

Methylene blue	0.8 g.
Azur I	0.5 g.
Na ₂ HPO ₄ (anhydrous) (Na ₂ HPO ₄ , 12 H ₂ O, 12.6 g.)	5.0 g.
KH ₂ PO ₄ (anhydrous) (KH ₂ PO ₄ , 2 H ₂ O, 8.0 g.)	6.25 g.
Distilled water	500 ml.

The phosphate salts are first dissolved in freshly boiled and still warm distilled water and the stain is then added. Set aside for twenty-four hours and filter before use.

Solution B (eosin):

Eosin	1.3 g.
Na ₂ HPO ₄ (anhydrous) (Na ₂ HPO ₄ , 12 H ₂ O, 12.6 g.)	5.0 g.
KH ₂ PO ₄ (anhydrous) (KH ₂ PO ₄ , 2 H ₂ O, 8.0 g.)	6.25 g.
Distilled water	500 ml.

The phosphate salts are first dissolved in freshly boiled and still warm distilled water, then the stain is added. Set aside for twenty-four hours and filter before use.

The stains are kept in covered jars, the level being maintained by the addition of fresh stain as necessary. The same solution may be used continuously for many weeks without apparent deterioration, but the eosin solution should be renewed when it becomes greenish from the slight carry-over of methylene blue (*vide infra*). If solutions show a growth of bacteria or moulds they should be discarded and replaced from stock solutions which, if stored carefully, will remain satisfactory up to a year.

METHOD OF STAINING

- (1) Dip the slide into the Solution A for one to two seconds only.
- (2) Remove slide and immediately rinse *gently* in a jar of clean distilled or tap water until the stain ceases to flow from the film and the glass of the slide is free from stain.
- (3) Dip the slide into Solution B for one to two seconds only.
- (4) Rinse *gently* for two to three seconds in clean water.
- (5) Place *vertically* against a rack to drain and dry.

The relative times may require slight adjustment to suit different batches of stain.

Films up to three weeks old may benefit from immersion in phosphate buffer solution (as used for dissolving the stains) until haemoglobin begins to diffuse out. The film is stained in the ordinary way. Unduly thick films should be similarly immersed before staining to

remove the greater part of the haemoglobin. The phosphate buffer solution may be used in place of water for rinsing between Solutions A and B.

Another method of staining thick blood films for malaria parasites is that of Simeons.¹

STAINING OF SPIROCHAETES

FONTANA'S METHOD

Solutions required:

(a) *Fixative:*

Acetic acid	1 ml.
Formalin	.	:	:	:	:	2 ml.
Distilled water	.	:	:	:	.	100 ml.

(b) *Mordant:*

Phenol	.	:	:	:	.	1 g.
Tannic acid	:	:	:	:	:	5 g.
Distilled water	.	:	:	:	.	100 ml.

(c) *Ammoniated silver nitrate:*

Add 10 per cent. ammonia to 0.5 per cent. solution of silver nitrate in distilled water until the precipitate formed just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not re-dissolve.

(1) Treat the film three times, thirty seconds each time, with the fixative.

(2) Wash off the fixative with absolute alcohol and allow the alcohol to act for three minutes.

(3) Drain off the excess of alcohol and carefully burn off the remainder until the film is dry.

(4) Pour on the mordant, heating till steam rises and allow it to act for half a minute.

(5) Wash well in distilled water and again dry the slide.

(6) Treat with ammoniated silver nitrate, heating till steam rises, for half a minute, when the film becomes brown in colour.

(7) Wash well in distilled water, dry and mount in Canada balsam.

It is essential that the specimen be mounted in balsam under a cover-slip before examination, as some immersion oils cause the film to fade at once.

The spirochaetes are stained brownish-black on a brownish-yellow background.

¹ Simeons, A. T. W. (1942), *Indian med. Gaz.*, 77, 725.

LEVADITI'S METHOD OF STAINING SPIROCHAETES IN TISSUES

Pyridine Modification

This method is more rapid than the original technique.

- (1) Fix the tissue, which must be in small pieces 1 mm. thick, in 10 per cent. formalin for twenty-four hours.
- (2) Wash the tissue for one hour in water and thereafter place it in 96-98 per cent. alcohol for twenty-four hours.
- (3) Place the tissue in a 1 per cent. solution of silver nitrate (to which one-tenth of the volume of pure pyridine has been added) for two hours at room temperature, and thereafter at about 50° C. for four to six hours. It is then rapidly washed in 10 per cent. pyridine solution.
- (4) Transfer to the reducing fluid, which consists of:

Formalin	4 per cent.	100 parts
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to which are added immediately before use:

Acetone (pure)	10 "
Pyridine (pure)	15 "

Keep the tissue in this fluid for two days at room temperature in the dark.

(5) After washing well with water, dehydrate the tissue with increasing strengths of alcohol and embed in paraffin (p. 144). Thin sections are cut and mounted in the usual way. After removing the paraffin with xylol the sections are immediately mounted in Canada balsam.

STAINING OF AMOEBAE AND OTHER INTESTINAL PROTOZOA IN FAECES

Fix wet smears in Schaudinn's fluid (p. 144) for five minutes or longer.

Wash the films in 50 per cent. alcohol and apply Gram's iodine for two minutes to remove the mercury salt, remove the iodine with alcohol and wash the films in water.

Stain with iron haematoxylin for ten to twenty minutes.

Iron Haematoxylin:

(a) Haematoxylin	1 g.
Absolute alcohol	100 ml.
(b) Liquor ferri perchlor. 30 per cent.	4 ml.
Concentrated hydrochloric acid	1 ml.
Distilled water	100 ml.

Mix equal parts of (a) and (b) immediately before using.

After staining, wash films in water, pass through alcohol, clear with xylol and mount in balsam, as in the treatment of tissue sections.

Preparations may be counter-stained with van Gieson's stain, fifteen to thirty seconds:

Saturated aqueous solution of acid fuchsin	1-3 parts
Saturated aqueous solution of picric acid	100 "

Dehydrate rapidly with absolute alcohol, clear in xylol and mount in balsam.

Note.—Fixed wet preparations must be treated in the same manner as sections and never allowed to become dry.

Dobell's Method¹

Fix films as above, and after washing in distilled water, mordant for ten minutes in 2 per cent. watery solution of ammonium molybdate.

Wash in distilled water and stain for ten minutes with 0.2 per cent. haematoxylin solution in water (the haematoxylin should be fresh, not "ripened").

Wash in distilled water and transfer to tap water for about thirty minutes, *i.e.* until the film assumes a blue colour. Dehydrate with alcohol, clear with xylol and mount in balsam.

STAINING OF FUNGI IN WET MOUNTS WITH LACTOPHENOL BLUE

Staining solution:

Phenol crystals	20 g.
Lactic acid	20 ml.
Glycerol	40 ml.
Distilled water	20 ml.
Cotton blue (or methyl blue)	0.075 g.

Dissolve the phenol crystals in the liquids by gentle warming and then add the dye.

"Needle-mount" Method

(1) Place a drop of the stain on a slide and in this gently tease a fragment of the culture with needles.

(2) Apply a cover-slip with little pressure, as far as possible eliminating bubbles. With blotting-paper remove the excess stain exuding at the edges of the cover-slip.

(3) After several hours or a day, seal the edges with cellulose lacquer to make a permanent preparation. (*Note.*—Some staining occurs rapidly, but the preparation improves on standing for several hours.)

Staining of slide culture preparations. Slides or cover-slips bearing

¹ Dobell, C. (1942), *Parasitology*, 34, 109.

fungus grown *in situ* by the slide culture method are mounted undisturbed with a drop of lactophenol blue.

STAINING OF VIRUS INCLUSION AND ELEMENTARY BODIES, AND RICKETTSIAE INCLUSION BODIES

For intranuclear and cytoplasmic inclusions Giemsa's stain, p. 132, is satisfactory when such forms are of a basophilic nature as in psittacosis. For acidophilic inclusion bodies other stains give more satisfactory results.

Mann's Methyl-Blue Eosin Stain

1 per cent. aqueous solution of methyl-blue	35 parts
" " eosin	45 "
Distilled water	100 "

Fix tissues in Bouin's solution (p. 144) or Zenker's fluid (p. 142), and cut paraffin sections in the usual way. Stain for twelve hours in the incubator at 37° C. Rinse the section in water, differentiate under the microscope in 70 per cent. alcohol to each ml. of which has been added one drop of saturated aqueous Orange G. solution, dehydrate and mount in balsam.

In Ford's modification the sections are stained for three hours at 37° C., treated with 40 per cent. formaldehyde (strong formalin) for five seconds, washed in water, differentiated and mounted as above. This method is especially useful for staining the Negri bodies in rabies.

ELEMENTARY BODIES

Giemsa's Stain

This has already been described on p. 132, and while satisfactory for the elementary bodies of vaccinia and psittacosis, it has been replaced by other methods, which are quicker, free from deposit and give more consistent results.

Gutstein's Method

This method is valuable for staining the elementary bodies of the variola-vaccinia group of viruses in smears made from scrapings of skin lesions and elsewhere.

Solution 1. Methyl violet	1 g.
Distilled water	100 ml.
Solution 2. Sodium carbonate	2 g.
Distilled water	100 ml.

Prepare films of infected material and if much protein is present rinse first in saline and then in distilled water. Fix in methyl alcohol

for twenty to thirty minutes. Place the slide, film facing downwards, supported on two pieces of capillary tubing in a Petri dish.

Mix equal volumes of solutions 1 and 2, filter, and run the stain under the slide in the Petri dish.

Cover the Petri dish, and incubate at 37° C. for twenty to thirty minutes. Remove the slide, rinse in distilled water, and leave to dry in air.

Paschen's Method

This method is especially recommended. Prepare films from the infected tissues, etc., on glass slides of a thickness suitable for critical high-power microscopical investigation (pp. 92, 98), and allow to dry. Place in distilled water for five minutes, dry in air, cover or immerse in absolute alcohol for five minutes and again dry in air. Treat with Löffler's flagella mordant:

Tannic acid, 20 per cent. aqueous solution	.	.	100 ml.
Ferrous sulphate, saturated aqueous solution	:	.	50 ml.
Basic fuchsin, saturated alcoholic solution	.	.	10 ml.

Gently heat for one minute and allow to remain on the slide for ten minutes. Wash off the mordant with distilled water and stain the film with carbol fuchsin solution, 1 part in 20 of distilled water, heating gently for a minute. Rapidly wash the film with water, dry between blotting-paper and mount in immersion oil.

Castaneda's Method

This method is also particularly useful for Rickettsiae.

Prepare a buffer formaldehyde solution as follows:

Dissolve 1 gram of monopotassium dihydrogen phosphate in 100 ml. of distilled water; dissolve 25 grams of disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$) in 900 ml. distilled water; mix the two solutions so that the pH is 7.5; add 1 ml. of strong formalin as a preservative.

The stain consists of a 1 per cent. solution of methylene blue in methyl alcohol.

Mix 20 ml. of buffer solution with 1 ml. formalin, and 0.15 ml. of the methylene blue solution. Apply the mixture to the film for three minutes and then decant without washing. Counter-stain for one or two seconds with

Safranin "O", 0.2 per cent. aqueous solution	.	1 part
Acetic acid, 0.1 per cent.	" "	3 parts

Wash in running water, blot and dry.

The Rickettsiae remain blue while the protoplasm and nuclei of the cells are red.

This stain can also be used for elementary bodies, e.g. those of psittacosis. It may be modified by using Azur II in place of methylene blue.

Macchiavello's Method for staining Rickettsiae

This method is very suitable for staining Rickettsiae in films from tissues.

Make a film in the usual way and dry in air. Warm the slide gently and stain for four minutes with 0.25 per cent. basic fuchsin (in distilled water) which has been adjusted to pH 7.2-7.4 with alkali and filtered through paper.

Then wash off the stain rapidly with 0.5 per cent. citric acid and after this with tap water.

Finally, stain with 1 per cent. watery methylene blue for a few seconds.

The Rickettsiae are coloured red, tissue cells blue.

STAINING WITH FLUORESCENT ANTIBODY

The specific combining affinity of immune antibody for its corresponding antigen is exploited in the staining of antigenic material with fluorescein-labelled antibody. This renders the antigen fluorescent and visible microscopically with ultra-violet illumination. It is thus possible to identify particular bacteria, viruses or antigens in tissue sections or dried smears,¹⁻⁴ and even to visualize the anatomical localisation of the different antigens within the bacterial cell (*e.g.* O, H and Vi antigens of *Salmonella*, p. 573).

The *direct method*³ involves the addition of fluorescein to antibody which is specific for the antigen to be identified; fluorescein isocyanate is coupled to the globulin fraction of specific antiserum and this labelled antibody globulin is applied to the specimen. The *indirect method*¹ involves a preliminary treatment of the specimen with non-fluorescent rabbit antiserum which is specific for the antigen and then a treatment with fluorescein-labelled antibody which is specific for rabbit globulin. Lissamine rhodamine B 200 (RB 200)⁴ which gives an orange fluorescence, is easier to use and may be substituted for fluorescein, which gives an apple-green fluorescence.

**FIXATION AND EMBEDDING OF TISSUES;
SECTION CUTTING**

As the ordinary routine bacteriological investigation of tissues is carried out almost exclusively with paraffin sections, this technique only will be described.

The fixed tissue is embedded in paraffin wax to support it during the cutting of the section, and the section is held together by the wax in the process of transferring it to the slide.

¹ Coons, A. H., & Kaplan, M. H. (1950), *J. exp. Med.*, **91**, 1.

² Carter, C. H., & Leise, J. M. (1958), *J. Bact.*, **76**, 152.

³ Weller, T. H., & Coons, A. H. (1954), *Proc. Soc. exp. Biol. Med.*, **86**, 789.

⁴ Chadwick, C. S., McEntegart, M. G., & Nairn, R. C. (1958), *Lancet*, **1**, 412.

The paraffin wax must completely permeate the tissue, but before it can do so, all water must be removed from the material and replaced by a fluid with which melted paraffin will mix.

Water, therefore, is first removed by several changes of alcohol; the alcohol is replaced by some fluid—such as xylol, benzol, acetone, chloroform—which is a solvent of both alcohol and paraffin wax, and the tissue is finally embedded in melted paraffin.

Before removing the water from the tissue preparatory to embedding, the tissue must be suitably fixed and hardened.

The essentials for obtaining good sections are:

- (1) The tissue must be fresh.
- (2) It must be properly fixed by using small pieces and employing a large amount of fixing fluid.
- (3) The appropriate fixing fluid must be employed for the particular investigation required.
- (4) The tissue must not remain too long in the embedding bath.

FIXATIVES

FORMALIN

Ten per cent. commercial formalin in normal saline solution is a good fixative for general use. Its advantages are: it is easily prepared, has good penetrating qualities, does not shrink the tissues, and permits considerable latitude in the time during which specimens may be left in it. Moreover, the subsequent handling of the material is much easier in our experience than in the case of mercuric chloride fixatives, such as Zenker's fluid. Formalin fixation is not so good as other methods where fine detail has to be observed, as, for example, in material containing protozoa. For general routine use, however, it is the most convenient and useful of fixatives. Tissue should be cut into thin slices, about 4 mm. thick, and dropped into a large bulk of fixative. The fluid may be changed at the end of twenty-four hours, and fixation is usually complete in forty-eight hours. Specimens are then washed in running water for an hour and transferred to 50 per cent. alcohol. In the latter fluid they may be kept for a considerable time without deterioration.

Formalin tends to become acid owing to the formation of formic acid. The strong formalin should be kept neutral by the addition of excess of magnesium carbonate. The clear supernatant fluid is decanted off when formalin dilutions are required.

ZENKER'S FLUID

Mercuric chloride	5 g.
Potassium bichromate	2.5 g.
Sodium sulphate	1 g.
Water	100 ml.

Immediately before use, add 5 ml. of glacial acetic acid per 100 ml. of fluid.

The fluid should be warmed to body temperature and only small pieces of tissue must be placed in it. Fixation is complete in twenty-four hours, and thereafter the pieces of tissue are washed in running water for twenty-four hours to remove the potassium bichromate and mercuric chloride. The tissue is then transferred to 50 per cent. alcohol.

It is essential that all the mercuric chloride should be removed, otherwise a deposit will appear in the sections. The bulk of it is removed by washing. The remainder can be removed with iodine during the dehydration stage in alcohol. The material after washing is transferred to 50 per cent., and later to 70 per cent. alcohol to which sufficient iodine has been added to make the fluid dark brown in colour. (It is convenient to keep a saturated solution of iodine in 90 per cent. alcohol in a drop-bottle, and add a few drops as required.) If the alcohol becomes clear more iodine is added until the fluid remains brown. This indicates that all the mercury salt has been dissolved out by the iodine-alcohol.

Cut sections fixed on slides can also be treated with iodine—*e.g.* Gram's iodine—for three to five minutes, to remove mercuric chloride.

Animal tissues fixed in Zenker's fluid are more difficult to cut, and sections are apt to float off the slide, particularly if fixation has been unduly prolonged.

ZENKER-FORMOL FLUID

This is similar to Zenker's fluid except that the acetic acid is omitted and 5 ml. of formalin are added per 100 ml. immediately before use. It is a useful general fixative for animal tissues.

MERCURIC-CHLORIDE-FORMALIN SOLUTION

Mercuric chloride, saturated aqueous solution	90 ml.
Formalin, commercial	10 ml.

Small portions of tissue must be used and fixation is complete in one to twelve hours. Then transfer to alcohol and iodine as after Zenker's fluid (*q.v.*). This fluid fixes with the minimum amount of distortion and the finer cytological details of the cells are retained.

"SUSA" FIXATIVE (M. Heidenhain)

Mercuric chloride	45 g.
Distilled water	800 ml.
Sodium chloride	5 g.
Trichloracetic acid	20 g.
Acetic acid (glacial)	40 ml.
Formalin (40 per cent. formaldehyde)	200 ml.

This is one of the best fixatives for both normal and pathological tissues. Pieces of tissue not thicker than 1 cm. should be fixed for three to twenty-four hours, depending on the thickness. The material should be transferred *direct* to 95 per cent. alcohol. Lower grades of alcohol, or water, may cause undue swelling of connective tissue. Add to the alcohol sufficient of a saturated solution of iodine in 95 per cent. alcohol to give a brown colour. If the latter fades, more iodine should be added.

The advantages of "Susa" fixative are rapid and even fixation with little shrinkage of connective tissue. The transference direct to 95 per cent. alcohol shortens the time of dehydration, while tissues thus fixed are easy to cut.

BOUIN'S FLUID

This fixative is useful for the investigation of virus inclusion bodies.

Saturated aqueous solution of picric acid	75 parts
Formalin	25 "
Glacial acetic acid	5 "

This solution keeps well. Use thin pieces of tissue not exceeding 10 mm. thick. Fix for one to twelve hours according to thickness and density of tissue. Wash in 50 per cent. alcohol (not water), then 70 per cent. until the picric acid is removed.

SCHAUDINN'S FLUID

Absolute ethyl alcohol	100 ml.
Saturated aqueous solution of mercuric chloride .	200 ml.

This is an important fixative for protozoa. It may be used cold or warmed to 60° C., when it is more quickly penetrating.

EMBEDDING AND SECTION CUTTING

After fixation by any of the above-mentioned methods and transference to 50 per cent. alcohol, *small pieces* of tissue are treated as follows:

- (1) Place in 90 per cent. alcohol for two to five hours.
- (2) Transfer to absolute alcohol for two hours.
- (3) Complete the dehydration in fresh absolute alcohol for two hours.
- (4) Transfer to a mixture of absolute alcohol and chloroform (equal parts) till tissue sinks, or overnight.
- (5) Place in pure chloroform for six hours.
- (6) Transfer the tissue for one hour to a mixture of equal parts of chloroform and paraffin wax, which is kept melted in the paraffin oven.
- (7) Place in pure melted paraffin in the oven at 55° C. for two hours, preferably in a vacuum embedding oven.

The tissue is embedded in blocks of paraffin. These are cut out,

trimmed with a knife, and sections $5\text{ }\mu$ thick are cut by means of a microtome. The sections are flattened on warm water, floated on to slides and allowed to dry. Albuminised slides are useful where the staining process involves heating, and where animal tissue is used, especially after fixation with Zenker's fluid. The slides are coated with albumin either by means of a small piece of chamois leather or by the finger tip. The albumin solution is made by adding three parts of distilled water to one part of egg-white and shaking thoroughly. The mixture is filtered through muslin into a bottle, and a crystal of thymol is added as a preservative. It is usual to coat a number of slides and, after drying, these are stored until required. The albuminised side may be identified by breathing gently on the slide; it is not dimmed by the breath, whereas the plain side is.

For further treatment of sections, see Staining Methods.

For additional details, reference must be made to works on histology.

CHAPTER VIII

STERILISATION

STERILISATION is the freeing of an article from all living organisms, including bacteria and their spores. The sterilisation of culture media, containers and instruments is essential in bacteriological work for the isolation and maintenance of pure cultures. In surgery and medicine, the sterilisation of instruments, drugs and other supplies is important for the prevention of infection. The efficacy of sterilisation procedures in hospitals and elsewhere may at times require confirmation by bacteriological tests.

There are three main methods of sterilisation: (a) killing of the organisms by heat, (b) killing by disinfectants and (c) removal by filtration. Heat is usually employed, since it is generally the simplest and most reliable means of sterilisation. Chemical disinfectants are not used for bacteriological culture media and containers; the presence of a chemical which destroyed contaminants would prevent the growth of subsequently inoculated bacteria. Chemical disinfectants are generally less reliable than heat; they are used mainly for disinfecting the skin, floors, furniture and other articles which can not be heated effectively without damage. Bacteria-stopping filters are employed to sterilise liquids which would be spoiled by heat, e.g. blood serum, and in which contamination by filter-passing viruses is improbable or unimportant.

Articles which are required sterile must be kept in a sterile condition between their removal from the steriliser and their use. They must be protected from contact with unsterile objects and from exposure to the infected dust which settles from the air in occupied rooms (if exposure to air is unavoidable, it should be limited to a few seconds). The articles are usually enclosed in a dust-proof container or wrapping before placing in the steriliser; they are sterilised in this container or wrapping and subsequently kept in it until required for use (p. 176 for details).

STERILISATION BY HEAT

Heat is applied in two forms: (1) *dry heat*, i.e. in hot air in an oven at 140°–180° C., or in a flame, and (2) *moist heat*, i.e. in saturated steam in a steamer at 100° C. or autoclave at 105°–135° C., or in boiling water at 100° C. Moist heat is more "efficient" than dry heat, sterilising at lower temperatures in a given time and in shorter times at the same temperature. Moist heat kills microbes probably by coagulating and denaturing their essential enzymes and structural proteins, and water participates in this process. *Sterilisation, i.e. killing of the most resistant spores, generally requires moist heat at 121° C. for 15–30 minutes.* Culture media, since they contain water, must be sterilised by moist heat.

Dry heat kills microbes probably by promoting a destructive oxidation of essential cell constituents. *Killing of the most resistant spores by dry heat requires a temperature of about 160° for 60 minutes.* This high temperature causes slight charring of paper, cotton and other organic materials. Dry heat is employed mainly for glassware, syringes, metal instruments and paper-wrapped goods which are not spoiled by the high temperature and are required dry. It is also used for anhydrous fats, oils and powders which are impermeable to moisture and thus incapable of sterilisation by moist heat.

FACTORS INFLUENCING STERILISATION BY HEAT

The factors influencing the killing of microbes by heat include the temperature, time of exposure, presence of water (moist or dry heat), number of microbe cells, species and strain of microbe, presence of spores and nature of the infected material.

(1) The *temperature* and *time* required for killing are inversely related, shorter times sufficing at higher temperatures. Thus, the minimal moist-heat sterilising times for the most resistant kind of spores known (of a thermophilic *Bacillus* species) were found in tests of corn juice at pH 6.0 containing 200,000 spores per ml. to be: 22 hours at 100° C., 11½ hours at 105° C., 3¾ hours at 110° C., 84 minutes at 115° C., 23 minutes at 120° C., 8 minutes at 125° C., 3½ minutes at 130° C. and 1½ minutes at 135° C. Published findings on resistant spores show many discrepancies, but their collation suggests that in practice the following may be taken as equivalent minimal sterilising exposures:

Moist Heat		Dry Heat	
Temperature	Sterilising Time	Temperature	Sterilising Time
100° C.	20 hours	120° C.	8 hours
110° C.	2½ hours	140° C.	2½ hours
115° C.	50 minutes	160° C.	1 hour
121° C.	15 minutes	170° C.	40 minutes
125° C.	6½ minutes	180° C.	20 minutes
130° C.	2½ minutes		

For surgical and bacteriological sterilisation, most authorities consider that a 10–12 minutes' exposure of the organisms to moist heat at 121° C. is generally sufficient. This ensures killing of all pathogenic sporing organisms and all saprophytes except for some strict thermophiles which cannot grow at less than 40° C., and so cause no trouble in media stored and used at lower temperatures (*e.g.* incubated at 37° C.).

The minimal sterilising times just recommended are the times for which the microbes themselves are held at the appropriate temperature, *and do not include heating-up time*. When an article is being sterilised by exposure in hot water, steam or air, the total duration of the exposure must include time for the article to become heated up to the sterilising

temperature, in addition to the recommended minimal sterilising time at that temperature. The amount of time to be allowed for heating up will be discussed later for the individual methods.

(2) The *number of microbes and spores* affects the rapidity of sterilisation. The susceptibility and duration of survival on heating varies considerably among the individual cells, even in a pure culture. The number of survivors diminishes exponentially with the duration of heating, and the time for complete sterilisation increases with the number initially present. Thus, when an exposure of 23 minutes at 120° C. was required to sterilise a suspension of 200,000 spores per ml., only 17 minutes was required for one of 2000 spores per ml. In practice it is advantageous to minimise bacterial contamination by cleansing procedures before applying heat for the purpose of sterilisation.

(3) The *species, strain and spore-forming ability* of the microbe greatly affect its susceptibility to heat. The amount of heat required to kill a given variety is normally stated in terms of the temperature and time of exposure, either as the *thermal death point*, i.e. the lowest temperature to give complete killing in aqueous suspension within 10 minutes, or, preferably, as the *thermal death time*, i.e. the shortest time for complete killing at a stated temperature. The tests are made under strictly standardised conditions, e.g. with sealed 9 mm. diameter hard glass tubes containing 1-2 ml. of a suspension of 50,000,000 organisms per ml. in a phosphate buffer solution at pH 7.0.

Susceptibility to Moist Heat.—The vegetative forms of most bacteria, yeasts and fungi, and most animal viruses, are killed in 10 minutes by a temperature between 50° C. (e.g. *N. gonorrhoeae*) and 65° C. (e.g. *Staph. aureus*). Extreme susceptibility is shown by *Tr. pallidum* which is killed in 10 minutes at about 43° C., and extreme resistance by thermophilic saprophytic bacilli, e.g. *B. stearothermophilus*, whose vegetative forms can grow at temperatures of up to nearly 80° C. A few animal viruses are more resistant than the majority: that of poliomyelitis may require heating at 75° C. for 30 minutes and that of homologous serum hepatitis, when in serum, 60° C. for 10 hours. Many bacteriophages are more resistant than their host bacterium, and it is often possible to kill the latter by heating at 60° C. for 15-30 minutes without affecting the phage; these phages are killed by temperatures in the range 65°-80° C.

The spore forms of actinomycetes, yeasts and fungi are more resistant than the vegetative forms, though not as highly resistant as bacterial spores. The more susceptible kinds are killed at 70° C. in 5 minutes and the more resistant at 80°-90° C. in 30 minutes.

The spores of bacterial species are killed by moist heat in 10 minutes at temperatures mainly in the range 100°-121° C. Their resistance may vary considerably between different strains of the same species. Thus, spores of most strains of *Cl. tetani* and *Cl. welchii* are killed by boiling at 100° C. for 10 minutes, but exceptional strains of either species may resist boiling for 1-3 hours. These *tetani* strains are the most resistant pathogens capable of infecting wounds and their degree of resistance

thus determines the minimum standards for surgical sterilisation: *i.e.* 121° C. for 10 minutes or 115° C. for 30 minutes, exclusive of heating-up time. *Cl. botulinum*, the cause of botulism food-poisoning, forms spores which at pH 7.0 may resist boiling at 100° C. for up to 8 hours and autoclaving at 115° C. for 10–40 minutes; these limits determine the standards of heat processing employed in the preservation of non-acid foods by canning.

Susceptibility to Dry Heat.—For vegetative bacteria, dry heat at 100° C. for 60 minutes is required to kill strains that would succumb to moist heat at 60° C. in 30 minutes. Fungal spores are killed in hot air at 115° C. within 60 minutes, and bacterial spores at temperatures in the range 120°–160° C.

(4) The *nature of the material* in which the organisms are heated may affect the rate of killing. A high content of organic substances generally tends to protect spores and vegetative organisms against the lethal action of heat. Proteins, gelatin, sugars, starch, nucleic acids, fats and oils all act in this way. The effect of fats and oils is greatest with moist heat, since they prevent access of moisture to the microbes. The presence of an organic or inorganic disinfectant has the opposite effect and facilitates killing by heat. The pH is important; the heat resistance of spores is greatest in neutral media (pH 7.0) and diminished with increasing acidity or alkalinity. Thus, *tetani* spores whose killing required moist heat at 100° C. for 29 minutes at pH 7.2, were killed in only 11 minutes at pH 10.2 or pH 4.1. The effect of acidity (*e.g.* pH 4) in enhancing killing of *botulinum* spores, as well as its inhibitory effect on growth, explains the safety of acid fruits preserved by brief heating at 100° C. The effect of alkali may be put to practical use in the sterilisation of metal instruments; boiling in water containing 2 per cent. sodium carbonate gives as effective killing in 10 minutes as boiling in plain water for several hours.

The conditions under which sporulating bacteria are grown may influence the heat resistance of the spores. Thus, spores formed by soil and intestinal bacteria in artificial cultures are sometimes less resistant than those formed in the organism's natural habitat.

METHODS OF STERILISATION BY DRY HEAT

(1) *Red Heat.*—Inoculating wires, points of forceps and searing spatulas are sterilised by holding them in the flame of a Bunsen burner until they are seen to be red hot.

(2) *Flaming.*—This method is used for sterilising scalpels, needles, the mouths of culture tubes, cotton-wool stoppers, and glass slides and cover-slips. It involves passing the article through the Bunsen flame without allowing it to become red hot. When a slide or other glass article is heated sufficiently for sterilisation, it is apt to crack if placed at once on a cold surface. Needles, scalpels and basins are sometimes treated by immersing them in methylated spirit and burning off the

spirit, but this does not produce a sufficiently high temperature for sterilisation.

(3) *Hot-Air Oven*.—This is the main means of sterilisation by dry heat. The oven is usually heated by electricity and has a thermostat which maintains the chamber air constantly at the chosen temperature. Preferably, it should have a fan or turbo-blower to assist the circulation of air and so ensure rapid, uniform heating of the load.¹ A temperature of 160° C. for 1 hour is generally employed.

This is the best method of sterilising dry glassware such as test-tubes, Petri dishes, flasks, pipettes and instruments such as forceps, scalpels, scissors, throat swabs and assembled *all-glass* syringes. Before sterilisation, test-tubes and flasks should be plugged with cotton-wool stoppers; other glassware, e.g. pipettes, may be wrapped in kraft paper (p. 177). Certain brands of cotton-wool give off volatile substances during sterilisation, which condense on the glass and later may interfere with the growth of sensitive bacteria, e.g. pneumococcus. Slip-on metal caps (p. 245) may be substituted for cotton-wool. Although screw-capped bottles themselves will withstand the temperature of the hot-air oven, the rubber liners or washers in their caps will not, and bottles already capped should therefore be autoclaved (see p. 161).

The hot-air oven is also used for sterilising dry materials in sealed containers, and powders, fats, oils and greases which are impermeable to moisture. These materials are penetrated very slowly by heat and must therefore be sterilised in small lots or shallow layers, e.g. in packets not exceeding 10 g. and in layers not exceeding 0.5 cm. depth in a Petri dish.

Glassware should be perfectly dry before placing in the sterilising oven; wet glassware is liable to be cracked and should first be dried in a "drying oven" at about 100° C. The sterilising oven must not be overloaded and spaces must be left for circulation of air through the load. It may be cold or warm when loaded, and is then heated up to the sterilising temperature in the course of 1-2 hours. The *holding period* of 1 hour at 160° C. is timed as beginning when the thermometer first shows that the oven air has reached 160° C. Finally, the oven is allowed to cool gradually during about 2 hours before the door is opened, since glassware may be cracked by sudden or uneven cooling.

A holding period of 1 hour at 160° C. is generally considered sufficient for loads of a kind that will heat up rapidly and reach 160° C. soon after the oven air does so; e.g. loosely packed loads of simple glassware and metal instruments, especially if the oven is equipped with a fan. An exposure of 2-2½ hours at 160° C. is preferable for loads likely to require a longer heating-up time: e.g. heavy loads in an oven without a fan, assembled *all-glass* syringes packed in test-tubes and slowly heating materials such as powders, oils and greases. Two hours at 170° C. is sometimes used.

¹ Darmady, E. M., & Brock, R. B. (1954), *J. clin. Path.*, **7**, 290.

METHODS OF STERILISATION BY MOIST HEAT

Three methods are in common use: (1) boiling in a water-bath at 100° C. for 5–10 minutes, (2) steaming in free steam at atmospheric pressure and 100° C. either for 90 minutes or intermittently for shorter periods, and (3) autoclaving at 121° C. for 15–45 minutes in pure saturated steam at 15 lb. per square inch above atmospheric pressure. Only the third procedure fully ensures sterilisation and killing of the most highly resistant spores. Killing by moist heat requires contact of the hot water or steam with the microbes, and if these are protected from wetting, as by grease or by a sealed impervious container, they will be subject only to the weaker effect of dry heat at the same temperature.

Boiling at 100° C. in a Water-bath.—A suitable form of boiling bath is the fish-kettle type made of enamel-ware or tinned copper. It should have a removable tray provided with a raised edge to prevent cylindrical instruments from falling off. Boiling at 100° C. for 5–10 minutes is sufficient to kill all non-sporing and many, but not all, sporing organisms, e.g. not the spores of exceptional *Clostridium tetani* strains which may survive boiling for 1–3 hours. The method thus does not ensure sterility, but it has been found satisfactory for certain purposes in bacteriology and medicine where absolute sterility is not essential or better methods are unavailable. It may be used for tubing, pipettes, measuring cylinders, rubber stoppers, instruments such as scalpels, forceps and scissors, and syringes of the metal and glass type which do not stand higher temperatures (p. 178). If the water supply is "hard", distilled water should be used, otherwise the instruments on removal become covered with a film of calcium salts. Sterilisation may be promoted by the addition of 2 per cent. sodium carbonate to the water.

The instruments and other articles are removed from the boiling water with long-handled forceps which have been stored in 3 per cent. lysol (saponated cresol) solution to a level approaching the finger grips. Before taking into the hand, which should be dry, the newly boiled instrument is held by the forceps for a few moments while it dries by evaporation. If it were taken into the hand while still wet, its working end (e.g. scalpel blade or syringe needle) would be liable to contamination with skin bacteria floating down from the fingers in the film of water.

The interior of a test-tube may be sterilised quickly for ordinary purposes by boiling water in it.

STERILISATION BY STEAM

Saturated steam is a more efficient sterilising agent than hot air, partly because it provides the greater lethal action of moist heat and partly because it is quicker in heating up the exposed articles and in penetrating porous materials such as cotton-wool stoppers, paper and cloth wrappers, bundles of surgical linen and the interstices of hollow apparatus. When the steam contacts the cooler surface of

it condenses into a small volume of water and liberates its large latent heat to that surface (e.g. 1600 ml. steam at 100° C. and atmospheric pressure condenses into 1 ml. water at 100° C. liberating 518 calories of heat). The contraction in volume causes immediate suction of more steam to the same site and the process thus continues rapidly until the article is raised to the temperature of the steam. The condensation water ensures the effective "moist" conditions for killing of the exposed microbes. Pure steam is used and the presence of air avoided, since air hinders penetration by the steam.

Steam sterilisation is especially suitable for culture media and aqueous solutions, since the atmosphere of steam prevents the loss of water by evaporation during heating. To avoid drenching of cotton-wool stoppers in a steamer or autoclave, the stoppers should be covered with kraft paper; thus a wire basket of test-tubes is covered by a single sheet of paper turned down at the edges.

STEAMING AT 100° C.

Pure steam in equilibrium with water boiling at normal atmospheric pressure (760 mm. Hg) has a temperature of 100° C.; at the lower pressures found at high altitudes the temperature is slightly less, e.g. 99° C. at 1000 feet, 97° C. at 3000 feet and 95° C. at 5000 feet. Because of its convenience, "steaming" at 100° C. is commonly used for the sterilisation of culture media such as broth and nutrient agar, although it is not as certainly effective as autoclaving. A Koch or Arnold steam steriliser ("steamer") heated by steam, gas or electricity is employed. In its simplest form this is a vertical metal cylinder with a removable conical lid (having a small opening for the escaping steam) and containing water which is boiled by a heater under the cylinder; but various modifications are available. A perforated tray situated above the water bears the articles to be sterilised. The apparatus is inexpensive and simple to operate. Bottles of medium may be introduced or removed while steaming is in progress, but unnecessary opening of the steamer, with the consequent introduction of cool air, should be avoided. Sterilisation may be effected in two ways:

(a) *By a single exposure at 100° C. for 90 minutes.*—The spores of some thermophilic and rare mesophilic bacteria can survive this treatment, but in practice it seldom fails to sterilise. The steaming period of 90 minutes includes the time required for the tubes and bottles of media to be heated up from room temperature to 100° C. This may be about 15–20 minutes for tubes or bottles containing up to 100 ml., 30 minutes for bottles of 600 ml. and 45 minutes for a flask of 5 litres. For the larger volumes it is advisable to increase the total steaming period by an appropriate amount.

(b) *By intermittent exposure at 100° C., e.g. for 20–45 minutes on each of three successive days.*—The principle of this intermittent method of sterilisation, or "Tyndallisation", is that one exposure suffices to

¹ Draught organisms; between the heatings the spores, being

in a favourable nutrient medium, become vegetative forms which are killed during the subsequent heating. The duration of each steaming should be sufficient to heat up the medium to 100° C., i.e. 20 minutes for lots up to 100 ml. and longer for larger volumes (see (a) above). The method is used for media containing sugars which may be decomposed at higher temperatures (p. 202), and for gelatin media (p. 194) which after prolonged heating fail to solidify on cooling. Thermo-phobic, anaerobic and other bacteria whose spores will not germinate in the particular medium and under the conditions of storage between the heatings, may escape being killed.

STERILISATION IN THE AUTOCLAVE

The autoclave provides a means of applying saturated steam, and thus moist heat, at temperatures above 100° C. Autoclaving at 121° C. for 15-45 minutes is the most certain means of sterilising culture media and should be employed for all media that can withstand the high temperature. The exact duration of the holding period in steam at 121° C. depends upon the nature of the load, particularly on the volume of medium per container, since it must include sufficient time for this to become heated up to 121° C. (p. 160). Surgical supplies also are commonly autoclaved at 121° C. for 15-45 minutes, but with modern high pre-vacuum machines time may be saved by autoclaving instead at 135° C. for only 3 minutes.

Water boils when its vapour pressure equals the pressure of the surrounding atmosphere. This occurs at 100° C. for normal atmospheric pressure (i.e. 760 mm. Hg, 14.7 lb. per square inch absolute pressure or 0 lb. per sq. in. "gauge pressure"). Thus, when water is boiled within a closed vessel at increased pressure, the temperature at which it boils, and that of the steam it forms, will rise above 100° C. The exact temperature depends upon the pressure employed and also on whether any air is present with the steam. The temperature of the steam at different pressures, when all, half or none of the original air content has been discharged from the autoclave chamber, is shown below:

Gauge Pressure i.e. pressure above atmospheric (lb./sq. in.)	Temperature in Degrees Centigrade		
	Complete air discharge	Half air discharge	No air discharge
0	100	—	—
5	109	94	72
10	115	105	90
15	121	112	100
20	126	118	109
25	130	124	115
30	135	128	121

Thus, for the temperature of 121° C., autoclaving is carried out at a "gauge pressure" of 15 lb. per sq. in. above atmospheric pressure (*i.e.* at an absolute pressure of 29.7 lb. per sq. in.).

The Importance of Air Discharge.—All the air must be removed from the autoclave chamber and articles of the load, so that the latter are exposed to pure steam during the period of sterilisation. There are three reasons for this: (1) the admixture of air with steam results in a lower temperature being achieved at the chosen pressure; (2) the air hinders penetration of the steam into the interstices of porous materials and the narrow openings of containers, syringes, etc.; and (3) the air, being denser than the steam, tends to form a separate and cooler layer in the lower part of the autoclave, and so prevents adequate heating of the articles there (*e.g.* in an autoclave with no air discharge, a temperature of only 70° C. was recorded at the bottom when that at the top was 115° C.).

There is one exception to the necessity for complete air discharge from the load. Hermetically sealed bottles and ampoules of aqueous solutions and culture media are satisfactorily sterilised in spite of the presence of some air within them. The contained water provides the conditions for moist-heat sterilisation, making unnecessary the entry of steam for this purpose, and the contents are heated to the same temperature as the chamber steam, though to a higher pressure, by conduction of heat through the container walls.

The Simple Non-jacketed Laboratory Autoclave (p. 157)

The simplest form of laboratory autoclave, the so-called "pressure-cooker type", consists of a vertical or horizontal cylinder of gun-metal or stainless steel in a supporting frame or case. The size may be up to about 18 inches in diameter and 30 inches in length. The lid (or door) is fastened by screw clamps, and is rendered air-tight by means of an asbestos washer. The cylinder contains water up to a certain level (*e.g.* 3½ in. for a vertical autoclave of 19 in. internal height) and this is heated by a gas burner or electric heater below the cylinder. The bottles, tubes, etc., to be sterilised are placed on a perforated tray situated above the water-level. The apparatus is furnished on its lid or upper side with a discharge tap for air and steam, a pressure gauge and a safety valve which can be set to blow off at any desired pressure.

Directions for Using the Simple Autoclave.—See that there is sufficient water in the cylinder. Insert material to be sterilised and turn on the heater. Place the lid in position, see that the discharge tap is *open* and then screw down the lid. Adjust the safety-valve to the required pressure; in some varieties of autoclave this adjustment has to be determined previously by trial. As steam rises from the boiling water, it mixes with the air in the chamber and carries this out through the discharge tap. *Allow the steam and air mixture to escape freely until all the air has been eliminated from the autoclave.* A means of testing this is to lead a rubber tube from the discharge tap into a pail of cold water.

The steam condenses within the water, while the air rises in bubbles to the surface. When the latter cease, the air discharge is seen to be complete. After some trials it will be known what period of discharge to allow under normal operating conditions.

Now close the discharge tap. The steam pressure rises until it reaches the desired level, *e.g.* 15 lb. per sq. in. for 121° C., when the safety-valve opens and allows the excess steam to escape. From exactly this point begin the *holding period*, continuing exposure at 15 lb. pressure for the appropriate time, *i.e.* usually 15 minutes for aqueous media in lots up to 100 ml. and longer for the large volumes which heat up more slowly (see p. 160). Then turn off the heater and allow the autoclave to cool until the pressure gauge indicates that the inside is at atmospheric pressure (0 lb. per sq. in.). At once open the discharge tap slowly to allow the air to enter the autoclave. If the tap is opened while the chamber pressure is still high, and the pressure is reduced too rapidly, liquid media will tend to boil violently and spill from their containers. On the other hand, if the tap is not opened until the pressure has fallen much below atmospheric pressure, an excessive amount of water will be evaporated and lost from the media. (When spontaneous cooling is too slow, *e.g.* taking about 1 hour, the discharge tap may be opened very slightly so as to cause a gradual reduction to atmospheric pressure during 15–30 minutes; see p. 160.)

Deficiencies of the Simple Autoclave.—The simple form of laboratory autoclave is effective when carefully operated, but has several important disadvantages. The method of air discharge is inefficient, especially for a large and heavily loaded chamber, and it is difficult to decide when the discharge is complete. If, as a result, the discharge tap is closed and the holding period begun while there is still some air present in the chamber and load, the temperature produced at 15 lb. pressure will not be as high as 121° C. (see table, p. 153). This failure to achieve the proper temperature is likely to pass undetected, since these simple autoclaves are not furnished with a thermometer showing the temperature in the lowest and coolest part of the chamber. The operation of the autoclave is controlled solely by the pressure gauge and it is very common for such gauges to become inaccurate.

The simple autoclave also lacks means for *drying* the load after sterilisation. This is desirable for apparatus wrapped in paper or cloth, and essential for surgical linen and dressings. Although dry when put into the autoclave, these articles are moistened by the condensation of steam. When damp, paper and cloth wrappings, even in several layers, are unable to prevent the entry of contaminating bacteria. *It is therefore important to avoid placing the sterilised articles in contact with unsterile objects until their wrappings are dry.*

A wide variety of autoclaves are manufactured which incorporate various devices to overcome these and other difficulties, some being specialised for particular purposes. Many autoclaves at present in hospitals and laboratories have been badly designed or wrongly installed, and cannot ensure sterilisation without damage to heat-sensitive

materials.¹ The following description is given of an autoclave suitable for either laboratory or surgical purposes.

Steam-Jacketed Autoclave with Automatic Air and Condensate Discharge

This is a horizontal cylinder (*e.g.* 20 in. diam. by 30 in.) of rustless metal (*e.g.* Monel metal). At the front is a swing door fastened by bolts and nuts, or preferably by a "capstan head" which operates radial bolts and automatically remains locked while the chamber pressure is raised. A pressure-locked safety door is a valuable guard against the possibility of a dangerous explosion through premature opening by the operator.

The autoclave (see figure) also possesses: (1) a supply of steam from an external source, *e.g.* an independent boiler beside the autoclave or the main steam supply of the building; (2) a steam jacket which heats the side walls independently of the presence of steam in the chamber and so facilitates drying of the load; (3) a channel for discharging air and condensate by gravity from the bottom of the chamber, with a thermostatic valve ("steam trap") to control this discharge automatically; (4) a thermometer indicating the temperature in the discharge channel above the steam trap, *i.e.* approximately that of the lowest and coolest part of the chamber; (5) a vacuum system which may be used to assist drying of the load; and (6) an air-intake with a self-sterilising filter for introducing warm sterile air into the chamber. It may also have (7) a cooling system to hasten the cooling of liquids without violent boiling, and (8) an automatic control system which carries through exactly a pre-selected cycle of sterilisation, including heating-up, holding, cooling and drying stages, without requiring attention from the operator.

Steam Supply.—The steam supplied to the autoclave should be *dry*, *i.e.* free from excess water in the form of suspended droplets, and *saturated*, *i.e.* not superheated above the phase boundary of equilibrium with water boiling at the same temperature and pressure. Steam that is piped a long distance from the boiler tends to become "wet" through cooling and condensation, and such wet steam is undesirable because it soaks porous materials and so hinders further penetration. However, the pressure of the main steam supply should be between 40 and 75 lb. per sq. in. (ideally 55 lb. per sq. in.), and the steam is passed through a reducing valve and pressure regulator so that it enters the autoclave at a pressure of 15 lb. per sq. in. This reduction dries the steam usually to a sufficient extent.

The employment of saturated steam is necessary in order to maintain the conditions of "moist heat" and prevent evaporation of condensation water from the articles of the load during the period of sterilisation. Superheated steam is unsatisfactory because it abstracts water from the

¹ Bowie, J. H. (1955), *Pharm. J.*, 174, 473; Howie, J. W., & Timbury, M. C. (1956), *Lancet*, 2, 669.

exposed material and so brings about the less lethal conditions of "dry heat". Superheated steam cannot be produced in a simple autoclave wherein water is boiled to generate the steam, but may be produced

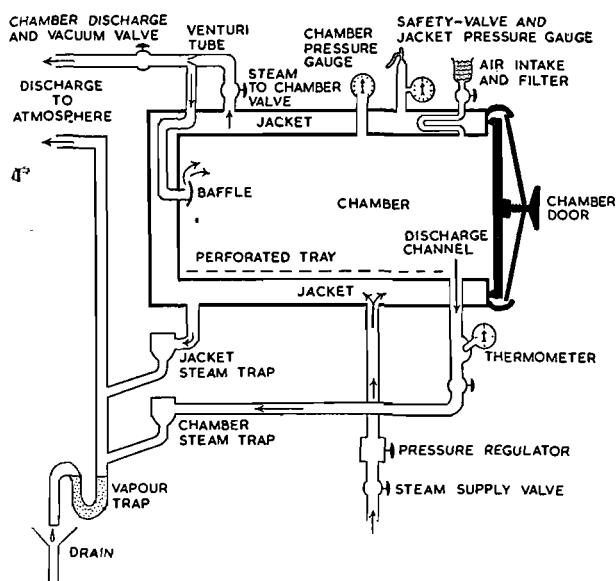
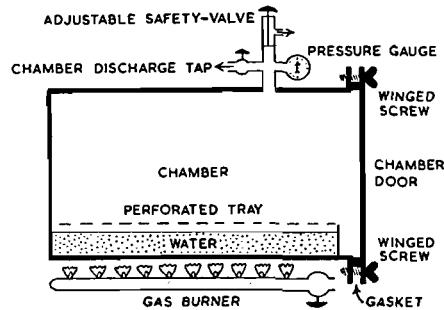


FIG. 7

AUTOCLAVES. *Above:* Simple non-jacketed autoclave. *Below:* Steam-jacketed autoclave with automatic gravity discharge of air and condensate, and system for drying by vacuum and intake of filtered air.

under some conditions in a jacketed autoclave supplied with steam from an external source: e.g. if the steam pressure in the jacket is raised above that in the chamber or if air remains mixed with the steam in the chamber.

Starting of Autoclave and Heating of Jacket.—The steam is first introduced into the jacket, a space between the double side walls of the chamber. The jacket is kept filled with steam at 121° C. throughout the whole day, both during and between the successive steamings in the chamber. Some steam is continually condensing into water on the walls of the jacket and this condensate is drained away through a jacket discharge channel controlled by a thermostatic "jacket steam trap". Care must be taken to ensure that there is no obstruction to this discharge.

Loading of Chamber.—When the jacket is heated, the load is packed into the chamber. Articles requiring different treatment should not be included in the same load, e.g. aqueous media in unsealed containers together with wrapped goods requiring drying. The articles should be arranged loosely to allow free circulation of steam and displacement of air. For further details see pages 162.

Heating-up and Air-displacement Period.—The door is closed and steam allowed to enter the chamber through a baffle high up at the back. The steam for the chamber is drawn from the jacket, which thus acts as a reservoir between the chamber and the supply line; the same pressure must be maintained in the chamber as in the jacket. The steam tends to float as a layer above the cooler and denser air, and as more is introduced it displaces the air downwards through the articles of the load and out through the chamber discharge channel which leads from the bottom of the chamber near the front. The condensation water formed on the cool load and chamber door also drains through this channel. The channel's thermostatic steam trap remains open while steam mixed with air and condensate passes through it to the drain and atmospheric vent, but as soon as all free air has been eliminated and the arrival of pure steam raises the trap's temperature to 121° C., it automatically closes and prevents further escape. About 5 or 10 minutes may be taken for this displacement of air by steam.

Holding Period of Sterilisation.—The holding period at 121° C. is timed as starting when the thermometer in the discharge channel first shows that this temperature is reached. The exact duration of the holding period is decided according to the nature of the load, particularly the time which must be allowed for this to become heated throughout to the temperature of the steam (see pp. 160, 163). During the early part of the holding period some residual air may gradually be displaced from the interior of a porous load; this air together with excess condensate collects in the discharge channel above the steam trap, cools to 120° C. or less, and so causes the trap to open momentarily and allow its escape. (A "near-to-steam" trap is essential, i.e. one which opens when the temperature falls by only 1° C. below that of the pure steam.)

Since a pressure gauge is liable to become inaccurate, the steam-pressure regulator is ultimately adjusted so as to produce a thermometer reading of 121° C. after the completion of air discharge. This control by the thermometer in the discharge channel ensures that autoclaving is carried out at the correct temperature, and is greatly preferable to control

pressure readings. Any obstruction to the discharge of air and condensate is indicated by the temperature falling below 121° C. while the pressure remains at 15 lb. This may occur if a bottle of agar breaks during sterilisation and the agar reaches and solidifies in the steam trap of the chamber discharge channel; the autoclave may then become half-filled with condensed water which is unable to escape. The discharge channel and trap must be kept clear and the removable strainer in the mouth of the discharge channel should be cleaned daily.

Cooling and Drying Period.—At the end of the holding period the supply of steam to the chamber is stopped, while that to the jacket is maintained. The steam left in the chamber begins to cool by loss of heat through the unjacketed door and its pressure falls accordingly. The management of this stage depends on whether drying of the load is required, as for wrapped apparatus or surgical dressings, or must be avoided, as for aqueous media in loosely stoppered containers. Details of the methods of cooling and drying are given below (pp. 160, 163).

AUTOCLAVING OF AQUEOUS SOLUTIONS AND CULTURE MEDIA

The autoclaving of aqueous solutions and culture media must be managed in such a way that the exposure to heat is sufficient for sterilisation, but not so excessive or prolonged as to damage heat-sensitive ingredients. Culture media vary in heat sensitivity. Some, such as gelatin media, will not stand autoclaving and are sterilised by intermittent steaming at 100° C. (p. 152). Many other media can withstand autoclaving at 121° C. for 15 minutes, but are spoiled if heated at this temperature for 30–45 minutes, or if, after a 15-minute exposure, they are cooled so slowly as to be maintained above 100° C. for a further 30–60 minutes. Thus, sugars such as glucose, maltose and lactose may be partially decomposed to form acids, peptones may be broken down, and agar, especially in acid media, may lose its ability to form a firm gel. The media should therefore be autoclaved for the minimum period sufficient for sterilisation and then cooled as rapidly as possible (*vide infra*). Bottles of media should be of such a size that their whole contents are used on one occasion, so as to avoid the need for repeated sterilisation or melting. Agar medium should be sterilised when first made and melted, to avoid the extra heating needed to melt it on another occasion.

An exposure of the microbes to 121° C. for 10–12 minutes is generally thought sufficient for sterilisation. The holding-period of sterilisation, which is timed to begin when the chamber steam first reaches 121° C. at 15 lb. pressure, must include not only this 10–12 minutes but also a time sufficient for the bottles, tubes, etc., and their contents to become heated up to the same temperature as the steam. The length of the heating-up period depends on the nature of the container, the volume of its contents and the mode of operation of the autoclave. Thus, it might be only 1–2 minutes for 10-ml. volumes in test-tubes loosely placed to allow free circulation of steam, and as much as 45 minutes for

a flask of 9 litres. Ideally, the exact time should be determined by trial for the particular kind of container and volume of contents; the autoclave is fitted with a thermocouple and this is inserted in a test container to reveal its temperature throughout the course of autoclaving (p. 164). In general, the following are recommended as the total holding periods in steam at 121° C.: 12 minutes for 10-ml. volumes in loosely packed test-tubes, 15 minutes for 10-ml. volumes in tubes tightly packed in wire baskets, 15 minutes for volumes up to 100 ml. in bottles or flasks, 20-25 minutes for 500-ml. volumes, 25-30 minutes for 1000-ml. volumes and 35-45 minutes for 2000-ml. volumes. It is a bad practice to autoclave large and small volumes in the same load, since with the same holding period (*e.g.* 20 minutes) the former (*e.g.* 2000 ml.) may not be sterilised, yet the latter (*e.g.* 10 ml.) be damaged by overheating.

The bottles, tubes, etc., should not be filled to more than 75-80 per cent. of their capacity lest the contents overflow on expansion during heating. The containers may be loosely stoppered, *e.g.* with cotton-wool plugs or loosely applied screw caps, or else hermetically sealed, *e.g.* sealed ampoules and bottles with tightly applied screw caps. In sealed containers the aqueous content provides the conditions for moist-heat sterilisation and the presence of some air does not interfere with this (p. 154). Hermetic sealing is an advantage in preventing loss of water from the contents by evaporation or violent boiling during the cooling period when the steam pressure is being reduced. It also makes possible the autoclaving of solidified egg medium without disruption by bubble formation (p. 214). However, except for the smallest bottles, the tight application of a screw cap increases the liability to breakage during autoclaving and makes cooling-down slower.

For these reasons, it is usual to autoclave aqueous media in containers stoppered with cotton-wool, with loose metal caps or with screw caps which are loosened slightly. This practice necessitates careful management of the cooling period. When the steam supply to the chamber is stopped at the end of the holding period, the steam already in the chamber gradually cools and diminishes in pressure. This induces evaporation of water from the medium in the container and escape of the vapour through the loose stopper. The evaporation is the main means of cooling of the containers and contents. With the correct management the loss of water from the medium is only 3-5 per cent. It is therefore usual to prepare aqueous media and solutions for autoclaving by adding an extra 5 per cent. of distilled water, so that their concentration will be correct after the autoclaving. The cooling process should be managed in such a way that the chamber pressure diminishes gradually from 15 lb. per sq. in. to atmospheric pressure in the course of 10-30 minutes, the optimal time varying with the volume of medium per container (15-20 minutes is usually most satisfactory). The rate at which the chamber steam spontaneously cools and loses pressure, *i.e.* without opening of the chamber discharge valve, varies with the type of autoclave, the load and other conditions. The reduction to atmospheric pressure may occur in the desired time (*e.g.* 15-20 minutes),

or may take up to an hour or longer. The slower cooling is undesirable since it may result in damage to heat-sensitive materials. If necessary, therefore, the chamber discharge valve should be opened slightly so as to bring about a gradual reduction to atmospheric pressure over the proper period of time. Too rapid a reduction must be avoided, since the media would then boil explosively. Some modern autoclaves, specially designed for sterilising aqueous media, incorporate a device which effects rapid cooling without violent boiling; this may involve the replacement of chamber steam with air at the same pressure, or spraying of the load with condensate at a temperature slightly below that of the steam.

When the pressure gauge shows that the steam in the chamber has reached 0 lb. per sq. in. (atmospheric pressure), air is at once admitted into the chamber, through the air inlet and filter if available, or through the chamber discharge tap, or by opening the chamber door slightly. If this is not done until the pressure has fallen below atmospheric, more water will be lost by evaporation from the containers. When sufficiently cool, e.g. below 70° C., the containers are removed from the autoclave and their screw caps tightened firmly.

Autoclaving in "Free Steam".—When a Koch or Arnold steamer is not available, an autoclave may be used to sterilise culture media at a temperature of 100° C., or just over. The door of the chamber is tightly closed, the steam supply turned on and the air expelled through the open discharge tap. After expulsion of the air, the steam supply is adjusted so that an adequate amount continues to escape through the open tap, and a pressure of 1-2 lb. (above atmospheric) is maintained in the chamber during the holding period; this may be less than in the ordinary steamer (e.g. 60 minutes).

AUTOCLAVING OF EMPTY BOTTLES AND IMPERVIOUS CONTAINERS

Empty and dry containers must not be tightly stoppered, since steam would be excluded and sterilisation by moist heat impossible. They should be placed on their sides in the autoclave to allow a horizontal path for the entry of steam and escape of air. If unstoppered, they will be sterilised quickly, but if stoppered even loosely, as with cotton-wool or a loosened screw cap, the displacement of air is slow and the holding period at 121° C. should be extended to at least 30 minutes. Because of the uncertainty of air displacement from stoppered empty containers, it is better to sterilise them in the hot-air oven.

STERILISATION OF WRAPPED DRY GOODS AND SURGICAL DRESSINGS¹

Dry porous goods such as paper- or cloth-wrapped apparatus, and surgical linen and dressings, require special attention in autoclaving,

¹ For further details see Perkins, J. J. (1956), *Principles and Methods of Sterilisation*, Illinois, Thomas.

firstly to ensure the complete displacement of air from their interior by steam, and secondly to dry them before removal from the autoclave (p. 163).

In conventional sterilisers the air is removed by the *gravity displacement method*, being driven downwards through the load by the lighter steam accumulating above it (p. 158). This method requires that the load be carefully packed so that adequate spaces are left for circulation of steam between the packs and that a free downward movement of air is possible through the materials of each pack. Dry materials must not be enclosed in sealed impervious containers which prevent the entry of steam and escape of air. Glass and metal containers are left open or covered only loosely, and placed on their sides. Metal drums and caskets must be provided with air ports and these must not be obstructed by the contents being packed against them. It is preferable to pack materials in a wrapper of porous cloth or paper, since this allows a much freer passage of air and steam. For surgical packs, a wrapping of at least two layers of good muslin is recommended. A single layer of coarse brown (kraft) paper is also satisfactory, though more likely to be torn in the handling of large packages. A paper wrapping is commonly used for small articles of laboratory apparatus (p. 177).

Surgical dressings and other cloth articles should be arranged in packs no bigger than $12 \times 12 \times 20$ inches, and these should be placed on edge in the autoclave so that the layers of cloth are vertical. Rubber gloves are powdered and packed loosely in muslin wraps, with pads of muslin in the palm and folds to allow access of steam to all parts. Tubing is wetted inside with water just before placing in the autoclave. Instruments and syringes must be free of oil and grease, jointed instruments open, and syringes either disassembled or else moistened internally with water. The autoclave chamber must not be overloaded nor the perforated tray removed to make more room.

Vacuum Removal of Air.—The venturi vacuum device which is commonly incorporated in autoclaves to assist drying can draw a partial vacuum only and remove no more than a third to a half of the air from the chamber. This vacuum is sometimes applied before the admission of steam, but the partial evacuation of air is of little value and, if prolonged, may lead to overdrying and injurious superheating of the load. On the other hand, vacuum removal of air is employed to great advantage in modern "high pre-vacuum" sterilisers (p. 163).

Avoidance of Damage by Superheating.—Cloth and rubber articles are liable to be damaged by excessive heating. When very dry cloth is first exposed to steam, it adsorbs and condenses an excessive amount of it and receives the corresponding excess of latent heat; this may raise its temperature to 25° - 100° C. above that of the autoclave. Freshly laundered fabrics contain sufficient moisture to prevent this and so should not be stored overlong in a place of low humidity before being sterilised, nor be subjected to drying by pre-heating in the steriliser or a prolonged application of vacuum. Heating in steam mixed with some

air is more damaging than heating in pure steam, especially in the case of rubber articles.

The Duration of the Holding Period at 121° C.—The holding period is timed as beginning when the discharge-channel thermometer first indicates 121° C. All free air has then been displaced from the chamber, but some still remains trapped in the interior of the porous load. The further time required for the steam to penetrate all parts, displace the air and heat the load throughout to 121° C., may extend to 30 minutes or even longer. This "steam penetration time" must be added to the "sterilising time" of 12 minutes in computing the holding period. The following are generally recommended as the total holding times at 121° C.: 15–20 minutes for muslin- or paper-wrapped instruments, rubber gloves and open metal or glass containers; 30 minutes for muslin- or paper-wrapped packs of surgical linen and dressings, wrapped syringes, and loosely stoppered metal or glass containers; 45 minutes for metal surgical dressing drums with muslin liners.

Drying of the Load.—The load is dried during the cooling period. The supply of steam at 121° C. is maintained in the jacket, while that to the chamber is cut off. The chamber steam is allowed to escape rapidly through the discharge tap until zero gauge pressure is reached. The moisture of the load is then evaporated by the residual heat of the articles and radiant heat from the jacketed walls of the chamber. The drying is assisted to completion by the removal of vapour from the chamber by one of two methods. (1) "*Cracked-door*" method. When the chamber has been exhausted to atmospheric pressure, the door is "cracked", i.e. opened about $\frac{1}{2}$ cm. only. This is enough to allow the escape of vapour without overcooling the chamber. Cool air enters at the bottom and warmed air mixed with vapour escapes at the top. After about 20 minutes the load is sufficiently dry for it to be removed. (2) *Vacuum and air method*. When the chamber steam is exhausted to atmospheric pressure, the venturi device is used to suck warmed, filtered air into and through the chamber; this carries away the vapour and dries the load in about 15 minutes. The air allowed into the chamber must be drawn through an efficient filter to free it from dust-borne bacteria which otherwise might enter the sterilised packages.

Without the concurrent admission of air, the application of the partial vacuum obtainable by the venturi device is quite inadequate to effect drying. Some modern sterilisers, however, are fitted with electrically driven water-sealed pumps which draw a very high vacuum and can dry the load in a few minutes.

On removal from the autoclave, wrapped packs should not at once be placed on a cold flat surface, since residual moisture will condense on this, make damp the muslin or paper wrapping, and so permit the entry of contaminating bacteria.

HIGH PRE-VACUUM STERILISERS

The most advanced surgical sterilisers are equipped with electrically driven pumps capable of exhausting the chamber to an almost perfect

vacuum (*e.g.* to an absolute pressure below 20 mm. Hg for oil-sealed pumps and below 50 mm. Hg for water-sealed pumps). A high pre-vacuum is drawn before admission of steam to the chamber and the absence of air then enables the steam very rapidly to penetrate and heat up all parts of the interior of the load. *Even a tightly packed load is heated rapidly and uniformly to the sterilising temperature.* This makes it feasible to employ a higher sterilising temperature for a shorter time, namely 135° C. for 3 minutes (*i.e.* jacket and chamber steam at 30 lb. per sq. in. gauge pressure). The total operation time is greatly shortened and damage to heat-sensitive materials through exposure to injurious air-steam mixtures or prolonged heating in the outer parts of the load, is avoided.

The chamber is loaded with as tight packing as desired and the vacuum drawn to remove all air from the chamber and load within 5–10 minutes. (Automatic control, *vide infra*, obviates the possibility of the evacuation being unduly prolonged, with resultant overdrying and superheating.) Steam is admitted to the chamber and heats the whole load to 135° C. within two or three minutes. The holding period is continued for 3 minutes from the time the thermometer first reaches 135° C. The load is then dried within a few minutes by exhaustion of the chamber to a high vacuum with a water-sealed pump, and the vacuum is finally broken by admission of air through a filter.

AUTOCLAVE CONTROLS AND STERILISATION INDICATORS

Automatic Process Control.—It is advantageous for the steriliser to be furnished with an automatic control system which carries through the whole sterilisation cycle, including the heating-up, holding, cooling and drying stages, according to a pre-selected scheme for the duration, temperature and pressure of each stage. After the chamber has been loaded and the process started, no further attention is required until the load is ready for removal. Apart from saving the time of a skilled operator, automatic control is a valuable safeguard against error due to negligence or distraction. A monitoring system ensures that if the temperature at any time falls below that selected, the operation will be repeated.

Recording Thermometer.—This desirable adjunct makes a graphic timed record of the temperature changes in the chamber discharge channel and thus, in the absence of automatic control, helps the operator to avoid errors in timing the holding period.

Thermocouple Measurement of Load Temperature.—This is the method of discovering the heating-up time required for a given kind of load. A thermocouple is inserted deeply inside a test article in the autoclave chamber, *e.g.* a bottle of liquid or a pack of dressings, and its wire leads are carried out under the chamber door to a potentiometer. The latter indicates the temperature inside the test article during the course of autoclaving.

Chemical Indicators may be placed inside the load, which show a

change of colour or shape after exposure to a sterilising temperature. Thus, a pellet of sulphur in a small glass tube will show a change of shape by melting when exposed at 120° C. for a few minutes. Browne's steriliser control tubes¹ contain a red solution which turns green when heated at 115° C. for 25 minutes (type 1) or 15 minutes (type 2), or at 160° C. for 60 minutes (type 3). They must be stored at less than 20° C. to avoid deterioration and premature colour change.

Spore Indicators.—A preparation of dried bacterial spores is placed within the load in the autoclave and after autoclaving is tested for viability. Commonly used strains include *Bacillus subtilis* var. *globigii*, which is killed at 105° C. in about 15 minutes, and *Bacillus stearothermophilus*, which is killed at 121° C. in about 12 minutes. The former is cultivated at 30°–35° C. and the latter at 55°–60° C. A culture grown aerobically on nutrient agar for 5 days is suspended in sterile water to a concentration of 1 million spores per ml., or greater. Small strips of filter paper, e.g. 5 × 1 cm., are soaked in the suspension, dried at room temperature and placed in paper envelopes which are then sealed. A few of these packets are placed in different parts of the load. After autoclaving, the envelope is cut with sterile scissors and the strip transferred with sterile forceps to a tube or flask of "recovery medium", e.g. thioglycollate broth (p. 235) or cooked-meat medium (p. 233); it is necessary to take rigorous precautions against contamination while making this transfer. The tube is incubated for 7 days at the appropriate temperature and then examined for growth. An unautoclaved spore strip is cultured as a positive control and an uninoculated tube of medium as a negative control. The results should be reported in terms of the degree of heat-resistance of the spore preparation used. This may be determined by holding small sealed tubes of the spore suspension for varying periods in a water-bath at 100° C. or in an oil-bath at higher temperatures, and then testing for viability by culture. Instead of spore strips, envelopes containing about 1 g. of dried earth may be used, since samples of earth almost always contain highly resistant spores.

Recovery Media.—Bacteria and spores that have been damaged by heat, may require special cultural conditions to allow their recovery and growth. They may lie dormant for several days when placed in a culture medium, and incubation should be continued for at least a week to give them the opportunity of growing. Moreover, certain enriched media may permit their growth when ordinary media fail. Enrichment with yeast extract, starch, glucose, blood or milk has been found beneficial. Thioglycollate broth (p. 235) and cooked-meat medium (p. 233) are suitable for recovery of both aerobic and anaerobic bacteria.

The chemical or spore indicators are placed in the centre of the largest and most densely packed items of the load, and some near the bottom of the chamber where air tends to collect. The results may reveal the failure of some parts of the load to become adequately heated and thus draw attention to a fault in the construction, loading or operation of the steriliser. Successful tests, on the other hand, give no

¹ A. Browne Ltd., Chancery Street, Leicester.

assurance that the steriliser and technique are reliable, since heating might yet be inadequate in other parts of the load or under different conditions of loading. The essential guarantee of sterilisation is that a properly designed and properly loaded autoclave be operated so as to show the correct sterilising temperature on the discharge-channel thermometer for the appropriate time.

OTHER METHODS OF STERILISATION BY HEAT

The sterilisation of serum or body fluids containing coagulable protein can sometimes be effected by heating for one hour at 56° C. on several successive days. The principle is the same as in the case of intermittent sterilisation at 100° C. (p. 152). It may be necessary to repeat the heating eight times to ensure complete sterilisation. Care must be taken not to allow the temperature to rise above 59° C., as inspissation may occur. The exposure to 56° C. is best carried out in a water-bath, but a 56° C. oven may be used. This procedure is not always effective—*e.g.* if certain resistant types of sporing organisms are present.

Vaccines.—Vaccines prepared from cultures of non-sporing bacteria are sterilised in a special water-bath ("vaccine bath") at a comparatively low temperature, one hour at 60° C. being *usually* sufficient. Higher temperatures may diminish the immunising power of the vaccine.

The *vaccine bath* consists of a copper container sometimes lagged with linoleum or other non-conducting material and is heated either by gas or electricity. The bath is fitted with a suitable removable rack for holding the tubes, while the lid has a hole into which a thermometer is placed. The bath is maintained at a constant temperature, usually 60° C., by means of a thermostatic control, which is either a "capsule", similar to that in an incubator, or a bimetallic device. When the temperature rises above the desired level, the thermostatic control diminishes the gas supply or cuts off the electricity. When the temperature falls the heating is resumed. By means of an adjusting screw, the temperature can be regulated to $\pm 1^\circ$ C. of that required. All water baths should be inspected at weekly intervals and any loss of water, due to evaporation, etc., restored.

STERILISATION AND DISINFECTION BY CHEMICALS

(1) *Volatile Antiseptics*, *e.g.* *Chloroform*.—This method is sometimes used in the sterilisation and preservation of serum (for culture media), and the chloroform, which is added in the proportion of 0.25 per cent., can later be removed by heating at 56° C. If the serum is to be used for making a coagulated serum medium (*e.g.* Löffler's medium) the chloroform will be removed by the heating applied for coagulation. Chloroform is used also for preserving culture media in bulk.

(2) *Antiseptics of the Phenol Group*.—Liquor cresolis saponatus (lysol) and cresol are powerful antiseptics. Their chief use in a laboratory is for sterilising surgical instruments and discarded cultures, and for disinfecting the hands and killing cultures accidentally spilt by the worker. Lysol is generally used in a 3 per cent. solution. Phenol, 0.5 per cent., or *p*-chloro-*m*-cresol, 0.1 per cent., is used for preserving sera and vaccines.

(3) *Metallic Salts or Organic Compounds of Metals*—e.g. mercuric chloride (perchloride of mercury) is sometimes used as a disinfectant in a 1 in 1000 solution. "Merthiolate", a proprietary name for sodium ethylmercurithiosalicylate, is used in a dilution of 1 in 10,000 for the preservation of antitoxic and other sera.

(4) *Formaldehyde*.¹—This irritant water-soluble gas is highly lethal to all kinds of microbes and spores, killing bacterial spores almost as readily as the vegetative forms. It is cheap, and non-injurious to cloth-fabrics, wood, leather, rubber, paints and metals, and can thus be used to disinfect rooms, furniture and a wide variety of articles liable to damage by heat (e.g. woollen blankets and clothing, shoes, respirators, hairbrushes, gum-elastic catheters). It is applied as an aqueous solution or in gaseous form.

Disinfection by Aqueous Formaldehyde Solution.—Commercial "formalin" is a 40 per cent. (w/v) solution of formaldehyde in water containing 10 per cent. methanol to inhibit polymerisation. A dilution containing 5 or 10 per cent. formaldehyde is a powerful and rapid disinfectant when applied directly to a contaminated surface.

Bacterial cultures and suspensions are commonly killed and fixed by addition of formaldehyde to a concentration of 0.04–1.0 per cent., e.g. for preservation prior to counting or other measurements, and in preparation of a killed vaccine or agglutinable suspension.

Cleaned metal instruments may be sterilised by overnight immersion in a borax-formaldehyde solution:

Sodium tetraborate	50 g.
Formaldehyde, 4 per cent. in water . . .	1000 ml.

Disinfection by Formaldehyde Gas. Gaseous disinfection is required for articles that cannot be wetted completely with solution, or are damaged by wetting, but care is required to provide the proper conditions for action of the gas. Thus, the atmosphere must have a high relative humidity, over 60 per cent. and preferably 80–90 per cent., and a temperature of at least 18° C. Moreover, the materials must be arranged to allow free access of the gas to all infected surfaces, since its penetration into porous fabrics is slow.

The gas is liberated by spraying or heating formalin, or by heating solid paraformaldehyde. When spraying cold formalin, an equal volume of industrial spirit (ethanol) may be added to prevent polymerisation. The best method is by boiling formalin diluted with sufficient water to produce an adequate atmospheric humidity. Because

¹ *Monthly Bull. Minist. Hlth Lab. Serv.* (1958), 17, 270.

of the tendency of the gas to polymerise to paraformaldehyde, the maximal vapour concentration attainable at 20° C. is about 2.0 mg. per litre of air; it is desirable to achieve this concentration. Higher concentrations, which may be potentially explosive, are attainable at higher temperatures. | After disinfection, the article may contain sufficient paraformaldehyde to give off irritant vapour over a long period; this paraformaldehyde can be neutralised by exposure to ammonia vapour.

Small articles, such as instruments, shoes and hair-brushes, are disinfected by exposure for at least 3 hours to formaldehyde gas in an airtight cabinet of metal or painted wood. The gas is introduced into the air in the cabinet by boiling formalin in an electric boiler to the extent of 50 ml. of 40 per cent. formaldehyde per 100 cubic feet of air space. Blankets and the surfaces of mattresses are disinfected similarly in a large cabinet, where they are hung unfolded; to allow for absorption by the fabric, a much greater amount of formalin is used, namely 500 ml. per 100 lb. of fabrics. The vapour is finally vented to the open air. Folded blankets and clothing can be disinfected only if some heat is applied. They are packed in the chamber of a steam-jacketed autoclave (p. 156), 100 ml. of formalin per 100 cubic feet is placed in the bottom of the chamber and the chamber is heated at 100° C. for 3 hours by passing free steam through the jacket. The vapour is finally vented through the autoclave vacuum system.

*Disinfection of Rooms by Formalin Spraying.*¹—This is probably the most effective means of disinfecting the interior and furniture of a room, e.g. one infected with anthrax, tuberculosis or smallpox organisms. The room is first well sealed by covering cracks, ventilators, fireplaces, etc., with brown paper and adhesive tape. An operator protected by an efficient anti-gas respirator thoroughly moistens all surfaces of the walls, floor and furniture with a spray of 10 per cent. formaldehyde solution (1 volume of formalin and 3 volumes of water), and finally saturates the atmosphere by spraying undiluted formalin to the extent of 1 litre per 1000 cubic feet. The room is closed by sealing the door and left for 24 hours. A basin of ammonia solution is then introduced and left to evaporate for several hours (1 litre S.G. 880 ammonia solution mixed with 1 litre of water per litre of 40 per cent. formaldehyde used). This neutralises the formaldehyde and paraformaldehyde, and the excess ammonia is readily removed by ventilation.

Disinfection of Rooms by Formaldehyde Vapour.—The room is sealed as described above and heated, if necessary, to above 18° C. Formalin is boiled within the room in an electric boiler having a safety plug which kicks out when the vessel boils dry and a time switch set to cut off the current just prior to this; 500 ml. of 40 per cent. formaldehyde plus 1000 ml. water are boiled per 1000 cubic feet of air space. The room is kept sealed for 4–24 hours and an operator wearing a respirator then introduces a cloth soaked in ammonia solution (250 ml. per litre of formalin used). This is left for 2 hours to neutralise the formaldehyde.

¹ Jack, R. P. (1952 and 1954), *City of Edinburgh Public Health Reports*.

(5) *Ethylene Oxide*.—This gaseous disinfectant is also highly lethal to all kinds of microbes and spores, but is capable of much more rapid diffusion into dry, porous materials. Its handling requires care, since it is toxic on contact with the skin and on inhalation of odourless concentrations, and it forms inflammable, explosive mixtures with air. A non-explosive mixture of 10 per cent. ethylene oxide in carbon dioxide, or in halogenated hydrocarbon, is used to sterilise articles liable to damage by heat, e.g. plastic and rubber articles, blankets, pharmaceutical products and complex apparatus. These are exposed in a sterilising cabinet from which the air is first removed by drawing a high vacuum, and the gas then introduced to a pressure of 5–30 lb. per sq. in. above atmospheric pressure. The cabinet should be maintained at 45°–55° C. and water introduced, if necessary, to give a relative humidity of 30 per cent. After exposure for several hours or overnight, the gas is removed by drawing a high vacuum.

(6) *Disinfection of Blankets*.¹—The bedding of a patient or carrier is liable to become heavily contaminated with pathogenic bacteria, e.g. *Staph. aureus* and *Strept. pyogenes*, and, when disturbed, liberates large numbers of these into the air. Cotton and linen sheets, and blankets made from cotton or synthetic polyester fibre (Terylene), may be sterilised by boiling during laundering. Woollen blankets, however, shrink on boiling. They may be laundered at 40° C. and disinfected in a cold rinse containing quaternary ammonium compounds which kill nearly all the vegetative bacteria. Woollen blankets may be sterilised by low-pressure autoclaving, but this seriously damages them if repeated. Formalin disinfection can be used, but this requires special equipment. Application of oil to the blankets in the last stage of the laundering process reduces the subsequent scatter of organisms.

(7) *Disinfection of Skin*.—Skin is contaminated with micro-organisms derived from the environment and these may be removed from the hands by thorough washing and scrubbing in warm water with soap or some other surface-tension reducing agent, e.g. a disinfectant cationic detergent such as cetrimide ("Cetavlon") or benzalkonium chloride ("Roccal"). Commensal organisms such as *Staph. epidermidis* grow in the superficial layers of keratin, and in the glands and hair follicles of the skin, and are continually dispersing over the surface. Washing with soap and water, with or without the addition of disinfectants, will thus only temporarily clear the surface. After the hands are washed, they may be rinsed in 70 per cent. alcohol, which dries rapidly and has a transient bactericidal effect.

Where strict asepsis is required, as in surgery, it is necessary to wear sterile rubber gloves which are impervious to the organisms on the skin. As there is always a danger of the gloves being perforated during use, it is important before donning them to remove as many of the bacteria from the hands as possible. This may be done as above. A sustained

¹ For further details consult Blowers, R., Potter, J., & Wallace, K. R. (1957), *Lancet*, 1, 629; Gillespie, E. N., & Robinson, W. (1959), *J. clin. Path.*, 12, 351; Thomas, C. G. A., & Liddell J. (1958), *Brit. med. J.*, 2, 1336.

sterilising effect has recently been claimed for hexachlorophene, which can be added (1-3 per cent.) to the wash water containing soap or other detergent.

When only a single application is required, as in preparing the skin of a patient or animal for incision or puncture, stronger disinfectants may be used, *e.g.* tincture of iodine or 5 per cent. phenol solution (as when obtaining blood for culture, see p. 260), or 0.1 per cent. merthiolate or a strong solution of chloroxylenol (*e.g.* "Dettol").

STERILISATION BY FILTRATION

This can be effected by the use of filters of unglazed porcelain (Chamberland) or of diatomaceous earth (Berkefeld), the pores of which are so small that ordinary bacteria are prevented from passing through. Seitz filters, in which filtration is effected through asbestos disks, are likewise very useful and reliable.

Such filters are also employed in separating viruses. The principles of this application are considered on p. 748.

TYPES OF FILTER

The various types of filter used in bacteriological work are considered here, but some are clarifying filters and do not remove bacteria.

- (1) Earthenware candles, *e.g.* Berkefeld, Chamberland, Doulton.
- (2) Asbestos disks, *e.g.* Seitz.
- (3) Glass filters made of finely ground glass fused sufficiently to make the small particles adhere, as in the sintered glass filters of Schott and Genossen of Jena, and now made in England.
- (4) Collodion membranes, for estimation of the size of virus particles.
- (5) Sand-and-paper-pulp filters, for removing larger particles and clarifying exudates, tissue emulsions, etc.

BERKEFELD FILTERS

These are made from kieselguhr, a fossil diatomaceous earth found in deposits in Germany and other parts of the world. Filters made from this material are coarse—that is, have relatively large pores owing to the size of the granules forming the substance of the filter. They are made in three grades of porosity—namely V (viel) the coarsest, W (wenig) the finest, and N (normal) intermediate. Of these, the Berkefeld V is the one usually employed, and it should not pass a small organism such as *Serratia marcescens*.

A similar type to the Berkefeld is the Mandler filter, manufactured in the United States.

(The coarser grades of the Chamberland filters, *vide infra*, are similar in porosity to the Berkefeld types.)

STERILISATION

CHAMBERLAND FILTERS

These are made of unglazed porcelain and are produced in various grades of porosity. The finer grades will pass only certain viruses or extreme minuteness, such as the viruses of foot-and-mouth disease and of fowl plague. The most porous, L₁, allows many organisms to pass, being merely a clarifying filter. The next three, L_{1a}, L₂ and L₃, are comparable with the Berkefeld V, N and W candles respectively. The porcelain filters are used for the removal of organisms from fluid cultures in order to obtain the bacterial toxin.

SEITZ FILTERS

This type consist of a disk of an asbestos composition through which the fluid is passed. The disk is inserted into a metal holder which ensures a tight joint being made. After use the asbestos disk is discarded and a new one employed for each filtration. Various sizes for laboratory work are available. The large size of Seitz filter, with 14 cm. diameter disk, can be recommended for the sterilisation of large amounts of serum to be used in the preparation of medium (p. 206). The disks are supplied in three grades—termed clarifying (K), normal and "special EK". The normal and EK grade of disk do not allow the ordinary test bacteria, e.g. *Serr. marcescens*, to pass.

Similar disks are made in England, and are as reliable and efficient as the foreign ones. The grade GS corresponds to the EK, and the FCB to the K disks.¹

COLLODION FILTERS

Elford² has devised a technique for preparing collodion membranes of graded porosity which he terms *gradocol* membranes, since they are products of graded coagulation of collodion. Collodion films show two types of structure:

- (a) *microgel*, which has a coarse structure visible microscopically, and
 - (b) *ultragel*, the structural elements of which are not resolvable by the microscope, but which is built up of particulate matter.
- Hitherto, these membranes, although uniform, have not been sufficiently permeable for filtration work.

The *gradocol* membranes possess the permeability of the microgel type, but have the ultragel structure and are very uniform in their porosity. They are made from an acetone solution of collodion (nitro-cellulose) diluted with an ethyl-alcohol-ether mixture to which are added varying amounts of amyl alcohol. 75 ml. of the mixture are poured into a shallow cell 20 cm. in diameter in a constant-temperature

¹ Supplied by A. Gallenkamp & Co. Ltd., London, and John C. Carlson, Ltd., Weir Mills, Mossley, Lancs.

² For details of technique see Elford, W. J. (1931), *J. Path. Bact.*, **34**, 505; and Elford, W. J. (1933), *Proc. roy. Soc. B.*, **112**, 384.

room (22.5° C.), allowed to evaporate for varying periods of one to three hours, and then washed over an extended period with distilled water. By varying the amount and composition of the collodion mixture, and the conditions of evaporation, permeable filters of average-pore size (A.P.S.) ranging from 3μ down to $10 m\mu$ or less have been prepared. It is possible to reproduce accurately at any time filters of any desired permeability and porosity. The technical details are elaborate and of extreme importance.

By means of these collodion membranes it has been possible to determine the size of many of the viruses.

TECHNIQUE OF FILTRATION

As fluids do not readily pass through the above described filters by gravity, it is necessary to use positive or negative pressure. Suction is the most convenient method of filtration, the fluid being drawn through the filter into a sterile container, usually a "filtering flask" which is a conical flask of thick glass with a side-arm.

The smallest negative pressure that produces satisfactory filtration should be used, commencing with a small pressure and gradually increasing as filtration proceeds. It should be noted, however, that the time of filtration should not be prolonged as with a slight pressure over a prolonged time small motile flexible organisms such as spirochaetes and slender vibrios may pass through the filter. A high negative pressure must be avoided as small particles are rapidly forced into the pores of the filter, thus preventing further filtration. For ordinary purposes a negative pressure of 100–200 mm. of mercury is usually sufficient.

When using a filter of the Berkefeld type the earthenware "candle" is fitted by means of a screw and washers into a cylindrical glass mantle, and the metal tube of the filter passes through a rubber stopper which is fitted into the neck of the flask. The side-arm of the flask is connected with an exhaust pump by pressure tubing. The fluid is poured into the mantle and after filtration is collected into the flask. The necessary suction is obtained by the usual form of water pump or by a mechanical air pump. The negative pressure is estimated by means of an attached mercury or other type of manometer.

Similarly, when using a Seitz filter the metal tube may be inserted into a rubber bung which fits into a filtering flask.

A disadvantage of the filtering flask is that the filtered fluid has to be transferred later to another container, and where it is desired to store filtered fluids, e.g. serum or culture media, contamination may occur in the process. It has also been our experience that rubber bungs are not resilient after one autoclaving and do not again fit satisfactorily so that it is necessary to tie the bung to the filter flask and seal the joints with wax.

As an alternative to a filtering flask a simple fitting attached to a screw-capped bottle can be recommended. It consists of a straight piece

of metal tubing, 6–7 mm. external diameter, surrounded by a wider piece of tubing to which is fitted a side-arm. The tubes are fitted into a metal screw-cap furnished with a washer to secure an air-tight joint (figure, 8). The fitting is made preferably of stainless steel. Any of the screw-capped bottles can be used according to the amount of fluid to be filtered. As several sizes of bottles may fit one size of screw-cap (p. 185), a few different sizes of cap will cover a range from a few ml. up to 4 litres. The filter employed—*e.g.* Berkefeld, Seitz, sintered glass or paper pulp—is connected to the top of the fitting by pressure rubber tubing.

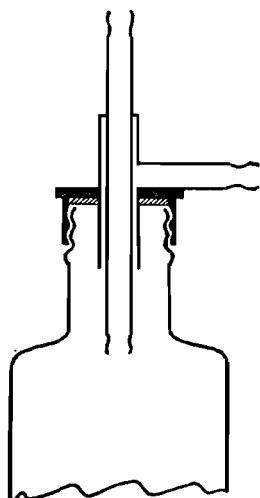


FIG. 8

If it is desired that the fluid should not come in contact with metal, a piece of glass tubing is passed through the central metal tube and is held in position and an air-tight joint secured by means of a piece of rubber tubing at each end of the metal tube. A wide central tube is necessary for this. Alternatively, an attachment without the central metal tube can be used; the glass tubing takes the place of the central metal tube and is secured to the upper end of the wider tube by a short length of rubber tubing which grips both.

One of the advantages of the metal screw-cap fitting is that when the filtrate has to be stored, *e.g.* toxin, serum, etc., it need not be removed from the container. An ordinary screw-cap for the bottle is wrapped in kraft paper and sterilised with the remainder of the apparatus. After filtration the filter and screw attachment are removed, the ordinary cap is taken from its sterile wrapper and screwed on. Where the filtrate is to be kept for some time a viskap (p. 262) over the screw-cap is recommended to exclude dust or obviate unauthorised opening.

Berkefeld Filters.—Before setting up the filter it should be tested for gross leaks by passing air through it under pressure while the candle is under water. If large bubbles of air escape, the filter is unsuitable. Faults usually occur at the junction of the candle and metal holder, but cracks may be seen in the earthenware portion. Water should be drawn through the filter both ways before it is put into use.

The procedure with the small size ($2\frac{1}{2}$ in. $\times \frac{1}{2}$ in.) when using a filtering flask is as follows. In order to secure the maximum amount of filtrate a test-tube slightly wider and longer than the candle should be inverted over it. This ensures that the whole of the candle is covered with fluid almost to the end of the filtration. Before sterilisation, the glass mantle, the candle and covering test-tube are loosely assembled without tightening up the screw and washers. The open end of the glass cylinder is plugged with cotton wool, and the metal tube of the candle is inserted into the rubber stopper which fits the filter flask. The whole is wrapped in kraft paper and sterilised by steaming or autoclaving. The filter flask is plugged with cotton wool, over which is tied a piece of kraft paper, and a small air filter, as figured on p. 203, is attached to the side-arm by means of rubber tubing. The flask is sterilised in the steamer or autoclave. It is advisable, where several filters and flasks are kept sterilised, to have corresponding numbers on the filters and flasks, to ensure the rubber stopper attached to the filter candle being used with the appropriate flask. When filtration is to be carried out the filter is unwrapped, the bung inserted into the flask and the washers are tightened up. The air filter is connected to the vacuum supply, fluid is poured into the mantle and gentle suction commenced. After filtration, the filter and rubber bung are removed and the filtrate is transferred to a sterile container by means of a pipette.

When using the screw attachment with a screw-cap bottle, the mantle, filter and covering tube are loosely assembled. The metal tube of the filter is connected by pressure tubing with the screw attachment, to the side-arm of which is attached an air filter (Figure, p. 203) by means of rubber tubing. The screw-capped bottle is *loosely* inserted into the screw fitting. The whole is wrapped in kraft paper or a piece of cloth and sterilised in the steamer or autoclave. An ordinary cap wrapped in kraft paper is sterilised with the outfit.

For filtration the apparatus is unwrapped, the bottle screwed tightly on, the filter and bottle are held securely in a retort stand and the air filter is connected with the vacuum supply. After filtration the bottle is unscrewed from the screw attachment and the ordinary cap screwed on.

Large Berkefeld filters are fitted into tall, narrow cylinders, and the outlet-end connected with rubber tubing to a glass tube which passes through the rubber bung of a large filtering flask, or the filter may be connected to the top of a screw attachment which fits a narrow-mouth one-gallon bottle. The fluid to be filtered should always cover the porous part of the candle.

After use the filters should be brushed with a stiff nail-brush and then boiled in distilled water. Before sterilising again, distilled water should be run through them to show that they are pervious. When the pores of earthenware or porcelain filters become clogged with organic matter they should be heated to redness in a muffle furnace and allowed to cool slowly.

Chamberland Filters.—The large sizes are used in the same manner as the larger Berkefeld filters. The smaller candles, such as sizes 6 and 7 of Nos. L_{1a}, L₂ and L₃, are fitted into a rubber bung so that the open end of the candle projects just above the top of the bung and the candle itself hangs down in the filtering flask. The fluid to be filtered is placed in the open end of the candle, and filtration occurs from the inside to the outside of the filter.

Seitz Filters.—The filter is loosely assembled with the asbestos disk in position and the delivery tube passed through a rubber bung when a filtering flask is used. The whole is wrapped in kraft paper and sterilised in the steamer or autoclave. The filtering flask is plugged and fitted with an air filter as described under Berkefeld filters.

When a screw fitting is used, the filter is assembled with an asbestos disk, and the delivery tube connected by pressure tubing with the screw attachment previously described, to the side-arm of which is attached an air filter. (When small amounts of sterile fluid have repeatedly to be removed, e.g. sugars, serum, etc., the apparatus figured on p. 203 may be used.) The bottle is loosely screwed into the screw attachment, the whole wrapped in kraft paper and sterilised in the steamer or autoclave. When required for use the screw-cap is tightened up on the rubber washer and filtration proceeded with as above. After filtration the bottle is unscrewed and an ordinary sterile screw-cap fitted to it. The used asbestos disk is discarded. When using Seitz filters it is advisable to moisten the disk with sterile saline and then screw down tightly the upper part of the metal on the softened asbestos before pouring in the liquid to be filtered.

Sintered Glass Filters.—A special grade for sterilisation purposes is manufactured by supporting a specially fine ("grade 5") filter on a coarser ("grade 3") layer, and is known as the "3/5" type. These filters are attached to the filtering apparatus and sterilised in the same way as the Seitz filter, but care must be taken that extremes of temperature are avoided. After use they are washed with running water in the reverse direction. They should be cleaned with warm sulphuric acid to which has been added a quantity of potassium nitrate, and not with sulphuric-acid-bichromate mixture.

Sand-and-Paper-Pulp Filters.—Before filtering nasal washings or other tenacious materials through Berkefeld or Chamberland filters, it is advisable to pass them through a sand-and-paper-pulp filter. This can easily be made from a piece of glass tubing, 8 in. long by 1 in. diameter, with the lower end drawn out to a $\frac{1}{4}$ -in. bore. At the bottom of the wide portion of the tube is a small perforated porcelain disk or plug of cotton wool, and on the top of this is placed about $\frac{1}{2}$ in. of

paper pulp which has been well shredded and soaked in water. This is gently pressed down, and on the top is placed about 1 in. of fine washed silver sand. On top of the sand a further $\frac{1}{2}$ in. of paper-pulp is placed, and the whole three layers are pressed down. The lower end of the filter fits into a bung and filtering flask or is connected by rubber tubing to the screw attachment figured on p. 173. The whole is wrapped in kraft paper and sterilised in the steamer. Filtration is carried out by suction in the usual manner.

This preliminary filtration removes coarse particles, mucus, etc., which would be liable to clog the pores of an earthenware filter.

Filtration of Small Amounts of Fluid

With the smaller sizes of Berkefeld or Seitz (3-cm.) filters, a small test-tube is arranged inside the filtering flask so that the delivery tube of the filter projects into the open end of the tube and the filtered fluid is collected directly in the small tube instead of the flask itself. Similarly, with the Chamberland filter the lower end of the candle fits into a small test-tube inside the flask.

Centrifugal Filter.—A small amount of fluid is conveniently filtered with a simple filter holder (supplied by H. A. Jones, Beaumaris, Anglesey) carrying a sterile screw-topped $\frac{1}{2}$ -oz. or 1-oz. bottle at either end. The fluid is placed in one bottle and the holder, which is fitted with a Seitz filter pad (1.8 cm.), is screwed on to the top of the bottle. The second bottle is screwed on to the other end of the holder and the assembly then placed in a bucket of a centrifuge so that the empty bottle will be outermost. It is necessary to match balanced pairs of these filters in opposite buckets. Filtration is usually complete within ten minutes of centrifugation.

MAINTENANCE OF STERILITY

It is necessary that apparatus, after sterilisation, should be kept sterile.

Test-Tubes and Flasks.—The interiors of test-tubes, flasks, bottles, etc., must be carefully protected from bacterial contamination due to access of air, dust, etc., before and after the addition of medium and during the subsequent cultivation of organisms. This has usually been done by means of cotton-wool stoppers. These should be $1\frac{1}{4}$ – $1\frac{1}{2}$ in. long, $\frac{3}{4}$ –1 in. being inserted into the mouth of the tube, etc., and the remainder projecting. They should fit firmly, but not so tightly as to render their removal difficult.

Stoppering of Tubes.—Long-fibre cotton-wool is essential and must be free from short broken fibres and dust. Non-absorbent cotton is preferable, because, after steaming, plugs tend to remain moist, and if the medium is to be kept for any length of time and absorbent wool

is used, moulds will grow through the stopper and contaminate the medium. A sufficient amount of cotton-wool (*vide supra*) should be forced into the tube with a rod or pair of forceps, but should not be twisted in, as creases are formed along the sides of the glass and create channels for contaminating organisms.

Instead of the ordinary roll of cotton-wool being used, it is recommended that the non-absorbent wool be obtained in the form of a long thin ribbon known as "rope wool" or "neck wool" of the type used by hairdressers. It is kept in a tin container with a hole in the lid, and the appropriate amount of wool for the stopper is easily obtained without waste.

When tubes or flasks have to be stored for some time the stoppers or tops of the crates or boxes should be covered with sterile kraft paper, kept in place by means of fine string or a rubber band. Sterile rubber stoppers may, in some cases, be used instead of cotton-wool, particularly where the contents of the flask or tube have to be kept a considerable time, as in the case of immune sera; this also applies to vessels which have to be transported by post or by messenger.

Screw-capped Bottles.—Flasks for storing culture media have now been replaced by screw-capped bottles of 3-, 5- and 10-oz. capacity, while the smaller bottles of $\frac{1}{2}$ -, $\frac{1}{4}$ - and 1-oz. capacity are employed instead of test-tubes. Their use is referred to later.

Petri Dishes.—Each individual dish should be wrapped in kraft paper before sterilisation, and kept in the paper until used. For a 4-in. dish the size of paper should be 12 in. square. The dishes may also be sterilised (unwrapped) and kept in cylindrical tinned-copper boxes.

Pipettes.—1-ml. and 10-ml. graduated pipettes should be wrapped in a long strip of kraft paper, which is wound round them in a spiral manner before sterilising in the hot-air oven. Bulb pipettes (10 ml., 50 ml., etc.) are also covered with kraft paper. Under these conditions pipettes remain sterile in their wrappers for considerable periods of time.

Capillary pipettes are sterilised in large test-tubes 15 in. \times $2\frac{1}{2}$ in., having a gauze or cotton-wool stopper, or in tinned-copper boxes. The former method is preferable. Alternatively, 8-in. lengths of 5-mm. glass tubing are plugged with cotton-wool at both ends, wrapped in batches of a dozen in kraft paper, sterilised and stored. When capillary pipettes are required, the middle of the tubing is heated in a Bunsen or blowpipe and pulled out, the ends of the two pipettes being sealed in the making.

Ampoules are sterilised in the hot-air oven with the necks sealed, and are kept in metal boxes. If unsealed ampoules are used, they should be plugged with cotton-wool before sterilisation.

It must be emphasised that a wrapping of kraft paper or double-thickness muslin is effective in excluding contaminating bacteria *only when it is dry*. If the wrapped articles are sterilised in an autoclave instead of a hot-air oven, they must be dried before placing on an unsterile surface.

STERILISATION OF SYRINGES¹

As syringes play an important part in the work of a bacteriological laboratory, particular attention must be given to their use, care and sterilisation.

It is recommended that all-glass syringes should be used in preference to the glass-metal syringe of the "Record" type, over which they have many advantages. The glass-metal type is more difficult to clean, and is more likely to break on heating owing to the difference of expansion of glass and metal. It cannot be sterilised when assembled and is more difficult to keep sterile until ready for use. The solder uniting the glass and metal parts may melt in the hot-air oven, and may even do so in the autoclave. The all-glass syringe should be of the "Luer" type and preferably according to British Standards Specification No. 1263 (1945).² Syringes of 5 ml. capacity and upwards should have eccentric nozzles. The needles should be of stainless steel of the best quality. The mounts of the needles must fit accurately to the nozzle of the syringe.

All-glass Syringes.—Before being put into use, new syringes must be well washed in soap and water with a test-tube brush or burette brush according to size. After washing in clean, warm water, both barrel and piston are dried.

In a laboratory it is convenient to have the syringes assembled, wrapped and sterilised, ready for use, and to have a supply of these sterilised syringes always on hand. When this service is not available, all-glass syringes may be sterilised just before use by boiling in a fish kettle or saucepan. If the tap water is hard it is best to use distilled water. The syringe is dismantled and the barrel and piston placed in cold water, which is brought to the boil and kept boiling for not less than five minutes. The perforated tray is removed from the steriliser, the water poured off and the tray returned to the steriliser, which is covered immediately. When cool enough to be handled, the barrel and piston are assembled with sterile forceps or clean, dry fingers, touching only the outside of the barrel and the top of the piston. The sterile syringe should be used immediately and not placed in stock "sterile" water or alcohol. The needle should be boiled at the same time, and it is an advantage to thread it through a piece of lint to protect the point. The needle is affixed to the nozzle by means of sterile forceps.

It is much better, however, to sterilise all-glass syringes in the hot-air oven as follows. New syringes are cleaned as above in soap and water, washed and dried. The piston is lightly smeared with liquid paraffin, the paraffin being well rubbed into the ground glass with the finger, inserted into the barrel, and moved backwards and forwards several times so that the syringe works evenly and smoothly. Excess of liquid

¹ Medical Research Council War Memorandum, No. 15 (1945), *The Sterilisation, Use, and Care of Syringes*, London, H.M.S.O.

² Obtainable from British Standards Institution, 28 Victoria Street, London, S.W.1.

paraffin is to be avoided. The assembled syringe is placed in a stout glass tube of such diameter that the barrel of the syringe fits loosely and the flange rests on the top of the tube. The tube should be of such length that it accommodates the syringe with needle fitted. The tube containing the syringe is then wrapped in clear transparent cellophane or similar material, a strip of material of the following sizes being used: for 1-ml. and 2-ml. syringes 3 in. \times 9 in., for 5-ml. 4 in. \times 11 in., for 10-ml. and 20-ml. 5 in. \times 14 in. The cellophane is rolled in a spiral fashion round the tube, commencing at the bottom with a fold and turn-in, and finishing above the piston of the syringe with a firm twist. If cellophane is not available, kraft paper can be used, but the disadvantage of this is that the syringe cannot be seen, and relevant information, *e.g.* size of syringe, etc., must be written in pencil on the paper.

The assembled and wrapped syringe is sterilised in the hot-air oven at 160° C. ($\pm 2^\circ$ C.) for not less than one hour (p. 150). Under these conditions the cellophane turns slightly brown, indicating to the user that the syringe has been subjected to sterilisation. The syringe remains sterile indefinitely in its wrapping and is always ready for use.

Needles are sterilised in 3 in. \times $\frac{1}{2}$ in. test-tubes plugged with cotton-wool. In order to protect the point of the needle, a piece of 5-mm. glass tubing 2 in. long is placed in the tube and the point of the needle passed down it so that the mount of the needle rests on the tubing. The cotton-wool plug keeps the needle in place. The tubes with contained needles are individually wrapped in cellophane and sterilised as above at 160° C. for one hour.

After the syringe has been used, *e.g.* for blood culture, aspiration, etc., it is *immediately* washed out in a cold solution of 2 per cent. lysol, which should always be ready for the purpose. Blood must never be allowed to clot in the syringe, otherwise it will be difficult to remove the piston. Hot fluid must not be used, otherwise it will coagulate the protein and the piston will stick. If the needle has been removed before the blood, etc., is expelled, it must immediately be cleaned after the syringe has been washed out by affixing it to the syringe again and washing it through with the lysol solution. After washing, syringe and needle are returned to the tube in which they were sterilised.

Before re-sterilising, the syringe is thoroughly cleaned in soapy water, a brush being used, then washed in clean, warm water and dried. It is finally lubricated with liquid paraffin, assembled and sterilised as described above.

The needle is washed in warm water, the bore of the needle cleared with a stilette, and the mount of the needle cleaned with a piece of cotton-wool on a swab-stick to remove any blood, etc. After washing it with warm water it is run through with alcohol and allowed to dry. Before being sterilised the point is touched up on a fine Arkansas slip-stone (size 4 in. \times $1\frac{1}{4}$ in.), lubricated with thin machine oil or liquid paraffin, and examined with an 8 \times hand-lens to see that the point is really sharp. It is then run through with the stilette, washed in alcohol and dried.

Glass-Metal ("Record") Syringes.—These cannot be sterilised as above described because the solder-cement joining the glass and metal parts together may melt in the hot-air oven. Moreover, they cannot be sterilised, while assembled, by any heat method as the unequal expansion of glass and metal causes cracking of the barrel. In order to sterilise "Record" type syringes they must be taken apart. The "Record" type syringe is usually sterilised by boiling for five minutes as described above for all-glass syringes. Alternatively, the piston and barrel can be wrapped separately in kraft paper and sterilised in the autoclave, although it should be noted that the solder-cement in some makes may melt even at this temperature.

Glass-metal syringes are washed out immediately after use as described above, and the needles are cleaned and sharpened as for all-glass syringes.

SYRINGE SERVICE FOR A HOSPITAL¹

It is convenient in a hospital to have all the syringes used for withdrawal of blood, injection of serum, or antibiotics, sterilised centrally and under bacteriological control. The all-glass syringes are assembled by the nursing staff as detailed above and brought to the laboratory, where they are sterilised under proper control and returned to the central stock from which they are distributed to the wards. After use the syringes are washed out with 2 per cent. lysol as previously described, and returned to the central point for cleaning and sterilisation. Where this system has been instituted it has proved efficient and economical.

In a large hospital the work of collecting, cleaning, sorting and replacement of broken tubes may be considerable and transparent film envelopes² made of cellophane or similar materials have been used. Different sizes are available according to the size of the syringe and whether the needle is attached or not. The syringe after cleaning and lubricating with liquid paraffin is placed in the envelope, which is sealed with cellophane adhesive like an ordinary gummed envelope. The needles can be sterilised separately in a small transparent envelope size $1 \times 3\frac{1}{2}$ in. The needles are protected, whether in the small envelope or attached to the syringe, by a tube $1\frac{1}{2}$ in. long cut from transparent drinking-tubes. The syringes in their envelopes are laid in a flat wire tray and sterilised in the hot-air oven at 160°C. ($\pm 2^{\circ}\text{C.}$) for one hour. After the syringes have been sterilised they must not be touched for a few hours. On removal from the oven the envelopes are very brittle, but they soon absorb moisture from the air and become pliable again. The envelopes with their syringes can then be handled without cracking and can be packed and distributed safely. To remove the syringe a cut is made with scissors at the side of the envelope just above the top of the piston and the envelope torn across.

¹ See reference in footnote on p. 178 for details.

² See "The Use of Transparent Film Envelopes for a Syringe Service", McCartney, J. E. (1951), *Lancet*, 1, 509.

PREPARATION OF BACTERIAL VACCINES

The method to be described refers mainly to the preparation of vaccines on a small scale, such as *autogenous vaccines*—i.e. consisting of the organism or organisms isolated from a particular patient and used for the treatment of the case. Therapeutic vaccines are now little used and have been superseded by chemotherapeutic and antibiotic substances in the treatment of bacterial infections.

In preparing stock bacterial vaccines for prophylactic use, e.g. typhoid-paratyphoid (T.A.B.) vaccine, it is essential that the strains used should be carefully selected, as pathogenic bacteria when maintained in laboratory culture for any length of time may undergo variation in antigenic characters and so lose their specific immunising properties. Further references to this question are made later in Part III.

The organism must be isolated in pure culture, and then several cultures are made on appropriate solid medium so as to yield sufficient growth after twenty-four to forty-eight hours' incubation, according to the amount of vaccine to be prepared and the abundance of the growth on the particular medium. The growth is emulsified in sterile saline solution (0.85 per cent. sodium chloride) so as to form a fairly dense suspension. This should be free from fragments of medium; if present, they can be removed by centrifuging the suspension for two or three minutes or by allowing them to sediment by gravity and then decanting the supernatant fluid. The bacterial suspension must be rendered as uniform as possible by shaking in a tube or bottle with glass beads. A special shaking machine is generally used for this purpose. *All manipulations involved in preparing the suspension must be carried out with strict precautions to avoid contamination.*

Standardisation

It is necessary at this stage to estimate the *approximate* number of bacteria per ml. of the suspension. Various methods are available for this purpose; the turbidity of the suspension may be compared with standard opacity tubes¹ or the bacteria themselves may be counted in a haemocytometer. (See Counting bacteria, pp. 301, 305.)

Sterilisation of the Bacterial Suspension

The suspension is sterilised at relatively low temperatures, e.g. 60° C. for one hour in a water-bath. To ascertain whether the organisms have been killed, several loopfuls are transferred to a tube of *suitable* medium and incubated for forty-eight hours. (For further sterility tests, *vide infra.*)

¹ Brown, H. C. (1919), *Indian J. med. Res.*, 7, 238.

Preparation of the Vaccine for Administration

Any series of doses consisting of a certain number of organisms (computed in millions) can be prepared in volumes of 1 ml. by making appropriate dilutions in carbol-saline (0.85 per cent. sodium chloride + 0.5 per cent. phenol) from the original standardised suspension. Graduated pipettes, as used in serological work, are employed for the purpose. The dilutions are made in sterile tubes and each dose is transferred to a sterile vaccine ampoule which is then sealed.

The most convenient method of supplying the vaccine for actual use is to prepare, from the stock suspension, concentrations of 50, 100, 500 or 1000 million organisms per ml. (according to the doses required) in quantities of 20 ml. The dilutions are placed in 25-ml. "vaccine bottles" with special tightly fitting thick rubber caps which are covered with a layer of paraffin wax, or in 1-oz. bottles with perforated screw-caps, like that of the blood-culture bottle, and covered with a viskap before issue. The required dose can be obtained by puncturing the cap with the hypodermic syringe and withdrawing the appropriate amount.

When a vaccine representing more than one type of organism is required, e.g. from mixed infections, pure cultures of each organism must be obtained and separate standardised suspensions prepared. Appropriate concentrations of each are then combined in the final preparation.

In preparing dilutions from the stock vaccine all manipulations, etc., must be carried out with strict precautions to prevent contamination. Pipettes, tubes, ampoules, bottles, caps, etc., must be absolutely sterile.

Before supplying the diluted vaccine it is essential to carry out further sterility tests with the contents of two of the ampoules, or 2 ml. withdrawn from the bottled vaccine with a syringe. One-half of this sample is tested for aerobic organisms and one-half for anaerobes by appropriate cultural methods.

Earlier regulations under the Therapeutic Substances Act applicable to the manufacture for sale of various therapeutic substances laid down the following sterility tests which may be adopted in the case of bacterial vaccines preserved with a phenolic antiseptic:—

"The tests shall be made on fluid media, the quantity of medium contained in each tube or other vessel used in the test being such as to secure that any phenolic antiseptic present in the sample is diluted to less than 0.01 per cent. In the case of a test for aerobic organisms the medium shall consist either of a meat extract with the addition of 1 per cent. of peptone, or of such an equivalent as can be prepared by the tryptic digestion of muscle. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by pH=7.2 and pH=7.8. In the case of a test for anaerobic organisms the medium shall consist of a nutrient broth similar to that used in testing for aerobic organisms, with the addition

of heat-coagulated muscle of an amount sufficient to occupy a depth of not less than 1 centimetre at the bottom of the tube. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by pH=7.2 and pH=7.8. Before the test inoculation the medium shall be heated to 100° C. for a period sufficient to free it completely from dissolved oxygen, and then cooled to 37° C. or lower. The inoculated tubes shall be incubated at 37° C. for five days. . . ."

If a vaccine has been prepared from an organism which does not grow readily in ordinary media, a similar test must be carried out with media specially suitable for the growth of the particular organism, or the vaccine may be tested by injection of an animal of a species known to be susceptible to infection by that organism.

For details of the present requirements in the application of sterility tests to therapeutic substances and the methods to be used, reference should be made to the latest Therapeutic Substances Regulations.

CHAPTER IX

CULTIVATION OF MICRO-ORGANISMS

CULTURE MEDIA

ONLY in exceptional cases can the identity of a bacterium be established by its morphological characters (p. 18). It is therefore essential to obtain a *culture* by growing the organism in an artificial *medium*, and if more than one species or type are present each requires to be carefully separated or isolated in *pure culture* (p. 29). In this process there are three distinct operations:

- (1) The preparation of suitable culture media.
- (2) The removal of other organisms from the medium and its containers, *e.g.* glassware, etc., by sterilisation. Bacteria are ubiquitous, and are present in the material and on the articles used for making media. These contaminating organisms must be destroyed so that the culture medium is rendered sterile.
- (3) The cultivation of the organism and its isolation from others present in the material to be examined. It is only occasionally that organisms can be grown directly from the body in pure culture.

Containers for Medium and Cultures

In the past, flasks and test-tubes, stoppered with cotton-wool, have been universally employed as containers for medium and cultures. Recently, however, improved methods for the distribution and storage of culture media have been introduced, in which screw-capped bottles of varying capacity and shape are used for these purposes and substituted for the original types of container. Thus, medium can be distributed and preserved in hermetically sealed bottles on the same principle as the canning of foodstuffs. The designation "bottled" has been applied to media preserved in this way. These methods are particularly valuable in large laboratories where culture media are prepared in quantity for distribution. Among other advantages, the medium is not liable to dry out during storage and need not be stored in a cold room.

*Note.—Glassware must be thoroughly cleaned before using as containers for culture media, and new glassware requires special treatment (see p. 291 *et seq.*).*

Bottled Culture Media¹

This system consists essentially in placing the medium in a container, which is then hermetically sealed and thereafter sterilised. The result

¹ McCartney, J. E. (1933), "Screw-capped Bottles in the Preparation and Storage of Culture Media", *Lancet*, 2, 433.

is, that the container being completely closed, the contents remain sterile indefinitely. The principle is applied to media not only in bulk but also in smaller quantities, and small screw-capped bottles can be substituted for test-tubes for bacteriological purposes.

For culture media in bulk, screw-capped plain white bottles are employed. They are supplied in various sizes, ranging from 2 oz. to 20 oz., but the sizes most commonly used are 3 oz., 5 oz. and 10 oz., in which are placed 50, 100 and 250 ml. of media respectively. As a substitute for test-tubes small round screw-capped bottles of $\frac{1}{4}$ -, $\frac{1}{2}$ - and 1-oz. capacity are used. The bottles are made of clear white flint glass and the neck has an external screw thread. The screw caps are made of aluminium (the smaller sizes are sprayed with white cellulose paint) and the washer is made of special black rubber which is non-inhibitory to bacterial growth.

Types of Screw-capped Bottles.—Screw-capped bottles are now extensively used in general laboratory work, and the following range, which covers practically all needs, indicates the most useful types.

Details of the caps are given to show how comparatively few varieties are needed for a complete series. It should be noted that six of the smaller bottles (which are the most used) need only two sizes of caps, which is economical in supplying and maintaining stocks. Bottles may be obtained with perforated caps (for blood cultures, p. 262).

Bottle	Capacity in ml.	Cap	Washer
1 gallon, narrow mouth .	4600	Special, to fit "Compo" cork and "resistol"	
80 oz. Winchester series .	2400	KN31	
140 oz. " "	1190	Ditto	
120 oz. " "	600	Ditto	
10 oz. " "	290	KN134	
5 oz. round . .	140	KN133 sprayed white	
3 oz. " . .	85	KN133 " .	
2 oz. medical flat . .	60	KN132 " .	black rubber, 3 mm. thick
21 oz. round (H 53) . .	28	KN133 " .	
$\frac{1}{2}$ oz. " . .	15	KN132 " .	
$\frac{1}{4}$ oz. " (bijou) . .	6	KN132 " .	
1 oz. Universal container	28	KN86	aluminium lacquered

These bottles are made by the United Glass Bottle Manufacturers, Ltd., and may be bought only from retailers. With the exception of the first two listed, they are supplied cleaned and washed by a special process which removes the surface alkali. The caps are already fitted,

¹ These bottles are also used for intravenous infusion solutions, e.g. saline, glucose-saline, etc.

² See Fig. on p. 247.

the rubber washers having previously been well boiled before insertion. No further treatment is necessary before they are used, and culture media can be added to them after which they are capped and the contents sterilised in the appropriate manner. The introduction of this process of cleaning and washing by the makers saves all the tedious work of preparing new glassware in the laboratory, which is time-consuming and expensive. It is of especial value where large quantities of culture media are produced. Normally the cleaned bottles are supplied in cardboard cartons which not only keep the bottles clean and facilitate storage before use but are very useful for storing and despatching the culture medium after it has been made.

The round bottles can be autoclaved with the caps tightly screwed on, either empty or containing media, with little risk of breakage. (If dry empty bottles are autoclaved, the caps should be slightly loosened to admit steam.) Care must be taken that the bottles are placed in the steriliser loosely, and not packed tightly in a wire crate or other container, otherwise breakages will inevitably occur. They must not be sterilised in the hot-air oven with the caps on, as the temperature will injure the rubber washer. *As "medical flat" bottles with the cap screwed on tightly may crack when autoclaved, a common practice is to have the caps slightly loosened during autoclaving and to tighten the caps after removal from the autoclave.*

When the bottles are cleaned for re-use they must be fitted with new caps and washers, and the old ones discarded. Owing to the difficulty of complete cleansing, *caps preferably should not be used a second time.*

In addition to the bottles, the following screw-capped containers are useful in laboratory work:

2-oz. "pomade pot", p. 433.

8-oz. pot, p. 433.

$\frac{1}{2}$ -lb. jar } same size

1-lb. jar } of cap.

$\frac{1}{2}$ -gallon wide-mouth jar } for the preparation of culture media.

1-gallon " " "

PREPARATION OF CULTURE MEDIA

The majority of the organisms to be studied are pathogenic, and in order to obtain suitable growths the artificial culture media should approximate in certain respects to the composition and also to the H-ion concentration of the tissues and body fluids in which these organisms grow. The general subject of bacterial nutrition and conditions for growth has been dealt with in Chapter II.

Dehydrated Culture Media

There are available, from certain commercial firms, culture media in dehydrated form. These are convenient when only small quantities of media, or certain specialised media, are required, and are suitable

for small laboratories where space is limited and facilities for medium making are inadequate. The dehydrated medium is dissolved in water according to the directions supplied, and the resulting preparation which is of the correct composition and pH is canned or bottled, and sterilised in the usual way. While such media are satisfactory for most purposes, they are not equal in quality to freshly made culture media, particularly those made from fresh meat such as digest broth and digest agar (p. 192).

Broth, Nutrient Gelatin and Agar

The basis for the media ordinarily employed in the study of the common pathogenic bacteria is nutrient broth. Agar or gelatin is added merely to solidify it. Nutrient broth is usually prepared as a mixture of the commercial products "peptone" and "Lab.-Lemco" meat extract; however, a *meat infusion broth* may be prepared by making a watery extract of lean meat, and a *digest broth* by digesting meat with a proteolytic enzyme.

Nutrient Broth

Ingredients:

Commercial bacteriological peptone	10 g.
Meat extract (Lab.-Lemco)	10 g.
Sodium chloride	5 g.
Water (tap water)	1 l.

Mix the ingredients in these proportions and allow to dissolve. Solution may be facilitated by brief heating in a steamer. When cool, adjust the pH to 7.5-7.6 as described on p. 277; a precipitate of phosphates may then appear and this may be removed by filtering through filter paper.¹ (If clarity of the broth is essential, the mixture should be adjusted to pH 8.0 instead of pH 7.5, heated at 100° C. for thirty minutes to precipitate most of the phosphates, cooled, filtered through filter paper and finally adjusted to pH 7.5.)

Dispense the broth in large bottles for storage or in tubes and small bottles for use. Then sterilise these by steaming at 100° C. for ninety minutes or, preferably, by autoclaving at 121° C. (15 lb. per sq. in.) for fifteen minutes. If the bottles have screw caps, these should be slightly loose during the sterilisation process and tightened on cooling. The heating causes a slight fall of pH to the required value of about 7.4. The medium should be clear and free from deposit. The presence of a deposit, generally of phosphates, does not interfere with the nutritive value of the medium, but it may hinder the recognition of slight bacterial growth by observation of a developing turbidity.

¹ For filtration of hot liquid media and melted agar media, it is desirable to use a grade of hardened filter paper which is rapid in passing fluid and strong when wet. A recommended grade is "Hyduro" 904½, supplied by J. Barcham Green Ltd., Maidstone, England.

Tap water is usually suitable for making broth and other ordinary culture media; if for any reason the local supply is found unsuitable, distilled water may be used.

*Commercial peptone*¹ consists of water-soluble products obtained from lean meat or other protein material (e.g. heart, casein, fibrin or soya flour) by digestion mainly with a proteolytic enzyme such as pepsin, trypsin or papain. The important constituents are peptones, proteoses, amino acids, a variety of inorganic salts (including phosphates, potassium and magnesium) and certain accessory growth factors (including nicotinic acid and riboflavin). Peptone is supplied as a golden granular powder with a low moisture content (preferably under 5 per cent.) and usually a slightly acid reaction (giving a pH between 5 and 7 in a 1 per cent. solution). It is hygroscopic and soon becomes sticky when exposed to air; stock bottles should therefore be kept firmly closed and weighing of loose powder rapidly completed. According to the starting materials and mode of preparation, the brands of peptone supplied by different manufacturers show appreciable differences in composition and growth-promoting properties; moreover, variations may occur between different batches of one brand.

The essential requirements of a good peptone have not yet been fully defined, but include the ability to support the growth of moderately exacting bacteria from small inocula (e.g. *Staph. aureus*, *Strept. pyogenes* and *Sh. dysenteriae* type 1), the absence of fermentable carbohydrates, a low content of contaminating bacteria and a very low content of copper. Small amounts of copper contaminating culture media are highly inhibitory to bacterial growth. Apart from the standard grades of bacteriological peptone, some manufacturers supply special grades of peptone recommended for particular purposes, e.g. "Neopeptone", "Proteose peptone", mycological peptone, etc.

The analysis of a suitable bacteriological peptone (Oxoid) has been supplied by the makers as follows:

Total nitrogen	14.5 per cent.
Total proteose nitrogen (sat. ZnSO ₄)	1.9 per cent.
Primary proteose nitrogen (half-sat. ZnSO ₄)	0.14 per cent.
Amino acid nitrogen (formol titration)	1.7 per cent.
Tryptophane	1.2 per cent.
Ash	5.5 per cent.
Chloride	1.0 per cent.
Phosphate (as P ₂ O ₅)	1.3 per cent.
Calcium	0.13 per cent.
Magnesium	0.07 per cent.
Copper	0.0010 per cent.

¹ Information on the preparation and composition of commercial products used in culture media, including peptone, meat extract, yeast extract, malt extract, gelatin and agar, is given in a report of the Society of General Microbiology, "Constituents of Bacteriological Culture Media", edited by G. Sykes, Cambridge University Press, 1956.

Iron	0.0075 per cent.
Zinc	0.0025 per cent.
Sulphur (total)	0.68 per cent.
Ether-soluble extract	0.03 per cent.
Nicotinic acid	75 µg. per g.
Riboflavin	50 µg. per g.
Carbohydrate (fermentation test)	nil
Moisture	less than 5 per cent.
pH of a 1 per cent. solution	5.9-6.1.

(Indole production test, good reaction).

Meat Extract.—A commercially prepared meat extract known as Lab.-Lemco is used as a substitute for an infusion of fresh meat. Meat extract is manufactured by a method derived from that invented by Liebig. Finely divided lean beef is held in boiling water for a short time while its readily soluble constituents pass into solution and form the unconcentrated extract. This is freed of excess fat and then concentrated by evaporation to a dark viscous paste containing 70-80 per cent. of solids. The product contains a wide variety of water-soluble compounds, including protein degradation products, e.g. gelatin, albumoses, peptones, proteoses and amino acids, and other nitrogen compounds such as creatine, creatinine, carnosine, anserine, purines and glutathione (total N about 10 per cent.); it also contains many mineral salts (KH_2PO_4 and NaCl most abundantly), accessory growth factors (e.g. thiamine, nicotinic acid, riboflavin, pyridoxine, pantothenic acid and choline) and certain amounts of carbohydrates. The required quantity of the sticky extract is conveniently weighed on a piece of clean paper and put with the paper into the water for solution; the paper is subsequently removed from the broth.

Nutrient broth may be prepared according to other formulae than that noted above. Thus, with less highly exacting bacteria, good growth can be obtained in weaker media, e.g. containing 3 grams Lab.-Lemco and 5 grams peptone per litre. When using a high-quality peptone, the meat extract may be omitted altogether (see Peptone Water, p. 199). Some formulae omit the addition of sodium chloride; this does not reduce the nutritive value of the medium, but renders it unsuitable as a base for preparing blood agar (p. 211) since the red cells lyse in the weakly saline solution. For certain purposes a yeast extract may be substituted for part or all of the meat extract, and casein hydrolysate or soya hydrolysate for the ordinary peptone.

Yeast Extract.—Commercial yeast extract is prepared from washed cells of brewers' or bakers' yeast. These are allowed to undergo autolysis which is initiated by mild heating (e.g. at 55° C.) or, in some cases, are hydrolysed with hydrochloric acid or a proteolytic enzyme. After removal of the cell walls by filtration or centrifugation, the extract is evaporated to a thick dark paste containing about 70 per cent. of solids. It contains a wide range of amino acids (amounting to nearly 50 per cent. of its mass), growth factors (especially of the vitamin B group) and

inorganic salts (particularly potassium and phosphate); over 10 per cent. of carbohydrates are present, including glycogen, trehalose and pentoses. Yeast extract is used mainly as a comprehensive source of growth factors and may be substituted for meat extract in culture media. It is used thus in media for growing and counting the bacteria in milk, rinse waters from food utensils, etc. (see Yeast Extract Agar, p. 197).

Casein Hydrolysate.—This consists largely of the amino acids obtained by hydrolysis of the milk protein "casein". It also contains phosphate and other salts, and certain growth factors. Hydrolysis is effected either with hydrochloric acid, the product being neutralised with sodium carbonate and thus rich in sodium chloride, or with a proteolytic enzyme (trypsin). The acid hydrolysate is the poorer nutritionally since tryptophane is largely destroyed during the hydrolysis and some other amino acids are reduced in amount; tryptophane must therefore be added to the medium to make it suitable for tryptophane-requiring bacteria. The more expensive enzymic hydrolysate contains abundant tryptophane and the full range of amino acids, and does not require such supplementation. Casein hydrolysate may be substituted for peptone in broth and other media. A special advantage applies to its use in experimental work where a nearly defined medium is required, since its composition is more constant and more fully known than that of other peptones. Thus it may be added to a minimal synthetic medium (see p. 199) to render it suitable for growth of exacting bacteria.

Broth may be enriched or modified by the addition of various substances, e.g. glucose.

Glucose Broth.—This is broth *plus* 1 per cent. of glucose (or 0.1 or 0.25 per cent.). The glucose promotes luxuriant growth of many organisms; it also acts as a reducing agent and may enable growth of anaerobes.

Glycerol Broth.—This is broth *plus* 5–7 per cent. glycerol. It is sometimes used for cultivation of the tubercle bacillus.

Infusion Broth

The first stage in the preparation is the making of a watery extract of meat. The type of meat used is an important factor in the quality of the broth obtained. Freshly killed (not frozen) lean beef or ox heart should be used. (Horse-flesh is cheaper, but is usually not so fresh, and coming from older animals is more fibrous than beef. In addition, it contains a higher percentage of fermentable sugar, which may make the broth unsuitable for many purposes, such as the preparation of toxins.) The meat is carefully freed from fat, minced as finely as possible, and added to tap water in the proportion of 500 grams to 1 litre. After extraction for twenty-four hours at a low temperature—*e.g.* in the refrigerator—the mixture is strained through muslin to keep

back the small particles of meat, and the meat residue expressed. The fluid is bright red in colour and there is often a thin layer of fat on the surface, which may be removed by skimming with a piece of filter paper. It is boiled for fifteen minutes, or steamed in a steam steriliser for two hours, when it becomes brown in colour and turbid on account of the alteration of the haemoglobin and the small particles of coagulated protein. It is now filtered and the clear fluid is made up to the original volume by the addition of distilled water. The unheated material contains soluble proteins along with other extractives. As a result of the heating, the meat proteins are coagulated, and removed by filtration. The finished extract should be clear and light yellow in colour, but is not yet suitable as a culture medium owing to the lack of nitrogenous material. Uncoagulable protein derivatives in the form of commercial peptone¹ are added in the proportion of 1-2 per cent., and the salt content is increased by the addition of sodium chloride (0.5 per cent.). These are dissolved by heat and the extract is again filtered. Owing to the sarcolactic acid present in the meat, the reaction of the extract is acid, and this reaction is unsuitable for the growth of most organisms. When the reaction has been adjusted to the optimum pH (p. 277), the medium is sterilised in the autoclave or steam steriliser, and the resulting preparation is designated Infusion Broth.

In the preparation of culture media it should be noted that very small quantities of copper salts are inimical to the growth of many organisms. Copper utensils should be avoided, but heavily tinned copper articles, e.g. funnels, containers, are safe to use. Should the tinning show signs of wear the article must be re-tinned.

It was shown by H. D. Wright that broth may be unsuitable for the cultivation of the more delicate organisms if the peptone is incompletely reduced. When the peptone is added to the meat and water and the whole boiled together, the resultant broth gives good results, particularly with the pneumococcus. The broth, however, is not so suitable for the cultivation of many anaerobes such as the tetanus bacillus. *Wright's method* is as follows. To 1 litre of distilled water add 10 grams of peptone, 5 grams of sodium chloride, and 500 grams of meat, preferably veal, finely minced after removal of excess of fat. Mix well and heat for twenty minutes at 68° C., stirring at intervals. Shake well and steam in the steam steriliser for thirty minutes, filter through paper and adjust the reaction to pH 7.8-8.0 (p. 277). Again steam for thirty minutes and filter through paper. Check the reaction of the filtrate (pH 7.6-7.8) and add 1.5 grams of glucose per litre. Distribute as required and sterilise by autoclaving for ten minutes at 10 lb. pressure. This broth can be used for ordinary purposes, when the glucose may be omitted.

Infusion broth and nutrient agar made from it are identified by means of a YELLOW bead in the container (p. 244).

¹ For ordinary purposes "commercial" peptone is satisfactory, but for special purposes sugar and indole must be absent.

Todd-Hewitt Broth (Modified)

(for use in typing streptococci—p. 487)

Add 450 grams of fat-free minced beef to 1 litre of tap water; mix well and place in the cold overnight. The following morning heat to 85° C. for thirty minutes. Filter through filter paper and add Eupeptone 2 per cent. Adjust the reaction to pH 7.0 with 10 N NaOH (about 3 ml. per litre required), then add:

NaHCO ₃	0.2 per cent.
Glucose	0.2 per cent.
NaCl	0.2 per cent.
Na ₂ HPO ₄ , 12H ₂ O	0.1 per cent.

Boil the mixture for fifteen minutes, filter through Chardin paper, bottle and autoclave at 10 lb. pressure for ten minutes.

The final pH is 7.8. The medium does not require any further adjustment, but the pH should be checked.

DIGEST MEDIA

In the ordinary media, digested protein is added in the form of commercial peptone, but in the preparation of "digest" media meat is digested by the action of trypsin, and the products of this digestion constitute the basis of the medium. Digest media are valuable for obtaining luxuriant growths of exacting organisms, but the cultures tend to die out rapidly and these media are not suitable for maintaining stock cultures.

A useful form of "digest" medium for general use is:

Hartley's Broth

Ox heart or lean beef (free from fat and minced)	1500 g.
Tap water	2500 ml.

Mix together and heat in the steam steriliser until a temperature of 80° C. is reached. Then add:

Sodium carbonate (anhydrous) 0.8 per cent. solution (cold)	2500 ml.
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Cool to 45° C. and add:

Pancreatic extract ¹	50 ml.
Chloroform	50 ml.

¹ COLE AND ONSLOW'S PANCREATIC EXTRACT

Fresh pig pancreas (fat-free and minced)	500 g.
Distilled water	1500 ml.
Absolute alcohol	500 ml.

Industrial methylated spirit may be used instead of absolute alcohol (see note on p. 109).

Shake the mixture thoroughly in a large stoppered bottle and allow to stand for three days at room temperature, the shaking being repeated occasionally. Strain through muslin, and filter through paper (e.g. as in footnote, p. 187).

The mixture is then incubated at 37° C. for six hours, or 45° C. for three hours, the liquid being frequently stirred. After digestion is completed, add 40 ml. of pure strong hydrochloric acid, steam for half an hour, and then filter. The broth is stored in an acid condition. When required for use adjust the reaction to pH 8.0 with normal caustic soda, and steam for one hour to precipitate phosphates. Filter while hot and allow to cool. Adjust the reaction to pH 7.6 (p. 277) and distribute in 250 ml. amounts in 10-oz. screw-capped bottles, or in smaller amounts, *e.g.* in blood-culture bottles (p. 262) or in 1-oz. and $\frac{1}{2}$ -oz. bottles according to requirements. The caps are tightly screwed on and the bottles sterilised.

If the broth is to be stored in bulk, it is left acid, and when cool distributed into one-gallon screw-capped bottles. Add 0.25 per cent. of chloroform and shake vigorously. Shake the bottles frequently during the next two or three days and store in a cool dark place.

Digest broth and the nutrient agar made from it are identified by a BLACK bead in the container (p. 244).

Horse Flesh Digest Medium

This medium is specially suitable for cultivating haemolytic streptococci when an abundant growth is required.

Mix 2 lb. minced horse flesh with 1500 ml. cold water and raise temperature to 80° C.; add 2 litres cold water and 12 grams sodium carbonate (anhydrous). Adjust pH to 8. Add 0.5 per cent. pancreatin and keep at 56° C. for six hours; then add 20 ml. pure hydrochloric acid, boil for half an hour to arrest digestion, and filter. Incorporate in the digest 1 per cent. high-quality peptone and adjust to pH 8; add 0.125 per cent. calcium chloride; steam and filter when cold. Add 0.2 per cent. sodium bicarbonate and filter through a Seitz filter; store in bottles; incubate at 37° C. to test for sterility.

SOLID CULTURE MEDIA

Broth being a fluid medium has certain disadvantages:

- (1) growths do not exhibit specially characteristic appearances in this medium, and therefore it is of little use in identifying species;
- (2) organisms cannot be separated from mixtures by growth in this medium (p. 252).

Measure the filtrate and add pure strong hydrochloric acid in the proportion of 0.1 per cent. This causes a cloudy precipitate which settles in a few days and can be filtered off.

Filtration of the extract is not essential and it can be kept exactly as mixed, with the addition of hydrochloric acid.

This extract keeps for about two months in stoppered bottles in the cold. If used at once the hydrochloric acid need not be added as its action is to retard the slow deterioration of the trypsin.

For these purposes solid media are normally used. Nutrient broth may be converted into a solid (gelatinous) medium by the addition of 15 per cent. of gelatin or 1·2-2·0 per cent. of agar-agar.

Nutrient Gelatin

Gelatin is a protein prepared from the collagen of skin, hide, sinew or bone by partial hydrolysis with hot water at 55°-80° C. The extract is filtered and vacuum-dried to yield the final product in sheet or powder form. For bacteriological use it is best to use an edible grade of gelatin, since this is free from preservatives and inhibitory amounts of heavy metals. Gelatin will not by itself support microbial growth and nutrients must be added. In practice it is added to a liquid nutrient medium in a concentration of 10-20 per cent. to convert it into a firm gel.

In preparing nutrient gelatin it is important to expose the medium to a high temperature for the minimum amount of time, otherwise it will not solidify when cooled, thus rendering it useless for bacteriological purposes.

Steam the required amount of stock digest broth (acid condition) to remove chloroform, filter, allow to cool and add gelatin, 15 per cent. Place in the cold overnight.

Next day dissolve the gelatin at 45° C., adjust to pH 8.4, and return to the steamer for ten minutes. Cool quickly to 45° C. and slowly add the beaten white of egg (*i.e.* white of two eggs, or 10 grams egg albumin dissolved in 50 ml. water, per litre of medium); this helps to clear the medium of colloidal particles, which otherwise interfere with its transparency. Steam for thirty minutes, stirring occasionally. Filter through paper pulp or filter paper (see footnote, p. 196). The reaction is approximately pH 7.6, but may require a little adjustment to this figure. Bottle in 12 ml. amounts.

Sterilise in the autoclave for ten minutes in free steam and then at 10 lb. pressure for ten minutes.

Remove from the autoclave as quickly as possible and keep at a low temperature.

The resulting medium is perfectly transparent when solid, and should be of firm consistence, yet not so stiff that it is split by the wire when inoculated (p. 250).

The proportion of gelatin used varies with the time of year, but 15 per cent. is a suitable average. Gelatin at this strength melts at about 24° C., and is therefore fluid at incubator temperature. Organisms, however, can be grown at 37° C. and liquefaction then tested by placing the culture in cold water.

Gelatin is a protein, and therefore is digested and liquefied by the proteolytic ferments of many bacteria. This property of liquefying gelatin is used as a means of differentiating certain organisms. As gelatin is not frequently used in routine work it should be kept in small screw-capped bottles (p. 185). When the medium is made it is dis-

tributed into the bottles, the caps are tightly screwed on, and the whole batch is sterilised. Under these circumstances the medium keeps indefinitely. After the gelatin has been inoculated, the cap is again screwed on, which prevents evaporation during growth of the organism.

Nutrient Agar

Agar-agar, or "Agar" for short, is a long-chain polysaccharide substance derived from certain seaweeds. It gives a firm gel in watery solutions at concentrations of about 2 per cent. This remains firm and unmelted at incubation temperatures of 37° C., or even higher (e.g. 50° C.); it is not decomposed or liquefied by any but a few rare varieties of bacteria. Having these advantages over gelatin, agar is the substance usually employed for solidifying culture media. It is not intended to add to the nutritive properties of the medium and a suitable preparation of agar should be free of growth-promoting as well as of growth-inhibiting substances.

Nutrient agar is nutrient broth solidified by the addition of agar. It should be noted that nutrient agar is frequently referred to as "agar", the context making clear that the agar-broth mixture is meant and not the pure, non-nutritive agar itself.

Agar is prepared in several countries from a variety of seaweeds, *Gelidium*, *Eucheuma*, *Pterocladia* and others, the weed being dried, extracted by hot-water processes, clarified, dried and finally supplied as the dried strands or as a powder. There are considerable differences in the properties of the agars manufactured in different places, and even between different batches from the same source. Japanese and New Zealand agars are the most generally used. Japanese agar yields a gel of suitable firmness at a concentration of about 2 per cent., and New Zealand agar at about 1·2 per cent. The exact concentration to be used may require some adjustment according to the batch of agar and also according to the other constituents of the medium. *In the formulae for media given in this book, the amount of agar is stated as for Japanese agar powder: if New Zealand agar is used, the amount will be barely two thirds of this.*

Apart from the polysaccharide, which seems mainly composed of *d*-galactopyranose units, agar contains a variety of impurities including inorganic salts, a small amount of protein-like material and sometimes traces of long-chain fatty acids which are inhibitory to growth. The minerals present are mainly magnesium and calcium, and agar is thought to exist as the magnesium or calcium sulphate esters of the polysaccharide.

At the concentration normally used (about 2 per cent.), most bacteriological agars melt at about 95° C. and solidify only when cooled to about 40° C. In preparing nutrient agar, therefore, the appropriate amount of agar powder or fibre is added to the nutrient broth and the mixture is placed in a steamer at 100° C. for one hour to effect solution. To

ensure clarity of the final product, it is necessary at this stage to remove particulate impurities and excess phosphates.

Small quantities of medium may be treated with egg albumin as described for nutrient gelatin (p. 194). Larger quantities are usually clarified by filtration alone. The hot agar solution, preferably first adjusted to pH 8.0 and held at 100° C. for thirty minutes to precipitate phosphates, is filtered before it can cool and solidify. Paper pulp or cellulose wadding¹ enclosed in muslin is used as the filter, or for smaller amounts of medium a suitable filter paper (see footnote, p. 187). For quantities of 5–10 litres it is convenient to use a porcelain Buchner-type filter funnel (*i.e.* with flat perforated platform) about 10 in. (25 cm.) in diameter. Cut out two 10-in. disks of cellulose wadding, place these one on top of the other on a large (24 in. square) sheet of muslin and fold over the excess muslin to enclose the wadding completely. Invert the disk-like bundle and press into position on the platform of the filter, ensuring a good fit at the edges. Pour some hot water through the filter and then heat it at 100° C. in the steamer (at the same time as heating the agar solution) so that it is hot for the time of its use. Without allowing any time for cooling, pour the hot agar solution through the filter. The whole should pass quickly without assistance by suction and the filtrate still be liquid for the final adjustment of pH (see below).

If a large Buchner filter is not available, quantities up to 5 litres may be filtered using a 10-in. conical glass funnel, the lower third of which is filled with pebbles or glass beads to form a supporting platform for the filter material. The latter, paper pulp or cellulose wadding wrapped in muslin to form a bundle about 2 in. deep, is pressed into position on this platform and fitted closely to all sides of the filter funnel. Moist pulp may be superimposed to ensure that the filter is not leaky. A fresh filter element must of course be prepared for each occasion.

After filtration the nutrient agar must be adjusted to pH 7.5. It is remelted by steaming if necessary and hydrochloric acid added in an amount known from previous experience to effect the required adjustment. After thorough mixing, two test-tubes are filled with 10 ml. of the medium, phenol red indicator is added to one, and after cooling the pH is determined by use of the Lovibond comparator as described on p. 277. If the correct pH has not been achieved, a further addition of hydrochloric acid (or sodium hydroxide) is made to the bulk of the medium, still hot and liquid, and further samples are taken to check the pH.

The molten agar is finally dispensed into screw-capped bottles and sterilised by steaming at 100° C. for ninety minutes, steaming at 100° C. for thirty minutes each day for three successive days, autoclaving at 109° C. (5 lb. per sq. in.) for thirty minutes, or autoclaving at 121° C. (15 lb. per sq. in.) for fifteen minutes.

¹ A suitable paper pulp is "White Heather" brand, or T. B. Ford's filter pulp. Both these are sold in slabs. A suitable cellulose wadding is "Cellosene" supplied in sheet form by Robinson & Sons, Ltd., Chesterfield.

Nutrient agar made from infusion broth is distinguished by a YELLOW bead in the container, whereas nutrient agar made from digest broth (Hartley's broth) is identified by a BLACK bead (p. 244).

Glucose Agar is made by the addition of 0.1–1 per cent. of glucose, and *Glycerol Agar* by adding 5–8 per cent. of glycerol to ordinary nutrient agar. The former is used chiefly for deep stab cultures of anaerobes (p. 677).

✓Yeast Extract Agar

This is a form of nutrient agar in which yeast extract is used in place of meat extract. It is employed particularly for making plate counts of the viable bacteria in drinking-water supplies. The formula prescribed for this purpose in the Ministry of Health Report No. 71 is as follows:

Yeast extract	3 g.
Peptone	5 g.
Agar	15 g.
Distilled water	1 l.

Dissolve the yeast extract and peptone in the water at 100° C., cool to room temperature and adjust to pH 7.4. Weigh out the agar, place it in a muslin bag, wash in running water for fifteen minutes and express excess moisture before adding it to the broth. Autoclave at 121° C. for twenty minutes and filter hot through paper pulp. Test the reaction of the filtrate at 50° C. and adjust to pH 7.0 to give a final pH of 7.2 when cool. Tube in 10-ml. amounts and sterilise by autoclaving at 121° C. for twenty minutes (The recommended brand of yeast extract is "Yeastrel", supplied by the Brewers' Food Supply Co. Ltd., Edinburgh.)

Yeast-Extract Milk Agar

This is prepared in the same way as yeast extract agar, but with 10 ml. of fresh whole milk being added per litre of broth at the same time as the washed agar is added. The medium is used for making plate counts of viable bacteria in milk supplies and rinse waters from dairy and food utensils, the milk in the medium promoting growth of a larger number of bacteria than would grow on yeast extract agar alone. For examination of milk supplies in Scotland it is specified in the Milk (Special Designations) (Scotland) Order, 1951, that the medium be prepared from "Yeastrel" yeast extract and "Peptone of a type approved by the Secretary of State".

Semi-Solid or Sloppy Agar

Agar is added in smaller concentrations to media used for special purposes: at 0.2–0.5 per cent. to yield semi-solid media through which

motile but not non-motile bacteria may spread, and at 0.05–0.1 per cent. to prevent convection currents in media used for anaerobic and microaerophilic organisms (*e.g.* Brewer's thioglycollate medium, p. 234).

Detection of Motility by Cultivation in Semi-Solid Nutrient Agar

In semi-solid agar media, motile bacteria give diffuse spreading growths which are easily recognised by the naked eye. Motility may thus be detected more easily than by the microscopical, "hanging drop" method.

The exact optimal concentration of agar depends on the particular brand used and must be determined by trial; usually it is about 0.4 per cent. of Japanese agar or 0.25 per cent. of New Zealand agar. This is dissolved in nutrient broth or peptone water. It is important that the final medium should be quite clear and transparent. Dispense 10-ml. amounts in test tubes and leave to set in the vertical position. Inoculate with a straight wire, making a single stab down the centre of the tube to about half the depth of the medium. Incubate under the conditions favouring motility. Examine at intervals, *e.g.* after six hours and one, two and six days when incubating at 37° C.

Non-motile bacteria generally give growths which are confined to the stab-line, have sharply defined margins and leave the surrounding medium clearly transparent. Motile bacteria typically give diffuse, hazy growths which spread throughout the medium rendering it slightly opaque. The outgrowth may reach the walls of the tube after a few hours and the foot of the tube after one or two days. It is best observed by contrast while there is still some transparent medium not yet invaded. With a non-motile strain which yields motile variants, a discrete line of growth is formed along the stab and diffuse outgrowths then fan out from one or two points. Sharply defined finger-like outgrowths may be given by some kinds of poorly motile bacteria, and also by some kinds of non-motile bacteria; these doubtful cases may be resolved by use of the "hanging drop" method.

Defined Synthetic Media

Chemically defined media are used for various experimental purposes, especially in studying the nutritional requirements of bacteria. They are prepared exclusively from pure chemical substances and their exact composition is thus known. The ingredients must be of analytical reagent quality (*e.g.* "Analar") and are dissolved in distilled or demineralised water (p. 288).

Minimal Medium of Davis and Mingozi:

Glucose	2 g.
K ₂ HPO ₄	7 g.
KH ₂ PO ₄	3 g.
Na ₃ -citrate, 2H ₂ O	0.5 g.
MgSO ₄ , 7H ₂ O	0.1 g.
(NH ₄) ₂ SO ₄	1 g.
Agar (if required)	20 g.
Distilled water	1 l.

This medium is suitable for growth of a wide variety of enterobacteria. Since glucose is partly decomposed when autoclaved in the presence of phosphate, the medium should be prepared and sterilised without the glucose; a 10 per cent. glucose solution is sterilised separately and 20 ml. of this added per litre of medium just before dispensing for use.

If required, other sugars may be substituted for the glucose and the citrate may be omitted. Particular amino acids or growth factors may be added, or a mixture of the essential amino acids in the form of a vitamin-free casein hydrolysate. Essential minerals other than those added are likely to be present in sufficient amounts contaminating the agar, water and other ingredients. If necessary, 5 ml. of the following trace-element solution may be added per litre of medium: 0.5 g. FeSO₄.7H₂O, 0.5 g. ZnSO₄.7H₂O, 0.5 g. MnSO₄.3H₂O, 10 ml. 0.1 N H₂SO₄ and 1000 ml. distilled water; also 1 ml. of a 1 per cent. CaCl₂ solution may be added per litre of medium. The large phosphate content is required to buffer the acid that is formed by fermentation of glucose, the mixture shown giving a pH of 7.1. If it is required that the phosphate content of the medium be low, a citrate or bicarbonate buffer system may be used (p. 281). Thus, a pH of 7.1 is obtained by incorporation of 0.3 per cent. of NaHCO₃ in the medium and incubation of the culture in an atmosphere containing 20 per cent. (v/v) of CO₂.

Peptone Water

This is a simple medium, consisting of

Peptone 1 per cent.

Sodium chloride 0.5 per cent.

dissolved in warm water and then filtered. It is sterilised in the autoclave. It is used chiefly as the basis for sugar fermentation media, since broth and nutrient agar, being made from meat, may contain a small amount of muscle sugar, and it is essential that the basic medium, to which various carbohydrates are added for fermentation tests, should be free from natural sugar.

Peptone water is best made up in bulk and distributed in 250 ml. amounts in 10-oz. screw-capped bottles. The pH should be adjusted to 7.4-7.5, as on sterilising there is some reduction of pH. The caps are tightly screwed on and the whole batch of bottles is sterilised in

the autoclave at 5 lb. pressure for thirty minutes. The peptone water is later distributed into tubes or small bottles as required.

Plain peptone water *without indicator* is distinguishable by a WHITE bead in the container, while peptone water *with indicator* is identified by a BROWN bead (p. 244).

Peptone water is used to test the formation of indole (p. 608), and also for the enrichment of *Vibrio cholerae*, when isolating this organism from infected material. In the latter case the medium should be adjusted to a reaction neutral to phenolphthalein (approximately pH 8.4), as *V. cholerae* grows better in such alkaline medium.

Peptone Water Agar.—This consists of peptone water solidified with 2 per cent. agar, and is used as a basis for solid media containing sugars (*vide infra*).

Sugar Media

Under the designation of "sugars" are included a variety of fermentable substances, chiefly carbohydrates, which are used in the identification and classification of organisms. These are fermented with the formation of acid, and in many cases gas is formed in addition. The substances most commonly employed are the following:

Monosaccharides:

(a) *Pentoses*—

- (1) Arabinose (from gum acacia; and from sugar beet boiled with dilute sulphuric acid).
- (2) Xylose (from corn cobs boiled with dilute acid).
- (3) Rhamnose obtained by hydrolysis of quercitrin (from dyer's oak).

(b) *Hexoses*—

- (1) Glucose (dextrose or grape sugar).
- (2) Fructose (or laevulose; from many plants; formed in the inversion of cane sugar).
- (3) Mannose (from the ivory nut).
- (4) Galactose (made by the hydrolysis of lactose).

Disaccharides:

- (1) Sucrose (saccharose or cane sugar).
- (2) Maltose (malt sugar).
- (3) Lactose (milk sugar).
- (4) Trehalose (from ergot and several species of yeasts and fungi).

Trisaccharide:

- Raffinose (from cotton-seed meal and sugar-beet residues).

Polysaccharides:

- (1) Starch (soluble starch is usually prepared from potato starch).
- (2) Inulin (from dahlia tubers).

- (3) Dextrin (made by the partial hydrolysis of starch).
- (4) Glycogen (from the livers of mammals and lower animals; occurs also in yeasts and certain fungi).

Alcohols:

- (a) *Trihydric*.—Glycerol (glycerin; from hydrolysis of fats).
- (b) *Tetrahydric*.—Erythritol (erythrone; from *Protococcus vulgaris*, also present in many lichens).
- (c) *Pentahydric*.—Adonitol (adonite; from *Adonis vernalis*).
- (d) *Hexahydric*.—
 - (1) Mannitol (mannite; from manna).
 - (2) Dulcitol (dulcite; from dulcitol-manna and various plants).
 - (3) Sorbitol (sorbitane), produced from glucose by treatment with hydrogen under pressure.

Glucosides (vegetable products which on hydrolysis yield a sugar):

- (1) Salicin (from the bark and leaves of some willows and poplars).
- (2) Coniferin (from coniferous woods, and asparagus).
- (3) Aesculin (from the inner bark of the horse-chestnut tree).

Non-carbohydrate Substance:

Inositol (inosite—a benzene derivative; widely distributed in plants; extracted from walnut leaves and mistletoe).

The medium consists of peptone water, to which the fermentable substance is added in the proportion of 0.5 or 1 per cent. An indicator is incorporated to detect acid change. This may be Kubel-Tiemann litmus solution (now rarely used), neutral red (0.25 per cent. of a 1 per cent. solution), Andrade's indicator (1 per cent.),¹ or phenol red (about 0.01 per cent.). If acid is produced, the litmus turns bright red; the neutral red, pink; Andrade's indicator, reddish pink; and phenol red, yellow. It has been found that Andrade's indicator fades fairly rapidly when stored and should not be used unless the media can be utilised within a few months. Phenol red is recommended when bottled media may not be used for some time. It is best made up in a 0.2 per cent. solution prepared as described in the footnote on p. 277, except that the phenol red is ten times as strong. For use 5 ml. are added to each 100 ml. of medium.

In order to detect gas, a small inverted tube is placed in each culture tube (Durham's fermentation tube). During the process of sterilisation the heat drives out the air from the inverted tubes, which when cool should be completely filled with liquid and contain no air bubbles.

The original method of making the medium is as follows.

The stoppered test-tubes containing the small inverted tubes are sterilised by dry heat in the hot-air oven. The peptone water (with

¹ Made by adding 1 N sodium hydroxide to a 0.5 per cent. solution of acid fuchsin until the colour just becomes yellow.

the indicator added) is sterilised by autoclaving. The sugars are made up separately in 10 per cent. solutions in distilled water, which are sterilised in the steamer or by filtration. The requisite amount of sugar solution is added to the peptone water. The medium is tubed (p. 245), and steamed for twenty minutes on three successive days.

The various sugar media in tubes can be distinguished by having the cotton-wool stoppers of different colours. It is better to employ wool dyed in bulk rather than to colour white-wool stoppers with various stains (p. 243).

The following alternative method of making sugar media is recommended.

Peptone water with an indicator is tubed, the Durham fermentation tube inserted, and the test-tubes are stoppered with coloured cotton-wool. It is preferable to use small screw-capped bottles (p. 185). They are then sterilised in the steam steriliser, or in the autoclave for half an hour at 5 lb. pressure. The sugars are made up separately in 10 per cent. solutions in distilled water, and are sterilised, preferably by filtration (Seitz filter), or in the steamer. The sterile sugars are kept conveniently in 10-oz. screw-neck bottles fitted with a siphon and hooded pipette, as shown in the diagram.

This method of obtaining small quantities of sterile fluid from bulk can be applied to serum as well as to sugars (p. 206).

The apparatus recommended consists of a special stainless-steel metal fitting adapted to a 10-oz. bottle with a screw neck. It consists of a straight piece of tube (A), with a curved side-arm (B); around this is a slightly wider tube (C), with a side-arm (D), and fitted to a screw-cap (E) which screws on to the bottle, a rubber washer (F) ensuring an air-tight joint. To the upper end of tube A is connected a Seitz filter by means of a short piece of pressure tubing furnished with a screw-clamp, while attached to the lower end, by means of a short piece of rubber tubing, is a glass tube, 5 mm. in diameter, reaching to the bottom of the bottle. To the side-arm B is connected a piece of rubber tubing furnished at the other end with a pinchcock and hooded pipette. The hooded pipette is closed by a coloured cotton-wool stopper¹ containing a small glass test-tube to cover the delivery tube. The side-arm D is connected by pressure tubing to a cotton-wool air filter, the other end of which is attached to a filter pump.

The whole apparatus, as figured, is connected up (the joints being bound by tinned copper wire), wrapped in kraft paper and sterilised in the autoclave.

Filtration of the sugar is accomplished in the usual manner, and before the pressure is released, the pressure tubing to the Seitz filter is closed by means of the screw-clamp. The filter is then removed, and the end of the pressure tubing plugged with a piece of glass rod. The filter pump is now disconnected, when the air pressure forces the solution down the siphon tube as far as the pinchcock, so that the siphon is in operation as soon as the pinchcock is opened. Alternatively, air

¹ The colour denotes the particular sugar used.

may be forced through the cotton-wool filter on D by means of a rubber blowball to start the siphon action. In use, the neck of the bottle is held by a clamp at the top of a tall retort stand. The stem of the hooded pipette is held below the bottle by means of another clamp, at a height

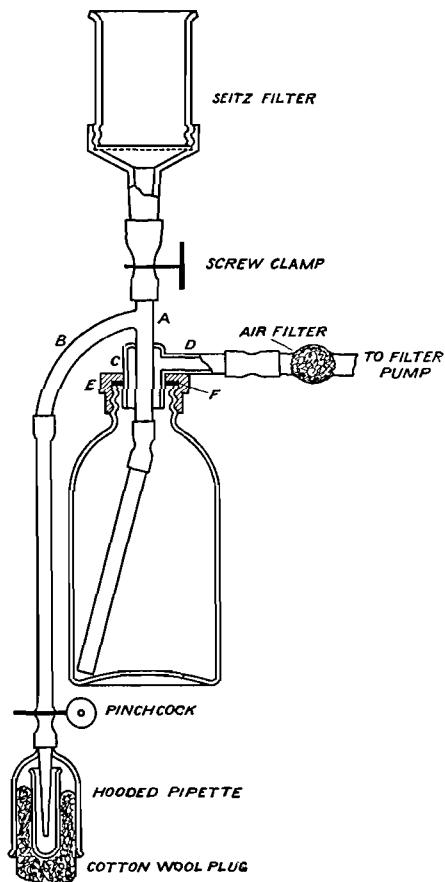


FIG. 9

convenient for placing a test-tube (or bottle) under it to receive the sugar solution. The cotton-wool stopper is removed and the inside of the pipette flamed. After use, the stopper is replaced and the hooded pipette fastened to the neck of the bottle by a piece of copper wire. The number of drops per ml. delivered from the pipette is determined, so that the amount of sugar solution required for the volume of peptone water is easily estimated. Thus, if a pipette delivers 18 drops per ml., then 9 drops (0.5 ml.) of 10 per cent. sugar solution per tube of 5 ml. peptone water gives a final concentration of 1 per cent. sugar.

Expensive sugars should be made up in 10 ml. amounts of a 10 per cent. solution in $\frac{1}{2}$ -oz. screw-capped bottles and sterilised by submerging the whole bottle, including cap, in a water-bath at 60° C. for one hour. This procedure we have found satisfactory and obviates the loss occasioned by the filtration method (*vide* method of withdrawal on p. 209).

It is recommended that sugar media as above be distributed in 3 ml. amounts into small screw-capped bottles, $\frac{1}{4}$ oz. capacity with a 19 mm. Durham tube. By this means the medium can be stored without risk of contamination or alteration in the concentration of the ingredients. This is particularly useful in the case of certain sugars which are only occasionally required. Moreover, sugar media in these bottles can easily be transported without leakage or spilling. As a result of shaking during transit, air may enter the Durham fermentation tube, but it is easily removed. The bottle is merely inverted, the Durham tube drops into the neck of the bottle, and the amount of fluid is such that the open end of the tube is below the surface. The bubble of air escapes and on turning the bottle to the proper position the Durham tube falls to the bottom of the bottle full of liquid and without any air.

When the bottle has been inoculated the cap should be *loosely* screwed on to allow access of air.

In order to identify the various sugar media the caps are painted with cellulose paint, of which many colours are available. The screw-caps, as received from the makers, are already sprayed white, and the added colours on the tops of the small screw-capped bottles indicate the same sugars as the coloured cotton-wool plugs. Thus, with the culture media supplied to Ministry of Health laboratories, green=glucose, red=lactose, mauve=mannitol, etc. (p. 243).

After the bottles have been prepared, the appropriate colour is painted on the caps. A batch of a gross takes only twenty minutes, and the paint is dry within thirty minutes. When two colours are used together for identifying media, e.g. yellow and mauve for starch, each half of the cap is painted.

Litmus Milk

Used in testing for the fermentation of lactose and clotting of milk.

Fresh milk is steamed for twenty minutes and then allowed to stand for twenty-four hours in order that the cream may separate. The milk is siphoned off and litmus is added in the proportion of 2½ per cent. of an alcoholic solution.¹ The medium is distributed in 5 ml. amounts

¹ *Litmus solution.*—Litmus granules 80 grams, 40 per cent. industrial spirit 300 ml. Grind up the granules and place in a flask with 150 ml. of the spirit and boil for one minute. Decant the fluid and add remainder of spirit to the granules; then boil for one minute. Decant the fluid and add to the first quantity of the extract. Make up to 300 ml. with 40 per cent. spirit and add 1 N HCl drop by drop, shaking continuously till the fluid becomes purple. To test for correct reaction, take a tube of tap water and one of distilled water, boil both and add one drop of the solution to each; the tap water should be blue and the distilled water mauve.

in screw-capped bottles or tubes and then sterilised by steaming for twenty minutes each day on three successive days. If bulk-amounts (e.g. 250 ml.) are put up it is advisable not to add the litmus solution until the milk is redistributed in smaller amounts, as the colour fades on storing.

Use of *p*-Aminobenzoic Acid in Culture Media

In cases treated with sulphonamide compounds, there may be enough sulphonamide in the blood stream to prevent the growth of bacteria when blood culture is carried out. As the sulphonamide is antagonised by *p*-aminobenzoic acid (p. 398), the addition of the latter substance to the medium will prevent the bacteriostatic action of the sulphonamide; *p*-aminobenzoic acid is added to the broth in the proportion of 5-10 mg. per 100 ml. before the blood-culture bottles are made up (p. 262). It is quite stable and withstands autoclaving.

As sulphonamide compounds are now widely used, the addition of *p*-aminobenzoic acid in the above concentration will be found valuable not only in blood culture but in the media used for the isolation of pathogenic cocci. Even if no sulphonamide has been administered *p*-aminobenzoic acid improves the nutritive qualities of the medium.

SERUM AND BLOOD MEDIA

These may be divided into two classes:

- (1) Where the medium consists mainly of serum or blood, which can be coagulated by heat (above 60° C.) so that a solid medium results.
- (2) Where the serum or blood is added in fluid form to enrich simpler media.

Supply of Blood and Serum

Sterile defibrinated¹ or oxalated² horse blood and sterile horse serum can be obtained commercially and such supplies are convenient for general use in preparing culture media. Alternatively, blood may be collected from rabbits and other laboratory animals, sheep, oxen and horses at the abattoir, or humans. The purpose for which the blood or serum is required determines whether it is necessary to maintain sterility during its collection. Maintenance of sterility is unnecessary if the blood or serum is to be incorporated in a medium which is subsequently sterilised by heating, e.g. Löffler's coagulated serum medium (p. 207), Hiss's serum water (p. 208) and coagulated blood medium (p. 208); it is advisable, nevertheless, to keep bacterial contamination to a minimum and thus the risk of introducing highly heat-resistant organisms. When sterile unheated and uncoagulated serum is required,

¹ Evans Medical Supplies Ltd., Liverpool.

² Burroughs Wellcome, Ltd.

it may be prepared by filtering unsterile serum through a Seitz filter (p. 175). When, however, sterile unheated blood is required, this must be collected from the animal with aseptic precautions adequate to exclude bacterial contamination and preserve the blood in its original sterile condition. This sterile unheated blood is required for making blood agar (p. 211) and other media containing unaltered red cells, since any attempt to sterilise contaminated blood by heat would lead to disintegration of the cells, coagulation of cell and serum proteins, and denaturation of the red haemoglobin to brown derivatives.

Unsterile Serum (for Löffler's and other media sterilised by heat).—A sterile wide-mouthed stoppered bottle is taken to the abattoir at a time when animals, preferably sheep, are being killed. After the neck vessels have been severed, the blood is allowed to flow for a short time and then the stream from the carotid artery is allowed to spurt directly into the bottle. When filled, the bottle is stoppered and returned carefully to the laboratory. The clot is then separated from the sides of the bottle by means of a stiff sterile wire. The blood is kept overnight in the refrigerator and the clear serum pipetted off. At some abattoirs, serum is prepared in bulk from blood allowed to coagulate in open trays; this is liable to greater bacterial contamination, but may be used when large quantities are required.

Serum may also be prepared from unsterile defibrinated or oxalated blood which does not clot on standing. The blood is collected from horse, sheep or ox as just described. For defibrination, a bottle containing glass beads is half filled with the blood, stoppered at once and shaken continuously for five minutes. It is then stored overnight in the cold to allow settling of the corpuscles, and the serum is siphoned off.

Oxalated blood is prepared by bleeding the animal into jars containing 10 ml. of a 10 per cent. solution of neutral potassium oxalate per litre of blood. The corpuscles are allowed to settle overnight in the cold and the plasma is siphoned off into a Winchester quart bottle. After warming the plasma to about 37° C., 22.5 ml. of a 4 per cent. solution of calcium chloride is added per litre and the bottle shaken on a machine until the fibrin has separated.

Quantities of serum prepared in these ways may be stored at 3°–5° in the refrigerator until required for use. It is advisable to sterilise the serum prior to storage. This is done by filtration through a Seitz filter (*e.g.* using a sterilising grade of Ford's Sterimat asbestos-cellulose disk). It is convenient to store the serum in a large sterile screw-capped bottle of 1–5 litres capacity, fitted with siphon delivery tube and hooded pipette, as described on p. 203.

Sterile Uncoagulated Serum (*e.g.* for serum agar medium).—This may be obtained as just described by filtration of unsterile serum. Smaller quantities may be prepared without filtration from sterile blood obtained by bleeding a rabbit under aseptic conditions (*vide infra*). The blood is collected in a sterile tube or cylinder stoppered with cotton-wool. (Free contraction of the clot is assisted if the tube is lined with agar; 10 ml. of melted 1.5 per cent. agar in physiological

saline is spread inside an 8×1 in. boiling tube by rotating the tube until the agar sets as a thin layer.) The blood is allowed to coagulate and, when fully separated, the serum is removed with a sterile pipette. The serum can be stored in sealed bottles after heating at 56° C. for one hour on each of three successive days (this does not cause coagulation), but fresh serum yields much better results than heated serum in the culture of certain pathogens—e.g. gonococcus.

Sterile Blood (for blood agar medium).—The blood must be rendered non-coagulating by defibrination or oxalation; the former is recommended.

Small amounts may be obtained from rabbits, up to 20–30 ml. from the *ear vein* and about 50 ml. per kilogram body-weight by *cardiac puncture*. The procedures for withdrawing blood aseptically in these ways are described on p. 420. The blood should be collected into a sterile bottle containing glass beads; as soon as this is half full, it is stoppered and shaken for five minutes to separate the fibrin.

Very large quantities of sterile blood are obtained from sheep or horses. A cannula or wide-bore needle is inserted into the external jugular vein. If a sheep is selected, the wool is clipped from the side of the neck and the part shaved. Contamination can be minimised by placing a bag made of waterproof material over the head of the animal. It is best to use a cannula connected by rubber tubing to a screw-capped bottle (p. 185), the whole being enclosed in kraft paper and sterilised. The vein may be made prominent by pressure on the lower part of the side of the neck. The skin over the vein is carefully sterilised with soap and water and then alcohol. The cannula is inserted into the vein and the requisite amount of blood removed. Horses are treated similarly except that it is advisable to make a small incision with a sharp knife in the skin over the vein. The cannula is then more easily introduced.

The sterile defibrinated horse blood is immediately distributed in 10 ml. amounts into sterile $\frac{1}{2}$ -oz. screw-capped bottles and stored in the refrigerator. In smaller laboratories amounts of 5 ml. will probably suffice. The defibrinated blood thus stored will keep for periods up to two months. The blood must not be allowed to freeze in the refrigerator or the corpuscles may be lysed.

Media consisting mainly of Serum or Blood

Löffler's Serum Medium.—To ox, sheep or horse serum is added one-third of its volume of 1 per cent glucose-broth. The mixture is added to stoppered sterilised tubes which are laid on a sloped tray and placed in the serum inspissator. The temperature is then slowly raised to 80°–85° C. and maintained for four hours, when the serum coagulates to a yellowish-white solid. The tubes are thereafter sterilised by heating at 85° C. in the inspissator for twenty minutes on each of three successive days. If sterile serum is used, only two hours' inspissation at 85° C. is necessary. If an inspissator is not available, the serum may be coagulated by placing the slanted tubes at the top of a steam steriliser

(where the temperature is a little below 100° C.) for a period of five to seven minutes. The slants are allowed to cool and then are sterilised by heating in the same way at just less than 100° C. for twenty minutes on each of three successive days. Further heating is detrimental, and overheating causes expansion of air bubbles and the formation of steam from the fluid droplets in the partially solidified material, which leads to disruption of the medium.

Löffler's medium is best made up in the small screw-capped bottles ($\frac{1}{4}$ -oz.). The requisite number of bottles fitted with caps (as received from the makers) are autoclaved at 15 lb. pressure for twenty minutes. The sterile serum-glucose-broth mixture is added to the bottles in 2.5 ml. amounts under sterile conditions. The caps are then tightly screwed on, and the bottles carefully laid in a slightly sloping position in the inspissator. The temperature is slowly raised to 80°–85° C. and maintained for two hours. The culture medium should be allowed to cool before being handled. The medium can be stored for long periods without drying. The caps should be only loosely screwed on during incubation.

Löffler's medium is especially useful for the growth of the diphtheria bacillus. Not only does it produce a luxuriant growth in a short time (twelve to eighteen hours), but it is also valuable in eliciting the characteristic staining reaction of the organism by Neisser's method (p. 120).

Inspissator.—This apparatus is used for the preparation of Löffler's serum medium, and Dorset's egg medium (*vide infra*). It consists of a water-jacketed copper box, the temperature of which can be regulated automatically. The serum or egg medium is tubed and placed in special racks, so that the tubes are at the correct angle for forming slopes. The temperature used is generally 80°–85° C. At this temperature the protein material is completely solidified, but the temperature is not so high as to cause bubbles of steam to disrupt the surface of the medium. As medium in tubes is apt to dry if kept in the inspissator for any time, a small opening should be present in the inner wall communicating with the top of the water-chamber above the level of the water. Water-vapour can enter the interior of the inspissator and the medium is kept moist. Electric inspissators without a water-jacket do not yield such satisfactory media if tubes with cotton-wool stoppers are used.

Coagulated Blood Medium.—This medium is useful for growth and short-term storage of exacting pathogenic bacteria such as the pneumococcus and the meningococcus. Mix three volumes of defibrinated blood with one volume of 1 per cent. glucose broth, tube in the slanted position, inspissate and sterilise in the same way as for Löffler's medium.

Hiss's Serum-Water.—As certain pathogenic organisms—e.g. streptococcus, pneumococcus—will not grow well in ordinary sugar media, it is necessary for fermentation tests to use a medium containing serum.

One part of serum is mixed with three parts of distilled water, and

1 per cent. of Andrade's indicator, or preferably 5 ml. of a 0.2 per cent. solution of phenol red per 100 ml. of medium (p. 277), are added. Some samples of horse serum may give fallacious results and batches should be tested before use. Sheep or ox serum is suitable. (Some workers prefer to substitute 0.1 per cent. peptone water for the distilled water). The various sugars are incorporated in the proportion of 1 per cent. This medium, if not acid, does not coagulate on heating, and may be sterilised in the steamer in the same way as other sugar media—namely, twenty minutes each day on three successive days.

Alternatively, the mixture of serum, distilled water and indicator is sterilised in the steamer, and the appropriate amount of the requisite sugar is added as described on p. 202.

When phenol red is used as the indicator, the reaction of the medium is adjusted to pH 7.6 before sterilisation.

Fermentation is indicated by the production of acid, which alters the indicator and causes coagulation of the medium.

Small screw-capped bottles may be used most conveniently as containers for this medium. The method recommended is similar to that for peptone-water sugar media (p. 204).

The Hiss's serum-water is made up with indicator, but without any carbohydrate added. The medium is distributed in 2.5 ml. amounts in $\frac{1}{4}$ -oz. bottles. The caps are tightly screwed on, and the whole batch is sterilised in the steamer for twenty minutes on three consecutive days. When a batch of any particular carbohydrate medium is required, the requisite amount of sterile 10 per cent. sugar is added from the siphon-filter bottle described on p. 203. The caps are then painted with cellulose paint, according to the sugar used (p. 243). Alternatively, the rarer sugars in 10 per cent. aqueous solutions may be sterilised (p. 204) and stored in $\frac{1}{2}$ -oz. screw-capped bottles fitted with a perforated cap and rubber washer similar to the blood-culture bottles described on p. 262. When the sugar solution is required it is withdrawn from the bottle by perforating the rubber with the needle of a sterile syringe.

Hiss's Serum-Water Starch Medium.—This medium, which is used for differentiating the *gravis* type of *C. diphtheriae*, does not keep well as the starch undergoes gradual hydrolysis forming glucose which is fermented by all types of *C. diphtheriae* (p. 516). It is essential therefore, to make up the starch solution only when required and add it to the serum-water medium immediately before use.

A convenient method sufficient for about two dozen small bottles (holding about 3 ml. of medium) is as follows. Weigh out 0.15 gram of soluble starch and place it in a sterile universal container or other sterile 1-oz. screw-capped bottle. Add 5 ml. distilled water, screw on the cap and shake vigorously. Place the bottle in a small sauce-pan or enamel mug of water, bring to the boil, and boil for about five minutes, shaking at intervals to ensure that all the starch is in solution and the contents are homogeneous. When the starch solution is cool add 0.15 ml. with a sterile 1-ml. pipette, or five drops from a sterile

capillary pipette, to each of the fermentation bottles. After the starch has been added the medium should be used within a few weeks.

Media enriched with Serum or Blood

These media are used for certain delicate pathogens, such as the pneumococcus, gonococcus and meningococcus, which usually grow feebly or not at all on ordinary media.

Serum Agar.—Ordinary nutrient agar *plus* 10 per cent. of sterile uncoagulated serum (p. 206). The agar is first melted and then cooled to about 55° C. The serum is usually added to the agar in tubes or bottles and, after it is incorporated, the medium is either solidified in the form of slopes, or poured into Petri dishes (p. 247). As serum agar is indistinguishable from ordinary agar, the tube should be marked “+S” or plugged with coloured cotton-wool (blue and white). If in a small bottle, a blue line is painted across the white cap.

Sterile hydrocele fluid or sterile ascitic fluid, withdrawn aseptically, may be used instead of serum.

Serum-smeared Agar.—This is made by running a few drops of sterile serum on the surface of an agar slope or plate (p. 247). This medium is not so satisfactory as the preceding one, but is useful in an emergency.

Sugar Medium for the Gonococcus, Meningococcus, Etc.:

Peptone	:	:	:	:	:	20 g.
Sodium chloride	:	:	:	:	:	5 g.
Distilled water	:	:	:	:	:	900 ml.

Dissolve in steamer for thirty minutes. Make just alkaline to phenolphthalein and steam for a further thirty minutes. Filter through Chardin filter paper and adjust reaction to pH 7.6. Add 100 ml. digest broth of the same pH. Add 25 grams agar powder (*i.e.* 2.5 per cent.) and autoclave for forty-five minutes in “free steam”, and fifteen minutes at 5 lb. pressure. Filter through paper pulp, and bottle in 100-ml. amounts. Add 2 ml. of 0.2 per cent. phenol red solution¹ to each bottle and sterilise for one hour in “free steam” and five minutes at 5 lb. pressure.

For use 100 ml. of the agar are melted, cooled to 55° C., and to this are added 5 ml. guinea-pig or rabbit serum (not horse), and 10 ml. of a 10 per cent. sterile solution of the required sugar. (This gives a concentration of 5 per cent. serum and 1 per cent. sugar.) The mixture is immediately distributed into sterile tubes or $\frac{1}{2}$ -oz. bottles, allowed to solidify in the sloped position and tested for sterility by incubation.

The sugars generally used are glucose, lactose, sucrose and maltose.

It should be noted that when the sugar is fermented by the organism and acid is formed, the colour of the medium changes from purple-pink to yellow.

¹ Made as described in the footnote on p. 277, except that the phenol red is ten times as strong. See also p. 201.

Blood Agar.—This is an important medium and is specially suitable for the gonococcus, the haemophilic group of bacteria, e.g. *H. influenzae* and other delicate pathogens. Moreover, it is used to observe the haemolytic properties of bacteria such as *Strept. pyogenes* (p. 479).

Either human or animal blood is suitable; horse blood is most commonly used. The blood is added to melted nutrient agar at 55° C. in the proportion of 5–10 per cent., as in preparing serum-agar (*q.v.*).

A considerable saving of blood can be effected if blood-agar plates are made in the following manner. Pour a thin layer (about 5 ml.) of melted plain agar into a 4-in. Petri dish and allow to set. Make 10 per cent. blood agar by adding defibrinated blood to melted agar at 55° C., and pour a similar quantity on the surface of the agar in the dish and allow it to set. A fairly thick layer of medium is required to prevent excessive drying during incubation, and if this were entirely 10 per cent. blood agar, the medium would be almost opaque when viewed by transmitted light through the dish, and the haemolytic effects of organisms would be difficult to see.

It is advisable to prepare several plates at one time as follows. Two 100-ml. screw-capped bottles of agar (p. 195), 10 ml. defibrinated horse blood in a $\frac{1}{2}$ -oz. screw-capped bottle (p. 185), and fourteen sterile 4-in. Petri dishes are required. The agar is melted in the steriliser and both bottles are cooled to 55° C. The contents of one bottle are distributed into the Petri dishes and the agar is allowed to set. Into the other bottle are poured the 10 ml. of blood from the $\frac{1}{2}$ -oz. screw-capped bottle. No pipette is necessary as the screw cap keeps the lip of the bottle sterile. The cap is again screwed on, the bottle inverted several times to mix thoroughly the agar and blood. The blood agar is now distributed into the Petri dishes on the surface of the first layer of agar. Any bubbles caused by the mixing can easily be removed by drawing a Bunsen flame quickly across the surface of the medium in the dish.

For special purposes, amounts of blood up to 50 per cent. may be added.

Heated Blood Agar ("Chocolate Agar").—This medium is suitable for cultivating *H. influenzae* and certain other organisms, such as the pneumococcus. To 5 ml. melted digest agar medium at 55° C. add 0.5 ml. (9 or 10 drops) of defibrinated rabbit blood. Heat the mixture by immersing the tube for exactly one minute in boiling water, and allow the medium to solidify in the sloped position. If a plate is required, 12 ml. of agar and 1.5 ml. of blood are used. If a large amount of medium is made at one time, e.g. 100 ml., it should be heated in the steamer at 100° C. for about ten minutes.

EGG MEDIA

Dorset's Egg Medium

This medium is used for growing the tubercle bacillus. Four "new laid" eggs are beaten up and 25 ml. distilled water then added. The mixture is strained through muslin to remove air bubbles, run into

sterile tubes or screw-capped bottles (p. 245), and solidified in the sloped position in the serum inspissator at 80°–85° C. for four hours. The tubes are then sterilised at 85° C. or at the top of the steam steriliser for twenty minutes each day on three successive days.

All apparatus used should be sterile, and the eggs, before they are broken, should be placed for a few minutes in alcohol; on removal the alcohol is allowed to evaporate. !

The addition of sufficient basic fuchsin to the medium to render it pale pink is advisable, as early growths of the tubercle bacillus are thus more easily seen.

As the tubercle bacillus takes some weeks to grow, the culture must be protected against drying. It is most convenient to use the medium in $\frac{1}{2}$ - or 1-oz. screw-capped bottles, the cap being kept firmly tightened during incubation. If tubes are used, they should be sealed after inoculation by pushing down the cotton-wool stopper below the top of the tube and pouring in a little melted paraffin wax.

A useful *modification of Dorset's medium* is the following.

Break into a sterile bowl fresh eggs which have been washed in soap and water and then dried, and beat thoroughly with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese-cloth over a filter funnel, and to every 75 ml. of egg mixture (two to three eggs, depending on the size) add 25 ml. of sterile digest broth and 1 ml. of a 1 per cent. aqueous solution of crystal violet. Distribute the medium in small sterile bottles (avoiding the formation of air bubbles) and coagulate in a slightly sloped position in the inspissator at 80°–85° C. Sterilise by heating in the inspissator at 80°–85° C. for two hours the next day. The material to be inoculated should be well rubbed over the surface of the medium.

Glycerol-Egg Medium (for growing the human type of tubercle bacillus) is prepared as above, but with the addition of 5 per cent. of glycerol to the digest broth and egg mixture.

Löwenstein-Jensen Medium (for the cultivation and differentiation of human and bovine types of the tubercle bacillus).

(1) Mineral Salt Solution

KH ₂ PO ₄ (Analar)	0.4 per cent.
MgSO ₄ (Analar)	0.04 "
Magnesium citrate	0.1 "
Asparagine	0.6 "
Glycerol (Analar)	2.0 "

in distilled water.

Heat to dissolve.

Boil the solution or place it in the steamer for two hours and allow it to cool overnight. 600 ml. is a convenient quantity to prepare.

(2) Salt-Starch Solution

To each 600 ml. of mineral salt solution add 30 grams of potato starch.

Mix and heat in a flask in a water-bath which is slowly raised to

70° C.; keep at this temperature for one minute and then remove from the bath. Shake the salt-starch solution from time to time.

(3) *Egg Fluid*

Hens' eggs are used and they must be less than one week old. 1 litre of egg fluid is required for each 600 ml. of salt-starch solution and 20 eggs are usually sufficient, but if they are small, 22 eggs should be used.

Cleanse the eggs thoroughly in 5 per cent. soft-soap solution, wash well in running water and place in industrial spirit for five minutes.

Remove each egg one by one, flame it, break into a sterile cup, mix the yolk and white, and pour into a sterile vessel containing glass beads. Shake well and filter through sterile gauze into the salt-starch solution. Mix thoroughly.

(4) *Malachite Green Solution*

Make a 1 per cent. solution of malachite green in distilled water and place in the incubator for one to two hours. To each 1600 ml. of prepared substance (1 litre egg fluid + 600 ml. salt-starch solution) add 40 ml. of 1 per cent. malachite green.

Distribute the medium in 4.5 ml. amounts in $\frac{1}{2}$ -oz. bottles (p. 185) and screw the caps tightly on. Lay the bottles horizontally in the inspissator and heat at 80°–85° C. for half an hour. Allow them to remain in the inspissator overnight, and heat again the following day at 75°–85° C. for half an hour.

The medium will keep for some months in screw-capped bottles, but if slopes are made in test-tubes they must be stored in the cold and used within a month.

On this medium the human type of tubercle bacillus grows very luxuriantly, in the form of large heaped-up dry yellow colonies, while the bovine type shows small discrete colourless colonies.

This medium will show good primary growth of tubercle bacilli (*e.g.* from sputum after treatment with 4 per cent. caustic soda, p. 547) in ten to twelve days. It can be strongly recommended, particularly for the human type.

Petragnani's Medium for growth of the tubercle bacillus

Mix together in a beaker 300 ml. of fresh milk, 2 grams of peptone, 12 grams of potato meal (not flour) (B.D.H.), and 2 finely grated potatoes each about the size of a hen's egg. Place the beaker in a water-bath at 100° C., stirring the mixture constantly for ten minutes. Allow the beaker to remain in the water-bath for one hour; remove and cool the mixture to 60° C. Now add 8 whole eggs, 2 egg-yolks, 24 ml. of glycerol and 20 ml. of a 2 per cent. aqueous solution of malachite green. Stir well to ensure thorough mixing, filter through sterile muslin and distribute in $\frac{1}{2}$ - or 1-oz. bottles. Slant and coagulate the medium at 90° C. for one hour; sterilise at 80° C. for twenty minutes on two successive days.

Egg Medium for preservation of cultures

This is prepared in a similar way to Dorset's egg medium, but omitting the broth and dye. Mix 3 volumes of egg fluid (yolk and white beaten together) and 1 volume of 0.85 per cent. sodium chloride solution. Strain through muslin to remove air bubbles and dispense in 2-3 ml. amounts in $\frac{1}{4}$ -oz. (6-ml.) screw-capped bijou bottles. In�ssipate in the slanted position at 80°-85° C.¹ for two to four hours. Sterilise the slants, with screw-caps tightened, by steaming at 100° C. for ninety minutes or autoclaving at 121° C. for fifteen minutes. If the screw-caps are loose, some of the slants may be disrupted by bubbles of steam.

Egg medium is recommended for the prolonged storage of cultures at room temperature (below 20° C.). Organisms of the enterobacteria group may be kept alive on this medium for one or two years without subcultivation; they survive longer than on nutrient agar, and are less liable to vary to the "rough" state (p. 574). The culture is grown overnight at 37° C. and the screw cap of the bottle is firmly tightened before storing in a dark, cool cupboard. Survival is dependent on the culture not being allowed to dry out.

OTHER MEDIA FOR SPECIAL PURPOSES

Dubos's Medium for the Cultivation of the Tubercle Bacillus¹

Dubos and his associates² have developed a synthetic medium for the cultivation of the tubercle bacillus. In this medium casein hydrolysate provides the necessary nitrogenous material; another ingredient, "Tween 80", is a wetting agent and has a dispersive effect on the ordinary granular growth of the organism. Bovine albumin is added, and, in addition to its nutritive quality, it protects against substances which are toxic to the tubercle bacillus. In this medium the tubercle bacillus grows quickly and diffusely, forming an even suspension.

The medium may be used for testing strains of tubercle bacilli for sensitivity to streptomycin.

Basic Medium

KH ₂ PO ₄	1.0 g.
Na ₂ HPO ₄ , 12H ₂ O	6.25 g.
Sodium citrate	1.25 g.
MgSO ₄ , 7H ₂ O	0.6 g.
Asparagine	2.0 g.

¹ See *Lancet* (1948), 2, 862.

² Dubos, R. J., & Davis, B. D. (1946), *J. exp. Med.*, 83, 409.

Dissolve one at a time in distilled water, then add:

Tween 80, ¹	10 per cent. solution	5 ml.
Casein hydrolysate, 20 per cent. ²	solution	10 ml.
Distilled water to		1 l.

The medium should have a pH of 7.2.

Distribute the medium either in 100 ml. amounts in 5-oz. screw-capped bottles, or in 2.5 ml. amounts in $\frac{1}{4}$ -oz. (bijou) bottles, and sterilise in the autoclave at 10 lb. pressure for ten minutes. The 100 ml. amounts are intended for large laboratories and distribution into the smaller containers as required.

Bovine Albumin

Prepare a 9 per cent. solution of bovine albumin³ (fraction V, Armour and Co.). Add 5 N NaOH drop by drop until pH is 7.4. Filter through a Seitz disk and distribute in 8.5 ml. amounts under aseptic conditions into $\frac{1}{2}$ -oz. screw-capped bottles.

For use, add 0.1 ml. to each 2.5 ml. of basic medium. This gives a final concentration of about 0.3 per cent. bovine albumin. The 8.5 ml. is sufficient for two 100 ml. amounts after distribution into bijou bottles.

Modified Kirschner Medium

(for the tubercle bacillus)

Na ₂ HPO ₄ , 12H ₂ O	19.0 g.
KH ₂ PO ₄	2.5 g.
MgSO ₄	0.6 g.
Sodium citrate	2.5 g.
Asparagine	5.0 g.
Glycerol ⁴	20.0 ml.
Phenol red (0.4 per cent.)	3.0 ml.
Distilled water	1.0 l.

The pH of this medium is approximately 7.4–7.6. No adjustment of pH is necessary. Bottle 9 ml. amounts in 1-oz. bottles and autoclave at 10 lb. pressure for ten minutes.

Before use add 1 ml. of sterile horse serum containing 100 units

¹ Obtainable from Messrs. Honeywill & Stein, Ltd., 21 St. James's Square, London, S.W.1.

² Casein hydrolysate. To 200 grams of commercial casein in a litre conical beaker add a mixture of 170 ml. of concentrated hydrochloric acid with 110 ml. of distilled water. Stir quickly with a glass rod to obtain a uniform suspension before the casein swells and becomes solid. Autoclave at 120° C. for three-quarters of an hour. Cool and add 40 per cent. sodium hydroxide till neutral (about 180 ml.); cool again and filter through pulp on a Buchner funnel. Dilute to 1 litre, place in a Winchester quart bottle, and add 1 per cent. chloroform. Shake vigorously immediately, and at intervals, to emulsify the chloroform. Store in the dark.

³ Obtainable from Armour Laboratories, Ltd., Hampden Park, Eastbourne, Sussex.

⁴ All the salts are dissolved before the glycerol is added.

penicillin per ml. to each 9 ml. of medium. The penicillin is added to reduce contamination to a minimum.

Potato Media

Select large potatoes, wash carefully and peel. With a potato borer (or large cork borer) cut out a cylinder of potato and wash it in water to remove excess of starch. Cut the cylinder obliquely in two and place each half in a test-tube with the thick end resting on a plug of cotton-wool or in a special potato tube, or in a wide-mouth screw-capped 1-oz. bottle.

Fill the tubes with sterile water and place in the steam steriliser for half an hour. Pour off the water and then autoclave the tubes at 10 lb. pressure for twenty minutes.

ALKALINE POTATO MEDIUM.—Prepare as above, but instead of filling the tubes with water add 0·7 per cent. sodium bicarbonate solution. The subsequent treatment is the same.

GLYCEROL POTATO MEDIUM.—Prepare as above, using a 5 per cent. solution of glycerol instead of water. This medium is useful for differentiating the eugonic and dysgonic types of the tubercle bacillus.

MacConkey's Bile-Salt Neutral Red Lactose Agar

This is a useful medium for differentiating intestinal organisms of the coliform, *Salmonella* and dysentery groups. It is a peptone solution solidified with agar, to which bile salt, 0·5 per cent., and lactose, 1 per cent., are added, with neutral red as the indicator.

Dissolve by heat in tap water, peptone, 2 per cent., and sodium taurocholate (commercial), 0·5 per cent. Then add 2 per cent. agar and dissolve in the steamer or autoclave. Clear with white of egg (p. 194) or filter. (Large quantities should be filtered through paper pulp in the same way as agar—p. 196.) Add a sufficient amount (about 0·7 ml. per 100 ml.) of a freshly prepared 1 per cent. watery solution of neutral red to give the medium a distinct reddish-brown colour. If the medium is acid, and assumes a rose-pink colour, add caustic soda solution until the colour becomes definitely reddish-brown. (It is preferable to adjust the reaction beforehand to pH 7·5 which gives the correct colour with neutral red.) Sterilise the medium in the steamer and when cool add 1 per cent. lactose (previously sterilised separately in a 10 per cent. watery solution). Sterilise the completed medium as in the case of other sugar media.

Organisms which produce acid from lactose—e.g. *Esch. coli*—form rose-pink coloured colonies, whereas the colonies of non-lactose-fermenters—e.g. *S. typhi*—are colourless.

MacConkey's medium immediately after being filtered is bottled in 100 ml. amounts in 5-oz. screw-capped "round" bottles, and sterilised as above. The 100 ml. of medium when melted will be sufficient for seven Petri dishes.

When MacConkey's medium is stored for any length of time the

neutral red indicator tends to fade. In order to overcome this, the medium is made up as above without neutral red, and the pH is adjusted to 7.5 so that the correct shade of colour is obtained when the indicator is added.

The MacConkey agar without indicator is indistinguishable from ordinary nutrient agar, but is identified by a RED bead (p. 244). The neutral red is made up in a 2 per cent. solution in 50 per cent. alcohol, and 0.3–0.4 ml. per 100 ml. of medium is the average quantity used. The neutral red solution should be well shaken before use. When plates are to be poured the bottle of MacConkey agar is melted, the indicator added, the screw cap replaced, and the contents are thoroughly mixed before pouring.

Brilliant Green MacConkey Agar is prepared as MacConkey's agar but with the addition of 0.04 gram of brilliant green per litre. This dye is inhibitory to *Esch. coli* and renders the medium selective for *Salmonella* organisms.

MacConkey's Bile-Salt Liquid Medium

(1) Single Strength

Sodium taurocholate (commercial)	5 g.
Peptone (any good make)	20 g.
NaCl	5 g.
Lactose	10 g.
Distilled water	1 l.

Dissolve the taurocholate, peptone and sodium chloride, steam for two hours and add the lactose during the last fifteen minutes of steaming. Cool and transfer to the refrigerator overnight. Filter while cold, adjust the reaction to pH 7.4 and add Andrade's indicator 12.5 ml., (see footnote, p. 201) or 1 per cent. aqueous neutral red solution 10 ml. Distribute in 5 ml. amounts in 1-oz. bottles or $6 \times \frac{1}{2}$ in. test-tubes with Durham tubes and sterilise at 100° C. for forty-five minutes.

(2) Double Strength

Make as above, but with double the amounts of the ingredients, including indicator. Distribute in 50 ml. amounts in 5-oz. bottles using $3 \times \frac{3}{4}$ in. test-tubes as Durham tubes and in 10 ml. amounts in 1-oz. (H53) bottles using $2 \times \frac{1}{4}$ in. Durham tubes.

Note.—With Andrade's indicator, the single-strength medium should be used within two to three months and the double strength within six to eight weeks.

Brilliant Green Bile-Broth

Bacto ox gall	30 g.
Bacto peptone	15 g.
Lactose	15 g.
Bacto brilliant green (1 per cent. w/v)	2 ml.
Distilled water to	1.5 l.

✓ **Glucose-Phosphate Medium**

(for Methyl-red and Voges-Proskauer Tests)

Peptone	5 g.
K ₂ HPO ₄ (anhydrous)	5 g.
Distilled water	1 l.

Steam until the solids are dissolved; filter through Chardin paper, and adjust the reaction at room temperature to pH 7.5. Add 5 grams of glucose, mix thoroughly, distribute in 5 ml. quantities in $6 \times \frac{1}{8}$ in. test-tubes or $\frac{1}{2}$ -oz. bottles and autoclave at 10 lb. for ten minutes.

✓ **Citrate Medium**

(for differentiation of coliform bacilli)

NaCl	5.0 g.
MgSO ₄	0.2 g.
Ammonium dihydrogen phosphate (NH ₄ H ₂ PO ₄)	1.0 g.
K ₂ HPO ₄ (anhydrous)	1.0 g.
Distilled water	1 l.

Dissolve, add 2 grams of citric acid and bring back the reaction to pH 6.8 with 1 N NaOH solution when the mixture forms a clear colourless solution. Tube or bottle in 5 ml. quantities, and autoclave at 15 lb. for ten minutes. (See alternative formula, p. 609.)

Desoxycholate-Citrate-Agar

(for the isolation of organisms of the Salmonella and dysentery groups)

Modification by M. Hynes of Leifson's medium:

Agar	22.5 g.
Lab.-Lemco	5.0 g.
Peptone (Difco proteose or Evans)	5.0 g.
Lactose	10.0 g.
Sodium citrate	8.5 g.
Sodium thiosulphate	8.5 g.
Ferric citrate	1.0 g.
Sodium desoxycholate	5.0 g.

Neutral red (as indicator)

Tap water to 1 l.

It is convenient to make up a four-litres batch as follows:

Dissolve 20 grams Lab.-Lemco in 200 ml. water over the flame; make just alkaline to phenolphthalein with 50 per cent. NaOH, boil and filter. Adjust the pH to 7.4, make up the volume to 200 ml. and add 20 grams of peptone. Dissolve 90 grams agar in 3700 ml. tap water by one hour's steaming. Filter the agar, add the Lab.-Lemco

peptone solution and mix. Add 5 ml. 2 per cent. solution of neutral red and 40 grams lactose, and mix. Bottle in accurate 100-ml. lots, and sterilise by free steam for one hour and then at 5 lb. pressure for ten minutes.

Solution A

Sodium citrate (Analar, $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)	17 g.
Sodium thiosulphate (Analar, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$)	17 g.
Ferric citrate (scales)	2 g.
Distilled water	100 ml.

Dissolve by heat or by standing at room temperature for two days.

Solution B

Sodium desoxycholate	10 g.
Distilled water	100 ml.

Sterilise these solutions at 60° C. for one hour.

For use, melt 100 ml. of the agar base, and add 5 ml. each of solutions A and B in this order, using separate pipettes and mixing well between. Pour plates *immediately* and dry the surface.

The medium should be poured and cooled as soon as possible after the addition of the desoxycholate, otherwise it tends to become very soft. The desoxycholate must be pure and samples tested with known positive specimens before purchase is made.

The medium is pale pink in colour and should be quite clear. Some coliform strains and particularly *Aero. aerogenes* grow on it, producing deep pink opaque colonies about 2 mm. in diameter, and causing (by acid-production) a precipitation of desoxycholate in the surrounding medium. The colonies of pathogens are colourless.

Colonies of *Sh. sonnei* are round, about 2 mm. diameter, with well-defined edges and no appearance of "roughness". They may be pale pink, or become so, on further incubation or storage. Rough variants of this organism do not grow on the medium. *Sh. flexneri* colonies are similar, but may have a narrow plane periphery surrounding a central dome. Colonies of *S. paratyphi B* and *Salmonella* food-poisoning organisms are larger, 2 to 4 mm. diameter, often with a central black dot. *S. typhi* yields a flat round colony.

Of non-pathogenic non-lactose fermenters, only *Proteus* strains grow freely; the colony is usually glossy and more translucent than those of the pathogens; some strains produce a central black dot. There is no tendency to spread, but the characteristic "fishy" odour of *Proteus* cultures is present.

Inoculate plates *heavily* with faeces or rectal swabs in a way that will ensure discrete colonies; incubate for eighteen to twenty-four hours. Re-incubation for another twenty-four hours is occasionally necessary if there are no non-lactose-fermenting colonies present after twenty-four hours' incubation or if the colonies are very small.

Slide-agglutination with colonies picked directly from the plate is

satisfactory provided the usual precautions are taken to obtain a fairly heavy and uniform suspension.

For fermentation reactions colonies are subinoculated with a straight wire into peptone water, and subcultures are made in the appropriate sugars after four to six hours' incubation. At the same time a subculture should be plated on MacConkey's medium to test the purity of the peptone-water culture.

This medium is particularly suited for the isolation of the dysentery organisms, the *Salmonella* food-poisoning group, and *S. paratyphi B*. It is not quite so selective for *S. typhi* though superior to MacConkey's medium.

Wilson and Blair's Bismuth Sulphite Medium

(*for the isolation of typhoid and paratyphoid bacilli*)¹

Prepare a stock bismuth-sulphite-glucose-phosphate mixture as follows:

Dissolve 30 grams bismuth-ammonio-citrate scales in 250 ml. boiling distilled water. Add to this a solution obtained by boiling 100 grams anhydrous sodium sulphite in 500 ml. distilled water, and then while the mixture is boiling add 100 grams sodium phosphate crystals ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$). To the bismuth-sulphite-phosphate mixture when cool add a solution of glucose obtained by dissolving 50 grams of commercial glucose in 250 ml. boiling distilled water. This mixture will keep for months.

Prepare an iron-citrate-brilliant-green mixture consisting of

1 per cent. solution of iron citrate scales (ferric citrate scales) in distilled water	200 ml.
1 per cent. brilliant green in distilled water	25 ml.

This mixture will keep for months.

Make up the medium as follows:

Nutrient agar, 3 per cent. (melted and cooled to 60° C.)	100 ml.
Stock bismuth-sulphite-phosphate-glucose mixture	20 ml.
Iron-citrate-brilliant-green mixture	4.5 ml.

Pour into Petri dishes.

The use of this medium depends on the property of *S. typhi* to reduce the sulphite to sulphide in the presence of glucose, and the inhibition of *Esch. coli* by brilliant green and by bismuth sulphite in the presence of an excess of sodium sulphite. Isolated colonies of *S. typhi* and *S. paratyphi B* are black, the former usually appearing within twenty-four hours and the latter within forty-eight hours.

¹ Wilson, W. J. (1938), *J. Hyg. (Lond.)*, **38**, 507, and personal communication.

Enrichment Media for the Isolation of the Typhoid-Paratyphoid Bacilli

These are fluid media which incorporate substances that inhibit coliform bacilli while permitting the typhoid-paratyphoid organisms to grow freely; thus, an enriched culture of the latter can be obtained from faeces and sometimes an almost pure growth.

Two examples are described:

- (a) Tetrathionate broth.
- (b) Selenite F medium.

The latter in our experience gives very satisfactory and uniform results.

Tetrathionate Broth.—The medium is prepared as follows. To 90 ml. of ordinary broth add 2.5 grams of chalk (previously autoclaved at 10 lb. pressure and then dried) and sterilise the mixture by steaming for half an hour. Add to the chalk-broth 10 ml. of a 60 per cent. solution of crystallised sodium thiosulphate solution (sterilised by steaming for thirty minutes) and 2 ml. of iodine solution (prepared by grinding in a mortar 6 grams of iodine and 5 grams of potassium iodide and dissolving in 20 ml. distilled water). Distribute in 10 ml. amounts in tubes or screw-capped bottles. A tube or bottle of the medium is inoculated with faeces and incubated for eighteen to twenty-four hours when a sub-inoculation is made on MacConkey's or desoxycholate-citrate medium.

As tetrathionate broth does not keep for more than several weeks it is convenient to prepare the solutions and make up the medium as required.¹

A. Sodium thiosulphate. Weigh out 24.8 grams of $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ and dissolve in distilled water to make a final volume of exactly 100 ml. This gives a 1 M solution. Sterilise by steaming.

B. Iodine. To about 50 ml. of warm distilled water add 20 grams of potassium iodide. Add 12.7 grams of iodine and make up to a final volume of 100 ml. This gives a normal or 0.5 M solution.

To prepare the tetrathionate broth (100 ml. amount) add 2.5 grams of chalk to 78 ml. nutrient broth and sterilise by steaming. When cool, add 15 ml. of the thiosulphate solution, 4 ml. of the iodine solution, and 3 ml. of 0.02 per cent. solution of phenol red in 20 per cent. alcohol as indicator. Distribute in 10 ml. amounts in bottles. Keep in the refrigerator and the medium will last several weeks.

Selenite F Enrichment Medium

Sodium acid selenite	4.0 g.
Peptone	5.0 g.
Lactose	4.0 g.
Na_2HPO_4	9.5 g.
NaH_2PO_4	0.5 g.
Distilled water	1.0 l.

¹ Knox, R., Gell, P. G. H., & Pollock, M. R. (1942), *J. Path. Bact.*, **54**, 469.

Distribute the yellowish solution in 10 ml. amounts in screw-capped bottles. Sterilise by steaming at 100° C. for thirty minutes (excessive heat is detrimental and autoclaving must not be used). A slight amount of red precipitate may form but this does not interfere with the action of the medium. The pH of the medium should be 7.1, and the phosphates may be varied slightly if necessary to attain this.

A bottle of the medium is inoculated with two or three large loopfuls of faeces and incubated overnight. Sub-inoculations are made on desoxycholate-citrate medium.

Composite Media for Preliminary Identification of Enterobacteria¹

The following method² is a reliable substitute for the conventional method described on p. 601 to determine the biochemical identity of non-lactose-fermenting colonies prior to confirmation by serological typing.

Method:

Composite medium I

Beef extract	2 g.
Proteose peptone No. 3 (Difco)	15 g.
Yeast extract	2 g.
Glucose	1 g.
Mannitol	10 g.
Agar	16 g.
Indicator mixture	26.5 ml.
Distilled water.	1 l.

Adjust pH to 7.2

After autoclaving at 10 lb. for fifteen minutes and cooling to 60° C., 50 ml. of a sterile 20 per cent. urea solution are added and the medium is distributed aseptically in sterile test-tubes to a depth of 6.5 cm. and allowed to solidify in a sloped position so as to provide a butt of 2.5 cm.

*Indicator mixture.*³ Three separate 0.2 per cent. indicator solutions are made up.

<i>Indicator</i>	<i>grams</i>	<i>ml. 0.05 N NaOH</i>	<i>Add ml. water</i>
Bromo-thymol blue	0.20	6.4	100
Cresol red	0.20	10.6	100
Thymol blue	0.20	8.6	100

The final indicator is obtained by mixing the individual solutions in the following proportions:

Bromo-thymol blue	12.5 ml.
Cresol red	4 ml.
Thymol blue	10 ml.

¹ Kohn, J. (1954), *J. Path. Bact.*, **67**, 286.

² Gillies, R. R. (1956), *J. clin. Path.*, **9**, 368.

³ Singer, J. (1950), *Amer. J. clin. Path.*, **20**, 880.

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Composite medium II

Agar	3 g.
Bacto-peptone (Difco)	10 g.
Tryptone (Difco)	10 g.
NaCl	5 g.
Na ₂ HPO ₄ , 12H ₂ O	0.25 g.
Sucrose	10 g.
Salicin	10 g.
Bromo-thymol blue	0.01 g.
Na ₂ S ₂ O ₃ , 5H ₂ O	0.025 g.
Distilled water	1 l.

Adjust pH to 7.4

The medium is distributed into test-tubes in 8 ml. amounts, autoclaved at 15 lb. for fifteen minutes and allowed to set with the tubes in the vertical position.

Lead acetate papers. Strips (5 mm. × 50 mm.) of filter paper are impregnated with saturated lead acetate solution and dried in an oven at 70° C. *Indole test papers* are similarly impregnated with the following solution:

<i>p</i> -dimethyl-aminobenzaldehyde	5 g.
Methanol	50 ml.
<i>o</i> -phosphoric acid	10 ml.

and are dried at 70° C. for a minimum period.

The two media are inoculated with a long straight wire charged from colonies of the organism to be identified; medium I is inoculated by both smearing the slant and then stabbing to the base of the butt; medium II is then inoculated by a single stab into its upper $\frac{1}{2}$ inch; finally, the two test papers are suspended above the latter medium and held by the cotton-wool stopper.

Results

In medium I the fermentation of glucose is indicated by the butt changing from deep green to yellow and that of mannitol by the development of a yellow slant. Urease production produces a deep blue colour throughout the medium. Gas production appears in varying degrees from a slight splitting along the wire track to disruption of the medium. In medium II, fermentation of sucrose or salicin or both changes the medium from light blue to yellow and accompanying gas production causes bubbles to form. Non-motile organisms grow only along the line of inoculation, whereas motile species show either a diffuse even growth spreading from the inoculum or more rarely localised outgrowths which are usually fan-shaped or occasionally nodular. H₂S production causes blackening of the lead acetate paper and the formation of indole gives a red colour in the yellow test paper.

IDENTIFYING PATTERNS OF ORGANISMS BY THE COMPOSITE MEDIA
(18 hours incubation at 37° C.)

Organism	Fermentation of		Urease production	Fermentation of Sucrose/Salicin	Motility	Production of H ₂ S	Formation of Indole
	Glucose	Mannitol					
S. typhi	+	+	-	-	+	+	-
Other Salmonellae	+	+	-	-	+	V	-
Sh. sonnei	+	+	-	-	-	-	-
Sh. flexneri	+	+	-	-	-	-	+
Sh. schmitzi	+	-	-	-	-	-	+
Proteus group	(-)	(-)	+	V	+	V	V

Key to table: Fermentation tests +; acid and gas produced.

±; acid only for glucose; acid for mannitol (gas production not observable).

-; no reaction.

V; variable.

++; positive.

--; negative.

V; variable.

(-); apparent negative reaction.

Christensen's Medium

(for the detection of urea-splitting organisms)¹

This medium is useful for the identification of *Proteus*, almost all strains of which break up urea. Certain strains of staphylococci also split urea, and when such organisms are found in an infected urine they are frequently associated with calculus in the urinary tract.

A. Make up a basic medium as follows:

Peptone	1 g.
NaCl	5 g.
KH ₂ PO ₄	2 g.
Phenol red	0.012 g.
Agar	20 g.
Distilled water.	1 l.

¹ Christensen, W. B. (1946), *J. Bact.*, 52, 461.

Filter, add glucose 1 gram, and distribute in accurate 5 ml. amounts in 1-oz. screw-capped bottles. Sterilise in the autoclave.

B. Make up a 20 per cent. solution of urea. Filter through a Seitz filter, preferably into a bottle fitted up as shown on p. 203).

For use, melt 5 ml. of the agar medium, cool to below 50° C. (this is important) and add 0.5 ml. of 20 per cent. urea.

Solidify in the sloped position.

The organism to be tested is inoculated on the surface of the slope. If urea is split, ammonia is formed, which turns the indicator purple-pink.

Elek's Medium

(*for the rapid identification of Proteus*)¹

This method depends on the ability of the *Proteus* group to split urea and liberate ammonia which is tested for with Nessler's reagent.

Medium

Prepare a buffer solution of pH 7.2 containing 2 per cent. urea as follows: Add 35 ml. of 0.2 N NaOH and 4 g. urea to 50 ml. of 0.2 M monopotassium phosphate, and make up the volume to 200 ml. with ammonia-free distilled water. Sterilisation of this substrate is not necessary, and it can be stored in a stoppered bottle (with the stopper vaselined) in the refrigerator. Freshly prepared substrate should be checked with a known urea-splitting organism, and for the test a negative control and an uninoculated blank must be included. The glassware must be scrupulously clean but not necessarily sterile.

Method

Emulsify sufficient of a 24-hour culture of the organism to be tested, in 0.5 ml. of the substrate in a $3 \times \frac{3}{4}$ in. tube. The fluid should be distinctly opalescent. Place the tube in a water-bath at 37° C. for exactly three hours. Remove the tube and add 0.1 ml. of Nessler's reagent, and a similar amount to the negative control and blank tubes. Read the result three minutes after adding the Nessler's reagent. Both negative and control tubes must be absolutely colourless. A positive reaction is shown by a colour ranging from a pale but distinct yellow to a dark-brown precipitate. The time of incubation is important and should be strictly adhered to.

When isolated non-lactose-fermenting colonies are to be examined, the volume of substrate is reduced to 0.3 ml. and only one drop of Nessler's reagent used. Readings are taken four to five minutes after nesslerisation.

Dieudonné's Medium—Blood-Alkali-Agar

(*for the isolation of the cholera vibrio*)

Mix equal parts of defibrinated ox blood and 1 N sodium hydroxide and heat for one and a half hours in the steam steriliser. At this stage

¹ Elek, S. D. (1948), *J. Path. Bact.*, **60**, 183.

the mixture is unsuitable for immediate preparation of the medium and if so used would inhibit the cholera vibrio. The blood-alkali is first subjected to repeated steaming (e.g. one and a half hours on eight successive days) in a flask of sufficient size that a large surface of the fluid is exposed to air, and then allowed to stand at room temperature for about ten days. In this process the volatile ammonia is removed and carbon dioxide is absorbed from the air. For use add three parts of the blood-alkali to seven parts of 3 per cent. agar. This product will grow *V. cholerae* abundantly while inhibiting coliform bacilli and *Proteus*. The blood-alkali can be kept in bulk for a considerable period without loss of its selective properties. The original method was to incorporate the blood-alkali in agar immediately after its preparation and to "ripen" the finished medium in plates kept at room temperature for two days, the ammonia being removed in this way and carbon dioxide absorbed. It is more convenient, however, to ripen the blood-alkali in bulk so that plates can be poured ready for immediate use.

Aronson's Medium

(*for the isolation of the cholera vibrio*)

Materials required

(1) Na_2CO_3 (anhydrous)	10 per cent. solution.
(2) Na_2SO_3	10 per cent. ,
(3) Sucrose	20 per cent. ,
(4) Dextrin	20 per cent. ,
Make up all the above in distilled water and heat at 100° C.					for thirty minutes.
(5) Basic fuchsin					3.5 per cent. solution in 90 per cent. alcohol.

Method

Add 20 ml. of solution (1) to 300 ml. of melted nutrient agar and steam for thirty minutes. Then add 15 ml. each of solutions (3) and (4), 1.2 ml. of solution (5) and 6.5 ml. of solution (2), and again steam for twenty minutes.

A precipitate forms, but rapidly sediments, and the clear supernatant material is used for making plates. Colonies of the cholera vibrio appear in twelve hours, and after twenty-four hours these are relatively large with red centres, while coliform bacilli generally produce small slightly pink colonies.

Milk Agar

(*for isolation and identification of Staphylococci*)

Fresh milk, after heating to 60° C. and shaking, is sterilised by autoclaving at 10 lb. pressure for fifteen minutes; repeated sterilisation should be avoided as this produces caramelisation with alteration of colour. Suitable sterilised milk may be obtained commercially. Two volumes of nutrient agar (containing 3 per cent. of agar), melted and

cooled to 56° C., are mixed with one volume of the milk, and the mixture is then used for pouring plates, or making slopes.

On this medium, growth of staphylococci is rapid, and large characteristic colonies are produced within twenty-four hours. Pigmentation is particularly marked and easily recognised against the opaque white background of the medium.¹

Salt Medium for the Selective Cultivation of Staphylococci²

It has been observed that staphylococci grow in the presence of relatively high concentrations of sodium chloride which are inhibitory to many other bacteria. This has been made use of in the preparation of selective media for the isolation of *Staphylococcus aureus* where this organism is likely to be present in small numbers, e.g. in faeces.

One of the most satisfactory of such media is the cooked meat medium modified in its preparation to contain 10 per cent. sodium chloride instead of 0.5 per cent. (p. 233). Such medium is less inhibitory to staphylococci than ordinary nutrient or digest broth containing salt, and enables very small numbers of these organisms to be detected.

The medium is inoculated with the material for examination, and subcultures are made after 24 and 48 hours on a solid medium for identification of individual colonies.

Direct isolation of *Staphylococcus aureus* from swabs or exudate containing other bacteria may be facilitated by the use of solid media to which 10 per cent. sodium chloride has been added. The salt has been regarded as increasing chromogenesis.

Salt milk agar is a suitable solid medium; this is made as milk agar (see above) with 8–10 per cent. of sodium chloride added.

Ludlam's Medium³

(for selective cultivation of *Staphylococcus aureus*)

Lab.-Lemco	10 g.
Peptone	10 g.
K ₂ HPO ₄ (anhydrous)	5 g.
LiCl	5 g.
Mannitol	10 g.
Distilled water	1 l.

Heat to dissolve, adjust to pH 9.2 using thymol blue as indicator, add 25 grams agar, dissolve in the steam steriliser, distribute in screw-capped bottles in 100 ml. amounts and autoclave for twenty minutes.

¹ Christie, R., & Keogh, E. V. (1940), *J. Path. Bact.*, **51**, 189.

² Hill, J. H., & White, E. C. (1929), *J. Bact.*, **18**, 43; Chapman, G. H. (1945), *J. Bact.*, **50**, 201; Maitland, H. B., & Martyn, G. (1948), *J. Path. Bact.*, **60**, 553; Ludlam, G. B. (1949), *Monthly Bull. Minist. Hlth Lab. Serv.*, **8**, 15; Fairbrother, R. W., & Southall, J. E., *ibid.*, **9**, 170.

³ Ludlam, G. B. (1949), *Monthly Bull. Minist. Hlth Lab. Serv.*, **8**, 15.

Before use add sterile 0·25 per cent. potassium tellurite solution to the melted medium to give a final concentration of 0·005 per cent.

On this medium *Staph. aureus* produces after forty-eight hours' incubation dark-grey or black smooth shiny colonies, 3–4 mm. in diameter, usually with a narrow pale border, sometimes showing slight golden pigmentation. *Staph. albus* either does not grow or produces relatively small pale colonies.

The medium also inhibits the growth of diphtheroid and coliform bacilli, some sarcinae and aerobiotic sporing bacilli. It is specially useful for isolating *Staph. aureus* from air, dust, clothes and faeces.

Pike's Medium¹

(modified; for transport and enrichment of *Strept. pyogenes*, pneumococci and *H. influenzae*^{2, 3})

A tube containing blood-agar with 1 in 1,000,000 crystal violet and 1 in 16,000 sodium azide is used. The newly taken throat or nose swab is stabbed into the medium and sent to the laboratory. The swab is withdrawn from this medium and used to inoculate a plate of crystal-violet blood-agar medium; the swab is replaced in the stab which is incubated overnight and then used to inoculate a second plate. Enrichment culture increases the number of positive isolations of β -haemolytic streptococci, pneumococci and haemophili in specimens from nasal carriers and to a lesser extent in throat swabs from healthy individuals.

Sodium Azide Medium

(for isolation of *Streptococcus faecalis*)

Peptone	10 g.
NaCl	.	"	5 g.
K ₂ HPO ₄	.	"	5 g.
KH ₂ PO ₄	.	"	2 g.
Glucose	5 g.
Yeastrel yeast extract	3 g.
Sodium azide (Na N ₃)	0.25 g.
Bromo-cresol purple 2 ml. of 1·6 per cent. alcoholic solution									
Distilled water	1000 ml.

¹ Pike, R. M., *Proc. Soc. exp. Biol.*, N. Y., 1944, **57**, 186.

² Holmes, M. C., & Lermit, A., *Monthly Bull. Minist. Hlth Lab. Serv.*, 1955, **14**, 97.

³ Masters, P. L., Brumfitt, W., Mendez, R. L., & Likar, M., *Brit. med. J.*, 1958, **i**, 1200.

McLeod's Medium*(for the diphtheria bacillus)¹*

This medium consists essentially of heated blood agar (chocolate agar) containing 0.04 per cent. of potassium tellurite. It differs from other culture media for the diphtheria bacillus in that the meat extract which it contains is never heated above 75° C. and is sterilised by filtration.

Add 1½ to 2 lb. of minced meat to 1 litre tap water at 48° C. and keep at this temperature for one hour. Squeeze out the juice through lint or muslin, leave this in the ice-chest overnight and filter through filter paper.

To 1 litre filtrate add 20 grams peptone (Parke, Davis & Co.) and 5 grams sodium chloride; warm at 45° C. until dissolved.

In order to adjust the reaction, take 50 ml. and heat it to 80°–90° C. for fifteen minutes. Filter through paper. Determine the amount of 0.1 N NaOH required to bring 10 ml. to pH 7.6 in the usual way. Add to the bulk of the fluid an amount of alkali calculated on the basis of this titration.

Filter through a Seitz K clarifying disk.

Refilter this filtrate through a Chamberland candle previously sterilised in the autoclave.

Distribute into flasks and tubes. One or two tubes should be incubated for three days at 37° C. to control sterility. The remainder should be stored in the cold until required.

Mix equal parts of this broth and melted 5 per cent. agar in water.

Add 7–10 per cent. of freshly drawn defibrinated rabbit blood, and 0.04 per cent. of potassium tellurite.

Mix and heat at 75° C. for ten to fifteen minutes before pouring into Petri dishes.

Hoyle's² Medium

1. Lab.-Lemco	10 g.
Peptone (Difco proteose, or Evans)	10 g.
Sodium chloride	5 g.
Agar	20 g.
Water	1 l.

Adjust to pH 7.8 and autoclave. Bottle in 100 ml. quantities in screw-capped bottles.

2. Sterile horse blood laked by freezing and thawing four times. Store in the cold, preferably frozen.

Instead of laking the blood by freezing and thawing, the use of

¹ Anderson, J., Happold, F.; McLeod, J. W., & Thomson, J. (1931), *J. Path. Bact.*, **34**, 667.

² Hoyle, L. (1941), *Lancet* **1**, 175.

saponin in a final dilution of 1 in 200 is more simple and convenient.¹

Prepare a 10 per cent. solution of saponin (white) in distilled water and sterilise in the autoclave at 10 lb. for thirty minutes. Use 0.5 ml. of this solution for each 10 ml. of blood. Place the blood in the incubator for fifteen minutes, add the saponin, and invert the bottle gently several times to ensure thorough mixing but avoiding the formation of bubbles. Replace the blood in the incubator for a further fifteen minutes when it should have an "inky" black appearance. Store in the refrigerator where it will keep for several months.

Similarly, sodium di-octyl-sulpho-succinate (Aerosol O.T. 100 per cent.²) in a final dilution of 1 in 500 can be used. A 1 per cent. solution in distilled water is made and the bottle left in the 60° C. water-bath overnight to effect solution and to sterilise it. The solution is stored at room temperature. 2 ml. is used for each 10 ml. of blood in the manner described above.

3. Potassium tellurite (K_2TeO_3)	0.7 g.
Water	20 ml.

Store tightly stoppered and in the dark.

To each 200 ml. of agar, melted and cooled to 55° C., add 10 ml. of laked blood and 2 ml. of the tellurite solution. Pour plates.

Good growth occurs after eighteen to twenty-four hours' incubation. Colony characteristics are described on p. 525.

Type differentiation is similar to but not quite so good as that on McLeod's medium.

This medium gives satisfactory results for routine examination.

Monckton's Enrichment Medium

(for the *diphtheria bacillus*)

(Vide p. 527 for method of use, etc.)

Mix together

Sterile Hartley's broth pH 7.7-7.8 (p. 192)	100 ml.
K_2TeO_3 3.5 per cent. aqueous solution	1.0 ml.
$CuSO_4$ (Analar) 10 per cent. aqueous solution	0.25 ml.
Laked horse blood	5.0 ml.

Lake the blood with saponin as described on p. 229 under Hoyle's medium. Make up the potassium tellurite and copper sulphate solutions in distilled water. No further sterilisation is necessary. This mixture keeps well.

Distribute in 2.5 ml. amounts into $\frac{1}{4}$ -oz. ("bijou") screw-capped bottles as required.

¹ Young, M. Y. (1942), *J. Path. Bact.*, 54, 263.

² Cyanamid Products, Ltd., Shootersway, Berkhamsted, Herts.

For use, inoculate the broth mixture with the throat swab or culture on Löffler's medium, and incubate for six hours.

Inoculate a blood agar plate with a full loopful of this culture. Incubate the plate overnight and examine the next morning.

Bordet-Gengou Medium

(*for Bordetella pertussis*)¹

The following modification has given excellent results and is recommended. Clean and pare potatoes and cut them into thin slices. To 500 ml. tap water add 250 grams potato and 9 grams sodium chloride. Boil until the potato slices fall to pieces. Make up the water lost in boiling, filter through linen, and adjust the reaction to pH 7.

To 1500 ml. tap water add 60 grams agar powder to give a final concentration of 3 per cent. Dissolve by heat and add 500 ml. of the potato extract, 20 ml. glycerol, and 20 grams proteose peptone (Difco). Distribute in bottles and sterilise in the autoclave with "free steam" for one hour, and then raise the pressure to 5 lb. for five minutes. Store until required.

For use, melt in the steamer for one hour and invert the bottle several times. Place in the water-bath at 55° C. for five minutes until the temperature of the agar has dropped to about 70° C. Place an equal amount of defibrinated horse blood in the 55° C. bath for two to three minutes to warm slightly. Add one part of blood to one part of glycerol-potato agar. Mix thoroughly and pour plates. The plates should not be dried in the incubator, but should be stored at once in the refrigerator, and may be used up to two or three days after preparation.

The layer of agar should be thick, e.g. 30-40 ml. medium per 3½-4 in. Petri dish, and the plates should be incubated preferably at 35° C. in a humid atmosphere.

Note: Peptone was not included in the original Bordet-Gengou medium, and may be omitted from that just described. Some brands of peptone are markedly inhibitory to growth of *Bord. pertussis*, probably due to their content of colloidal sulphur or sulphide.

The medium can be made more selective for *Bord. pertussis* by incorporation of 0.25 units of penicillin per ml.

Liver Infusion Agar²

(*for the Brucella group*)

Mince fresh ox liver and then pulp it in a mortar. Mix 1 lb. with 500 ml. of distilled water and keep in the cold for twenty-four hours.

¹ A selective medium for *Bord. pertussis*, containing a diamidine, sodium fluoride and penicillin, has been described by Lacey, B. W. (1954), *J. Hyg. (Lond.)*, 52, 273.

² Huddleson, I. F. (1939), *Brucelloses in Man and Animals*, p. 13. New York, Commonwealth Fund.

Steam for one and a half hours, and filter through wire gauze (60-mesh). Incorporate 20 grams of washed agar, 5 grams peptone, and 5 grams sodium chloride in 500 ml. of the liver extract, and then add 500 ml. distilled water. Adjust the pH to 7.2 at 60° C. and filter the medium through wire gauze as above. Distribute into containers and autoclave. The ultimate pH should be 6.6-6.8.

Willis and Hobbs' Medium¹

(for the isolation of clostridia)

This is a lactose egg-yolk milk agar which is rendered selective for various clostridia, including *Cl. welchii*, by the incorporation of neomycin. The recommended concentration of 250 µg. neomycin sulphate per ml. medium may inhibit certain other clostridia, usually inhibits strains of *Bacillus* and *Staphylococcus*, and greatly reduces the growth of coliform bacilli. Nagler-positive organisms produce zones of opalescence which can be specifically inhibited by the appropriate antiserum spread over half the medium in a plate and dried-in before inoculation. Certain clostridia produce a "pearly layer" (p. 698). Lactose fermentation is indicated by a pink halo in the medium around the colony and proteolysis by a clearing of the milk in the medium.

Basic Medium.

New Zealand agar	4.8 g.
Lactose	4.8 g.
Neutral red, 1 per cent. solution	1.3 ml.
Meat infusion broth (pH 7.0)	400 ml.

Autoclave mixture at 121° C. for twenty minutes, cool to 50°-55° C. and then add 15 ml. egg-yolk suspension (egg-yolk mixed with an equal volume of sterile 0.9 per cent. NaCl solution) and 60 ml. of sterile stock milk (the cream is removed from ordinary milk by centrifugation and the milk then autoclaved).

Sodium thioglycollate, 0.1 per cent., may be added with the egg-yolk suspension to assist growth of the stricter anaerobes.

Neomycin sulphate (Upjohn), 250 µg. per ml., is added if required and the plates are poured. Stock sterile solutions of neomycin sulphate may be stored in the refrigerator with little loss of potency. The antibiotic is not decomposed by exposure to 60° C. for twenty minutes.

Fildes' Peptic-Blood-Broth

(for the isolation of *Clostridium tetani*)

Mix together in a stoppered bottle 150 ml. normal saline solution, 6 ml. pure hydrochloric acid, 50 ml. defibrinated sheep blood and 1 gram of pepsin (B.P. granulated). Heat at 55° C. for two to twenty-four hours. Add sufficient 20 per cent. sodium hydroxide solution (usually

¹ Willis, A. T., & Hobbs, G. (1959), *J. Path. & Bact.*, 77, 511.

about 12 ml.) until a sample of the mixture diluted with water gives a permanganate red colour with cresol red indicator. Now add pure hydrochloric acid drop by drop until a sample of the mixture shows almost no change of colour with cresol red but a definite red tint with phenol red. It is important to avoid excess of acid. Add chloroform 0.25 per cent. and shake the mixture vigorously. This peptic digest of blood keeps well for months. For use, heat to 55° C. for thirty minutes to remove the chloroform and add to broth (or agar) in the proportion of 2 to 5 per cent.

Ellner's Medium¹

(for inducing spore-formation in *Clostridium welchii*)

Peptone (e.g. Proteose peptone, Difco)	.	.	.	10 g.
Yeast extract	.	.	.	3 g.
Soluble starch	.	.	.	3 g.
MgSO ₄	.	.	.	0.1 g.
KH ₂ PO ₄	.	.	.	1.5 g.
Na ₂ HPO ₄ , 12H ₂ O	.	.	.	67 g.
Distilled water	.	.	.	1 l.

Steam briefly to dissolve, adjust to pH 7.8 with NaOH, dispense in tubes and autoclave at 121° C. for fifteen minutes. Tubes should be half to two-thirds full and anaerobiosis may subsequently be ensured by heating at 100° C. for ten minutes and cooling just prior to inoculation. It is important that the inoculum of *Cl. welchii* culture should be adequate; 0.5 ml. of an actively growing 4–12 hr meat broth culture should be introduced with a pipette into the bottom of the tube of Ellner medium.

Cooked-Meat Medium

(for the sporing anaerobic bacilli)

The original medium is known as "Robertson's bullock-heart medium", but the following modification of Martin and Lepper is recommended.

Mince 500 grams of fresh bullock's heart, place in 500 ml. of boiling distilled water to which has been added 1.5 ml. of 1 N NaOH, and simmer for twenty minutes, by the end of which time the neutralisation of the lactic acid will be ensured. Drain off the liquid through a muslin filter and, whilst still hot, press the minced meat in a cloth and dry partially by spreading it on a cloth or filter paper. In this condition it can be introduced into bottles without soiling them. Place enough in each to occupy about $\frac{1}{2}$ in. of a 1-oz bottle (about 2.5 grams), and cover with 10 ml. of peptone infusion broth made as follows. Add peptone 0.5 per cent., sodium chloride 0.25 per cent. to the liquid filtered from the meat; steam for twenty minutes, add 1.0 ml. of pure

¹ Ellner, P. D. (1956). *J. Bact.*, **71**, 495.

HCl and filter; bring the reaction of the filtrate to pH 8.2, steam for thirty minutes and adjust reaction to pH 7.7-7.8. Use perforated screw caps (p. 262) for the bottles. Autoclave at 120° C. for twenty minutes. After sterilisation the pH of the broth over the meat is 7.4-7.5. Introduce the inoculum towards the bottom of the tube in contact with the meat.

If test-tubes are used it is usual to cover the surface of the medium with a layer of sterile liquid paraffin, $\frac{1}{2}$ -in. deep, although this is not necessary.

This medium is suitable for anaerobic cultures, and also for the preservation of stock cultures of aerobic organisms.

Thioglycollate Media

(*for anaerobes*)

It has been shown by Brewer¹ that the addition of sodium thioglycollate (0.1 per cent.) maintains the anaerobic condition which prevails for a short time after the sterilisation of culture media, and anaerobes can be grown in open tubes similar to aerobic organisms. The medium also contains 0.05 per cent. agar to prevent convection currents, glucose in amounts up to 1 per cent. (according to the organism grown), and methylene blue 0.0002 per cent. (1 in 500,000) to act as an oxidation-reduction potential indicator. The methylene blue remains decolorised except in the surface layer.

The sodium thioglycollate medium recommended by Brewer consists of:

Pork infusion solids	10 g.
Peptone (thio)	10 g.
Sodium chloride	5 g.
Sodium thioglycollate.	1 g.
Agar	0.5 g.
Glucose	0-10 g.
Methylene blue	0.002 g.
Distilled water	1 l.

The medium is placed in 12 ml. amounts in $6 \times \frac{5}{8}$ in. tubes making a column of about 7 cm. The tubes are stoppered with cotton-wool, autoclaved at 120° C. for twenty minutes and stored at room temperature. The medium is inoculated in the usual way.

Any suitable infusion or digest broth with the addition of sodium thioglycollate, glucose and methylene blue in the amounts stated above will serve satisfactorily.

Media for Sterility Tests

The media most used for this purpose (growing both aerobic and anaerobic bacteria) are cooked-meat medium (p. 233) and a thioglycollate

¹ Brewer, J. H. (1940), *J. Amer. med. Assoc.*, **115**, 598.

medium resembling Brewer's medium. The following is prescribed in the United States Pharmacopeia.

Fluid Thioglycollate Medium

Yeast extract (water soluble)	5.0 g.
Casein hydrolysate (pancreatic digest)	15.0 g.
Glucose	5.5 g.
L-cystine	0.5 g.
Agar	0.75 g.
Sodium chloride	2.5 g.
Sodium thioglycollate	0.5 g.
Resazurin sodium solution (1 in 1000), freshly prepared	1.0 ml.
Distilled water	1 l.

Mix the ingredients except the thioglycollate and resazurin. Steam to dissolve. Add the thioglycollate and adjust the pH so that after sterilisation the pH will be 7.1. If filtration is necessary, heat the solution without boiling and filter hot through moistened filter paper. Add the resazurin solution, mix, dispense in tubes and sterilise at 121° C. for twenty minutes. Cool at once to 25° and store in dark, preferably at between 20° and 30°. Do not use the medium after it has evaporated to the extent of affecting its fluidity. If more than the upper third shows a pink colour, restore the anaerobic conditions (once only) by steaming at 100° C. for a few minutes.

Whey Media

(for *Lactobacillus acidophilus*)

Whey Broth.—Add 10 per cent. hydrochloric acid to skimmed milk heated to 80°–90° C., in amount just sufficient to precipitate the casein. Filter through cotton-wool and adjust the pH to 6.8–7.0. Now add 0.5 per cent. peptone, autoclave at 15 lb. pressure for fifteen minutes, and filter.

Whey Agar.—Made from whey broth by the addition of 1.5 per cent. agar.

Lactobacilli Counts

The detection and estimation of lactobacilli is becoming of importance in dentistry, and the following simple medium gives good results in practice.

A lactobacillus count is the number of lactobacilli present per millilitre of a standard 10-ml. sample of activated saliva. The sample, after measurement of its volume, is serially diluted in broth or peptone water and distributed in 0.1 ml. quantities over a series of tomato-peptone-agar plates.¹ The colonies are counted after three or four days

¹ Hadley, F. F. (1933); *J. dent. Res.*, 13, 415.

incubation at 37° C. and the count is adjusted to represent the number of lactobacilli in a 10-ml. sample of saliva.

The saliva is activated by the patient chewing a small piece of paraffin wax vigorously, and the amount produced made up to 10 ml. with saline.

Hadley's Modification of Kulp's Medium,¹ Substituting Casein Hydrolysate for Peptonised Milk

(A)	Peptone	1 g.
	Casein hydrolysate	1 g.
	Tomato juice	40 ml.

(Note.—the tomato juice must be expressed from canned whole tomatoes. The tomato juice sold as a beverage is not suitable.)

Heat gently to dissolve, taking care to avoid overheating. Adjust to pH 5.0 with lactic acid.

(B)	Agar	2 g.
	Distilled Water	60 ml.

Heat to dissolve.

Mix A and B while both are hot. Filter through a thin layer of absorbent cotton-wool. Dispense in 100-ml. amounts and autoclave at 10 lb. for ten minutes.

(Note.—On cooling the tomato tends to flocculate. On remelting the agar the bottle must be inverted several times until the tomato goes into suspension.)

MEDIA FOR FUNGI

Sabouraud's Glucose Agar Medium

Glucose	40 g.
Peptone	10 g.
Agar	20 g.
Water	1 l.

Melt in the steamer or autoclave. Filter through cotton gauze. Adjust to pH 5.4. Dispense in stock bottles or tubes for use. Autoclave at 115° C. (10 lb. per sq. in.) for fifteen minutes. Pour tubes and slant for setting. A suitable peptone is "Oxoid" mycological peptone.²

Sabouraud's Broth

This is prepared in the same way, but omitting the agar.

Fungus Preservation Medium

Peptone	30 g.
Agar	20 g.
Water	1 l.

¹ Kulp, W. L., & White, V. (1932), *Science*, **76**, 17.

² Supplied by Oxo Ltd. (Medical Dept.), London, E.C.4.

This is prepared as for Sabouraud's glucose medium, adjusting to pH 5.4. It is used to prevent pleomorphic variation in stock cultures of ringworm fungi.

Malt Agar

Malt extract	40 g.
Agar	20 g.
Water	1 l.

This is prepared as for Sabouraud's glucose medium, adjusting to pH 5.4. The malt extract must not contain added sugar or cod-liver oil.

Malt extract is prepared commercially by extracting the soluble materials from sprouted barley in water at about 55° C. The liquor is strained and concentrated by evaporation at a temperature below 55° C. to yield a brown viscous material. It consists mainly of maltose (about 50 per cent.), starch, dextrins and glucose, and contains about 5 per cent. of proteins and protein break-down products, and a wide range of mineral salts and growth factors (*e.g.* thiamine, nicotinic acid, riboflavin, biotin, pantothenic acid, pyridoxine, folic acid and inositol). It is extensively used for the cultivation of saprophytic as well as parasitic yeasts and fungi, its high sugar content making it very suitable for this purpose. *Malt extract broth* consists of malt extract 17 grams, peptone (*e.g.* Oxoid mycological peptone) 3 grams, and water 1 litre, adjusted to pH 5.4.

Tellurite Malt Agar

This is prepared from malt agar by the addition of 0.036 per cent. potassium tellurite (*e.g.* 1.8 ml. of 2 per cent. potassium tellurite per 100 ml. medium). It is thus made more inhibitory to bacterial contaminants.

Penicillin-Streptomycin Blood Agar

This medium is used for selective cultivation of yeasts and certain dimorphic fungi, being inhibitory to bacterial contaminants. It is prepared from ordinary blood agar by addition of 30 units of penicillin and 30 micrograms of streptomycin per ml. medium.

Corn Meal Agar

This medium is used to investigate a yeast-like culture for the production of mycelium and chlamydospores.

Corn meal (ground yellow maize)	40 g.
Agar	20 g.
Water	1 l.

Heat the corn meal in the water at about 60° C. for one hour. Filter through filter paper or gauze. Add water to bring back volume to 1 litre. Add the agar and steam or autoclave to melt. Filter. Sterilise by autoclaving at 115° C. for fifteen minutes. The pH is about 6.8, requiring no adjustment.

MEDIA FOR SPIROCHAETES

Smith-Noguchi Medium

This medium was introduced by Theobald Smith and developed by Noguchi, who used it for the growth of pathogenic spirochaetes. It consists of a small piece of fresh sterile animal tissue, preferably rabbit kidney, covered with a long column of ascitic fluid on which is superimposed a layer of vaseline.

The medium is anaerobic, the function of the fresh tissue being to destroy by means of its catalase any hydrogen peroxide that might be formed by organisms, and be detrimental to their growth.

The ascitic fluid must be clear, free from bile, and of high specific gravity; it must be sterile from the beginning, as sterilisation either by heat ($56^{\circ}\text{ C}.$) or by filtration may render it unsuitable.

Technique.—Special long narrow tubes ($8 \times \frac{1}{2}$ in.) are employed.

Sterilise eight pairs of forceps and eight pairs of scissors. Anaesthetise a large healthy rabbit (1500–2000 grams in weight) and bleed by cardiac puncture (p. 420). Open the abdomen with strict aseptic precautions and remove the kidneys, fresh sterile instruments being used at each stage of the operation.

Cut up the kidneys into small pieces, using separate sterile instruments for each organ. Each kidney yields eight to eleven pieces, and place one piece in each $8 \times \frac{1}{2}$ in. tube. Run in sterile ascitic fluid by means of a 50-ml. bulb pipette until the tubes are half full. Melt vaseline (previously sterilised by autoclaving in a 250-ml. conical flask) and add by means of a 10-ml. pipette, forming a layer of about half an inch. Incubate the tubes for forty-eight hours and examine for contamination.

To inoculate the medium first melt the vaseline and then introduce the inoculum to the bottom of the tube by means of a capillary pipette and rubber teat. Material is similarly withdrawn to be examined. Growth is indicated by a clouding of the fluid at the bottom of the tube, which appears about the fourth to the tenth day of incubation. Turbidity, however, is not definite evidence of growth, as uninoculated control tubes also may show clouding just above the piece of tissue.

Leptospira Media

Blood serum is an essential constituent of the many culture media recommended for leptospires. Rabbit serum is generally found the most satisfactory, though the serum of some larger animals, e.g. sheep, horse and newborn calf, has been used successfully. The serum of certain individual rabbits is inhibitory to leptospires due to its content of agglutinins or other agents. For this reason, the suitability of sera from several rabbits should be tested individually by examining for agglutinins or by making separate trial batches of medium from each serum. The suitable animals are retained to supply serum as required.

Blood is collected from an ear vein and allowed to clot. The serum is pipetted off, inactivated by heating at 56° C. for thirty minutes and sterilised by passing through a Seitz filter. The clot may be used for preparing the haemoglobin solution employed in Korthof's medium.

Certain brands of peptone have been recommended for particular media, e.g. Witte peptone, Difco neopeptone, but any good brand is likely to be suitable. There may be considerable variations in the growth-promoting ability of different batches of the same brand, and a preliminary test should be made of each new batch before taking into use. All glassware must be perfectly clean and free from any trace of soap or other detergent, since this is lethal to the spirochaete. After the usual cleaning, the glassware should be very thoroughly rinsed, preferably by soaking for twenty-four hours in a pH 7.6 phosphate buffer solution (p. 283) and then rinsing in distilled water prior to sterilisation. To allow confirmation that the pH of the medium in each container is at the proper value, phenol red may be incorporated in the medium at a concentration of 0.001 per cent.

After the medium has been dispensed in tubes or screw-capped bijou bottles, these are tested for sterility by incubating at 37° C. for two days and then at 22° C. for three days, before using. To obtain satisfactory growths a large inoculum is used; about 10 per cent. of a previous culture should be introduced with a sterile Pasteur pipette.

Stuart's Medium (modified)¹

Basic Medium, less Phosphate:

	<i>Concentration</i>	<i>Volume in ml.</i>	<i>Final Concentration</i>
L-Asparagine (d-rot)	0.1 M	2	0.001 M
NH ₄ Cl .	0.1 M	10	0.005 M
MgCl ₂ .	0.1 M	4	0.002 M
NaCl .	0.1 M	.66	0.033 M
Thiamine hydrochloride .	0.1 per cent.	0.2	0.0001 per cent.
Phenol red solution (p. 277) .	0.02 per cent.	10	0.001 per cent.
Distilled water .	—	91	—

With a sterile 10-ml. pipette measure the quantities of the stock solutions as tabulated above into a sterile screw-capped bottle, rinsing the pipette with boiling water after each solution has been measured. Steam the mixture for thirty minutes to remove carbon dioxide. Add 16 ml. of Sörensen's buffer solution (pH 7.6) which has likewise been steamed, and sterilise the medium at 100° C. for one hour: 20 ml. of this buffer solution may conveniently be prepared by mixing 17.6 ml. of Na₂HPO₄.2H₂O, 11.876 grams per litre, and 2.4 ml. of KH₂PO₄, 9.078 grams per litre.

¹ Bryan, H. S. (1957), *Vet. Med.*, 52, 111.

Before use, add 10 per cent. of sterile, Seitz-filtered rabbit serum. Distribute the medium in carefully washed sterile screw-capped bijou bottles and place the bottles in a water bath at 60° C. for one hour to inactivate the serum.

Korthof's Medium (modified)¹

Peptone-salt Solution:

Peptone (any good make)	0.8 g.
NaCl	1.4 g.
NaHCO ₃	0.02 g.
KCl	0.04 g.
CaCl ₂	0.04 g.
KH ₂ PO ₄	0.24 g.
Na ₂ HPO ₄ , 2H ₂ O	0.88 g.
Redistilled water to	1 l.

Boil (on a water bath) for twenty minutes. Filter through Chardin-type or double-thickness Whatman No. 1 paper, bottle in 100 ml. amounts and sterilise in the autoclave. The pH should be approximately 7.2.

“Haemoglobin Solution”.—After the serum has been removed from clotted rabbit blood (see introductory paragraph, p. 239), add an equal volume of distilled water to the clot, and freeze and thaw repeatedly to haemolyse the corpuscles. Filter through a Seitz disk.

Preparation of Medium.—To 100 ml. peptone-salt solution add 8 ml. sterile inactivated rabbit serum, and 0.8 ml. sterile “haemoglobin solution”. Distribute aseptically in 2–3 ml. amounts in tubes or screw-capped bijou bottles.

We have found Korthof's medium excellent for the cultivation of leptospirae.

Fletcher's Agar Medium

Peptone	2 g.
Sodium chloride	1 g.
Agar	1 g.
Distilled water	1 l.

Mix the ingredients and adjust to pH 7.2. Steam to melt the agar, dispense measured quantities into sterile screw-capped bottles and sterilise by steaming at 100° C. for ninety minutes. Cool to 55° C. and add 15 per cent. sterile rabbit serum. The small concentration of agar prevents convection currents in the medium and keeps conditions more favourable for maintenance of stock cultures.

¹ Alston, J. M., & Broom, J. C. (1958), *Leptospirosis*, p. 303. E. & S. Livingstone, Edinburgh.

N.N.N. (Novy, MacNeal, Nicolle) Medium
(for trypanosomes and leishmaniae)

Make meat extract in the ordinary way with rabbit or beef flesh, using 125 grams to a litre of water. Add to this: peptone, 20 grams; sodium chloride, 5 grams; agar fibre, 20 grams; and 10 ml. of 1 N sodium carbonate solution. Tube, autoclave and cool to 55° C. Then add to the medium in each tube twice its volume of defibrinated rabbit blood (p. 421). Mix the contents of the tubes by rotation between the palms of the hands, and solidify in the sloped position, preferably on ice. It is important to obtain a large amount of water of condensation.

Before inoculation place the tube in the upright position and introduce the inoculum by means of a capillary pipette. The growth of leishmaniae occurs mostly in the water of condensation.

Nöller's Modification of N.N.N. Medium

Agar	25 g.
Glucose	20 g.
Slightly alkaline broth	1 l.

Prepare, filter and distribute into test-tubes in about 2 ml. amounts. When required for use melt the medium, and when cooled to 55° C. add an equal or double volume of defibrinated horse blood, or undefibrinated rabbit blood.

Boeck and Drbohlav's Medium
(for the cultivation of amoebae)

Wash thoroughly four fresh eggs with soap and water, wipe them over with alcohol, and break into a sterile bottle containing glass beads. Add 50 ml. Locke's solution (p. 291) and shake mixture well. Fill test-tubes with sufficient of the medium to form short slopes of 1-1½ in., and coagulate in the inspissator at 75° C. Sterilise by autoclaving. To each tube add a mixture of 8 parts sterile Locke's solution and 1 part of sterile human serum which has been heated at 55° C. for half an hour. Cover the solid medium with the fluid to a depth of ½ in., and incubate the tubes to test for sterility. Instead of the serum mixture a 1 per cent. solution of crystallised egg albumin in Locke's solution, sterilised by filtration through a Seitz filter, may be substituted. The initial reaction of the medium, which varies between pH 7.2 and 7.8, does not require adjustment.

Dobell and Laidlaw have modified the medium in the following manner.

Inspissate sterile horse serum in the sloped position for one hour at 80° C. Cover with serum or egg albumin solution as above. Add a small quantity of solid rice starch (sterilised in thin layers at 180° C.

for one hour in the hot-air oven). Amoebae grow luxuriantly in this medium, and the whole life-cycle, including encystment, may be observed.

Stuart's Transport Medium for the Gonococcus

This soft agar medium is used to maintain the viability of gonococci on swabs during their transmission through the post to a laboratory.

Preparation of Medium

(1) To 900 ml. distilled water add:

Thioglycollic acid	2 ml.
N NaOH solution (to bring to pH 7.2)	12-15 ml.
Sodium glycerophosphate, 20 per cent. in water	100 ml.
CaCl ₂ , 1 per cent. solution	20 ml.

(2) Agar	6 g.
Distilled water	1000 ml.
Dissolve by steaming.	

(Note.—It is essential that the distilled water be free from chlorine.
To ensure this, it should be passed through an ion-exchange resin column before use.)

Add mixture (1) to melted agar (2). If necessary, adjust to pH 7.3-7.4. Add 4 ml. of a 0.1 per cent. solution of methylene blue. Dispense in $\frac{1}{4}$ oz. screw-capped (bijou) bottles, filling nearly to capacity, and apply caps. Sterilise in steam at 100° C. for sixty minutes and immediately tighten caps. The medium should be colourless after cooling.

Preparation of Swabs

Make neat swabs of absorbent cotton-wool on applicator sticks and boil for five minutes in 0.07 M phosphate buffer solution at pH 7.4 (p. 283). Shake off excess moisture and immerse in a 1 per cent. suspension of *finely powdered* charcoal (*e.g.* activated charcoal, B.D.H.) in water. Remove, shake off excess moisture, place in test-tubes, plug these with cotton-wool, dry in oven and sterilise in oven at 160° C. for sixty minutes.

Use of Medium.—Take specimen of exudate on swab. Press swab into upper third of the medium in the bijou bottle. Cut off the protruding part of the swab stick with scissors and screw the cap on the bottle tightly. Send to laboratory without unnecessary delay. For culture, grasp the short swab stick with artery forceps, withdraw from the transport medium and inoculate on to blood agar or other culture medium in the usual way; take care to ensure that material from the swab itself, not merely adherent transport medium, is deposited on the culture medium.

IDENTIFICATION OF MEDIA

It is necessary to identify a culture medium after it has been made, and as many of the media are similar in appearance, *e.g.* the various

kinds of nutrient agar and the different sugar media used in fermentation tests, it is essential that there should be some simple but reliable system of identification. It has long been the custom to denote the medium contained in test-tubes by cotton-wool stoppers of different colours, but for flasks some sort of gummed label has been used. In the case of bottled fermentation media, the caps are painted in various colours to distinguish the different sugars, while coloured beads are used to identify the other types of media, thus avoiding gummed labels which become detached in the steamer when a solid medium is melted. The glass beads are the ordinary opaque beads for threading necklaces, 6-7 mm. in diameter. (Clear glass beads are not suitable.) Before use they are boiled twice in distilled water and dried in the incubator. The appropriate bead is dropped into the bottle before filling. Owing to the convexity of the bottom of the bottle, the bead remains at one side of the bottle and is very easily recognised no matter what type of culture medium is used. On tilting the bottle for pouring, the bead comes to rest on the shoulder and remains in this position, even when the bottle is almost completely inverted. The coloured beads can be used for identification of all kinds of media and reagents. In the case of small bottles which will not take a bead, or if beads are unobtainable, a dab of coloured cellulose paint on the side or bottom of the bottle will answer the same purpose.

It is recommended that a standard colour scheme be adopted and the following system is suggested, as it is already widely used.

Fermentation Media—“ Sugars ”

For tubes, coloured cotton-wool is used, and for screw-capped bottles cellulose paint is applied to the cap.

Where colours are mentioned for which there is no coloured wool, e.g. gold, silver, a small patch of cellulose paint is placed on the tube itself.

Adonitol	Silver	Inositol	Gold
Aesculin	Brown	Inulin	Yellow and white
		Lactose	Red
Arabinose	Black and yellow	Maltose	Blue and white
Dextrin	Red and mauve	Mannitol	Mauve
Dextrose (see Glucose)		Mannose	Black and green
Dulcitol	Pink	Raffinose	Red and white
Erythritol	Black and red	Rhamnose	Black and pink
Fructose (laevulose)	Yellow	Salicin	Pink and white
Galactose	Mauve and white	Sorbitol	Black and blue
		Starch	Yellow and mauve
		Sucrose	Blue
Glucose	Green	(saccharose)	
Glycerol	Brown and white	Trehalose	Mauve and green
Glycogen	Blue and yellow	Xylose	Red and green

Other Culture Media

Other media in bulk in 3-10-oz. bottles are identified by means of a coloured bead. For smaller quantities a dab of coloured paint is placed on the cap. It should be borne in mind that it is better to use a few outstanding colours alone or in combination if necessary, rather than different shades of a colour; thus, green, irrespective of the shade, whether it be light or dark, yellowish green or bluish green, always indicates glucose.

<i>Culture Medium</i>	<i>Colour of Bead</i>
Digest broth	Black
Nutrient agar made from digest broth	Black
Infusion broth	Yellow
Nutrient agar made from infusion broth	Yellow
MacConkey's agar medium	Red
Peptone water without indicator	White
Peptone water with indicator	Brown
Glucose media	Green
MacConkey's fluid medium—	
Single strength	1 red spot
Double ",	2 red spots
Sabouraud's medium	Light blue
<i>Solutions, etc.</i>	
Distilled water	White
Normal saline (0.85 per cent.)	Dark blue
Glucose in saline.	Blue and green

CHAPTER X

CULTIVATION OF MICRO-ORGANISMS

USE OF CULTURE MEDIA

ONLY general methods are described here. Special methods applicable for particular purposes are referred to in the appropriate sections, *e.g.* under special media, etc.

Storage and Distribution of Culture Media

Culture medium after being made is either stored in bottles (p. 185), in bulk, or distributed in small bottles, tubes, or Petri dishes (*vide infra*). It is convenient to store fluid media in 250 ml. amounts in 10-oz. screw-capped bottles. The bottles are sterilised with the caps tightly screwed on, so that the medium remains sterile and without evaporation. For solid media, storage in 100 ml. amounts in 5-oz. round screw-capped bottles is recommended. The 100 ml. amounts are not only useful for subsequent distribution into tubes or small bottles but also are convenient for pouring into Petri dishes—*e.g.* when 100 ml. nutrient agar is melted and cooled to 55° C., and 10 ml. defibrinated horse blood added, it will make sufficient blood agar for seven plate cultures in 4-in. Petri dishes (p. 247).

Use of Solid Media

For immediate use the medium is allowed to solidify in sterile stoppered test-tubes either by cooling after having been melted by heat, as in the case of agar or gelatin (p. 194), or by coagulation in an inspissator, as in the case of solidified serum or egg media (p. 211). The tubes are plugged with cotton-wool, or stoppered with slip-on metal caps, and sterilised in the hot-air oven before addition of the medium.

Alternatively, 1-oz., $\frac{1}{2}$ -oz. or $\frac{1}{4}$ -oz. screw-capped bottles (p. 185) can be substituted for test-tubes.

Tubing of medium is conveniently carried out by means of a sterile 6-in. glass funnel (fixed in a burette stand) with a short length of rubber tubing and glass delivery nozzle fitted to the stem and controlled by a pinchcock. During the tubing the funnel is covered with the lid of a large sterile Petri dish to avoid aerial contamination.

The latest improved model of automatic filler devised by T. H. Ayling can be recommended for tubing media.¹ It consists of a glass funnel 7 in. in diameter, connected by rubber-tubing to a metal 3-way stopcock which in turn is connected to an all-glass syringe of

¹ Supplied by R. B. Turner & Co., London.

15 ml. capacity (see figure). The syringe is of the three-piece type, but without the nozzle, and the plunger is hollow, as the head of liquid will not lift a solid glass piston. The barrel is graduated to 15 ml. by 0.5 ml., and the numbers are so engraved as to be readable when the syringe is vertical. The syringe is connected to the stopcock by means of a metal screw fitting. A clamp secures the lower end of the syringe. The amount of fluid delivered is determined by the adjustable screw. The action of the filler is simple. The head of

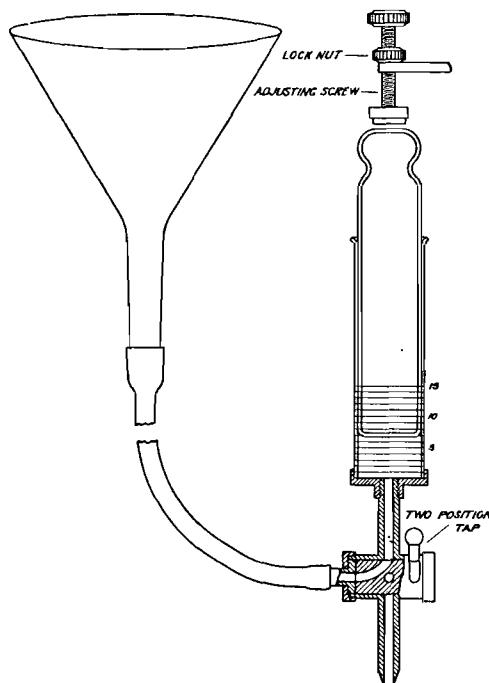


FIG. 10

medium in the funnel forces up the plunger until it is stopped by the adjustable screw. The handle of the stopcock is then turned and the syringe empties itself under the weight of the plunger. Air bubbles in the syringe are removed by first filling the apparatus, and emptying and filling the syringe two or three times, manipulating the piston by hand while this is being done. The adjustable screw is then turned to deliver the correct amount. If a smoothly working syringe is used, very little head of pressure is necessary, and the height need not be greater than 18 inches. Once set, the accuracy of the filler is much greater than that of an ordinary pipette, while media can be tubed with greater rapidity. It works equally well with melted agar or gelatin, provided that fresh hot supplies are available, and the syringe and stopcock are

washed out immediately after use. When tubing agar or broth with the filler, the medium is run into clean but not sterilised test-tubes or bottles. These are then stoppered and sterilised in the steamer or in the autoclave as indicated under the description of the various media.

Depending on the method of inoculation to be used, media are solidified in tubes or bottles as follows:

(1) *Upright for "puncture" or "stab" culture.*—The test-tube or small screw-capped bottle is half filled with the medium (about 12 ml.) which is allowed to solidify in the upright position. It is inoculated by plunging a long straight wire (p. 249), charged with the material, vertically down the centre of the tube. This method is used for anaerobic cultures in glucose agar, and for testing the liquefaction of gelatin.

(2) *Sloped, for "stroke" culture.*—This is often called a "slope" or "slant" and ensures a maximum surface of the medium exposed to the air. Quantities of 5 ml. of medium for ordinary $6 \times \frac{5}{8}$ in. tubes are sufficient. When a large number of agar tubes have to be sloped, special trays, which allow the tubes to be laid at the correct angle, are useful and, moreover, they can be stacked one upon another so that very little bench space is required during solidification. Fresh agar slopes after cooling contain "water of condensation" at the foot of the tube, and the tubes should be stored and handled in the vertical position to prevent the fluid from flowing over the surface of the medium or entering the cotton-wool stopper.

1-oz. or $\frac{1}{4}$ -oz. screw-capped bottles can conveniently be substituted for test-tubes. The aluminium cap should have a black rubber washer 3 mm. thick. 5 ml. amounts of the medium are added to the 1-oz. bottles, 2.5 ml. amounts to the $\frac{1}{4}$ -oz. (bijou) bottles, and the caps tightly screwed on. The bottles are placed at such an angle that there is a thick butt at the bottom, as with the test-tubes. Being tightly sealed there is no evaporation and the surface of the medium is always moist.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures (p. 252), the medium e.g. agar or gelatin, is allowed to solidify in the form of a thin layer in a Petri dish. For a dish of 4 in. diameter, 14 ml. of medium are ample. The *melted* medium is

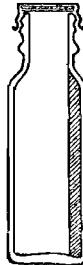


FIG. 11
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FIG. 12

poured into the dish with the necessary precautions to avoid contamination. Medium which has been bottled (*vide supra*) can be melted and used for pouring plates.

In separating organisms in mixed cultures by spreading the material on plates, it is essential that the surface of the medium should be dry.

When plates have been poured, the steam from the hot liquid condenses on the surface of the medium and this moisture is undesirable for cultural work. It is removed by drying the poured plates in the incubator at 37° C. for one hour. The lid of the dish is first laid in the incubator; the portion containing the medium is then inverted (so that the surface of the medium is downwards) and placed in the incubator with the free edge resting on the lid (*vide* diagram). If care is taken to avoid disturbing dust, there is very little risk of contamination of the medium by air organisms.

Shake Cultures.—Agar or gelatin medium in tubes, e.g. (1), *vide supra*, is inoculated in the melted condition at a temperature which keeps the medium fluid, but is not *immediately* lethal to the organisms inoculated, e.g. 45°–50° C. The contents of the tubes are mixed by rotation between the palms of the hands and then poured at once into a Petri dish, or left to solidify in the tube so that colonies may develop in the depth of the medium, as when separating anaerobes. In the latter case the test-tube is filed and broken, and the colonies picked out of the medium exposed in this way.

Use of Fluid Media

Fluid media are used in (1) test-tubes stoppered with cotton-wool or slip-on metal caps, the tubes being about half filled; (2) $\frac{1}{4}$ -oz. (bijou) bottles; broth or peptone water in 2.5 ml. amounts; and fermentation media in 3 ml. amounts; (3) 1-oz. screw-capped bottles, in 5 ml. amounts; (4) 3- or 5-oz. screw-capped bottles for blood culture (50–100 ml. amounts) (p. 262); or (5) stoppered or screw-capped bottles of larger capacity according to the quantity of culture required.

INOCULATION OF CULTURE MEDIA

According to the nature of the medium and the inoculum, various methods are employed for inoculation, and the following instruments are commonly used:

Wire Loop.—The original type of inoculating wire was of platinum, No. 23 S.W.G., 2½ in. long, but, owing to the high cost of platinum, "Nichrome" or "Eureka" resistance wire, No. 24 S.W.G., is now generally used. One end of the wire is fused into a glass rod, or inserted into a special aluminium holder. The other end is bent in the form of a loop, 2 mm. internal diameter, care being taken that the loop is flat and completely closed.

The wire is sterilised by holding it vertically in a Bunsen flame so that the whole length becomes red-hot at the same time. A wire charged with certain growths, e.g. of the tubercle bacillus, should be sterilised slowly in the cooler part of the flame. If rapidly burned, particles of unsterilised culture may "spurt" from the wire on to the bench. The loop is the most useful of the inoculating wires. It takes up a considerable amount of solid culture, and also a large drop of fluid.

Straight Wire.—This is similar to the foregoing, but without the loop. It is used for stab cultures, and also for picking off single colonies.

Long Straight Wire.—A wire $4\frac{1}{2}$ in. long mounted on a holder. It is employed for deep-stab inoculation when working with anaerobes.

Thick Wire, particularly with a loop, is very useful on account of its rigidity for lifting thick viscid sputum and tenacious growths.

Scalpel.—This instrument, sterilised by dipping in alcohol and flaming, is used for making inoculations with scrapings from tissues and ulcers, etc.

STERILE PIPETTES.—Bulb pipettes (10–100 ml.) are used when large amounts of fluid inoculum have to be added to a medium, and graduated 1-ml. or 10-ml. pipettes when the inoculum is between 0.1 and 10 ml. (e.g. in examination of water, p. 362). These pipettes are stoppered with a cotton-wool plug in their upper end to guard against contamination of their interior or accidental aspiration of their contents. Because of the danger of infection, they should not be placed directly in the mouth, but operated either by a rubber teat or by a glass mouth-piece which is attached by a length of rubber tubing and sterilised by flaming (see pp. 316 and 149 for details).

STERILE CAPILLARY PIPETTES.—These are made by heating the middle of a piece of glass tubing, 5 mm. bore and 8 in. long, and when melted pulling out the two halves; the capillary formed in the middle is broken after cooling and two pipettes thus obtained. The capillary ends, which should not be too thin, are sealed in the flame, and the other ends are plugged with cotton-wool. They are placed in a large test-tube (e.g. $8 \times 1\frac{1}{2}$ in.), which is then stoppered with cotton-wool, or covered with paper or aluminium foil, and sterilised by dry heat (p. 150). Before use, the tip of the capillary portion is broken off and a rubber teat fitted to the other end. An alternative method is to prepare the 8-in. lengths of glass tubing with cotton-wool plugs in each end, wrap them in bundles of 8–10 in kraft paper, sterilise in the hot-air oven, and draw the capillaries in a flame just before they are required for use. These pipettes are necessary for inoculating cooked-meat medium (*q.v.*) and certain other media, and are very useful in many bacteriological manipulations.

CAPILLARY PIPETTES DELIVERING MEASURED DROPS.—Small measured volumes are conveniently delivered by sterile capillary pipettes which have been prepared to give drops of a known volume. The pipettes are drawn from glass tubing as described above. When cool, the capillary is inserted into the appropriate hole of a Morse drill gauge and pressed through until it engages. For water drops of 0.020 ml. the hole used is Morse 59 (i.e. 0.041 in. diameter), for 0.025 ml. Morse 55 (0.052 in.), for 0.030 ml. Morse 52 (0.063 in.), for 0.035 ml. Morse 47 (0.078 in.) and for 0.040 ml. Morse 43 (0.089 in.). Exactly at its point of impaction in the hole, the capillary is scored with a glass-cutter (e.g. a vulcanite carborundum disk), and broken off squarely.

The pipettes are then plugged in their wide end with cotton-wool, packed in a large test-tube and sterilised in the hot-air oven. In use the pipette is fitted with a teat, filled with the liquid and held vertically with the tip downwards. For accurate work the drops should be expelled at the constant rate of about 40 per minute, *i.e.* taking about 1½ seconds for the gradual expulsion of one drop. The drop size may differ slightly in the case of liquids with different densities and surface tensions from that of water, and the pipette should be calibrated directly for the particular liquid by measuring the volume of 100 drops. For further details consult *A System of Bacteriology*, Medical Research Council, 1931, vol. 9, pp. 174-83.

Technique of Inoculating Tubes

The following routine methods are recommended.

Inoculation of one "slope" from another.—The two tubes are firmly held at their lower ends between the thumb and first two fingers of the left hand, with the sloped surface of the medium towards the worker. The tube containing the growth should be on the left and the uninoculated tube on the right. With the right hand loosen the cotton-wool stoppers by rotating them in the mouths of the tubes so that they may be removed easily. Take the holder of the inoculating wire at its end between the thumb and first two fingers of the right hand (as in holding a pen). Sterilise the wire by holding it vertically in the Bunsen flame. Remove the stopper of the tube from which the inoculation is to be made with the crooked third finger of the right hand, and flame the mouth of the tube. Pass the wire into the tube and touch a portion of the medium free from growth to ascertain if the wire is sufficiently cool. If too hot, the wire will melt the agar, causing a furrow, and might, of course kill the organisms in removing the growth. When the wire is cool, the growth is scraped from the surface, care being taken not to wound the agar. Withdraw the wire, remove the stopper from the other tube with the crooked little finger and flame the mouth of the tube. Insert the wire charged with the growth and lightly smear the surface of the agar. Withdraw the wire and sterilise it, flame the mouths of the tubes and replace the stoppers. The nature of the inoculated material and also the date should be written on the tube by means of a grease pencil, or on a gummed label which is then affixed to the tube.

For *stab cultures*, the tubes are held similarly and the straight wire charged with bacterial growth is plunged into the centre of the medium, care being taken to withdraw the wire in the same line and not to cause splitting of the medium.

In *inoculating a fluid medium*, such as broth, from a solid culture, the tube should be inclined almost to the horizontal and the growth on the loop deposited on the wall of the tube just above the surface of the liquid at the lower end of the tube. On returning the tube to the vertical position the inoculum is below the surface of the broth.

Technique of Inoculating Screw-Capped Bottles

When inoculating medium in screw-capped bottles, essentially the same procedure is carried out as above. Before the bottles are held in the hand it is advisable to loosen the screw cap, as this is usually tightly screwed before sterilisation to seal the bottle effectively.

If the caps are very tight they can easily be loosened by means of a bored-out rubber bung, a section of which is shown in the figure. A bung about 2 inches across is suitable, and by means of a cork-borer a number of holes are bored in a slanting direction round a diameter of $1\frac{1}{4}$ inches, so that the whole centre is removed, leaving a conical-shaped cavity. It is preferable to have the wall ridged, as it grips the cap more easily. The bung can be held in the hand, or suitably mounted just below the edge of the bench.

The bottles are held exactly as test-tubes, and the cap is held in the same way as the cotton-wool stopper. The bottles are then unscrewed from the cap, the wire is introduced and the inoculation made. The cap is now loosely screwed on, and when the bottle is ready for the incubator the screw-cap is tightened if considered necessary.

Inoculating Hood

It is advisable, as far as possible, to carry out certain inoculation procedures under a hood in order to minimise the chances of aerial contamination.

A suitable size of hood is 5 ft. wide, 5 ft. deep, 7 ft. 6 in. high. It fits over the bench to form a completely enclosed chamber and is entered by a sliding (not swing) door. All sides above the bench level consist of windows. Ventilation is secured by two holes in the roof; from the top of each is attached a vent pipe 3 in. wide and 18 in. long, and turned at right angles. The bench on which the hood is fitted should have a gas supply for the Bunsen burner, and it is convenient to have a pipe from the roof 3 in. in diameter with a funnel-shaped opening situated 24 in. above the bench top, under which the Bunsen burner is placed so that the gas fumes are led directly away. The hood may be lighted by an electric lamp suspended from the roof.

The table under the hood is covered by a towel soaked in 1:1000 mercuric chloride solution, so that any organisms deposited in dust are destroyed. The advantage of the hood depends on the relative absence of dust and air currents, which are liable to produce contamination of medium, etc., exposed in the process of inoculation. We have used the inoculating hood with advantage in the preparation of blood-agar plates and other highly nutritive media, and in conducting autopsies on animals under aseptic conditions.

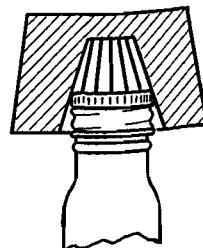


FIG. 13

A more simple inoculating box which is movable can easily be constructed as shown in the figure below. The frame is made of wood and it has a sloping glass window in front, and two apertures whereby the hands and arms can be inserted to carry out the necessary manipulation of the cultures. A convenient size is 3 ft. wide, 2 ft. deep and 3 ft. high.

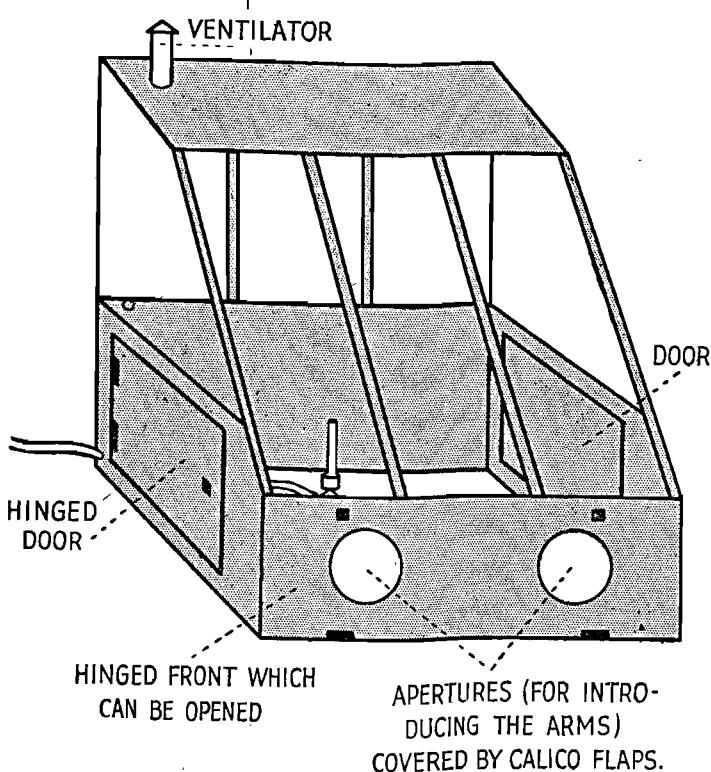


FIG. 14

SEPARATION OF MIXED CULTURES

1. **BY PLATING.**—The term “plating” is generally applied to the inoculation of medium in Petri dishes, usually by successive strokes or spreading. The wire loop is charged with the bacterial mixture, pus, fragment of tissue, etc., and several strokes in series are made on the surface of the medium in a Petri dish without re-charging the wire (p. 248). When a plate is not available separate colonies may be obtained by making successive strokes on one or two slopes in tubes or screw-capped bottles.

An alternative method for Petri dishes is to employ a spreader. This

is made by bending a piece of glass rod, 3 mm. diameter, at a right angle in the blowpipe flame, the short limb, used for spreading, being 1 in. long. A small amount of the bacterial mixture is placed on the plate with the inoculating loop or capillary pipette. By means of the spreader, previously sterilised by boiling and then cooled, the material is evenly distributed over the surface. The spreader is then transferred to a second plate, which is similarly inoculated. Thus the medium in the second dish is inoculated merely with the organisms carried over by the spreader from the first. (Spreaders may be sterilised more conveniently in the hot-air oven, a number being packed in a metal tin with a "press-on" lid loosely applied.)

By these methods the bacteria are gradually wiped off the wire or spreader so that they are ultimately deposited singly. Generally from each individual organism an isolated colony will grow; a single colony may be subcultured on fresh media and so yield a pure growth. *In order to ensure separation, the surface of the medium must be dry.*

2. BY PLATING DECIMAL DILUTIONS OF THE INOCULUM.—A series of tubes or bottles of melted agar or gelatin is inoculated with successive decimal dilutions of the infected material and then the medium in each tube is poured into a Petri dish and allowed to solidify. By dilution, the bacteria are separated from one another, and on incubation the resulting colonies are distributed singly throughout the solid media. (This method is also used when the number of viable organisms present in a fluid has to be ascertained, as in water and milk examinations, and in blood in cases of septicaemia.)

3. BY HEATING AND SUBSEQUENT PLATING.—This method is employed where the organisms to be obtained in pure culture are more resistant to heat than the remainder of the bacteria present. This method applies especially to spore-bearing organisms, such as the anaerobes (p. 697), the spores of which survive the heating. The mixture of bacteria is heated to 65° C. for half an hour and then plated. From the spores individual colonies develop, and these may then be picked off.

Plate cultures should have the nature of the material, and also the date, written on the glass of the Petri dish by means of a grease pencil. Agar plates are incubated in the inverted position, *i.e.* the lid of the plate is underneath and the grease-pencil writing should be on the portion of the dish containing the medium. On the other hand, gelatin, because it is liquefied by many organisms, is incubated with the lid uppermost on which the necessary pencil notes are made.

Care must be taken in *picking off single colonies*, particularly when they are very close to one another, that the point of the wire does not touch any of the neighbouring colonies. The culture should first be looked at through the medium by holding it up to the light. The lid should be removed and the dish held round the side by the thumb and middle finger of the left hand. The colonies selected should be marked by grease-pencil rings on the bottom of the dish. To pick off the colony, first sit down with both elbows on the bench. Hold the plate vertically with the left hand, then grasp the holder of the wire like a pen, with the

fingers quite close to the wire. Steady the right hand by placing the little finger on the left thumb in the way artists support the hand when painting. The selected colony is then easily removed without touching the others. Lay the plate on the bench, withdraw the right hand to the other end of the holder and inoculate the required medium in the manner previously described.

Plate Culture Microscope.—Several makers produce low-power binocular magnifiers which are extremely useful for examining plate cultures of organisms; they have a long working distance so that a colony can also be picked off the plate while using the instrument. When dealing with bacteria forming small delicate colonies, or where the colonies of the desired organism are few in number, the low-power binocular is invaluable. A magnification of ten diameters is useful for general work, but by interchangeable eye-pieces and objectives, magnifications from six to thirty diameters are available.

4. By SHAKE CULTURE IN TUBES (p. 248). This method is sometimes used in the separation of anaerobic organisms.

5. By THE USE OF SELECTIVE MEDIA.—Media such as desoxycholate-citrate-agar for the *Salmonella* and *Shigella* groups, Dieudonné's for the cholera vibrio, the tellurite media for the diphtheria bacillus, etc., have been devised so that the majority of the organisms other than those for which the media are used will not grow, and the isolation of pure cultures is thus facilitated.

6. By ANIMAL INOCULATION.—Advantage is taken of the fact that laboratory animals are highly susceptible to certain organisms—for example, the mouse to the pneumococcus. If a mixture of organisms containing the pneumococcus, e.g. sputum, be inoculated subcutaneously into a mouse, the animal dies of pneumococcal septicaemia in twenty-four to thirty-six hours, and from the heart blood the organism can be obtained in pure culture. Similarly the tubercle bacillus can be isolated from contaminating organisms by inoculation of a guinea-pig. The tubercle bacillus is found in a pure state in the resulting lesions.

INCUBATION

Students and others commencing work in a laboratory should familiarise themselves with the mechanism of the incubator, whereby any desired temperature may be constantly maintained. Incubators may be heated by electricity, gas or oil, according to the facilities of the laboratory.

All bacteriological laboratories have one or more incubators working at 37° C. This temperature, which is the optimum for practically all pathogenic organisms, is that referred to when speaking of incubation without mentioning the temperature.

Some laboratories have a warm room heated by gas or electricity, and kept at 37° C., in which large quantities of material can be incubated. The room has a regulating mechanism similar to the ordinary incubator to keep the temperature constant, and if electrically heated it should be

fitted with a device to cut off the current for the room at the main switch, if the temperature rises above 40° C.

Other temperatures for incubation are 30° C., used for cultivating staphylococci and leptospirae, and 22° C. ("cool incubator"), used for certain fungi and for gelatin cultures. (Gelatin medium melts at about 24° C.).

In order to prevent drying of the medium in test-tubes when prolonged incubation is necessary, as in the cultivation of the tubercle bacillus, the mouths of the tubes are sealed with paraffin wax, or covered with special rubber caps. Under these circumstances, however, we strongly advise that screw-capped bottles (p. 185) should be used instead of test-tubes.

METHODS OF ANAEROBIC CULTURE

Obligate anaerobes are defined as organisms that will grow only in the absence of free oxygen (p. 25). The method usually employed to establish anaerobic conditions is to remove oxygen from the atmosphere surrounding the culture, the oxygen being sometimes replaced by an inert gas (p. 256 *et seq.*).

The simplest method of securing anaerobiosis is by growing the organisms in solid media. Deep agar tubes are convenient and efficient for the purpose. The addition of 0.5 per cent. glucose to the medium is of value, particularly when cultivating the saccharolytic group of anaerobes. Glucose acts as a reducing agent, and further serves as a suitable pabulum for bacterial growth. The agar may be inoculated when solid by means of a long straight wire (p. 249). The colonies develop best in the depth of the tube, becoming fewer and smaller towards the surface. No growth is usually noted in the top half-inch of the medium. An alternative method is to melt the agar, cool it to 45° C. and introduce the inoculum by means of a capillary pipette. The contents of the tube are mixed by rotation between the palms of the hands. The agar is then rapidly solidified by placing the tube in cold water. The colonies develop in the deep portions of the tube, usually separated from one another.

A convenient method is the use of semi-solid agar medium. A fluid medium, *e.g.* nutrient broth, is heated in boiling water and to it is added one-tenth of its bulk of melted 2 per cent. nutrient agar. On cooling, a semi-solid "sloppy" medium results which can be used as it is, but is usually enriched with glucose, 0.5-1 per cent., or other reducing agent such as sodium thioglycollate, 0.1 per cent. (Brewer's medium, p. 234), or ascorbic acid, 0.1 per cent. If the semi-solid medium is tubed and kept for any length of time it should be placed in boiling water for ten minutes and allowed to cool before use.

Glucose-broth can easily be rendered completely anaerobic. Long tubes, 8×½ in., are half filled with the medium and are placed in the steamer for half an hour or in boiling water for five minutes. Sterile melted vaseline is then poured on the surface of the medium and the

tubes are rapidly cooled. The heating removes all oxygen, and the vaseline effectively seals the medium from the air. Inoculation is made by means of a capillary pipette after melting the vaseline. Gas-producing anaerobes should not be cultivated in this medium, as the gas formed will force out the vaseline seal.

Robertson's cooked-meat medium (p. 233) is also very useful for anaerobic work. The sterilised muscle tissue contains reducing substances, which are effective in maintaining anaerobic conditions at the bottom of the tube. The reducing activity of the meat is shown by the pink colour in the lower layers due to the reduction of haematin.

A convenient method of converting the usual laboratory media such as broth and peptone water for anaerobic use is by the addition of iron strips. These are cut from thin sheet iron (which is really a mild steel containing less than 0.25 per cent. carbon), 26 gauge and 25×3 mm. in size. (Ordinary iron nails or "tin-tacks", washed before use, are suitable and more easy to obtain.) The medium is heated in boiling water for ten minutes, cooled, and a sterile iron strip or tack (conveniently sterilised by flaming) is added. The medium is inoculated and incubated in the ordinary way, the iron strip ensuring anaerobic conditions. The strips fit conveniently the small $\frac{1}{4}$ -oz. (bijou) bottles commonly used for fermentation tests. Sugar reactions may be noted and tests for indole may be carried out (after 24-48 hours' incubation) with the anaerobes referred to on p. 674 *et seq.* and other organisms such as anaerobic streptococci and *Bacteroides* (p. 671). The results should be read before the heavy deposit of iron hydroxide masks the reaction.

For media in screw-capped bottles, the following method is very simple. Remove the screw cap and replace with a cap which has been perforated and fitted with a rubber washer as for blood-culture bottles (p. 262). (A supply of these caps individually wrapped in kraft paper and sterilised can always be kept available.) Connect a fine hypodermic needle by means of pressure tubing to a vacuum pump. Pass the needle through the perforated cap and washer and commence suction. After the air has been removed, and while *suction is still proceeding*, withdraw the needle from the bottle. The rubber will close and maintain the vacuum. If gas is produced by the anaerobe it will replace the vacuum.

McIntosh and Fildes' Jar

The principle of the apparatus is that spongy palladium or spongy platinum acting as a catalytic agent causes the slow combination of hydrogen and oxygen to form water. The jar itself (8×5 in.) is made of stout glass or of metal, and has a tight-fitting lid that can be clamped down. The lid is furnished with two tubes and taps, so that hydrogen may be introduced into the jar. Suspended from the lid by means of two stout wires, which are connected to terminals, is a small grooved

porcelain spool around which is wound a fine coil of resistance wire, the ends of which are connected to the two wires supporting the spool. Around the spool is wrapped a layer of palladinised asbestos. This is made by immersing asbestos in a solution of palladium chloride and allowing it to dry; on heating in the blowpipe, the palladium is deposited in a black amorphous spongy layer on the asbestos. When an electric current is passed through the resistance wire on the spool the spongy palladium is heated. The spool is surrounded by wire gauze which, on the principle of the Davy lamp, prevents an explosion of the hydrogen and oxygen mixture. The electric terminals must be connected to the mains through an appropriate resistance, such as the rheostat supplied for the purpose by certain manufacturers.

Petri dishes or tubes are placed inside the jar, and also an indicator to show that anaerobiosis is maintained. The Petri dishes are placed in the usual way with medium uppermost and lid downwards. It is advantageous to separate the lid from the rim of the dish about 1 mm. by the insertion between them of a bent pin or fragment of blotting-paper; this prevents sealing of the lid by condensed moisture. The indicator of anaerobiosis consists of a mixture in a cotton-wool plugged test-tube of equal volumes of (a) 0.1 N NaOH 6 ml., water to 100 ml., (b) 3 ml. $\frac{1}{2}$ per cent. watery methylene blue, water to 100 ml., (c) glucose 6 grams, water to 100 ml., and a small crystal of thymol; the mixture is boiled until it becomes colourless and is at once placed in the jar. This indicator, when in the jar, should remain colourless except for a slight tinge of blue at the top, which slowly disappears during the passing of the current.

An alternative indicator solution consists of 1 per cent. glucose in Hartley's broth (pH 8.5-9.0), coloured deeply with methylene blue and preserved by addition of 0.01 per cent. merthiolate. This becomes decolorised after about two to three hours anaerobic incubation at 37° C.; it may be used for several occasions.

It is advantageous to evacuate most of the air from the jar before admitting the hydrogen; this minimises the risk of explosion and prevents trapping of oxygen in Petri dishes and tubes which are too tightly closed. It is of course essential that the culture dishes and tubes should not be sealed hermetically.

The rim of the jar is smeared with vaseline and the lid clamped firmly in place. One tap is closed and the other connected by rubber pressure tubing to a water-pump or mechanical pump. This is used to evacuate at least three-quarters of the air from the jar, preferably reducing the remaining content to less than 60 mm. Hg absolute pressure. The tap is then tightly closed and the other tap connected by rubber tubing to a hydrogen supply (p. 259). Most conveniently, this is a large rubber bladder (*e.g.* a football bladder) which has been filled with hydrogen from a cylinder. Hydrogen is allowed to pass into the jar until no more will enter due to the jar having become filled. The electric current is now switched on so that the palladinised asbestos may be heated. The combination of hydrogen and oxygen takes place

quietly in the jar. Water is formed, and more hydrogen enters to take the place of the hydrogen and oxygen consumed. After about twenty minutes all the oxygen is used up, and the tap is then turned off and the hydrogen supply disconnected. The jar is placed in the incubator, and the indicator tube containing the methylene blue should remain colourless, showing that complete anaerobiosis is established. When a metal jar is used, the indicator should be found colourless when removed from the jar after incubation.

In spite of the wire gauze round the palladinised asbestos, explosions sometimes occur, and it is advisable always to place the anaerobic jar, if made of glass, inside a box while the current is passing through the spool.

Simple Form of McIntosh and Fildes' Jar

The original form of McIntosh and Fildes' anaerobic jar, described in 1916, may be prepared inexpensively and is satisfactory in use. The catalyst for combustion of oxygen is a well-dried "capsule" of palladinised asbestos, which is active at room temperature and does not require heating electrically.

A suitable size of commercial "tin" with a press-in lid (*e.g.* of the kind used to hold confectionery) is rendered gas-tight by soldering its seams. A small brass gas-tight tap is soldered into the centre of the lid and a folded strip of brass, for holding the capsule, to the underside of the lid.

The capsule is made up as follows. One gram of palladium chloride is dissolved in 10 ml. of distilled water containing a few drops of concentrated hydrochloric acid; 1.5 g. of asbestos wool is saturated with this solution and dried. The asbestos is teased out as finely as possible and heated in a smoky flame until black, and then roasted in the outer part of a Bunsen flame till the black material vanishes. The catalytic activity of the preparation may be tested by directing a fine jet of hydrogen on to it, when the asbestos should glow and ignite the gas. Each capsule is made by spreading palladinised asbestos in a thin layer, $1\frac{1}{2}$ inches square, on one-half of a sheet of 30-40 mesh brass or copper gauze, $1\frac{5}{8} \times 3\frac{1}{4}$ in., folding over the other half and turning in the edge of the two layers. The capsules should be stored in a dry place, preferably in a desiccator. Moisture deposited on the surface inhibits catalytic activity; it is removed before each use by heating the capsule in the outer part of a Bunsen flame. Inactive palladium sulphide may be formed from hydrogen sulphide evolved by cultures in the jar. To remove this, the capsules must be opened and the asbestos re-roasted.

Place the cultures and an indicator tube in the jar, insert the capsule in the holder and apply the lid firmly. Seal the lid by pressing plasticine between its edge and the jar rim. Connect the tap to a water-pump and pressure-gauge, and exhaust two-fifths of the air from the jar (*i.e.* to about 450 mm. Hg absolute pressure); this assists removal of oxygen and avoids the danger of explosion, but does not collapse the thin tin. Close the tap, connect to the hydrogen supply and open the tap to let

the jar fill with hydrogen. Shut the tap for a few minutes. On reopening the tap, a further amount of hydrogen is drawn into the jar to replace the hydrogen and oxygen that have combined due to the action of the catalyst; this second intake is evidence that the catalyst is active and the jar gas-tight. Finally, close the tap and transfer the jar to the incubator, where the catalysed combustion will continue, the volume of hydrogen now in the jar being sufficient to utilise all the remaining oxygen.

Hydrogen Supply

A hydrogen supply may be obtained from a Kipp's apparatus by the action of sulphuric acid on zinc. The gas must be purified by passing through three wash-bottles containing: (1) 10 per cent. solution of lead acetate, to remove sulphuretted hydrogen; (2) 10 per cent. solution of silver nitrate, to absorb arseniuretted hydrogen; and (3) a mixture of pyrogallic acid and caustic soda, to remove oxygen.

It is more convenient, however, to obtain hydrogen from a cylinder containing the compressed gas. The commercial hydrogen so obtained is suitable for use in the various anaerobic apparatus employed. The hydrogen cylinder cannot be connected directly to the McIntosh and Fildes' jar, as the pressure is too great. It should be fitted with a reducing valve to deliver hydrogen at a constant pressure (*e.g.* 2-3 lb. per square inch) which can be predetermined or altered at will. The gas is then passed through a small wash-bottle containing water in order that its rate of flow may be observed, and to detect when no further hydrogen is drawn into the anaerobic jar—a state which is reached when all the oxygen in the jar has combined with hydrogen.

A very simple alternative method is to attach an ordinary football bladder to the hydrogen cylinder. The gas is turned on and the bladder inflated. The gas is then turned off, the tube of the bladder closed by a screw clamp, and removed from the cylinder. The inflated bladder is connected by its tube direct to the anaerobic jar.

CULTIVATION IN AN ATMOSPHERE WITH ADDED CARBON DIOXIDE

It has been found that certain organisms will grow only when carbon dioxide is added to the atmosphere surrounding them, *e.g.* *Br. abortus*, and that some grow better in such atmospheres than in ordinary air, *e.g.* pneumococcus, etc. A convenient method¹ is to use tin containers, size 8 × 10 in., with press-on lids, and capacity of about 3½ litres.² (Any similar tin container which will accommodate Petri dishes may be used.) The carbon dioxide is generated in the tin itself from marble and hydrochloric acid. The cultures, either in Petri dishes (enclosed in a simple wire basket) or in tubes, are placed in the tin

¹ Gladstone, G. P., & Fildes, P. (1940), *Brit. J. exp. Path.*, 21, 161.

² Obtainable from A. Gallenkamp & Co., Ltd., London.

together with an open tube, 8×1 in., containing 10 ml. (excess) of 2*N* hydrochloric acid. A marble chip of about 0.7 gram (weight need only be approximate) is dropped into the acid and the lid pressed on. (Note—1 g. CaCO₃ treated with 10 ml. or more, of 2*N* HCl liberates 224 ml. CO₂ at normal temperature and pressure.) The slight increased pressure of the carbon dioxide is of no consequence. If the cultures are carefully removed and fresh ones added immediately there is no need to renew the marble and acid.

In using such closed containers there is a tendency for moisture to collect on the lid of the Petri dish. The same occurs in anaerobic jars (*vide supra*). It is recommended, therefore, before incubating to place in the lid of the dish a square piece of filter- or blotting-paper of such a size that it is just held in position by its four corners, *e.g.* for the usual 4-in. plate, a 3-in. square. The paper should not fill the top of the dish, as it would, when wet, act as a seal and prevent the access of carbon dioxide to the inside of the dish.

For larger proportions of carbon dioxide an anaerobic jar may be used. Air is withdrawn by means of a filter pump and replaced by carbon dioxide from a Kipp's apparatus or cylinder, as described above for hydrogen.

A measured volume of carbon dioxide may be added to a culture in liquid or on solid medium in a bottle which is tightly sealed by a perforated metal screw-cap with an unperforated thick rubber liner. A syringe is filled through its nozzle to the desired volume with carbon dioxide from a cylinder or Kipp's apparatus, a sterile hypodermic needle is applied, this is inserted into the bottle through the rubber liner and the gas is injected.

BLOOD CULTURE

In most bacterial infections of the blood in the human subject the organisms are not numerous, and it is essential for their demonstration by blood culture that a relatively large amount of blood, *e.g.* 5–10 ml., should be used as the inoculum. When such quantity of blood is added to a culture medium, its natural bactericidal or bacteriostatic action may readily interfere with growth and it is therefore essential that this effect should be annulled by diluting the blood with medium. Alternatively, the antibacterial effect may be prevented by some substance incorporated in the medium, *e.g.* trypsin. While it is not strictly necessary that the blood should remain unclotted in the medium, some workers prefer to add sodium citrate, ammonium oxalate or other anticoagulant.

Requisites:—

(1) A 10-ml. "all-glass" syringe (with a firmly fitting needle) sterilised in the hot-air oven as described on p. 178.

If a Record syringe is used it must be sterilised by boiling in water for fifteen minutes; the syringe must not come into contact with any

antiseptic; it should not be removed from the steriliser until it is immediately required, and the parts should be taken out of the steriliser and fitted together with the aid of forceps so that the needle, nozzle and piston are not touched by the fingers. The ordinary Record syringe cannot be sterilised in the hot-air oven as the cement or solder is apt to melt. Extraneous bacterial contamination of blood specimens is more frequent when the syringes are sterilised by boiling (*e.g.* 10 per cent. of specimens contaminated) than when they are sterilised in the hot-air oven (*e.g.* 1 per cent. contaminated). The syringes are liable to be contaminated with non-sporing bacteria while being assembled after their removal from the boiling water (p. 180).

(2) Gauze or cotton-wool, bandage, antiseptic (*e.g.* 2 per cent. iodine in 70 per cent. alcohol or 5 per cent. carbolic acid in water), methylated spirit, collodion, dissecting forceps, Bunsen burner or spirit lamp.

(3) 50 ml. sterile digest broth (*e.g.* Hartley's, p. 192) in a stoppered flask, or preferably the special blood-culture bottle described later. (0.2 per cent. sodium citrate or 1.0 per cent. ammonium oxalate may be incorporated in the medium.)

The blood is drawn by vein puncture. The skin of the patient's arm at the bend of the elbow is *thoroughly sterilised* by first washing with soap and water, then applying spirit and finally treating with the iodine solution. Alternatively, the skin is rubbed with 5 per cent. carbolic acid solution and a gauze swab soaked in the solution is then applied to the skin over the vein and held in position for two or three minutes. Thorough disinfection is necessary to obviate contamination of the culture with skin organisms—*e.g.* staphylococci. Several turns of a bandage are applied round the upper arm about the middle of the biceps to render the veins turgid, or a piece of rubber tubing firmly, but not too tightly, wound once round the arm and clipped with pressure forceps provides a convenient and easily released tourniquet for the purpose. The turgescence of the veins can be increased by the patient's alternately opening and clenching the hand. The needle of the syringe is inserted into a prominent vein and 5–10 ml. of blood are drawn into the syringe. The tourniquet is then released. The needle is now withdrawn from the vein and detached from the syringe by means of forceps so that the nozzle is not touched by the fingers. The flask of broth is unstoppered and the mouth of the flask flamed. The blood is added to the broth and the flask re-stoppered. The blood and broth are thoroughly mixed by rotation of the flask. These operations are all done at the bedside. A spirit lamp may be used for flaming. The flask is incubated at 37° C.

The patient should raise the arm after blood has been withdrawn and firm pressure should be applied to the site of the puncture to obviate haematoma formation. (*Note*—Carbolic acid is irritant on prolonged contact with skin; when it has been used to disinfect the skin, it should be removed by sponging with water as soon as the specimen has been taken.)

The syringe and needle should be washed out at once with 2 per cent. lysol solution. The puncture wound may be dressed with gauze or cotton wool, and collodion.

When the flask has to be transported some distance to the laboratory it is essential to stopper it with a rubber bung which has been sterilised by boiling, and inserted into the flask with flamed forceps, but under these circumstances a blood-culture bottle should be used (*vide infra*).

In suspected cases of *enteric fever*, 0.5 per cent. sodium taurocholate broth may be used. 5 ml. of blood are added to 50 ml. of this medium.

To obtain the best general results a range of media should be used. A medium containing saponin is of special value in isolating *Streptococcus viridans*; broth containing glucose and trypsin serves well for staphylococci. Cooked-meat medium (p. 233) is applicable for cultivating anaerobes and microaerophilic organisms from blood.

"Liquoid", a proprietary name for sodium polyethanol sulphonate, in the proportion of 0.05 per cent., is of value in annulling the bactericidal action of blood.

Saponin broth.—Broth with 0.2 per cent. sodium citrate and 0.1 per cent. white saponin (B.D.H.), the medium being sterilised by intermittent steaming. Blood is added in the proportion of 1 to 5 of the medium.

Glucose trypsin broth.—Broth with 1 per cent. glucose, and 1 part of filtered Liquor trypsini Co. (Allen & Hanbury) to 10 parts of the medium.

In cases treated with sulphonamide compounds there may be enough drug in the blood stream to prevent the growth of bacteria when blood culture is carried out. The addition of *p*-aminobenzoic acid to the broth in the proportion of 5 mg. per 100 ml. will prevent the bacteriostatic action of the sulphonamide. It is quite stable and withstands autoclaving.

Blood-culture Bottle.—Instead of using a flask of broth with a cotton-wool or rubber stopper, as described above, the following container is much more simple and convenient, especially when the patient is some distance from the laboratory.

It consists of a 3-oz. round bottle, with a screw cap, similar to that used for storing nutrient agar, etc., in 50 ml. amounts (p. 185). A hole is punched out of the cap and the rubber washer re-inserted. In order to protect the surface of the cap and the exposed portion of the rubber washer from contamination before use, the cap and neck of the bottle are covered by a "viskap",¹ such as is used for perfume bottles. This is a cellulose preparation which is slipped-on moist and allowed to dry. In so doing the viskap shrinks, moulding itself tightly to the cap and neck of the bottle.

The apparatus is fitted up as follows. The bottles are supplied in a carton already washed, cleaned and capped, so that no further preparation is required. The rubber washer is removed, a $\frac{5}{16}$ -in. hole

¹ Made by the Viscose Development Co., Ltd., Woldham Road, Bromley, Kent.

punched out of the centre of the cap by means of a hollow punch, and the rubber washer re-inserted. The medium in the bottle is a matter of choice, and the following range is useful. The different types are recognised by the colour of the viskap and the glass bead in the bottle:

- (1) plain broth (white cap and bead). To this can be added saponin 0.1 per cent., or "Liquoid" 0.05 per cent., if required;
- (2) broth + 0.1 per cent. glucose (green cap and bead);
- (3) glucose broth as in (2) *plus* 5 mg. per cent. *p*-aminobenzoic acid;
- (4) broth + 0.5 per cent. sodium taurocholate (yellow cap and bead).

The size of the viskap is No. 2 semi-opaque cut 1½ in. The top of the viskap is coloured with cellulose paint. The bead is to identify the medium on its return to the laboratory. If beads are not available a small dab of coloured paint is placed on the shoulder of the bottle. If large numbers of bottles are used, coloured labels are preferable and they obviate the use of coloured beads or painted caps.

50 ml. of the medium are placed in the bottle, the appropriately coloured bead added, and the perforated cap with rubber washer firmly screwed on. The bottle is now sterilised in the autoclave for fifteen minutes at 5 lb. pressure. When the bottle is cool the viskap is at once slipped on. Viskaps dry in a few hours and mould themselves to the cap and neck of the bottle. The broth can be stored without deterioration. For streptococci or the pneumococcus the glucose broth with added *p*-aminobenzoic acid will be found the most useful. The bile-salt broth is intended for cases of suspected enteric fever.

For use, the bottle is taken to the bedside of the patient. Just before the vein is punctured the viskap is removed. Blood (5 ml.) is now withdrawn from the vein, with the usual precautions, and immediately afterwards the needle is passed through the rubber washer and the blood is expelled into the medium. The needle is withdrawn and the puncture in the washer seals itself. The bottle is shaken to mix blood and broth, and sent to the laboratory for incubation. It is advisable to wipe the exposed portion of the washer with a little antiseptic (*e.g.* alcohol), in order to remove any infective material at the site of the puncture. If more blood than 5 ml. is removed, further bottles are inoculated with successive 5 ml. amounts.

When the culture is examined after incubation, the screw-cap is removed in the ordinary way.

It has been shown that, particularly with blood cultures in flasks, incubation in an atmosphere containing 5 per cent. carbon dioxide is of advantage (p. 259).

"Clot" Culture.—When blood samples from suspected enteric fever have been submitted for the Widal test (p. 313) it is useful as a routine to cultivate the clot after the serum has been removed. If blood is taken in the early stages of the disease the Widal reaction may be negative, but blood culture will probably be positive. Moreover, enteric organisms may be present in the blood stream at any time throughout the

illness, and isolation of the causative organism is the most satisfactory form of diagnosis.

If it is known that the blood has been withdrawn with strict aseptic precautions, the clot may be placed in a wide tube (8×1 in.) half filled with broth, or in a wide-mouth screw-capped bottle (8-oz. pot) containing 80 ml. of broth. Where, however, there is any doubt as to the presence of contaminating organisms, and this is always a possibility when specimens of blood are sent to the laboratory from a distance, the clot should be transferred directly to a tube of sterile ox bile. After incubation overnight the bile culture is examined for enteric organisms in the usual manner.

A method of clot culture with streptokinase has been recommended.¹ Take blood from the arm vein in the usual way and allow 5 ml. quantities to clot in sterile screw-capped Universal containers (p. 185). Remove the separated serum and add to each bottle 15 ml. of 0.5 per cent. bile-salt broth containing 100 units per ml. of streptokinase. The streptokinase causes rapid clot lysis with release of bacteria trapped in the clot. The cultures are then incubated.

Examination of Blood Culture.—After incubation for eighteen to twenty-four hours, films are made from the blood broth mixture and stained by Gram's method. If organisms are noted, sub-inoculations are made on a plate of agar or other suitable medium by the successive stroke method (p. 252). The subcultures are incubated and the organisms developing are identified as far as possible by their microscopic characters and colony appearances. If further investigations are required for accurate identification, single colonies are picked off on to slopes and the resulting cultures are studied.

Where an infection with the enteric group is suspected, it is convenient to plate directly from the blood culture on MacConkey's medium to obtain the characteristic colonies on this medium.

Even when no organisms can be detected in films from the primary blood culture, it is advisable to make sub-inoculations as scanty organisms may not be observed, but still develop colonies in subculture.

If no result is obtained after twenty-four hours' incubation, the blood broth should be incubated continuously for at least four days, films and sub-inoculations being made each day. If brucellosis is suspected cultures should be incubated for three weeks before being discarded as negative.

A convenient instrument for drawing aseptically a blood sample from a vein is the so-called *Behring-Venule*.² It also serves as a container for transmitting the sample to the laboratory, and provides medical practitioners with a simple means of carrying out the clinical technique of blood culture. It consists of an evacuated receiving-tube closed by a hollow rubber stopper, and a needle attached to a narrow glass connecting-tube which passes through the stopper and is kept closed by a simple valve arrangement (see diagrams). The needle

¹ Watson, K. C. (1955), *J. Lab. clin. Med.*, **46**, 128.

² These instruments can be obtained from Bayer Products Ltd., London.

is enclosed in a sealed extension of the connecting-tube. Both the needle and interior of the receiving-tube are sterile. The needle is exposed by breaking off the covering tube and is inserted into a vein; the valve is then opened by bending the "knee" formed by the receiving- and the connecting-tubes. Blood is automatically drawn into the receiving-tube and thereafter the valve is allowed to close. In addition to the venule for collecting ordinary blood samples, special venules are obtainable—e.g. containing broth for blood culture, glass beads to defibrinate the blood, citrate solution to obviate coagulation, etc.

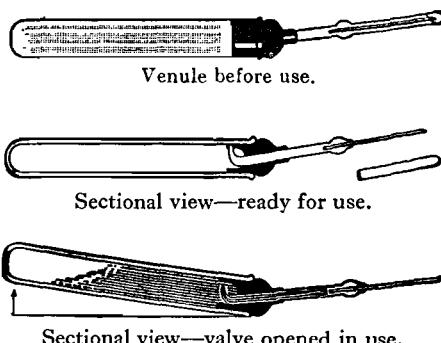


FIG. 15

Reproduced by permission of Bayer Products Ltd., London.

THE AGAR-BLOCK METHOD OF ØRSKOV FOR STUDYING THE MORPHOLOGY OF GROWING BACTERIAL CULTURES

This method has been applied by Ørskov¹ and others for the morphological study of *Actinomyces* and it allows the maintenance of living cultures under continuous observation.

Cubes of suitable size are cut out of an agar plate by means of a sterilised knife. These cubes should not exceed 3–4 mm. in thickness. They are transferred with the knife to a sterilised microscopic slide. The agar is now inoculated with the organism by a fine stroke. With first a low-power objective the stroke is defined and then with a higher power an area is found where the bacteria lie sufficiently scattered. With a suitable lamp and objective and closing down of the diaphragm young bacteria appear as strongly refractile and well-defined bodies.² The area is then registered by means of the vernier scales on the mechanical stage. The slide is removed and placed in a Petri dish

¹ Ørskov, J. (1923), *Investigations into the Morphology of the Ray Fungi*, Copenhagen.

² Phase contrast microscopy is recommended for this (p. 103).

with a piece of moist filter paper in the bottom, and the dish is incubated at a suitable temperature. The selected area is then examined at intervals and the changing features observed. In this way the development of individual bacteria can be studied and also that of colonies at each stage. (A microscope incubator, heated electrically, by means of which a colony can be observed microscopically throughout its period of growth is also very convenient for these and similar studies.)

DISPOSAL OF CULTURES

Cultures to be discarded should be killed by heat or antiseptics before the container is cleaned for re-use.

In the case of non-sporing organisms, it is sufficient to remove the cotton-wool plugs and immerse the tubes and plugs in a large basin of 3 per cent. lysol or cresol.

When screw-capped bottles are used the cap is completely unscrewed and both it and the bottle placed in the lysol solution.

Petri dish cultures are also similarly immersed in lysol solution.

Cultures of the tubercle bacillus and sporing organisms, such as Cl. tetani B. anthracis, etc., must be sterilised by autoclaving.

PERSONAL PRECAUTIONS IN BACTERIOLOGICAL LABORATORY WORK

It is essential to wear an overall while at work. If any material containing pathogenic organisms drops on the bench, floor, clothes, apparatus, etc., it should be sterilised at once with 3 per cent. lysol or 1 in 1000 perchloride of mercury solution. If the hands become contaminated they should be sterilised in a basin of dilute lysol or perchloride of mercury solution, and workers should make it a rule always to wash the hands thoroughly after completing any bacteriological work and particularly when leaving the laboratory.

It must be emphasised that in the laboratory labels must never be licked. There is always a grave risk of infection by this habit. Labels should be moistened either by a drop of water on the finger or by a pignon of wet cotton wool.

It is recommended that the worker refrains from smoking, particularly when dealing with cultures or infective material. No food should be consumed in a bacteriological laboratory.

Precautions to be taken in carrying out special methods are referred to later.

Bacteriological and pathological laboratory workers should consult Leaflet No. 64 issued by the National Association for the Prevention of Tuberculosis. This deals with the risk of tuberculous infection associated with work on material containing tubercle bacilli, not only in the laboratory but also in the animal house and post-mortem room; it summarises the precautions to be taken in the course of such work to avoid accidental infection.

COLD STORAGE

It is essential to have some form of cold storage in the laboratory for the preservation of blood, serum, culture media, cultures, vaccines, etc.

Mechanical refrigeration is now universally used, and refrigerators are available in a large number of sizes from $1\frac{1}{2}$ cubic feet capacity to cold storage rooms of several thousand cubic feet. For the smaller laboratory one of the domestic refrigerators of 4-7 cubic feet capacity is suitable, while larger laboratories require a correspondingly larger instrument, or an insulated cold room with the refrigerating plant outside. Mechanical refrigerators can be obtained to work with electricity, gas or oil, and most of them have provision for making small quantities of ice. The temperature should be maintained between 4° - 5° C. (39° - 41° F.). It should never be so low as to cause freezing, as this may be detrimental to vaccines, bacterial suspensions, red cells and certain sera containing a preservative.

It should be noted that with the domestic type of refrigerator an accumulation of ice, due to freezing of water vapour, surrounds the freezing unit, and at intervals (about 10-14 days) it is necessary to "de-frost" to remove this ice. The contents of the refrigerator are removed, the current (or gas) turned off and the doors opened. The melted water from the ice is caught in a suitable receptacle. When the ice is melted the interior of the refrigerator is wiped with a cloth, the contents replaced, and the refrigerating unit started again.

It is convenient also to have a refrigerator working at low temperatures for the preservation of sera, viruses, etc., and one of the commercial types of "deep freeze" refrigerators working at -10° to -40° C. is suitable for this purpose.

PRESERVATION OF CULTURES

Bacterial species vary greatly in the ability of their cultures to remain alive after the completion of growth (e.g. after twenty-four hours at 37° C.). Some species such as *Neisseria gonorrhoeae* and *Streptobacillus moniliformis* are poorly viable and their cultures usually die out within a few days, whether kept at 37° C., at room temperature or at 4° - 5° C.; thus, they must be subcultivated every two to four days for maintenance in the laboratory. Other species are much harder, especially sporing species which may remain viable for many years. There are also many non-sporing species (e.g. enterobacteriaceae) whose cultures, under suitable conditions, commonly remain viable for several months and often for as much as a few years. Their prolonged preservation requires the following: (1) that drying of the culture is prevented by hermetic sealing of the tube or bottle with a screw cap and new rubber liner, or with a cotton-wool plug soaked in paraffin wax, (2) that the culture is stored in the dark, and either at room temperature or in a refrigerator at 4° - 5° C., but not at 37° C., and (3) that the culture be grown on a

suitable "preservation medium", e.g. on a slope of coagulated egg medium (p. 214), though nutrient agar stab cultures are also generally satisfactory. It is desirable that at least a moderate proportion of the cells in the culture should remain alive; if only a few survive, these may be exclusively resistant mutants, all cells of the original type being lost. Frequent subcultivation also tends to replace the original type by mutants and must therefore be avoided as far as possible.

PRESERVATION OF CULTURES AND SERA BY FREEZE-DRYING *IN VACUO*

When dried and kept in the dry state under suitable conditions, bacterial cultures and virus suspensions may remain viable for several years, and antisera may be preserved without appreciable loss of antibody potency. If such materials are dried from the liquid state, a high salt concentration is produced in the later stages of drying, which causes denaturation of proteins, death of organisms and deterioration of serum. This is largely avoided by the "freeze-drying", or "lyophile" method, in which the culture or serum is dried rapidly *in vacuo* from the frozen state.¹ The material is frozen by a suitable method (*vide infra*) and then dried by sublimation of the ice. The sublimation is effected by exposure to an atmosphere of very low pressure (e.g. 0.01 mm. Hg. or less) which is dried by a chemical desiccant or refrigerated condenser. The dried material is preserved *in vacuo* in hermetically sealed ampoules which are stored in the dark, either at room temperature or, preferably, in a refrigerator at 4°–5° C. This is a convenient means of preserving stock strains of bacteria, guinea-pig complement serum and samples of antisera required for reference or standardisation purposes. On a larger scale, it is used for preserving therapeutic antisera, human plasma, antibiotics and vaccines.

Freezing must be very rapid, with the temperature lowered to well below 0° C. (e.g. to –20° C.), since slow freezing would prolong exposure to the denaturing influence of the suspending salt solution as it is concentrated to its eutectic level by the formation of pure ice crystals. The liquid should be frozen as a shallow layer with a large surface available for evaporation. Two methods of freezing are available: (1) *prefreezing*, i.e. before the drying process is begun, and (2) *evaporative freezing*, effected during the first stage of the drying process.

Prefreezing is generally employed for large volumes. The liquid is frozen as a layer, or "shell", lining the walls of the bottle, either by rotating the bottle while immersed nearly horizontally in a bath of ethyl alcohol and solid carbon dioxide, or of refrigerated coolant, or by spinning the bottle on its vertical axis in a current of refrigerated air.

Evaporative freezing is conveniently employed for the smaller

¹ Harris, R. J. C. (1954), *Biological Applications of Freezing and Drying*. New York, Academic Press.

quantities that generally suffice for laboratory purposes. The liquid is quickly frozen by the withdrawal of latent heat during its initial rapid evaporation when exposed to the vacuum applied for drying. Precautions must be taken against frothing and spilling during desolution of the atmospheric gases; the "centrifugal" and "degassing" methods have been developed for this purpose.

Greaves's Centrifugal Method of Freeze-Drying.—This is a most convenient method for preserving a number of cultures. Frothing is prevented by centrifuging the liquid during the first stage of evacuation and drying, until freezing is complete. A suitable centrifugal freeze-dryer¹ consists of a glass bell-jar and metal chamber containing the centrifuge and trays of desiccant for the primary drying, a manifold for the secondary drying, a rotary oil-sealed pump capable of drawing a vacuum below 0.04 mm. Hg. and a Pirani-type pressure gauge. The liquid cultures or suspensions (see p. 271) are dried in special hard-glass tubes or ampoules with a stem bore of 6 mm. The procedure is as follows.

(1) Place in each ampoule a small strip of filter paper on which has been typed the number designating the culture to be introduced. Plug the ampoules with cotton-wool and sterilise in the hot-air oven. With a sterile capillary pipette, put 0.25–0.5 ml. of liquid culture into each small "ampoule" (tube type) and up to 2.5 ml. into each of the larger ampoules. Discard the original sterility plug and insert a fresh, *loose* plug of sterile cotton-wool, pressing it wholly within the stem of the ampoule.

(2) Prepare the chamber for primary drying by charging the metal desiccant trays with fresh phosphorus pentoxide to the extent of at least 3 grams (about 10 ml.) of powder per ml. of water to be absorbed. (*Note*—Phosphorus pentoxide is corrosive and care must be taken to avoid spilling it on the skin, clothing or freeze-dryer. After each use of the dryer, the expended desiccant must be washed from the trays with an excess of water, taking care to avoid the corrosive fumes evolved, and the trays then thoroughly dried. The makers' instructions must be followed for cleaning the apparatus and removing spilt powder which may interfere with the working of the centrifuge motor.)

(3) *Primary Drying.*—Place the ampoules in the nearly vertical holes of the constant-speed centrifuge. Cover the centrifuge with the bell-jar and press this firmly into position on the sealing ring of the base plate. (The contact surfaces are previously cleaned with ether and lightly smeared with high-vacuum grease.²) While pressing down the jar, switch on first the centrifuge and then the rotary pump. Observe the rapid fall of pressure on the Pirani gauge. When a low pressure of about 0.1 mm. Hg has been achieved in two to five minutes, it can be assumed that the material is frozen as a thin layer on the outer wall of the ampoule. *Immediately switch off the centrifuge to avoid overheating.*

¹ Supplied by Edwards High Vacuum Ltd., Crawley, Sussex.

² Edwards High Vacuum Ltd., supply Apiezon "M" grease for glass joints and "N" grease for glass-metal and glass-rubber joints.

Leave the rotary pump running for a period sufficient to complete the primary drying: about three hours for 0.25 ml. volumes, six hours for 0.5 ml. volumes and eight hours for 2.5 ml. volumes. Then isolate the chamber, switch off the pump and leave the chamber evacuated until ready to remove the ampoules for the secondary drying. The ampoules must not be exposed to sunlight, nor, for more than a few hours, to weak daylight; if necessary, cover the apparatus to shield the ampoules from the light.

(4) Open the chamber air-release valve, lift the bell-jar and remove the ampoules. Draw out the stem of the ampoule to form a thin capillary neck near the open end. First flame the opening and press the loose cotton-wool plug about $2\frac{1}{4}$ in. (5.7 cm.) down the stem. While rotating the ampoule, heat the stem in a small gas flame at about $1\frac{1}{4}$ in. (3.2 cm.) from its open end, until the glass walls have softened and nearly doubled in thickness. Remove the ampoule from the flame and stretch it to form a capillary neck of about 2 mm. external diameter and 1 mm. bore. When cool, apply the open end to one of the rubber adaptors on the manifold used for the secondary drying; the manifold is placed so that the ampoules lie horizontally. Any adaptors not in use must be sealed by application of an empty ampoule.

(5) *Secondary Drying.*—Remove the expended desiccant from the trays and replace with fresh phosphorus pentoxide powder. Press the bell-jar into position, close the air-release valve and switch on the pump. Leave the pump running for a sufficient period to ensure complete drying, e.g. six to eighteen hours. Test the ampoules for vacuum tightness by briefly passing a high-frequency vacuum tester near the surface of the glass; a satisfactory vacuum is indicated by a blue-violet glow, failure of vacuum by long streaky discharges or absence of glow, and cracks in the glass by bright sparking. The Pirani gauge should show that a pressure as low as 0.01 mm. Hg is reached.

(6) Seal the ampoules while they are attached to the manifold with the pump running. Heat the capillary neck with a small gas flame; when the glass is melted, pull gently and allow the flame to sever the thin filament so formed. When all the ampoules are sealed and set aside, open the air-release valve and switch off pump and Pirani gauge. After standing for half an hour, lay the ampoules on a metal surface and retest them for vacuum with the high-frequency tester; discard any not showing the blue-violet glow.

Freeze-Drying by the Degassing Method using a Laboratory Desiccator and High-Vacuum Pump.—By this method, small volumes of culture may be freeze-dried with simple apparatus. From 0.25 ml. to 2.5 ml. of liquid culture is pipetted into a sterile glass tube or ampoule (stem bore 6 mm.) which is then stoppered with a *loose* cotton-wool plug. The ampoule is placed in a glass desiccator jar over phosphorus pentoxide and is supported in a sloping position so that its contents form a thin layer on one side. The rim of the desiccator jar is smeared with vacuum grease and the lid pressed firmly into position. A tap in the desiccator lid is connected by rubber

pressure tubing through a drying column of calcium chloride to a high-vacuum pump (*e.g.* Hyvac pump), all joints being sealed with vacuum grease (footnote, p. 269). The desiccator tap should be a three-way tap which will allow air to be admitted independently to the desiccator or the pump.

Start the pump and exhaust the desiccator only until the liquid is seen to begin to bubble slightly, and then maintain at this pressure for twenty to thirty minutes until the gentle degassing is complete. To do this, close the desiccator tap, admit air to the pump and switch off pump. If bubbling of the culture is marked, admit a very little air into the desiccator jar. When degassing is complete, reconnect the jar to the pump and rapidly exhaust it to high vacuum; the liquid in the ampoules is seen to freeze suddenly within a minute or so. Leave the pump running for about one hour, then close the tap, switch off the pump and leave for eighteen to twenty-four hours in the dark for drying to continue in the evacuated jar. Finally remove the ampoule from the desiccator, press the cotton-wool plug about $2\frac{1}{4}$ in. down the stem, heat the stem in a small gas flame at about $1\frac{1}{4}$ in. from its open end and draw it there to form a capillary neck about 2 mm. diameter (see p. 249). Attach the mouth of the ampoule stem to the rubber tubing leading to the vacuum pump and evacuate the ampoule to high vacuum for about one minute. Seal the ampoule by melting and severing the capillary neck in a small flame.

Rayner's Cellophane Method of Freeze-Drying.¹ Moderate quantities of agglutinating sera and guinea-pig complement serum may be preserved by this method. Disks of waterproof cellophane $2\frac{3}{4}$ in. in diameter are placed over upturned lids of about 2 in. diameter, *e.g.* the lids of 2-oz. waxed cardboard (sputum) cartons. The disks are sterilised individually in Petri dishes in the hot-air oven and placed on the waxed cardboard lids by means of sterile forceps. The serum is pipetted in 1-5 ml. amounts on to the cellophane disks. These are stacked in a desiccator over phosphorus pentoxide, and this is exhausted by means of a Hyvac pump. The serum rapidly freezes solid and dries in a short time, but is left overnight in the desiccator. The dried serum is detached quite easily by crumpling the cellophane, and is then placed in sterile $6 \times \frac{1}{2}$ in. test-tubes. These are heated above the middle and constricted to a capillary neck about 2 mm. in diameter. They are placed in the desiccator, which is again evacuated and left overnight. Finally, the tubes are connected individually to the Hyvac pump, evacuated to high vacuum and sealed by melting and severing the neck with a flame.

Suspending Media for Freeze-Drying of Bacteria

The survival of bacteria on freeze-drying is greatly influenced by the nature of the medium in which they are suspended. Nutrient broth containing 1 per cent. each of peptone and meat extract is a satisfactory

¹ Rayner, A. G. (1943), *J. Path. Bact.*, 55, 373.

medium for the most resistant organisms, e.g. *Strept. pyogenes* and *Staph. aureus*, and the moderately resistant, e.g. enterobacteriaceae and brucellae. Broth cultures of these may be dried directly.

Special protective suspending media are necessary for the poorly resistant organisms such as *Neisseria gonorrhoeae*, *Vibrio cholerae* and *Haemophilus influenzae*. Organisms from a fresh culture are suspended to a high density (e.g. equivalent to Brown's opacity standard No. 4, p. 305) in the sterile suspending medium just prior to freeze-drying. Various media have been advised, most of which contain sugar, peptone and a colloid (e.g. protein or dextran). Skimmed milk, containing lactose and protein, has been used with success. The most generally recommended at present is a serum broth containing 7.5 per cent. glucose; this is prepared as the following mixture:

Nutrient broth with 30 per cent. glucose	1 volume
Sterile inactivated serum (e.g. commercial sterile horse serum)	3 volumes.

CHAPTER XI

PHYSICAL AND CHEMICAL METHODS: I

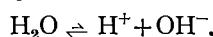
pH BUFFERS, OXIDATION-REDUCTION POTENTIALS, STANDARD SOLUTIONS: THE PREPARATION OF GLASSWARE

pH IN MICROBIOLOGY

MICRO-ORGANISMS, in common with other living organisms, are very susceptible to changes in the acidity or alkalinity of the surrounding medium. This is true with regard to both growth and survival. While many bacteria show vigorous growth within a fairly wide range of acidity or alkalinity, there are others which require the "reaction" of the medium to be adjusted within narrow limits before multiplication takes place. Moreover, all micro-organisms have a particular "reaction" at which growth is optimal. In order, therefore, to secure the best growth, particularly of highly parasitic organisms, it is necessary that the adjustment of the "reaction" should be made as accurately as possible. For this purpose, it is necessary to become familiar with the factors determining this "reaction", with the mode of its expression and with the methods used for its estimation.

The Meaning of the pH Scale

Pure water is very slightly dissociated into an equal number of hydrogen ions and hydroxyl ions.



According to the law of mass action, the following formula will hold at equilibrium:

$$K = \frac{[\text{H}^+] [\text{OH}^-]}{[\text{H}_2\text{O}]}.^1$$

But the amount of water ionised will be extremely small, so that the concentration of unionised water, $[\text{H}_2\text{O}]$, is virtually constant. Therefore, at equilibrium, the product of the concentration of hydrogen ions and hydroxyl ions will be a constant, which is termed the ion product of water K_w , i.e.

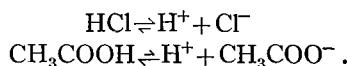
$$K_w = [\text{H}^+] [\text{OH}^-].$$

From conductivity measurements, it has been found that the concentration of hydrogen ions and hydroxyl ions in pure water at 22° C.

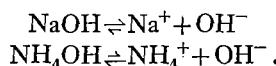
¹ The square brackets refer to the molar concentrations.

is 10^{-7} gram ions per litre. Therefore K_w at 22 C. $^{\circ}$ will be 1.0×10^{-14} . At a given temperature and in dilute aqueous solutions, the product of the molar concentrations of hydrogen ions and hydroxyl ions will always be the same, *no matter what other substances are present*.

Consider what happens when an acid is added to water. The acid will dissociate, liberating hydrogen ions, the amount of which depends on the amount of acid added and on the degree of dissociation of the acid. A strong acid will be largely dissociated in dilute solutions while a weak acid will be largely undissociated, *e.g.*



As a result of the liberation of hydrogen ions caused by dissociation of the acid, the number of hydroxyl ions must be decreased in order to maintain the ion product of water at a constant value. Similarly, when an alkali is dissolved in water, it also undergoes dissociation and ionisation with the liberation of hydroxyl ions, the amount of these being proportional to the amount of alkali and its degree of ionisation, *e.g.*



As a result of the liberation of hydroxyl ions, there must be a corresponding decrease in the number of hydrogen ions to keep the ionic product of water constant. It will be seen, therefore, that in spite of the fact that a solution may be alkaline, its reaction can still be expressed in terms of the hydrogen ions present, the stronger the alkali the smaller the concentration of hydrogen ions. A solution is *neutral* if $[\text{H}^+] = 10^{-7}$, a solution is *acid* if $[\text{H}^+]$ is greater than 10^{-7} and is *alkaline* if (H^+) is less than 10^{-7} . Since $[\text{H}^+]$ can be measured with considerable accuracy, it is convenient to express acidity and alkalinity in terms of $[\text{H}^+]$. For reasons of practical convenience, $[\text{H}^+]$ is usually expressed as a logarithmic or pH scale.

The pH value of a liquid is defined as the logarithm of the reciprocal of the hydrogen-ion concentration, *i.e.*

$$\text{pH} = \log \frac{1}{[\text{H}^+]}.$$

$$\text{For neutral water, pH} = \log \frac{1}{10^{-7}} = 7.$$

Two points should be borne in mind about the pH scale.

(1) Since it is a *logarithmic* scale, a change in one unit of pH is equivalent to a tenfold change in hydrogen-ion concentration, that is a tenfold change in acidity; thus a liquid of pH 5 is ten times more acid than one at pH 6, while a liquid of pH 9 is ten times more alkaline than one of pH 8.

(2) Since it is a *reciprocal* scale, the lower the pH, the greater will

be the acidity. A pH value of less than 7^1 indicates an acid solution, and greater than 7 indicates an alkaline solution.

IMPORTANCE OF pH MEASUREMENTS IN MICROBIOLOGY

Micro-organisms are sensitive in varying degrees to the pH of the external environment. Although this is important for survival, it is even more important for growth where there is an optimum, a maximum and a minimum pH. Media should be adjusted as far as possible to the pH optimal for the growth of the organism concerned. Most pathogenic bacteria have a fairly restricted pH range and grow best around pH 7.5, that is, at a slightly alkaline reaction. This may be a reflection of the fact that the pH of mammalian blood and tissues is of this order. For example, the pneumococcus has an optimum pH of 7.8, and a growth range between pH 7.3-8.3. On the other hand, commensal and saprophytic bacteria have a wider pH growth range. *Escherichia coli* has an optimum pH of 6.5, and a growth range between pH 4.4-7.8. Yeasts and fungi generally have an acid optimum and may grow at a pH of 2.0 or even lower. Not only should growth media be adjusted to the optimum pH, but all suspending fluids should be at a reaction giving the largest survival time (usually of the same order as the optimum pH).

METHODS USED IN pH MEASUREMENT

Two types of methods are generally employed for the measurement of pH in the laboratory. These depend either upon the use of pH indicator dyes or upon the use of electric pH meters.

Methods depending upon the use of pH indicator dyes

Indicator dyes are substances which will change in colour with variations in the pH of the solution in which they are dissolved. For example, phenol sulphone-phthalein (phenol red) is yellow in acid solution and red in alkaline solution. If alkali be gradually added to an acid solution containing phenol red, the change in colour will commence at pH 6.8, the yellow becoming redder until the final red is reached at pH 8.4; thus the "range" of the indicator is pH 6.8-8.4. Within this range, phenol red will have different colours for different pHs and this can be used to determine pH. The range of phenol red is particularly suitable for the adjustment of the pH of bacterial culture media. It must be emphasised that outside the range at which the colour is changing, an indicator can only show whether the solution is

¹ 1 N HCl has an approximate pH value of 0,
0.1 N HCl has an approximate pH value of 1,
0.01 N HCl has an approximate pH value of 2.

more acid or more alkaline than the indicator range. For example, phenol red is yellow at all pHs below 6.8 and is red at all pHs above 8.4. However, other dyes have their own different ranges in which colour change occurs, and there is now available a series of indicators which will cover the range from pH 1 to 11. The following are examples:

<i>Indicator</i>	<i>Range of pH</i>	<i>Colour change</i>
Thymol blue (acid range)	1.2-2.8	red to yellow.
Bromo-phenol blue	2.8-4.6	yellow to violet.
Bromo-cresol green	3.6-5.2	yellow to blue.
Methyl red	4.4-6.2	red to yellow.
Bromo-cresol purple	5.2-6.8	yellow to violet.
Bromo-thymol blue	6.0-7.6	yellow to blue.
Phenol red	6.8-8.4	yellow to purple-pink.
Cresol red	7.2-8.8	yellow to violet-red.
Thymol blue (alkaline range)	8.0-9.6	yellow to blue.
Phenolphthalein	8.3-10.0	colourless to red.
Thymolphthalein	9.3-10.5	colourless to blue.
B.D.H. "Universal"	3.0-11.0	red—orange—yellow —green—blue— reddish violet.

The simplest method of determining the pH of a solution is to use commercially available pH indicator papers. These papers are impregnated with an indicator which gives a change of colour over a specific or general range of pH. The paper can simply be dipped in the solution to be tested or, alternatively, a drop of the solution can be withdrawn by a wire loop or Pasteur pipette and placed on the paper. The resulting colour is compared with the chart supplied with the papers. One example of a good wide-range indicator is the "Universal Indicator" contained in test-papers supplied by Messrs. Johnson & Sons, Hendon, London, N.W.4. It must be emphasised, however, that these test-papers will only give, at the best, an approximate idea of the pH and the results should always be checked by a more accurate method.

The Comparator Method

The most convincing instrument of this type is the Lovibond Comparator.¹

The comparator normally consists of a bakelite case with two holes at the top for tubes of standard bore and of colourless glass. Tube A contains water if the untreated "unknown" solution is colourless, but some of the untreated "unknown" if it is coloured. The hinged door of the case holds a rotatable disk containing a series of standard coloured glasses corresponding to various pH values and each glass can be brought in front of tube A in turn and viewed through aperture A.

¹ Obtainable from British Drug Houses, Ltd.

It is possible to obtain disks for various indicators and the appropriate one can be inserted in the comparator. A solution of the indicator is added to tube B, which contains the unknown solution, and the disk is rotated until a match is obtained. The pH is then read in the aperture at the bottom of the apparatus. If the "unknown" is in the middle of the range of the indicator selected, it is possible to obtain a value accurate to within 0.1–0.2 pH units.

One of the commonest uses for a comparator in a bacteriological laboratory is in the adjustment of the pH of standard culture media.

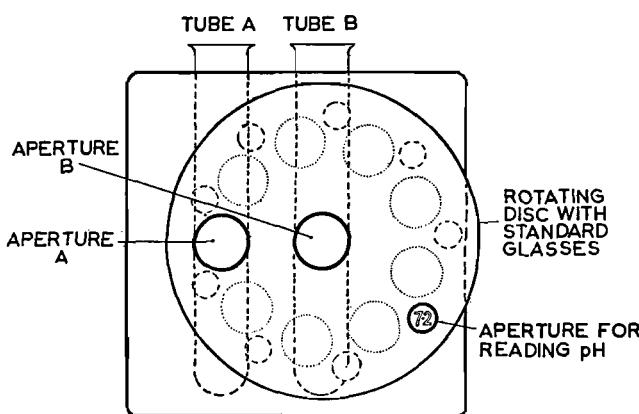


FIG. 16

As an example, the adjustment of nutrient broth to pH 7.5 using phenol red will be considered. For this purpose, the following are required in addition to the Lovibond comparator with a phenol red disk.

- (1) A solution of phenol red, 0.01 per cent.,¹ in distilled water.
- (2) 0.05 N NaOH made up as follows:

500 ml. 0.1 N NaOH
91 ml. 0.01 per cent. phenol red
distilled water to 1 l.

The indicator is incorporated into the standard alkali solution, so that when the medium is titrated, the actual concentration of the dye always remains constant.

¹ First prepare a stock 0.02 per cent. solution as follows. Weigh out 0.1 g. phenol red, add to this 10 ml. (accurately) of 0.1 N NaOH and 20 ml. of distilled water. Dissolve by gentle heat. Transfer the contents to a 500 ml. volumetric flask, washing out all the indicator into the flask. Now add accurately 10 ml. 0.1 N HCl, and fill up to the mark. The 0.01 per cent. solution of phenol red is made by diluting the stock solution with an equal part of distilled water.

(3) A burette, preferably a microburette, measuring to 0.01 ml.

To tube A is added 5 ml. nutrient broth and to tube B 5 ml. nutrient broth + 0.5 ml. of 0.01 per cent. phenol red solution. The 0.05 N NaOH solution is run into tube B until the tint produced is midway between the standard glasses of pH 7.4 and 7.6. The average of the two readings is taken and this gives the amount of 0.05 N NaOH required to bring 5 ml. of broth to the correct pH. From this, one can calculate the amount of 1 N NaOH required to bring the total amount of broth to the correct pH.

When media adjusted in this way by the addition of alkali are sterilised, it is common to obtain a precipitate of phosphates so that the medium has to be filtered again before use. It may be preferable when making media in bulk to have the reaction slightly alkaline and to adjust it for use by the addition of acid. The medium is first adjusted to a pH of about 8.0 with NaOH and steamed for thirty minutes. The precipitated phosphates are filtered off. The medium is then adjusted back to pH 7.5, using acid. The titration is carried out in exactly the same way as described previously except that, instead of NaOH solution, 0.05 N HCl containing phenol red is employed, and the calculated amount of normal hydrochloric acid is added per litre to obtain the desired reaction.

The standardisation of a solid medium such as nutrient agar presents greater difficulty than in the case of fluid media. The medium may be titrated when liquid, but the exact determination is not easy to obtain with any degree of accuracy. It has been found that agar of good quality has very little effect on the reaction of the broth to which it is added, but the reaction of the finished agar should be controlled by titrating the melted medium and then comparing the colour *when cold*. We have found the following method satisfactory. Mix together 0.5 ml. of the melted agar, 4.5 ml. of hot (neutral) distilled water and 0.5 ml. of 0.01 per cent. phenol red solution; cool and compare with the standard tubes. Gelatin may conveniently be adjusted if the medium is liquefied and kept at about 37° C.

The Capillator Method

A knowledge of the pH of bacterial cultures and of the pH changes which they undergo is often of importance and is sometimes of practical value (e.g. in the differentiation of *Streptococcus agalactiae* from *Streptococcus pyogenes*). When only small quantities of culture are available, one of the best methods of pH determination is the use of the capillator. The "B.D.H. Capillator Outfit"¹ is available with indicators and cards to cover the range from pH 1.2-11.0. Alternatively, separate sets can be obtained for each indicator.

The technique is as follows. The pH is first approximately determined by the use of a universal indicator. This can be done in two ways.

(1) A small quantity of the microbial culture is withdrawn with a sterile Pasteur pipette and transferred to a white tile and the appropriate amount of indicator added. From the resulting colour of the mixture,

¹ For full details see catalogue, British Drug Houses, Ltd.

the approximate pH can be obtained by comparison with the standard set of colours supplied with the indicator. The tile is appropriately sterilised after use with 3 per cent. v/v lysol solution.

(2) A universal pH test-paper can be used (see previously). After use the test-paper is destroyed by burning or is placed in disinfectant solution.

The pH is then determined more accurately, using a capilliator and choosing an indicator which acts over the desired range. The capilliator consists of a series of standard-sized capillary tubes filled with buffer solutions and indicator. These tubes show the colours corresponding to different pH values over the whole range of the indicator, and the pH value corresponding to each colour is marked on the card. The capilliator set is used as follows.

(a) A capillary tube, identical in diameter with those in the capilliator, is fitted with a rubber teat and is used for withdrawing a tube full of indicator (supplied with the capilliator set and of double the concentration occurring in the standard tubes). The indicator is then pipetted on to the small watch-glass provided.

(b) After washing, the same "pipette" is used to withdraw an equal volume of the microbial culture or unknown solution which is pipetted on to the same tile. If the culture is to remain uncontaminated, a sterile capilliator pipette must be used.

(c) The two fluids are mixed on the watch-glass by sucking in and out of the pipette, and finally the tube is filled with the mixture.

(d) The prepared tube is compared in colour with the standards and the pH value thus obtained.

Errors due to the colour of the culture or fluid itself can be corrected by using a compensation cell. Care should be taken when working with pathogenic cultures, and the used capillary tubes should be dropped into lysol solution.

The pH Meter

The methods described above, although simple and requiring relatively cheap apparatus, are generally not accurate. They are also very laborious if large numbers of estimations have to be carried out. Further, they all assume that the colour of an indicator is influenced only by the pH of the solution. This is not always so, since the dissociation of an indicator can be influenced by substances such as salts, ethanol and proteins in solution. These errors may be quite appreciable, although in the choice of indicators listed previously those with large errors have been discarded. The only accurate method of measuring pH is by using a pH meter, and in laboratories where numerous routine determinations of pH are required, this apparatus is a necessary piece of laboratory equipment. It is easy and quick to use although care must be taken in its maintenance.

A pH meter consists of an electrode pair which is sensitive to hydrogen-ion concentration and an electrical circuit which measures the e.m.f. developed across the electrode pair. Though it is not practicable to use

the hydrogen electrode for general laboratory use, a modified form known as the "quinhydrone electrode" is sometimes employed. However, almost all modern pH meters employ a glass electrode as it is easier to use and maintain. Consequently, the following discussion will concern the glass electrode pH meter using a calomel electrode as the other electrode. Only a brief description of the instrument and basic directions for its use can be given here. More detailed descriptions of theory can be found in appropriate textbooks and instructions are provided in the makers' pamphlets.

The e.m.f. developed between the glass electrode and the calomel electrode will depend upon the concentration of hydrogen ions and, hence, the pH. In order to measure this e.m.f., no current must flow in the electrode pair or the resultant chemical reactions at the cell boundaries will result in a "polarisation" of the electrodes so that the observed e.m.f. will be due to a combination of phenomena. For this reason, a high impedance circuit is used to detect the potential developed and a vacuum tube is used to drive the measuring meter. This meter can be a microammeter in a vacuum tube circuit as in most line-operated meters, or a null-type bridge circuit may be used as with battery-operated meters.

The following precautions should be observed in the use and maintenance of a pH meter to avoid damaging the instrument and to get an accurate pH value.

- (1) Always exercise extreme care in handling the electrodes, particularly the glass electrodes which usually have a very thin glass bulb. Do not allow this glass bulb to touch the beaker in which the measurements are taking place, or any other hard surface.
- (2) Before a series of pH measurements, ensure that the calomel electrode is filled with a solution of saturated KCl.
- (3) Make sure the instrument has been given sufficient time to warm up as specified by the manufacturers.
- (4) Make frequent standardisations of the meter against a standard buffer solution of known pH as near as possible to the pH to be measured.
- (5) Between measurements, wash the electrodes with a stream of distilled water using a wash bottle.
- (6) When a "drift" in the reading occurs, give the electrodes time to reach equilibrium. Gently stirring the solution often hastens equilibrium.
- (7) Never remove the electrodes from the solution when the measuring circuit is closed.
- (8) When the instrument is not in use, keep the electrodes immersed in water.

BUFFERS AND THEIR USES

Not only is it important to have the suspending fluids for micro-organisms within a certain pH range, it is also important to keep the pH within the same range. Most micro-organisms produce acids or alkalis

as a result of their metabolic activities and these must be prevented from altering the pH of the environment too radically. For example, bacteria when grown on a medium containing a sugar generally produce acid intermediate or end-products (*e.g.* formic, acetic, propionic, butyric, or lactic acids). This is particularly true of fermentation under relatively anaerobic conditions. If these acidic products were allowed to accumulate in an unbuffered medium, the organism would soon be killed by the low pH produced.

It is, therefore, preferable and often essential to include buffers in culture media and in suspending fluids. These buffers tend to resist changes in hydrogen-ion concentration. They are usually formed by mixing a weak acid with its salt, although a weak alkali and its salt can also be used. Buffering action is due to the fact that a weak acid is only weakly dissociated while its salt with an alkali metal is strongly dissociated. Thus, whereas 0.1 *N* acetic acid is only 1.35 per cent. dissociated, 0.1 *N* sodium acetate is 97 per cent. dissociated. If hydrogen ions are added to such a buffer solution, they will react with the high concentration of salt anions to form unionised acids. This weak acid, once formed, does not tend to ionise appreciably and, at the same time, its ionisation is opposed by the high concentration of anions present. Therefore hydrogen ions have been added, but have been removed leaving the pH of the solution only slightly altered.

Generally speaking, the buffering power of a mixture of a weak acid and its salt is greatest when the two are present in equimolar proportions. From such mixtures, buffers can be prepared covering a range of about 1 pH unit on each side of the pH given by an equivalent mixture (the pK of a buffer). Outside this range, the buffering capacity falls off very rapidly. Although the concentration of the buffer determines its ability to resist changes in hydrogen-ion concentration, the actual pH given by a certain mixture is only slightly affected by dilution.

Buffers suitable for use with biological material should have a pK around the optimal for this material and ideally they should also be non-toxic and non-physiological, *i.e.* not react with or affect the living organism or the component of the living organism to be studied. In practice, however, most buffers with useful pKs around 7 are physiologically active, and allowance must be made for this.

The following is a list of suitable buffer systems for use in microbiology. It should also be noted that some components of the complex organic growth media commonly used in microbiology are also buffers. This is particularly true of amino acids and peptides which, as well as providing nutrients, act as important buffers.

PREPARATION OF BUFFERS

Citrate Buffer

(1)

Stock Solutions

A: 0.1 *M* solution of citric acid (19.21 g. in 1000 ml.).

A: 0.1 *M* solution of sodium citrate (29.41 g. $C_6H_5O_7Na_3 \cdot 2H_2O$ in 1000 ml.).

x ml. of *A* + *y* ml. of *B*, diluted to a total of 100 ml.

<i>x</i>	<i>y</i>	pH
46.5	3.5	3.0
43.7	6.3	3.2
40.0	10.0	3.4
37.0	13.0	3.6
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
23.0	27.0	4.8
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	41.5	6.0
7.2	42.8	6.2

(2)

Acetate Buffer

Stock Solutions

A: 0.2 *M* solution of acetic acid (11.55 ml. in 1000 ml.).

B: 0.2 *M* solution of sodium acetate (16.4 g. of $C_2H_3O_2Na$ or 27.2 g. of $C_2H_3O_2Na \cdot 3H_2O$ in 1000 ml.).

x ml. of *A* + *y* ml. of *B*, diluted to a total of 100 ml.

<i>x</i>	<i>y</i>	pH
46.3	3.7	3.6
44.0	6.0	3.8
41.0	9.0	4.0
36.8	13.2	4.2
30.5	19.5	4.4
25.5	24.5	4.6
20.0	30.0	4.8
14.8	35.2	5.0
10.5	39.5	5.2
8.8	41.2	5.4
4.8	45.2	5.6

(3)

Citrate-Phosphate Buffer

Stock Solutions

A: 0.1 *M* solution of citric acid (19.21 g. in 1000 ml.).

B: 0.2 *M* solution of dibasic sodium phosphate (53.65 g. of

$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71.7 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml.).
 x ml. of *A* + y ml. of *B*, diluted to a total of 100 ml.

<i>x</i>	<i>y</i>	pH
44.6	5.4	2.6
42.2	7.8	2.8
39.8	10.2	3.0
37.7	12.3	3.2
35.9	14.1	3.4
33.9	16.1	3.6
32.3	17.7	3.8
30.7	19.3	4.0
29.4	20.6	4.2
27.8	22.2	4.4
26.7	23.3	4.6
25.2	24.8	4.8
24.3	25.7	5.0
23.3	26.7	5.2
22.2	27.8	5.4
21.0	29.0	5.6
19.7	30.3	5.8
17.9	32.1	6.0
16.9	33.1	6.2
15.4	34.6	6.4
13.6	36.4	6.6
9.1	40.9	6.8
6.5	43.6	7.0

(4) Phosphate Buffer

Stock Solutions

A: 0.2 *M* solution of monobasic sodium phosphate (31.2 g. NaH_2PO_4 , 2 H_2O in 1000 ml.).

B: 0.2 *M* solution of dibasic sodium phosphate (53.65 g. of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ or 71.7 g. of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ in 1000 ml.).

x ml. of *A* + y ml. of *B*, diluted to a total of 200 ml.

<i>x</i>	<i>y</i>	pH
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
73.5	26.5	6.4
62.5	37.5	6.6
51.0	49.0	6.8
39.0	61.0	7.0
28.0	72.0	7.2
19.0	81.0	7.4
13.0	87.0	7.6
8.5	91.5	7.8
5.3	94.7	8.0

(5) **Barbitone (Veronal) Buffer***Stock Solutions*

A: 0.2 M solution of sodium barbitone (sodium diethyl barbiturate).

B: 0.2 M HCl.

50 ml. of *A* + *x* ml. of *B*, diluted to a total of 200 ml.

<i>x</i>	pH
1.5	9.2
2.5	9.0
4.0	8.8
6.0	8.6
9.0	8.4
12.7	8.2
17.5	8.0
22.5	7.8
27.5	7.6
32.5	7.4
39.0	7.2
43.0	7.0
45.0	6.8

Solutions more concentrated than 0.05 M may crystallise on standing, especially in the cold.

(6) **Tris (hydroxymethyl) aminomethane (Tris) Buffer***Stock Solutions*

A: 0.2 M solution of tris (hydroxymethyl) aminomethane (24.2 g in 1000 ml.).

B: 0.2 M HCl.

50 ml. of *A* + *x* ml. of *B*, diluted to a total of 200 ml.

<i>x</i>	pH
5.0	9.0
8.1	8.8
12.2	8.6
16.5	8.4
21.9	8.2
26.8	8.0
32.5	7.8
38.4	7.6
41.4	7.4
44.2	7.2

(7) **Boric Acid-Borax Buffer***Stock Solutions*

A: 0.2 M solution of boric acid (12.4 g. in 1000 ml.).

B: 0.05 *M* solution of borax (19.05 g. in 1000 ml.; 0.2 *M* in terms of sodium borate).

50 ml. of *A* + *x* ml. of *B*, diluted to a total of 200 ml.

<i>x</i>	pH
2.0	7.6
3.1	7.8
4.9	8.0
7.3	8.2
11.5	8.4
17.5	8.6
30.0	8.8
59.0	9.0
115.0	9.2

(8) Bicarbonate—CO₂ Buffer

The pH of these buffers is markedly dependent on temperature. The following examples are for a temperature of 37° C.

Concentration of NaHCO ₃	Concentration of CO ₂ in gaseous phase		
	0.02 <i>M</i>	0.05 <i>M</i>	0.075 <i>M</i>
0.02 <i>M</i>	5%	10%	20%
0.05 <i>M</i>	7.4	7.1	6.8

(9) Carbonate-Bicarbonate Buffer

Stock Solutions

A: 0.2 *M* solution of anhydrous sodium carbonate (21.2 g. in 1000 ml.).

B: 0.2 *M* solution of sodium bicarbonate (16.8 g. in 1000 ml.).

x ml. of *A* + *y* ml. of *B*, diluted to a total of 200.

<i>x</i>	<i>y</i>	pH
4.0	46.0	9.2
9.5	40.5	9.4
16.0	34.0	9.6
22.0	28.0	9.8
27.5	22.5	10.0
33.0	17.0	10.2
38.5	11.5	10.4
42.5	7.5	10.6

Note.—These buffers are all made up to a final concentration of 0.1 *M* (with the exception of the bicarbonate-CO₂ buffers). The pH will not change appreciably on dilution. It should be noted, however, that there will be variation in the ionic strengths of the different buffers and of the same buffer at different pHs. If isotonic solutions are required, the concentration should be adjusted accordingly. All the buffers are given as a mixture of the sodium salts with the acid. Potassium salts may also be used.

OXIDATION-REDUCTION (REDOX) POTENTIALS

It has been stated previously that the oxidation-reduction conditions in a medium are very important in the growth of certain bacteria. Strict aerobes are able to grow only in presence of dissolved oxygen while strict anaerobes require reducing conditions and hence absence of dissolved oxygen. This may be related to the metabolic character of the organism, a strict aerobe obtaining its energy and intermediates only through oxidation involving oxygen as the ultimate hydrogen acceptor, a strict anaerobe utilising hydrogen acceptors other than oxygen while a facultative anaerobe can act in both ways. However, strict anaerobes may be actually poisoned by the presence of oxygen, possibly due to the production of toxic hydrogen peroxide which cannot be removed by catalase, or possibly due to the oxidation of certain essential groupings in the organism, e.g. the sulphhydryl groups of proteins.

We may consider oxidising agents as substances capable of taking up electrons and reducing agents as substances able to part with electrons. It is therefore possible to determine the intensity level of oxidising or reducing conditions in a system by the net readiness of all the components in that system to take up, or part with, electrons. This ability is usually expressed as the oxidation-reduction (redox) potential of the system.

Redox potentials can be best measured by virtue of the fact that when an "unattackable" electrode is immersed in a solution, an electrical potential difference is set up between the electrode and the solution, and the magnitude of this potential depends on the state of oxidation or reduction of the solution. This electrode potential (or, more shortly E_h) can be measured in millivolts, and the more oxidised a system, the higher (or more positive) is the potential; in more reduced systems the potential is lower (or more negative). By measuring the electrode potential it is possible to determine and follow the reducing conditions in cultures at different periods and to grade different systems in order according to their state of oxidation or reduction. This measurement can usually be carried out by coupling up a potentiometer with an electrode pair of a platinum electrode (the "unattackable" electrode) and a standard calomel electrode. The redox potential can then be measured by the millivolt scale provided on most commercial pH meters. It should be borne in mind that the redox potential of a system indicates the oxidation-reduction *intensity* of the system itself, and not its *capacity* to oxidise or reduce some other component or system. Further, it must be emphasised that for a micro-organism, not only is the redox potential of the system important, but the factors contributing to this redox potential may be equally critical. Thus a substance capable of giving up or taking in electrons may not necessarily affect a micro-organism unless it can spatially reach certain essential components of the cell. Further, a substance, like oxygen, which can actually be

metabolised by the catalytic action of enzymes in the cell, may be important through this metabolism as well as through its direct contribution to the redox potential.

Although the redox potential of a bacterial culture may be measured accurately by electrical methods, an approximate idea of the state of reduction may sometimes be obtained by adding various special dyes (oxidation-reduction indicators) and observing by the colour changes how much they are reduced. Such changes are in intensity of colour, not changes from one colour to another, as is the case with the indicators used for the measurement of pH. It is found that the state of oxidation or reduction of any particular dye depends on the electrode potential, so that at any given pH value, if we know the electrode potential of the solution, we can calculate the degree of reduction of the dye. Conversely, and this is more important practically, if the percentage reduction of the dye has been observed colorimetrically the corresponding electrode potential can be determined. Different dyes are reduced over different range of potential; for instance, methylene blue at pH 7 is 95 per cent. in the oxidised condition at $E_h + 50$ mv., and 99 per cent. reduced at $E_h - 50$ mv., whilst neutral red is still 87 per cent. oxidised at -300 mv., and 87 per cent. reduced at -350 mv. Theoretically it should be possible by suitable choice of indicators to measure any range of E_h , but in practice experimental difficulties arise due to poisoning,¹ catalytic effects and the toxicity of the dyes used towards bacteria, etc. Colorimetric E_h determinations do not reach the degree of accuracy and convenience attained in the case of pH indicators.

A few examples will suffice to illustrate the results obtained when the electrode potentials of growing bacterial cultures are measured. In a culture of *C. diphtheriae* the initial E_h of the medium, about +300 mv. falls gradually and reaches -200 mv. after some forty-eight hours' incubation, and the potential remains at this low level for some considerable time. With haemolytic streptococci, on the other hand, the potential falls from +300 mv. to -150 mv. in twelve hours, but then rises fairly rapidly, probably owing to the formation of hydrogen peroxide. In a dextrose broth culture of *Esch. coli*, in which gas formation occurred, the potential falls extremely rapidly, reaching -370 mv. after about one hour's incubation. The behaviour of staphylococci is roughly similar to that of *C. diphtheriae*, whilst pneumococci behave similarly to haemolytic streptococci. Strict anaerobes are unable to proliferate in ordinary aerobic culture media unless the E_h is lowered to some extent. This lowering of the E_h , or establishment of reducing conditions, may be effected in a variety of ways, such as removal of oxygen in an anaerobic jar or by means of a pyrogallol seal, or reduction may be effected by adding a reducing agent, e.g. thioglycollate.

Oxidation-reduction potentials and oxidation-reduction indicators are employed in the testing of sewage and sewage effluents, in cheese-making, in the keeping qualities of beer, etc. The metabolic activities

¹ Corresponds to the buffering effect in pH estimation.

of bacteria and other cells and tissues and the functioning of enzymes are followed by observing the reduction of methylene blue in Thunberg tubes. A commonly used application of this technique is in the grading of milk and testing the hygienic quality of milk samples. The milk samples are incubated under standard conditions with methylene blue, and the time of reduction is noted. Heavily contaminated milks show a rapid decolorisation, whilst with good quality milk there is a long lag period and reduction is slow (*vide p. 374*).

For full details of this important subject consult *Oxidation-Reduction Potentials in Bacteriology and Biochemistry*, by L. F. Hewitt, 6th edition, 1950 (E. & S. Livingstone Ltd., Edinburgh).

WATER

Tap water contains many impurities and is unsuitable for the preparation of defined culture media, for chemical solutions and for many other uses in the laboratory. These impurities can be largely removed by distillation or demineralisation.

Distilled Water

Normally distilled water is prepared in a commercial metal-lined still which will deliver it at the rate of $\frac{1}{2}$ -50 gallons per hour, depending on size. However, for some purposes this water is insufficiently pure, and it may be necessary to use an all-glass distillation apparatus which should be fitted with an efficient spray trap. It is often advisable to add a knife point of potassium permanganate and a few pellets of sodium hydroxide to the tap water before commencing distillation in order to oxidise steam volatile organic compounds which might otherwise be carried over into the distillate. For some experimental methods such as tissue culture it may be necessary to repeat the distillation in a glass still to give doubly glass distilled water.

It is useful to check the purity of distilled water at times by simple conductivity testers. Satisfactory distilled water should have a conductivity no greater than that given by 1.5 p.p.m. of NaCl, and preferably below 1.0 p.p.m.

Demineralised Water

Ion-exchange resins may be used to demineralise water. A simple apparatus consists of an anion and a cation exchanger in two columns of glass tubing about 2 m. tall and 3 cm. in diameter. A variety of resins are available for the purpose, e.g. Amberlite IR 120 (H) as the cation-exchange resin followed by Amberlite IRA 400 (OH) as the anion-exchange resin.¹ Tap water or distilled water is passed over each of the resins in turn. The columns must be periodically regenerated by rinsing with 10 per cent. aqueous HCl for the cation-exchanger and 10 per cent. aqueous NaOH for the anion-exchanger. After regeneration,

¹ Obtainable from British Drug Houses, Ltd.

the columns are rinsed with distilled water until the final product has a neutral reaction. Commercial demineralisers are available,¹ which have the advantage of being transportable and of requiring no external source of heat or electricity. Demineralised water should be equivalent to doubly glass-distilled water and should have a very low conductivity. However, it often carries dissolved organic compounds derived from the resins.

FLUIDS FOR CELL SUSPENSION AND DILUTION

A variety of fluids are used for the suspension of micro-organisms, blood cells or tissue culture cells. These fluids should preserve, as far as possible, the cells in their original condition. The following points should be noted.

(1) They should have an osmotic pressure nearly isotonic with the cell to be suspended. This is particularly true of mammalian cells (*e.g.* red blood corpuscles) where lysis readily occurs in non-isotonic media. Micro-organisms are generally more resistant to changes in the external osmotic pressure, but suspension in water or very dilute salt solutions may cause loss of viability.

(2) Suspension fluids should preferably contain a buffer to keep the cells at their optimum pH.

(3) Certain ions may be necessary for the optimal maintenance of cells, particularly with mammalian cells. Moreover, they may be required for certain *in vitro* reactions, *e.g.* agglutination, complement fixation, etc. In some cases a source of energy such as glucose may be required.

(4) Other additions may be made for specific purposes.

The following suspension and diluent fluids are commonly used. In all cases analytical grade reagents (when available) should be made up in distilled or demineralised water.

Physiological Saline

A solution of 0.85 per cent. NaCl in water. This solution is sometimes called normal saline, a term which should be discarded because of its chemical connotation. It is also often referred to as "saline". The solution has an osmotic pressure roughly equivalent to that of mammalian blood serum and can therefore be used for the suspension of blood cells as well as most micro-organisms. However, the solution has no buffer present and it is recommended that phosphate-buffered saline be used as a general suspension fluid in the laboratory.

Buffered Salines

As stated previously, it is preferable to have a buffer present in a suspending fluid or diluent and variety of solutions containing basically

¹ *E.g.* the "Portable Deminrolits" produced by the Permutit Co. Ltd., Gunnersbury Avenue, London, W.4.

NaCl but with a buffer added have been proposed. They should all have a final osmotic pressure roughly equivalent to that of physiological salines. A series of solutions can be prepared by diluting standard buffer solutions of the required pH (see pp. 281-5) with physiological saline to a strength of 0.01 M. If a greater buffering power is required, the concentration of buffer must be increased and of saline decreased.

The following types of buffered saline are recommended for various purposes:

(a) *Phosphate buffered saline:*

NaCl	8.00 g./l.
K ₂ HPO ₄	1.21 g./l.
KH ₂ PO ₄	0.34 g./l.

This solution gives a pH of about 7.3 and also provides potassium and phosphate ions. It is a very useful general diluent and suspending fluid.

(b) *Azide saline.*—Sodium azide at a concentration of 0.08 per cent. is added to physiological saline or buffered saline. The azide acts as a preservative preventing microbial decomposition and is often used for the dilution of serum, etc.

(c) *Borate-calcium saline:*

NaCl	8.0 g./l.
CaCl ₂	1.0 g./l.
H ₃ BO ₃	1.2 g./l.
Na ₂ B ₄ O ₇ . 10H ₂ O	0.052 g./l.

This solution gives a pH of about 7.3 and is used for haemagglutination experiments where calcium is required and phosphate should be absent.

(d) *Veronal-NaCl diluent:*

NaCl	8.5 g./l.
Barbitone (diethyl-barbituric acid)	0.575 g./l.
Sodium barbitone	0.20 g./l.
MgCl ₂ . 6H ₂ O	0.168 g./l.
CaCl ₂	0.028 g./l.

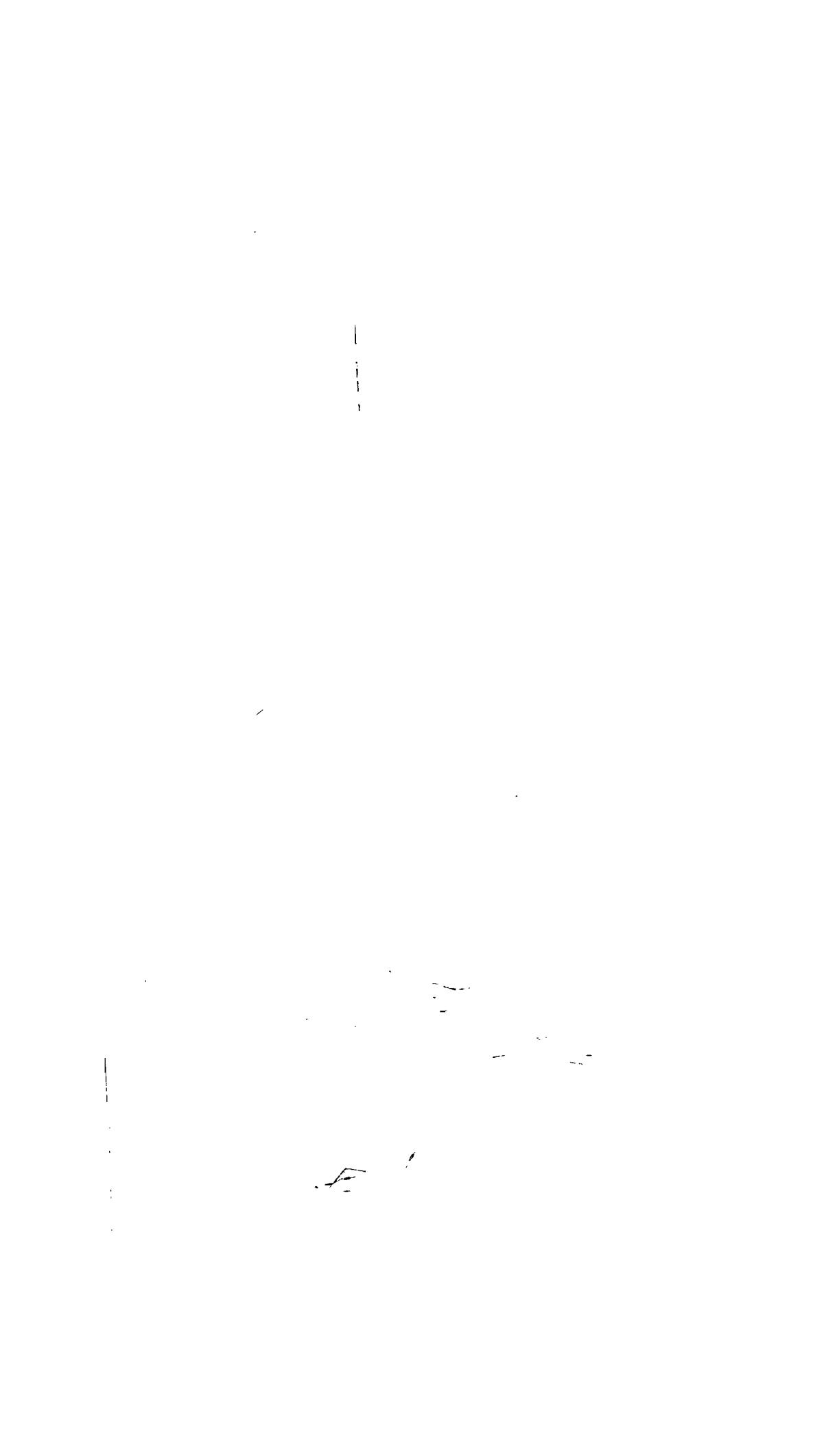
A stock solution concentrated $\times 5$ is made up by dissolving 5.75 g. barbitone in 500 ml. hot distilled water. Add 85 g. NaCl and make up to volume to about 1400 ml. Dissolve 2.0 g. sodium barbitone in 500 ml. distilled water and add it to the NaCl-barbitone solution. Make up to 2000 ml. Add 1.68 g. MgCl₂. 6H₂O and 0.28 g. CaCl₂. For use dilute 1 in 5 with distilled water.

This saline may be used for complement-fixation tests and gives more reproducible results than physiological saline. If glass tubes are used to contain the reaction mixtures, there may be some absorption of complement on to the glass surfaces. To reduce this absorption, add

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0.1 per cent. inactivated rabbit serum, 0.1 per cent. gelatin or 0.1 per cent. bovine serum albumin.

Complex Suspending Media

More complex media are required for the suspension and dilution of micro-organisms and other cells where optimum viability must be maintained. For example, in viable counts of many bacteria, physiological saline may be to some extent bactericidal and must be replaced by solutions containing other ions as well as a buffer. For these fluids, prepare the following solutions which are all isotonic with mammalian serum and can be mixed in any proportions. The mixtures, although of different composition, will remain isotonic.

	g./l.	Ringer	Locke	Krebs-Ringer		
				Plain	Bicarbonate ^a	Phosphate
NaCl	9.0	100	100	100	100	100
KCl	11.5	4	4	4	4	4
CaCl ₂	12.2	3	3	3	3	3
KH ₂ PO ₄	21.1	—	—	1	1	—
MgSO ₄ .7H ₂ O	38.2	—	—	1	1	1
NaHCO ₃	13.0	—	3	—	21	—
0.1 M phosphate buffer ¹		—	—	—	—	20
pH 7.4		—	—	—	—	—

To simplify preparation and handling, the first five solutions can be made up in concentrations five times those listed. They are stable for months when stored in the cold.

The Krebs-Ringer solutions seem to be the most generally useful for the suspension of mammalian cells and are also valuable for many bacteria. It is also possible to use Davis's minimal medium for bacterial suspension (see p. 199). If growth is to be avoided, leave out the nitrogen source (ammonium sulphate) or the carbon and energy sources (glucose and citric acid).

Suspending media for tissue culture work are described on p. 449.

PREPARATION AND CLEANSING OF GLASSWARE

New Glassware

New glassware requires special attention because of the resistant spores which may be present in the straw and other packing material and also because it tends to give off free alkali which may be sufficient to interfere with the growth of certain organisms. Consequently it

¹ 17.8 g. Na₂HPO₄.2H₂O + 20 ml. 1 N HCl diluted to 1 l.

² The solution should be gassed with 5 per cent. v/v. CO₂ in O₂, air or N₂.

should be placed in 1 per cent. HCl overnight, washed in tap water and distilled water and autoclaved.

Screw-capped bottles (described later) are subjected to a special cleansing process by the makers whereby surface alkali is removed, and the above treatment is unnecessary. The bottles may be used without further treatment, as received from the manufacturers.

Cleansing of Glassware for General Laboratory Use

Glass containers with discarded cultures can be placed in 3 per cent. lysol after use or transferred directly to boiling soap solutions. Containers with tubercle bacilli or spore-bearing organisms such as *B. anthracis*, *B. subtilis* or *Cl. tetani* must be autoclaved. The discarded cultures and their containers are then boiled for one hour in a 5 per cent. solution of a good quality soft soap in either tap water (if it is sufficiently soft) or distilled or demineralised water (if the tap water is hard). The glassware is cleansed with a test-tube brush (or other suitable brush) and well rinsed in hot and cold water. Again, if the tap water is hard and contains a considerable amount of calcium salts, rinsing in distilled or demineralised water is necessary. The glassware is then allowed to drain and is dried in a hot-air oven or cabinet.

Washing of Tissue Culture Tubes

Since tissue cells are particularly sensitive to minute traces of toxic substances, meticulous care is essential in cleaning glassware for tissue cultures and it is preferable to use hard glass (e.g. Pyrex) tubes, flasks and containers. The following cleaning method has been found satisfactory.

- (1) Autoclave with rubber bungs *in situ* after use.
- (2) Remove bungs and rinse tubes in hot rinsing tap water.
- (3) Boil for twenty minutes in demineralised water in a boiler with soapflakes (one handful to about five gallons). Small tubes are boiled in an enamel basin on a gas-ring. (Rinse water from the demineraliser may be used for this purpose.)
- (4) Brush the tubes as removed from boiler (preferably with a motor-driven nylon brush). Do this *while the tubes are hot* or serum remains.
- (5) Rinse in hot running tap water or in demineralised water if the local water is hard.
- (6) Transfer tubes into hot demineralised water containing an inorganic detergent. Thoroughly wash in this by emptying and filling.

The following solution can be used:

Sodium hexametaphosphate	40 g.
Sodium metasilicate (technical)	360 g.
Demineralised water	1 gallon

Dissolve and allow to stand overnight. Dilute 1 in 100 before use.

- (7) Rinse in hot running tap water at least four times.

Rinse three times in demineralised water.

- (9) Drain and dry in drying cabinet.
- (10) Dry-sterilise at 160° C. for three hours in racks with the tubes either metal-capped or covered with aluminium foil.

Rubber bungs should be treated as follows after autoclaving:

- (1) Rinse in hot tap water.
- (2) Boil for twenty minutes in 20 per cent. NaHCO_3 .
- (3) Rinse in hot tap water.
- (4) Boil for twenty minutes in 20 per cent. HCl.
- (5) Rinse in hot tap water.
- (6) Pack in layers separated by lint in tins or glass containers and autoclave.

Cleaning of Glassware for Biochemical Work

- (1) Remove any grams with petroleum. Wash with warm tap water.
- (2) Place in dichromate-sulphuric acid cleaning solution for twelve to twenty-four hours.
- (3) Remove, washing by rinsing in hot tap water at least four times and in distilled water twice.
- (4) Dry in oven if the glassware is not used for accurate volumetric purposes.

Dichromate-Sulphuric Acid Cleaning Solution

Dissolve 63 grams of sodium (or potassium) dichromate by heating with 35 ml. water. Cool and add concentrated H_2SO_4 to 1 litre. Technical grade reagents may be used.

This fluid should be handled with care. Preferably rubber gloves and an apron should be worn. If clothes or skin are splashed with the fluid, they should be immediately washed in water, and any residual acid neutralised with sodium carbonate solution. This, in time, is washed off with water.

Cleaning of Pipettes

- (1) If contaminated with infective material, discard the used pipette into a 3 per cent. v/v lysol solution and leave until convenient to wash. (The lysol solution is best contained in a rubber cylinder about 15 in. high and 4 in. in diameter. The points of the pipettes are not liable to be broken when dropped to the rubber bottom of the cylinder.)
- (2) Rinse in tap water.
- (3) If necessary, steep overnight in dichromate-sulphuric acid cleaning fluid.
- (4) Wash with tap water in an automatic pipette washer.
- (5) Connect the pipette to a water pump by rubber tubing and draw through distilled or demineralised water followed by acetone. Finally, suck through air until the internal surface is quite dry.
- (6) If required, the top end of the pipette is plugged with cotton-wool; this is pressed entirely within the end of the pipette so that there

are no protruding strands of cotton to prevent close fitting of a rubber teat or mouth-piece tube which may be later attached to operate the pipette.

(7) To sterilise the pipettes, pack them in copper cylinders with slip-on lids or in lengths of wide-bore glass tubing stoppered with cotton-wool. Place in a hot air oven at 160° C. for sixty minutes.

Note.—Accurately calibrated volumetric glassware should never be heated in an oven, since the expansion and contraction of the glass makes the graduations inaccurate. Such glassware should be kept separate from that intended for sterilisation.

CHAPTER XII

PHYSICAL AND CHEMICAL METHODS: II

CENTRIFUGES, PHOTOELECTRIC COLORIMETERS AND METHODS OF COUNTING BACTERIA, MEASURING BACTERIAL GROWTH AND PREPARING CELL-FREE EXTRACTS

CENTRIFUGES

THE best method for the separation of a micro-organism from its suspending fluid is that of centrifugation. This is carried out in the centrifuge, an apparatus for the separation of two substances of different density by centrifugal force.

The rate of settling r (cm./sec.) of spherical particles of density d_p and of radius a (cm.) in a medium of viscosity η (c.g.s. units) and of density d_m is given by Stokes' law:

$$r = \frac{2a^2G(d_p - d_m)}{9\eta},$$

where G is the acceleration due to gravity (981 cm./sec.²).

From this equation, it is evident that the rate of settling of a particle will be increased by the following factors:

(1) An increase in the size of the particle. Thus, larger micro-organisms like yeast and fungi will sediment faster than bacteria which, in turn, will sediment faster than viruses. Note that the size of the particles is squared in the equation and thus an increase of the radius of the particles by a factor of 2 will increase the rate of settling by a factor of 4.

(2) An increase in the difference between the density of the particles d_p and that of the medium d_m . Thus a capsulate bacterium will have a lower average density and be more difficult to sediment than its non-capsulate variant.

(3) A decrease in the viscosity of the medium. For example, when defibrinated blood is being washed (*vide p. 339*), the first sedimentation of the corpuscles from the viscous serum takes much longer than when the corpuscles are suspended in saline.

(4) An increase in the force due to gravity. This force is increased artificially in the centrifuge. The degree by which this force is increased is measured by the relative centrifugal force (R.C.F.) which can be obtained by the following formula:

$$\text{R.C.F. (in } G\text{)} = 1.118 \times 10^{-5} \times R \times N^2,$$

where R = the radius of the centrifuge in cm., being the distance from

the centre of the centrifuge shaft to the tip of the centrifuge tube; N = revolutions per minute (r.p.m.).

From this equation, it is evident that the speed of the centrifuge, being squared, is very important in determining the rate of sedimentation. Although an increase in the radius of the machine will increase the rate of sedimentation, it is more efficient and simpler practically to increase the speed. However, it is most important to express the efficiency of a centrifuge according to the maximum R.C.F. rather than the speed itself, which, without specification of the radius of the centrifuge, is meaningless. The calculation is simple. Thus, a centrifuge with a radius of 20 cm. and a speed of 4000 r.p.m. has an R.C.F. of $1.118 \times 20 \times (4000)^2 \times 10^{-5} = 1788$ G., say 1800 times the force of gravity. Consequently, particles will sediment in this centrifuge at a rate 1800 times faster than in a tube on the bench.

Types of Centrifuges

A variety of centrifuges is now available and the choice of a suitable model depends upon the following factors.

(1) The size of the particles to be sedimented. As shown previously, the smaller the particle, the greater will be the R.C.F. and time required for centrifugation. Machines can be obtained commercially with speeds up to about 60,000 r.p.m. and R.C.F.s of up to about 200,000 G. Generally speaking, yeasts and fungi require a centrifuge with a maximum R.C.F. at least 1000–2000 G., bacteria about 2000–4000 G., and viruses about 50,000–150,000 G. At higher speeds (R.C.F. above about 4000 G.), glass tubes are apt to break even if surrounded by a rubber sleeve or a layer of water. Stainless-steel tubes may be supplied for the most exacting strength requirements, that is for the very highest speeds and centrifugal forces and for maximum resistance to corrosion. However, for most purposes, plastic tubes of cellulose acetate, nylon or polythene can be used. In order to prevent deformation of these tubes under high centrifugal forces, caps should always be used and the tubes should be fully filled. The main disadvantage of plastic tubes in microbiological work is that they cannot be sterilised in a hot-air oven like glass tubes. Even boiling tends to cause deformation and it is recommended that the insides of the tubes and caps be exposed to ultra-violet light for sterilisation.

(2) The volume of material. Centrifuges can be obtained with capacities of up to at least 15 l. The fluid to be centrifuged is contained in tubes or buckets, the number and size of which is subject to a wide variation. For very large amounts of material, continuous-flow machines are available. The fluid to be centrifuged is normally continuously passed along the inside of a rotating tube. The particles sediment very quickly in the thin layer of liquid passing along the sides of the tube and the supernatant passes out of the machine to be collected. Continuous flow centrifuges (*e.g.* Sharples) of this type are common in industry, but are not often used in the laboratory.

(3) The ease with which the particles form a hard pellet at the bottom

of the tube. Many centrifuges are of the angle type in which the tubes, instead of being allowed to swing out and rotate in a horizontal plane, are fixed at an angle (from 20°–45°) on the rotating head. The advantage of the angular position is that particulate matter is rapidly separated and concentrated, with consequent saving of time. This is because the particles have to traverse only a short distance before deposition on the sides of the tube, after which they slide to the bottom. The tubes are encased in a metal head, which in its rotation offers slight resistance to air and so obtains greater speed and is less liable to warm up due to friction. A disadvantage is that a "line" of deposited material may remain adherent on the peripheral wall of the tube, and when the suspending fluid is removed it is difficult to avoid contamination caused by turbulence.

(4) The temperature required for centrifugation. In most biological systems it is advantageous and often essential to centrifuge at low temperatures. This prevents metabolism, loss of viability or enzyme activity during centrifugation. Consequently, refrigeration units are built into many of the larger centrifuges. This is particularly important in high-speed centrifuges where the temperature may rise due to friction unless refrigeration is used. In many machines it is possible to obtain temperatures down to about –15° C.

For the routine bacteriological laboratory, a small bench centrifuge taking 10–20 tubes of capacity 10–30 ml. at a maximum R.C.F. of about 3000 G. is essential. In the M.S.E. range of instruments,¹ a bench "Minor" centrifuge will take 6×15 ml. tubes on an angle or swing-out head at speeds of up to 4600 r.p.m. (R.C.F.=2700 G.). Alternatively, the angle Model 50 centrifuge will take 12×15 ml. tubes at speeds up to 4600 r.p.m. (R.C.F.=2650 G.). It is, however, convenient to have a centrifuge which will hold the standard 5-in. test-tube and stopper used in routine bacteriological culture, thus avoiding the need for transference to a proper centrifuge tube. For more general and research purposes, larger machines are available, with or without refrigeration. For example, the M.S.E. "Major" or "Major Refrigerator" are floor models which will take up to 4 litres total capacity in a variety of angle or swingout heads which will hold tubes ranging from 15 to 1000 ml. Speeds up to 6000 r.p.m. (R.C.F.=6200 G.) are obtainable and a Super-speed unit can be fitted which will give a R.C.F. of up to 20,000 G. for small quantities. The highest speed centrifuge in the M.S.E. range is the "Super-speed 25" centrifuge with a capacity of up to 400 ml. and speeds up to 25,000 r.p.m. (R.C.F.=75,000 G.). This machine can be used to centrifuge rickettsiae and the larger viruses. For the smaller viruses (*e.g.* the poliomyelitis virus) an ultracentrifuge must be used, such as the Spinco Model Preparative Ultracentrifuge.² This machine is provided with a variety

¹ For further details, apply to Measuring and Scientific Equipment, Spenser Street, London, S.W.1.

² For further details, apply to the Spinco division of Beckman Instruments, Inc., Belmont, California, U.S.A.

of heads with capacities up to 940 ml. and speeds up to 40,000 r.p.m. (R.C.F.=144,000 G.). For these high-speed machines (above about 20,000 r.p.m.), the centrifuging compartment must be held *in vacuo* in order to reduce friction, and vacuum pumps are included.

Method of using the Centrifuge

(1) Tubes must be put in the centrifuge in pairs which have been accurately balanced. The members of a pair of tubes must be placed diametrically opposite each other. If there is an odd number of tubes, a balance tube containing water must be prepared. If the buckets are removable from the centrifuge, they should be balanced with the tubes. With a pipette or plastic washing bottle add a little water to the lighter bucket, not the tube, until the two sides are balanced.

(2) Before putting tubes into the buckets, make sure that the rubber cushions or sleeves are in position at the bottom of the buckets. Otherwise breakages are liable to occur.

(3) Precautions must be taken to ensure that the cotton-wool plugs of culture tubes are not forced down into the tube during centrifugation. In a swing-out head, fold the upper portion of the plug over the mouth of the tube and secure it with a rubber band. With an angle centrifuge, it is sufficient to splay out the top of the plug. However, even when the cotton-wool plug is secured in this way, cotton fibres become detached and can be seen microscopically in the centrifugate. In order to avoid this, aluminium or stainless-steel caps can be used to keep the tubes sterile. Alternatively a screw cap without a washer can be placed over the mouth of the tube, the size being a loose fit. (For ordinary 15 ml. tubes, the M2 screw cap of a $\frac{1}{4}$ -oz. "bijou" bottle is convenient.)

(4) After the tubes have been placed in position, make sure that the metal buckets in a swing-out head are properly seated on the rings and are free to swing.

(5) Close the lid and make sure it is secure. The lid must not be removed when the centrifuge is running. Apart from the danger of an open lid, a decrease in speed due to "winding" will ensue.

(6) Make sure that the rheostat is back to the zero position. (Some centrifuges have an automatic switch-off unless this is so. This prevents strain by inadvertently switching on with the resistance out of circuit.)

(7) Start the motor and *gradually* increase the speed by taking the resistance out by means of the rheostat. Pause at intervals to allow the machine to gather speed until the required r.p.m. are reached. Unless this process is carried out slowly, the life of the centrifuge will be considerably curtailed. Some machines have a built-in revolution counter, while in others the rheostat must be calibrated by placing a normmeter on the rotating spindle.

(8) When the tubes have been centrifuged sufficiently, ^{partic} ^{the} ^{side} ^{off} the motor and *then* bring the rheostat back to the zero ^{on} ^{set} ^{point}. ^{rected.} Some centrifuges have an automatic timer built in which ^{sets} the required time-interval.

(9) Allow the centrifuge to come to a stop. N^e

head with your hand as brake. This will tend to redisperse the centrifugate due to turbulence and may cause serious injuries. Wait until the machine has stopped before attempting to remove the tubes.

(10) Periodically (*e.g.* once a week or once a fortnight) a centrifuge should be lubricated according to the maker's instructions.

The Washing of Bacteria and Other Cells: “Washed Suspensions”

The cell suspension is centrifuged at a suitable speed and preferably at a low temperature. Micro-organisms grown on a solid medium are first suspended in liquid by scraping off the surface of the agar with a curved glass rod into a small volume of a suitable suspending fluid.¹ The pellet of cells at the bottom of the centrifuge tube is resuspended and centrifuged. This washing process is repeated once or more to free the cells from the original suspending medium. The cells are finally made up to the required volume in the required solution.

For metabolic experiments, the cells are washed in a medium similar in composition to the culture medium but with one or more components omitted so that growth does not occur. The “washed suspension” so obtained is particularly suitable for experiments on catabolism; a substrate and buffer are added so that the breakdown of the substrate can be studied uncomplicated by growth processes or by the metabolism of other substrates. However, it must be realised that some activities “decay” rapidly after, or during, the preparation of the washed suspension.

PHOTOELECTRIC COLORIMETER AND SPECTROPHOTOMETER

One of the simplest and most accurate methods of measuring the quantity of a micro-organism depends upon a turbidity measurement just as many of the quantitative micro-methods used in biochemistry depend upon the measurement of the depth of colour in a solution. For such measurements, a photoelectric colorimeter or spectrophotometer is simpler and more accurate than visual comparison. It is also much quicker and free from many personal factors, such as eye fatigue, colour blindness, etc., which are inherent in visual methods.

The theory of the instrument as a colorimeter depends upon the application of Beer's Law, which states that the extent of diminution in light intensity on passing through an absorbing material depends upon the nature and concentration of the absorbing material and upon the length of the light path. This can be expressed as follows:

$$\log \frac{I_0}{I} = acl$$

where I is the intensity of the beam after passing through the solution,

¹ This suspension may be contaminated by lumps of agar which can be removed by filtration through cheesecloth.

I_0 is the incident intensity, α is the extinction coefficient depending upon the particular chromogen, c is the concentration of the chromogen and l is the length of the light path through the solution.

It is possible, therefore, to determine the concentration of a substance by measuring I_0/I in a vessel of standard dimensions. In photoelectric colorimeters and spectrophotometers, light intensity is measured by photoelectric response which can be made directly proportional to the quantity of light falling on the photoelectric cell.

Two main types of instrument are available.

(1) Single-cell Apparatus¹

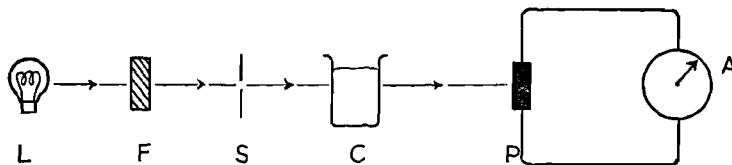


FIG. 17

(2) Twin-cell Apparatus²

The following points should be noted:

(1) It is essential that the intensity of the source of light L should remain constant during a reading. A mains supply is subject to sudden

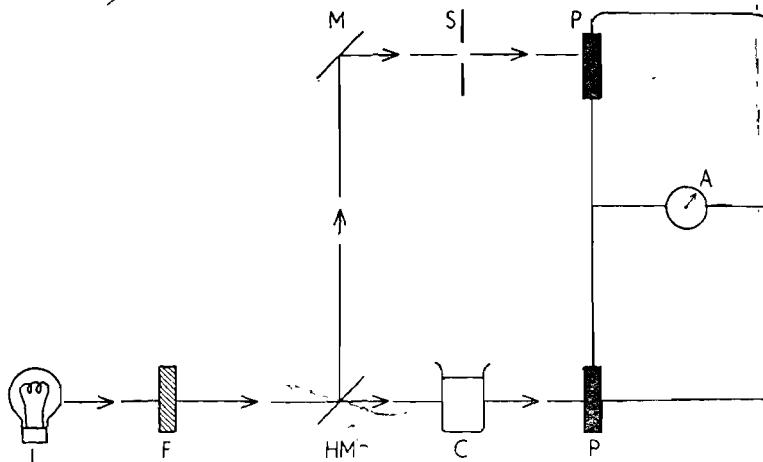


FIG. 18

changes of voltage and should be used only with a constant-voltage transformer. Alternatively, an accumulator can be used. With a

¹ E.g. the "Eel" photoelectric colorimeter. Details obtainable from Evans Electroseelenium Ltd., Harlow, Essex.

² E.g. the Unicam SP 600 Spectrophotometer. Details obtainable from Unicam Instruments (Cambridge) Ltd., Arbury Works, Cambridge.

twin-celled instrument, changes in light intensity affect both cells equally and therefore no error is involved. In a single-celled instrument it is necessary to check the reading with the blank solution after each determination.

(2) The colour filter *F* isolates the part of the spectrum where absorption by the chromogen is greatest. A filter is selected which gives a colour of light complementary to the colour of the chromogen. Thus, if the solution is blue, a red filter should be used. The narrower the range of wavelength transmitted, the more can interference by other compounds be eliminated. In a spectrophotometer, a prism is built into the machine and selects light of a small wavelength band.

(3) In the twin-celled instrument, a half-silvered mirror *HM* and a mirror *M* are used to split the light into two approximately equal beams.

(4) There is an adjustable slit *S* in the light path.

(5) The curvette or tube *C* containing the solution should be of standard length of light path.

(6) Light falls on the photoelectric cell or cells *P* and the current generated is measured by a microammeter *A* which is usually calibrated in a log scale permitting direct reading of $\log I_0/I$. In some two-celled apparatus,¹ a calibrated slit is placed on one side of the slit and is adjusted to give no deflection on a galvanometer.

Directions for the use of a particular machine can be obtained from the makers. In all cases a blank solution is used in which the chromogen would be dissolved. A calibration curve should be constructed of the reading of the instrument ($\log I_0/I$) against known amounts of chromogen. If Beer's Law is obeyed, a straight line will be obtained. Unknown samples are compared with the plot.

The use of such instruments for turbidimetric measurements of bacterial numbers is considered later (pp. 306-7).

COUNTING BACTERIA AND MEASURING BACTERIAL GROWTH

The method used for determining the amount of a micro-organism present in a suspension depends upon the kind of information required. In particular, since no constant relation exists between the ratio of increase in protoplasmic mass to rate of multiplication, it is necessary to distinguish clearly between methods which measure multiplication (e.g. total count) and those which measure growth (e.g. total nitrogen content, dry weight, etc.).

Methods of Counting Bacteria

(1) Total Count

A total count of the living and dead bacteria in a liquid culture or suspension is made microscopically using a slide counting chamber. A suitable chamber (as supplied by Hawksley Ltd., London) consists

¹ E.g. the "Spekker" photoelectric colorimeter. Details obtainable from Hiley and Watts Ltd., 98 St. Pancras Way, London, N.W.1.

of a thin glass slide with a flat, circular platform depressed exactly 0.02 mm. below the surface and surrounded by a deeper "trench". An area of 1 sq. mm. on the platform is marked with a Thoma-type grating of engraved lines into 400 small squares (each 0.0025 sq. mm.). The chamber is closed by a thick, optically-plane cover-slip. When the space between platform and cover-slip is filled with a bacterial suspension, the volume over each small square is 0.02×0.0025 c.mm., i.e. 0.000,000,05 ml. The average number of bacteria per square is calculated from counts made in sufficient squares (e.g. 100) to yield a significant total number of bacteria (e.g. 100-1000, preferably over 300). Counts are best made in preparations having between 2 and 10 bacteria per square (i.e. 40-200 million per ml.). For bacteria occurring in pairs, chains or clusters, an "individual cell count" may be made of all the cells, or a "group count" of the groups plus any isolated single cells.

Procedure.—(1) Fix the bacterial suspension by adding 2 or 3 drops of 40 per cent. formaldehyde per 10 ml. Mix thoroughly. If the suspension is too dense, prepare a measured dilution in the range 40-200 million bacteria per ml.

(2) Wash, rinse, drain and dry the counting chamber and cover-slip. Keep them covered until use, free from grit and dust.

(3) Place a small drop or loopful of the suspension on the centre of the chamber platform and apply the cover-slip. The size of the drop must be such that it will fill the whole space between platform and cover-slip, yet not extend across the "trench" to float the cover-slip from the slide. The cover-slip must be applied closely and evenly; it is pressed down until coloured "Newton's rings" are seen uniformly distributed over the areas of contact.

(4) Examine the preparation with a phase-contrast microscope, using the dry, $\frac{1}{2}$ in. objective; this shows the unstained bacteria clearly and enables their distinction from detritus. Alternatively, a dark-ground microscope may be used, or an ordinary microscope with the iris diaphragm closed or the condenser slightly defocussed (it may then be helpful to stain the bacteria by prior addition of freshly filtered methylene blue to a concentration of 0.1 per cent.).

(5) Count the bacteria in a sufficient number of squares to obtain a total of several hundred bacteria, selecting the squares in a pre-arranged pattern (e.g. all in every fifth row). Focus at different levels for the bacteria that have not settled; most settle on the platform in five or ten minutes, but some adhere to the cover-slip and a few remain in suspension.

(6) Calculate the average number of bacteria per square. Multiply this by 20,000,000 and by the dilution factor, if any, to obtain the count per ml. in the original suspension. Count two further preparations of the same suspension, and unless discordant, take an average of the three results.

If the original suspension contains much less than 40,000,000 bacteria per ml., a haemocytometer with a 0.1 mm. chamber may be used so



as to obtain a significant count in fewer squares. An ordinary microscope is used, the bacteria are stained and the preparation is left for twenty minutes before counting so that most bacteria may settle on the platform.

(2) *Viable Count*

The number of living bacteria or groups of bacteria in a liquid culture or suspension is counted by a cultural method such as the *pour-plate method*. A measured amount of the suspension is mixed with molten agar medium in a Petri dish. After setting and incubation, the number of colonies is counted. As a compromise between sampling and overcrowding errors, counts of pure cultures should be made on plates inoculated to yield between 50 and 500 colonies (ideally 200–400).

Procedure.—(1) Prepare serial tenfold dilutions of the bacterial suspension over a range ensuring that one dilution will contain between 50 and 500 viable bacteria per ml. Use a diluent suitable for the organism concerned, *e.g.* buffered saline, Ringer or Locke's solutions. Pipette 9.0 ml. amounts of diluent into each of several (6–9) sterile test tubes. Mix uniformly the bacterial suspension (vigorous shaking may disrupt cell groups and increase the viable count). With a sterile 1-ml. delivering pipette, transfer 1.0 ml. suspension into the first tube of diluent (fill and empty the pipette with suspension several times before withdrawing from the original container, remove any excess drop from the outside of the pipette and then slowly deliver its contents into the tube of diluent, touching the wall of the tube but not dipping into the diluent). With a fresh sterile 1-ml. pipette, mix the first dilution by filling and emptying several times, and then transfer 1.0 ml. into the next tube of diluent. Make the remaining dilutions in the same way, using a fresh pipette for each.

(2) Starting with the greatest dilution, pipette 1.0 ml. amounts of each dilution into each of three 4-in. Petri dishes. Then pour into each dish about 10 ml. of clear nutrient agar, melted and cooled to 45–50° C. At once mix by rapidly moving the plate, while flat on the bench, in a combination of side-to-side and circular movements in different directions; continue for about ten seconds, taking care not to spill any of the contents. Allow the agar to set and incubate inverted for two days at 37° C., or as most suitable for the species examined.

(3) Count the colonies in the three plates which were inoculated with the dilution giving between 50 and 500 colonies per plate (see p. 362 for counting methods). Multiply the average number per plate by the dilution factor to obtain the viable count per ml. in the original suspension.

A *surface viable count* is made when the bacterium is best grown in surface culture or on an opaque medium. Prior to inoculation, the plate of medium is dried for at least two hours at 37° C. with the lid ajar; it should then be able to absorb all the water of the inoculum within about fifteen minutes, *i.e.* before the bacteria can multiply. Tenfold dilutions of the bacterial suspension are made as for the pour-plate method. A suitable volume of each dilution, *e.g.* 0.1 ml., is pipetted

on to the surface of each of three plates and at once spread widely with a fine wire loop. The viable count is calculated from the average colony count per plate. Alternatively, by the method of *Miles and Misra*,¹ the inoculum is deposited as drops from a calibrated dropping pipette. Each drop, 0.02 ml. in volume, is allowed to fall from a height of 2.5 cm. on to the medium, where it spreads over an area of 1.5–2.0 cm. diameter. Each of six plates receives one drop of each dilution in separate numbered sectors. Counts are made in the drop areas showing the largest number of colonies without confluence (up to 20 or more); the mean of the six counts gives the viable count per 0.02 ml. of the dilution.

Because of variations in average cell size, bacterial counts do not bear a constant relationship to the amount of protoplasmic growth. The amount of protoplasm is better gauged by an opacity measurement, weighing or a total nitrogen estimation.

Methods of Measuring Growth

(1) Centrifugation

A specified volume of the suspension is centrifuged in a special tube, usually a capillary tube. The height of the packed organisms provide a measure of the total protoplasmic mass. However, the method is only useful if very thick suspensions of cells are available.

(2) Wet Weight

Amounts of culture for inoculation of animals are sometimes measured by wet weight. The moist surface growth on a solid medium is scraped from the medium and weighed at once. However, such estimations are inaccurate because of the difficulty of evaluating the relative contributions of water wetting the bacterial surface and intracellular water. Further, in bacteria forming capsules and slime, the wet weight may greatly overestimate the amount of protoplasm, since it includes the weight of these highly hydrated extracellular substances.

(3) Dry Weight

The weight of the dried solid matter of bacteria affords a better measure of their protoplasm. The cells from a known volume of culture are washed free of soluble salts, nutrients and waste products by centrifugation in distilled water (see p. 299). It is assumed that no lysis occurs during this process. The whole or a known proportion of the washed cells is placed in a weighed vessel and weighed again after drying to a constant weight by heating in an oven, e.g. at 120° C. for about three hours. Cool after each heating in a desiccator over P₂O₅ and weigh quickly to prevent absorption of water.

(4) Total Nitrogen

One of the most reliable and constant methods of measuring the amount of bacterial protoplasm for metabolic measurements is by an

¹ Miles, A. A., & Misra, S. S. (1938), *J. Hyg., (Lond.)*, **38**, 732.

estimation of the nitrogen present in the nitrogenous components of the cells, *i.e.* mainly proteins and nucleic acids (nitrogen content about 16 per cent.). The cells from a known volume of culture are washed by centrifugation to free them from nitrogenous constituents of the medium and from extracellular excretion products. The total nitrogen of the cells is then estimated by the micro-Kjeldahl method. The cells are digested with sulphuric acid using a $\text{CuSO}_4\text{-K}_2\text{SO}_4$ -selenium catalyst. The ammonia produced is removed after making the solution alkaline by steam distillation, in a suitable still (*e.g.* a Markham still), trapped in 2 per cent. boric acid and estimated after addition of Nessler reagent by the photoelectric colorimeter (see pp. 299, 306).

Instead of measuring the total nitrogen content of the cells, it may be preferable to measure the total non-dialysable nitrogen content. A measured volume of a washed bacterial suspension is placed in a length of dialysis cellophane tubing tied off at its lower end. The bacterial enzymes are inactivated and the cell membranes burst by immersing the sack in boiling water for a few minutes. The sack is then closed tightly on its contents by tying the upper end. It is placed for a period of twenty-four hours in a jar of running tap water, or in a large volume of distilled water. The fluid inside the dialysis sack is removed by cutting one end and the volume noted for any changes during dialysis. The nitrogen content is then determined as described previously.

(5) Turbidity

*Brown's Opacity Tubes*¹.—A simple method of determining the approximate number of bacteria in a suspension is by means of standard turbidity tubes such as the Brown series. This consists in comparing the opacity of the suspension with that of a series of ten standard tubes containing different dilutions of suspended barium sulphate. The suspension may be made up in liquid, in which case it must be well shaken before use. Alternatively a stable suspension in gelatin can be used provided a preservative is added. In making comparisons the bacterial suspension should, of course, be placed in a tube of similar dimensions to the standards. The matching is facilitated by reading printed letters through the suspensions.

The table gives the numerical equivalents of the opacity standards for certain organisms according to Cunningham and Timothy².

It must be realised, however, that these figures may be inaccurate owing to the method of counting that was used. Further, the opacity of a bacterial suspension will depend not only on the number of bacteria and the species of bacterium but also on the strain and the conditions of growth, which both affect cell size and density. It is advised that if opacity tubes are used, they should be calibrated for the particular organism and growth conditions being studied.

¹ Brown, H. C. (1919-20), *Indian J. med. Res.*, **7**, 238.

² Cunningham & Timothy (1924), *Indian J. med. Res.*, **11**, 1253.

*Showing the Relation of Opacity to the Numerical Equivalent of various
Bacteria estimated by means of the Haemacytometer Method*

Opacity Tube No.	<i>Staphylococcus aureus</i>	<i>Streptococcus (pyogenes)</i>	<i>Pneumococcus</i>	<i>Gonococcus</i>	<i>Esch. coli</i>	<i>S. typhi</i>	<i>S. paratyphi B</i>	<i>N. catarrhalis (on ord. agar)</i>	<i>H. influenzae</i>
10	3.8	3.0	7.1	3.6	3.8	4.6	4.2	3.6	11.4
9	3.4	2.7	6.3	3.2	3.4	4.1	3.8	3.3	10.3
8	3.0	2.4	5.6	2.9	3.0	3.7	3.3	2.9	9.1
7	2.7	2.1	4.9	2.5	2.7	3.2	2.9	2.5	8.0
6	2.3	1.8	4.2	2.1	2.3	2.7	2.5	2.2	6.8
5	1.9	1.5	3.5	1.8	1.9	2.3	2.1	1.8	5.7
4	1.5	1.2	2.8	1.4	1.5	1.8	1.7	1.4	4.6
3	1.1	0.9	2.1	1.1	1.1	1.4	1.3	1.1	3.4
2	0.8	0.6	1.4	0.7	0.8	0.9	0.8	0.7	2.3
1	0.4	0.3	0.7	0.4	0.4	0.5	0.4	0.4	1.1

The figures represent thousands of millions per ml.

Standard opacity tubes with the corresponding tables are supplied by Burroughs Wellcome & Co.

USE OF A PHOTOELECTRIC COLORIMETER OR SPECTROPHOTOMETER.—The turbidity of a suspension is caused by the light scattered by particulate matter during its passage through the suspension. Clearly, accurate measurements of turbidity and hence bacterial growth, can be obtained in two ways.

(1) By measuring the amount of light scattered directly, a procedure occasionally called nephelometry. This is rarely used in practice.

(2) By measuring the light lost from the beam by scattering. Light absorption is assumed to be absent. This loss can be measured accurately in a photoelectric colorimeter or spectrophotometer where a relation similar to Beer's Law applies. The expression is the same as that on p. 299 except that the term extinction coefficient is replaced by a constant called the turbidity coefficient. A standard plot can be made of $\log I_0/I$ against either the total nitrogen content or the dry weight. The concentration factor applies mainly to protoplasmic mass as the size of the organisms as well as their number determines turbidity.

The following points should be noted:

(1) The calibration curve applies only to *a particular organism grown under a particular set of growth conditions*. A new curve must be prepared if a change is made in either of these. It should be noted that the shape of an organism as well as its size will alter turbidity. Further, cells grown in a medium to give a high carbohydrate or fat content generally have a high turbidity per cell.

(2) Use a neutral or a blue filter. In a spectrophotometer use a

wavelength of 5400 Å. Light scattering increases very greatly as the wavelength decreases, although it is not advisable to use too low a wavelength since light absorption will become increasingly apparent.

(3) For the blank use the suspending fluid. The growth medium can be used provided the absorption is not altered by growth of the organisms. If it is altered, the cells must be washed and resuspended in fresh solutions.

(4) At low concentrations, a linear calibration plot should be obtained, but at higher concentrations a considerable departure from a straight line will normally occur. High cell populations cannot be determined unless they are first diluted to a suitable range.

(5) The suspending fluid must be the same as that used for the preparation of the calibration curve.

Turbidity estimations in this way are the easiest and the quickest way of calibrating a bacterial population and they are accurate for comparative studies provided the above points are borne in mind.

THE PREPARATION OF CELL-FREE EXTRACTS OF MICRO-ORGANISMS

In order to study the chemical and antigenic components of micro-organisms and particularly to study their enzymology, it is necessary to lyse the cell and to liberate their internal contents. This lysis will be accompanied by varying degrees of disintegration and solubilisation of cell components (*e.g.* the cell wall, cytoplasmic membrane, nucleus, etc.). It is preferable to use methods which avoid too much denaturation of the high molecular weight cell components. Thus, any raising of the temperature during lysis should be avoided. After lysis, intact cells and cell debris can be removed by centrifugation together with any abrasives added to aid distintegration. Before this process, it is often useful to add a small quantity of deoxyribonuclease to reduce the viscosity due to deoxyribonucleic acid.

Various methods have been devised and are discussed in more detail by Hugo.¹

(1) Autolysis

Many cells undergo autolysis under suitable environmental conditions. The rate of this autolysis varies according to the nature of the organism and the surrounding medium. However, due to their mechanically strong cell wall, autolysis in bacteria is usually slow and is rarely an effective method for preparing a cell-free extract.

(2) Induced Lysis

Various agents have been used to induce lysis in cells. The best methods for studying components of the protoplast depend upon the use of agents specific for cell-wall destruction. The enzyme lysozyme

¹ Hugo, W. B. (1954), *Bacteriol. Rev.*, **18**, 87.

obtainable from egg-white, tears, saliva and other body fluids breaks down links in the cell wall so that, in the absence of an osmotic stabiliser, the cell bursts. However, lysozyme only acts on a restricted range of bacteria. Other enzymes have also been used for this purpose as has bacteriophage "lysis from without" and complement in presence of the specific antibody.

(3) Extraction of Dried Cells

When a cell suspension is dried, the cytoplasmic membrane is usually damaged so that the permeability properties of the cells are changed and intracellular constituents can be extracted by suitable buffer solutions. Two methods of drying have been used commonly.

(a) Preparation of acetone powders. A thick bacterial suspension is added to at least ten volumes of acetone previously cooled to -10°C . Stir the mixture vigorously. Allow to settle for ten minutes and decant the supernatant. Filter on a Buchner funnel and wash with cold acetone and ether. Dry in air on a filter paper.

(b) Lyophilisation (*i.e.* freeze-drying; see pp. 268-272).

(4) Grinding with an Abrasive

A very simple method of producing a microbial cell-free extract is by grinding in a pestle and mortar with a suitable abrasive. A thick paste of bacteria is mixed with two to ten parts by weight of abrasive, the proportions varying with the organism concerned. Suitable abrasives are powdered Pyrex glass, polishing alumina or carborundum and the particles should have an average diameter of about 1μ . About 5 g. of the mixture is chilled in the mortar and is ground vigorously for about five minutes in a cold room or ice-bath. A suitable buffer is added before centrifugation.

There are also a number of machines available which will carry out a mechanical grinding by movement of the mixture of abrasive and bacteria between opposing glass surfaces.

(5) Shaking with Glass Beads

The cells are shaken vigorously with smooth glass beads. A suitable machine is the Mickle disintegrator¹ and suitable beads are those incorporated into beaded projection screens.² A roughly equal weight of glass beads and a fairly thick bacterial suspension are placed in the disintegrator tube and are shaken for fifteen to sixty minutes depending on the organism. It is preferable to place the whole disintegrator in a cold room for this stage. The beads can then be separated on a sintered glass filter.

This method, although it causes inactivation of many enzymes, is particularly suitable for the preparation of cell walls.

¹ Manufactured by H. Mickle, 4 Ormond Drive, Hampton, Middlesex.

² E.g. Ballotini beads manufactured by the English Glass Co. Ltd., Leicester.

(6) Extrusion through a Small Orifice under Pressure

If cells are driven through a narrow orifice under a very high pressure applied for a short interval of time, disruption and lysis often occurs. The Hughes' press¹ can be used for this purpose. A thick paste of cells prepared with or without a suitable abrasive, is placed in a stainless-steel block previously cooled to about -20° in a deep-freeze unit. A close-fitting piston is forced on to the cells with a series of blows on a fly-press.² The high pressure liquefies the frozen suspension and forces it through a narrow orifice into a reservoir chamber in the block, where it freezes again. During this process, the cells are disrupted at a low temperature, therefore causing a minimum of denaturation. The halves of the block are separated and the preparation is recovered.

(7) Exposure to Sound Waves

When a microbial suspension is subjected to sonic or ultra-sonic waves, breakage of cells occurs due to the creation of local areas of low and high pressure. Thick suspensions of bacteria can be disrupted within a short interval of time and at low temperatures. Ultrasonic disintegrators producing frequencies between 400 and 600 kilocycles/sec. have been largely replaced by magnetostricture sonic disintegrators giving about 8-20 kilocycles/sec. An example is the M.S.E.-Mullard Disintegrator³ giving a frequency of about 20 kilocycles/sec. A relatively thick suspension of bacteria is placed in a suitable tube (up to about 20 ml. at a time) and the probe of the disintegrator is placed so that it just touches the surface of the bacterial suspension. After tuning the instrument (see instructions provided with the machine), the apparatus is left running for between five and thirty minutes, depending upon the organism being disrupted. Cooling may be necessary and the tube can be placed in an ice-bath.

This method is one of the simplest and most reproducible methods of preparing cell-free extracts of bacteria and it is coming into increasing use in the laboratory.

(8) Freezing and Thawing

A series of freezing and thawing operations may cause lysis of a sensitive organism. Although this method has not been much used in the preparation of cell-free extracts, it has been useful in the liberation of toxins e.g. of *Bord. pertussis*.

¹ Obtainable from Shandon Scientific Co. Ltd., 6 Cromwell Place, London, S.W.7.

² E.g. Denbigh No. 4 Fly-press, manufactured by Thomas Ward, Ltd., Sheffield.

³ Details obtainable from the manufacturers, Measuring and Scientific Equipment, Ltd., Spenser Street, London, S.W.1.

METHODS FOR THE FRACTIONATION OF BACTERIAL COMPONENTS

In order to fractionate the components of a bacterial cell, it is first necessary to grow the organism in large amounts. It is then separated from the surrounding medium and washed by centrifugation or filtration. This surrounding medium will contain unutilised metabolites, intermediate metabolites produced in excess, the end products of metabolism (*e.g.* fermentation products) and the results of any autolysis which has gone on during growth. In addition, there will usually be high molecular weight substances which often have important biological properties and are usually of protein, polypeptide or polysaccharide nature (*e.g.* extracellular enzymes, exotoxins, antibiotics, levans, dextrans, type-specific polysaccharides, etc.).

The washed cells so obtained can then be fractionated in two general ways.

(1) Structural Fractionation

The cell is fractionated into its various morphological components, whose chemical and functional properties can then be determined. For example, methods have been developed for the separation and purification of the cell wall, the cytoplasmic membrane, the nuclear body and some of the many types of inclusion bodies (lipid and glycogen granules, ribosomes, chromatophores, etc.). The medical bacteriologist has been particularly interested in those surface structures which are responsible for the antigenic character of the organism, some of its toxic properties and its resistance to the antibacterial mechanisms of the host organism and to bacteriophage. Thus, the capsule, micro-capsule and cell wall have received particular attention.¹ Components of the capsule can be removed from the cell wall by treatment with solvents or enzymes, while a concentrate of the walls can be prepared after rupture of the cells by shaking with glass beads.

(2) Molecular fractionation

The various types of molecule which make up the bacterial cell can be fractionated and their structure and function determined in the pure isolated state. We may distinguish two types of compounds within the cell, those in solution separately within the cytoplasm and those integrated into more complex structures. The soluble substances can be separated by breaking down the cell wall and cytoplasmic membrane by one of the methods described in the previous section. If the resultant suspension is subjected to high-speed centrifugation, the complex structures will be found in the centrifugal deposit, *i.e.*

¹ For general details see Salton, M. R. J. (1960), in *The Bacteria*, p. 97, edited by I. C. Gunsalus and R. Y. Stanier. Academic Press, New York and London.

complexes of high molecular weight proteins, polysaccharides, lipids and nucleic acids. The supernatant will contain many of the enzymes, together with inorganic ions and a pool of organic metabolites and metabolic intermediates. Further methods are available which separate high and low molecular weight compounds. The most commonly used are:

(a) *Acid extraction.*—The cells are extracted with 5 per cent. trichloracetic acid or 10 per cent. perchloric acid in the cold so as to minimise hydrolysis. The low molecular weight intermediates together with some polysaccharides and lipids are obtained in solution after centrifugation.

(b) *Production of acetone powders.*—A thick suspension of cells is squirted rapidly into about 10 volumes of pure acetone cooled to -20° C . The high molecular weight compounds in the precipitate are filtered on a Buchner funnel and washed with cold acetone and ether.

In order to purify the components of the complex structures within the cell, it is necessary to break down the loose bonds joining the various molecules together. As an example, polysaccharides, lipids and lipopolysaccharides can be obtained by comparatively drastic procedures which denature much of the protein. Alcohol and alcohol-ether at 40° C . to 50° C . will extract much of the lipid, although a preliminary hydrolysis by sulphuric acid may be necessary for some lipids. The lipopolysaccharide components at the surface of the cell wall in Gram-negative bacteria can be extracted using trichloracetic acid¹ or 45 per cent. phenol solution.² These lipopolysaccharides have the toxic pyrogenic and immunologically specific properties of the O antigen of smooth-colonised cells, although the ability to produce antibody rests with the "complete" antigen which can only be extracted from the cell using mild procedures such as extraction with diethylene glycol in the cold. This O antigen consists of a high-molecular weight complex of protein, polysaccharide and two types of lipid.

Since proteins represent about 50 per cent. of the dry weight of the bacterial cell and comprise all the enzymes and many other biologically important compounds (toxins, antigens, etc.), much attention has been devoted to methods for their fractionation. The magnitude of the problem can be visualised when it is realised that there are over a thousand different proteins in a single cell, made up of the same amino acids and differing only slightly in their physico-chemical properties. Further, they are easily denatured by heat, extremes of pH, organic solvents and certain inorganic ions. It is not possible to do more than summarise the main fractionation methods.³

¹ Boivin, A., Mesrobeanu, I., & Mesrobeanu, L. (1933). *C. R. Soc. Biol. (Paris)*, **114**, 307.

² Westphal, O., Lüderitz, O., & Bister, F. (1952). *Z. Naturforsch.*, **76**, 148.

³ For further details, see *Methods in Enzymology*, vol. 1, edited by S. P. Colowick and N. O. Kaplan (1955). New York, Academic Press.

(a) Salt Fractionation.

If the inorganic salt concentration is increased to a sufficiently high level, many proteins precipitate from solution, some requiring higher concentrations than others. Ammonium sulphate is usually used for this purpose, the concentration being gradually increased while the protein precipitating out at each stage is collected by centrifugation.

(b) Precipitation by Organic Solvents

Proteins can be precipitated at a low temperature by certain organic solvents such as methanol, ethanol and acetone. Again, different proteins are precipitated by different solvent concentrations, as well as being influenced differently by the salt concentration and the pH of the medium.

(c) Ultracentrifugation

If sufficiently high centrifugal forces are applied, proteins can be sedimented, the rate of sedimentation varying with the size and shape of the molecule. The ultra-centrifuge can be used to separate and fractionate some of the larger protein molecules.

(d) Electrophoresis

Because of their different contents of basic and acidic amino acids, proteins have a characteristic charge and will move towards an anode or a cathode with different mobilities. This principle is made use of in electrophoresis, where a protein solution in buffer is placed between two electrodes. For small quantities, paper electrophoresis may be used, while on a larger scale a column, often supported by an inert solid such as cellulose, is used.¹

(e) Adsorption

Proteins are adsorbed to different extent on substances such as tricalcium phosphate and aluminium hydroxide. They can be adsorbed and then solubilised again by appropriate changes of pH or salt concentration.

(f) Chromatography

Methods for the chromatography of proteins have recently been developed and are gradually replacing many of the methods described above. Although adsorption chromatography and partition chromatography have been employed, the most successful methods use ion exchange resins and, in particular, the various cellulose derivatives which have been developed for this purpose.²

¹ Tiselius, A. (1958). From *Symposium on Protein Structure*, p. 93. John Wiley & Sons, Inc., New York.

² Sober, H. A., & Peterson, E. A. (1958), *Fed. Proc.*, 17, 1116.

CHAPTER XIII

IMMUNOLOGICAL AND SEROLOGICAL METHODS¹

THE WIDAL REACTION AND OTHER AGGLUTINATION TESTS; COMPLEMENT FIXATION, HAEMAGGLUTINATION INHIBITION, AND NEUTRALISATION TESTS

THE nature of the *Widal agglutination reaction* and its applications in the diagnosis of enteric fever are referred to on p. 581 *et seq.*

In general the underlying principle of the technique of agglutination tests is to examine serum *quantitatively* for agglutinins towards the particular organism. For this purpose the method usually adopted is to mix varying dilutions of serum (made up in saline solution) with a fixed quantity of a uniform and stable suspension of the organism, the mixtures being placed in narrow tubes, kept at 37° C. or 50°–55° C. in a water-bath for a certain length of time, and then examined for *visible* agglutination or flocculation of the suspension. The agglutinated organisms tend to sediment, and the reaction can also be gauged by the amount of deposit in the tubes and the clarity of the supernatant fluid. The strength of the reaction can be stated in terms of the highest dilution ("titre") which produces agglutination.

AGGLUTINATION TESTS

The *blood specimen* is taken by vein puncture (p. 261), so as to obtain a satisfactory amount of serum for the complete test. At least 5 ml. of blood should be obtained, and the blood immediately transferred from the syringe to a stoppered sterile tube or screw-capped bottle and allowed to clot. When the serum has separated, it is pipetted off into a sterile tube.

THE WIDAL TEST

In the routine Widal reaction the patient's serum is tested simultaneously with each of the organisms likely to be responsible for enteric fever in the particular region, *e.g.* in Great Britain at the present time,

¹ Only those immunological and serological methods which are applicable to routine bacteriological work are dealt with. For other methods, and the preparation and testing of therapeutic antisera, reference should be made to larger works on bacteriology and immunity.

S. typhi and *S. paratyphi B*. In other parts of the world *S. paratyphi A* or *C* may require to be included. As explained later, additional information can be obtained by testing separately for H and O agglutinins (p. 581). Thus, the Widal test generally involves parallel tests with different *Salmonella* group organisms, and also different forms of the same organism.

In addition to the tests with typhoid-paratyphoid organisms, it is the practice in many laboratories to test also for *Br. abortus* agglutinins, and, if considered necessary, with *Proteus X19* for typhus infection (p. 735), thus increasing the number of parallel tests carried out.

To simplify description a single test will be referred to.

Requisites:

1-ml. pipette graduated to the tip in 1/10ths and 1/100ths; 0.1-ml. pipette graduated to the tip in 1/100ths and 1/500ths; a rubber teat, or preferably a mouth-piece for pipetting by suction, i.e. 3 in. of 5-7 mm. bore glass tubing with 9-12 in. of rubber tubing attached which can be fitted to the top of the pipette (*vide infra*). The free end of the mouth-piece is "smoothed" in the Bunsen flame.

Sterile 0.85 per cent. saline; test-tubes $3 \times \frac{1}{2}$ in.; agglutination tubes $3 \times \frac{1}{4}$ in., or Dreyer's agglutination tubes with rounded bottoms (not conical as originally made); test-tube racks suitable for the tubes used; small beaker or similar container for saline solution; grease pencil for marking tubes; capillary pipette.

Bacterial Suspension

The strain used must be carefully selected and known to be suitable for the diagnostic agglutination test. It must be a motile "smooth" form of the particular type, and if the organism is "diphasic", e.g. *S. paratyphi B*, must represent the specific phase. Since the antigens of fimbriae (p. 604) are similar in different types of *Salmonella*, the use of suspensions prepared from fimbriate cultures may lead to confusing cross-reactions. Non-fimbriate cultures may be obtained by two or three successive subcultivations on well dried agar plates; liquid media should not be used.

It is now a general practice to use standard suspensions such as those described later; but if it is desired to prepare a small quantity of suspension for immediate use the following method can be adopted: add in fractions 5 ml. of physiological saline to a well-grown twenty-four hours' agar slope culture, and emulsify the growth with the aid of a wire loop. This suspension can be standardised to a suitable opacity, e.g. tube 1, Brown's opacity standards (p. 305). The suspension is decanted and allowed to stand for half an hour until bacterial clumps and fragments of agar have sedimented. Alternatively, it may be centrifuged for one minute.

Serum Dilutions

First make up a 1 in 15 dilution of the patient's serum, and from this prepare a series of doubling dilutions in small ($3 \times \frac{1}{2}$ in.) test-tubes:

(1)	(2)	(3)	(4)	(5)	(6)	(7)
1 in 15	1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	CONTROL- NO SERUM

The actual procedure is as follows.

In the rack place seven tubes as above; add 0.4 ml. saline to each of the tubes 2 to 7; in a separate tube add 0.1 ml. patient's serum to 1.4 ml. saline, *i.e.* a 1 in 15 dilution; wash out the pipette thoroughly in saline solution; add to tubes 1 and 2 0.4 ml. of the 1 in 15 dilution of patient's serum; the dilution of serum in tube 2 is now 1 in 30; after thorough mixing withdraw 0.4 ml. from tube 2 into tube 3, making now in tube 3 a 1 in 60 dilution of serum; withdraw 0.4 ml. from tube 3 into tube 4, *i.e.* 1 in 120, and so on till a dilution of 1 in 480 is obtained in tube 6; withdraw and discard 0.4 ml. from tube 6; tube 7 contains saline only.

0.4 ml. of the bacterial suspension is added to each tube and the pipette is then sterilised. The dilutions of serum are now:

(1)	(2)	(3)	(4)	(5)	(6)	(7)
1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	1 in 960	CONTROL- SUSPENSION -NO SERUM.

If the amount of serum available is insufficient to allow of 0.4 ml. being used of the various serum dilutions, a smaller volume, *e.g.* 0.3 or even 0.2 ml., may be employed with, of course, the same volume of bacterial suspension.

The mixtures are transferred with a capillary pipette to agglutination tubes, starting with tube 7.

An alternative method of preparing doubling dilutions of serum and making mixtures of serum and bacterial suspension is to use a "constant-volume" pipette made by slightly constricting a capillary pipette (p. 249) so that the volume of fluid contained in it from the tip to the constriction is about 0.25 ml., the capillary stem being $3\frac{1}{2}$ to 4 in. long and not too fine in calibre. This pipette is actuated by a teat, and with it the serum dilutions can be made directly in the narrow agglutination tubes; thus, after preparation of the 1 in 15 dilution, the constant volume measured with the capillary pipette is substituted in the directions above for the 0.4 ml. volume of serum dilution, saline or bacterial suspension measured with a graduated pipette. The contents of the tubes are mixed by gently bubbling air through the fluid by means of the pipette, starting with tube 7.

To observe agglutination of the H type (p. 66) it is usually sufficient to incubate at 37°C . for two hours and then leave for half an hour at

room temperature. (Some workers prefer incubation at 50–55° C.). "Large-flake" clumping or agglutination can easily be detected with the naked eye in a satisfactory light. The flocculi also sediment rapidly and the deposit is quite perceptible in the narrow tubes.

When agglutination of the O type (p. 66) is tested for, readings should be made after four and twenty-four hours as this form of reaction develops slowly. It was at one time considered advisable to incubate at 50°–55° C., but it has been shown that prolonged exposure of O agglutinins at this temperature level may weaken the agglutination reaction, and it is preferable therefore to incubate for 2–4 hours at 37° C. and then to keep the tubes at 4° C. in a refrigerator for 20–22 hours. The clumps are small and "granular" and observations are aided by the use of a hand-lens and a strong illuminant.

Pipetting with a Graduated Pipette and Mouth-Piece

The glass mouth-piece is held between the teeth at the right corner of the mouth, and the top of the pipette is supported between the second and third fingers of the right hand so that the rubber tube immediately above the end of the pipette can be compressed between the thumb and the first finger (see figure). The fluid is drawn up, e.g. from a test tube, into the pipette by suction until the column extends just above the required graduation mark. The end of the mouth-piece is then closed by the tongue, and the column of fluid is depressed to the particular level by gentle pressure on the rubber tubing between the thumb and fore-finger. *With the tongue still firmly applied to the mouth-piece* this exact volume of fluid can be transferred from the original tube and then expelled from the pipette into another tube.

This method, for which the necessary skill is soon acquired by practice, permits of *accurate* and *rapid* measurements of even small volumes.

The glass-tube of the mouth-piece can be sterilised by flaming.

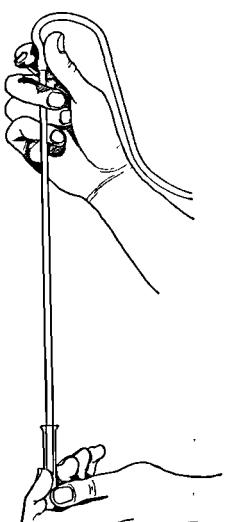


FIG. 19

Water-bath for Serological Tests

This instrument is constructed of non-corrodible metal and is thermally lagged on the outside. It is heated by gas or electricity and thermostatically controlled to $\pm 0.5^{\circ}$ C. of the desired temperature, e.g. 37° C., 50° C. or 55° C. The internal dimensions are 17 in. by 13 in., and the depth of water is 4 in. A bath of this size is usually

fitted with four removable metal racks, each holding thirty-six $3 \times \frac{1}{2}$ in. or $3 \times \frac{3}{8}$ in. tubes, but larger or smaller baths are available according to the particular requirements of the laboratory. The height of the racks is adjustable to take tubes of different lengths. Instead of one large lid over the bath, there is a hinged cover over each rack.

Measurement of Serum and other Fluids by Drops¹

Some serological workers prefer to make measurements of serum, saline, etc., in terms of drops delivered from a suitable dropping pipette. This consists in its simplest form of a piece of glass tubing drawn out to capillary dimensions (as in the capillary pipette, p. 249). Alternatively, special dropping pipettes can be purchased. The pipette is actuated by a teat. When used it is held vertically and the fluid in it is allowed to drop slowly from the capillary stem.

The following exemplifies the carrying out of an agglutination test by this method. In a suitable rack place a row of five Dreyer's agglutination tubes (p. 314) and a test-tube (about $3 \times \frac{3}{8}$ in.) which may be called the "dilution tube." Into this tube measure with the dropping pipette 18 drops of normal saline. Similarly, add 2 drops of the serum and mix. This yields a 1 in 10 serum-dilution. Saline, serum-dilution and bacterial suspension are now added to the five agglutination tubes as follows:—

Tube	Drops				
	1	2	3	4	5
Saline	0	5	8	9	10
Serum, 1 in 10	10	5	2	1	0
Bacterial suspension	15	15	15	15	15
Final dilution of serum	1 in 25	1 in 50	1 in 125	1 in 250	Control

The tubes are incubated and the observations then made (*vide supra*).

Bacterial Suspensions for Testing H and O Agglutinins

Formolised and alcoholised suspensions supply the necessary reagents for testing H and O agglutinins respectively,² and it is also advisable to use for such tests selected strains which are sensitive to H and O agglutination. Formalin interferes with O agglutination, and in the case of motile flagellate organisms, e.g. *S. typhi*, formolised suspensions show the large-flake agglutination characteristic of the

¹ For full details of the preparation and use of dropping pipettes see *A System of Bacteriology*, Medical Research Council, London, 1931, vol. ix., chap. 14.

² Standard suspensions for the Widal and other agglutination reactions may be obtained from the Standards Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

H antigen (p. 66). The reactivity of the H antigen can be annulled by alcohol, and if cultures are treated with alcohol a suspension representing the O antigen alone can be obtained.

H-agglutinable Formolised Suspensions are prepared by adding 0.1 per cent. of formalin (0.04 per cent. formaldehyde) to a twenty-four-hours' broth culture or by suspending a young agar culture in saline containing 0.1 per cent. formalin.

O-agglutinable Alcoholised Suspensions are prepared as follows: plate out the organism and select a smooth colony; subculture this on phenol-agar (1 in 800 phenol); scrape off the growth in the minimum amount of saline, emulsifying very carefully, and add about 20 times the volume of absolute alcohol; heat at 40°–50° C. for half an hour; centrifuge (if necessary) and suspend the deposit in saline to the proper density, with chloroform as a preservative. This emulsion keeps moderately well, but if an old suspension is used, it should be centrifuged and re-suspended in fresh saline. The original practice of keeping O suspensions in alcohol and diluting when ready for use is not recommended as the alcohol eventually annuls the agglutinability of the organisms.

The Typhoid Bacillus Vi Agglutination Test.—This test has not much application in the diagnosis of suspected cases of enteric fever, but is of value in the recognition of carriers of the typhoid bacillus.

The bacterial suspension should be prepared from a selected strain which responds only to the Vi agglutinin and is not acted on by the H and O agglutinins. It loses sensitiveness on keeping, and should not be used after two months. The test mixtures are made up and incubated in $3 \times \frac{1}{2}$ in. test-tubes. A series of doubling dilutions of the serum is prepared (p. 315), the initial dilution being 1 in 10 and the last tube in the series being 1 in 640. An additional tube is included, for control purposes, containing saline only. The amount of each dilution should be 1 ml. One drop (0.05 ml.) of the suspension is then added to each tube. Incubation is carried out at 37° C. for two hours and the tubes are then allowed to stand at room temperature overnight. To observe the result, the tubes are examined in ordinary daylight, being held somewhat tilted, and the type of sediment determined with the aid of a hand-lens. In the control the sedimented organisms should form a small, circular, well-defined, compact deposit. If marked agglutination has occurred, the deposit, consisting of clumped organisms, is scattered over the foot of the tube. Intermediate degrees are also observed. "Standard agglutination" is denoted by absence of the central deposit and bacterial clumps occupying about half the area of the foot of the tube. It has been pointed out that sera with haemolysed red cells may give false positive reactions in low dilutions.

In cases of suspected typhoid fever, standard agglutination in a titre of 1 in 10 is considered significant, but repeated tests and demonstration of a rising titre would make the result more conclusive. In suspected typhoid carriers a titre of 1 in 10 would also be regarded as suggestive.

OTHER AGGLUTINATION TESTS

The agglutination techniques described above are also applicable to diagnostic tests with various *Salmonella* group organisms, *Br. melitensis* or *Br. abortus*, *Proteus* X19 (Weil-Felix reaction of typhus fever), etc. These may be carried out at the same time and in parallel with the Widal test. The series of dilutions tested can, of course, be varied according to the range within which agglutination is likely to occur (pp. 582, 647, 736). It is essential in all cases to make these tests quantitative so that the "titre" or highest dilution in which agglutination occurs, can be estimated.

STREPTOCOCCUS M.G. AGGLUTINATION TEST (see p. 812)

Reagents.

(1) *Patient's serum.* 1.0 ml. is required. Two samples of serum should be tested: the first taken during the acute phase of the illness and the second after an interval of twelve to eighteen days.

Note. The sera must not be inactivated at 56° C. as this lowers the titre.

(2) *Streptococcus "M.G." Suspension.* Remove the growth from a 48 hour digest broth culture of the organism by centrifugation and wash it three times with sterile saline. Kill the suspension by heating in a water bath at 100° C. for thirty minutes. After one further washing with saline make the suspension up to a standard density (Brown's opacity tube No. 5, see p. 305) and add merthiolate 1 in 10,000 as a preservative.

(3) *Standard positive rabbit antiserum.*

(4) *Physiological saline.*

The Test.

Use $3 \times \frac{1}{2}$ in. tubes: Set up a rack containing 7 tubes. In the first tube place 0.8 ml. saline and 0.5 ml. in the remaining tubes. Add 0.2 ml. serum to the first tube, mix thoroughly and transfer 0.5 ml. of the mixture to the second tube and continue preparing doubling dilutions to the end of the row. Include a control tube containing 0.5 ml. saline only. To each tube add 0.5 ml. Streptococcus M.G. Suspension. Final serum dilutions are 1 in 10, 1 in 20, 1 in 40, 1 in 80, 1 in 160, 1 in 320, and 1 in 640.

With each batch of tests a titration of the positive rabbit serum should be included.

The tubes are incubated overnight in the water bath at 37° C.

A rising titre between acute and convalescent sera (at least four-fold) is regarded as significant.

Sera should not be screened by using a single low dilution tube method as a *prozone* is frequently observed.

BACTERIAL HAEMAGGLUTINATION TESTS

Bacteria or their components or products may cause the direct agglutination of red blood cells. These antigens may also adhere to the surface of the erythrocytes coating them partially or completely; when this occurs the cells are readily agglutinated by antibodies in sera developed against the antigens. The latter phenomenon is known as indirect bacterial haemagglutination and the antigens concerned are referred to as "erythrocyte sensitising substances". If complement is added to this system the erythrocytes are lysed. Occasionally it is necessary to treat the cells with tannic acid in order to make the antigens adhere to them. Antigens such as tuberculin, leptospiral and rickettsial extracts, pneumococcal polysaccharides etc. have all been used to sensitise cells for the development of diagnostic serological methods. The method has also been used to identify antigens in the body secretions. The techniques employed are often similar to the viral haemagglutination inhibition test described on p. 350. A full review of this subject is given by Neter.¹

"COLD AGGLUTINATION" REACTION

It has been shown that in cases of primary atypical pneumonia the serum may agglutinate at low temperatures human erythrocytes of the blood group O. This reaction is absent, unless in low dilutions of serum, in other types of pneumonia, other infections of the respiratory passages and normal individuals, and has been suggested as a means of confirming a diagnosis of atypical pneumonia. The reaction, however, tends to be late in its appearance during the illness. The test can be carried out quantitatively by preparing doubling dilutions of serum from 1 in 10 to 1 in 2560 and to each adding an equal volume of a 1 per cent. suspension of washed group O human red cells. The mixtures are placed in a refrigerator at 0°-4° C. for one hour when readings of agglutination are made. As the agglutinin is readily absorbed by erythrocytes at low temperature, the serum should be separated from the blood specimen at a temperature above 20° C. A titre of 1 in 80 (in terms of the final dilution of serum after addition of red cells) might be considered significant, but much higher titres have been recorded. The reaction, however, requires further study from the diagnostic standpoint.

THE PAUL-BUNNELL REACTION

During and after an attack of infectious mononucleosis an agglutinin for sheep red blood cells is present in the serum and is of diagnostic significance. The test is performed as follows. Heat the serum at 55° C. for twenty minutes. Make a series of doubling dilutions of the

¹ Neter E. (1956), *Bact. Rev.*, 20, 166.

serum with saline in 0·5 ml. amounts in 3-in. $\times \frac{1}{2}$ -in. tubes, ranging from 1 in 16 to 1 in 1024, as described on p. 315 for agglutination tests. A control tube containing only saline is included. Add to each tube 0·5 ml. of a 1 per cent. suspension of sheep red corpuscles in saline, washed as for the Wassermann test. Shake the tubes thoroughly and incubate at 37° C. for four hr. Note which tubes show agglutination of the red cells, and state the titre of the reaction in terms of the final dilution of the serum: 1st tube, 1 in 32; 2nd, 1 in 64; etc. Normal serum may agglutinate in low dilutions. A suggestive titre is 1 in 128. Repeated tests may reveal a rising titre. A significant titre is 1 in 256.

If a second reading of results is made after the tubes have stood overnight at room temperature or in the refrigerator, they should be replaced at 37° C. for one to two hr. This avoids fallacious results from "cold agglutination" (p. 320) which is reversible at 37° C. and, so far as is known, is not associated with infective mononucleosis.

It should be noted that the reaction is negative in tuberculosis, leukaemia and Hodgkin's disease.

In persons who have recently received an injection of a therapeutic serum (from the horse), an apparently similar heterophile antibody (Forssman's antibody) may be present in considerable amount in the blood, since horse serum contains the appropriate heterophile antigen and stimulates the production of an antibody for sheep red cells (p. 61).

It has been pointed out by certain authors that the type of antibody present in infective mononucleosis differs in certain respects from the Forssman antibody, and also from that found in normal serum, and that this difference can be determined by agglutinin-absorption tests as follows:—

Antibody	Treated with emulsion of guinea-pig kidney	Treated with ox red cells
Normal serum	Absorbed	Not absorbed
After serum therapy	Absorbed	Absorbed
Infective mononucleosis	Not absorbed	Absorbed

It may be found, however, that the antibody present after serum therapy is not absorbed by ox red cells and only partially absorbed by guinea-pig kidney tissue, *i.e.* more resembling the antibody in normal serum (Dempster).

The following method, a modification of Barrett's technique,¹ may be adopted for determining these absorption effects.

¹ Barrett, A. M. (1941), *J. Hyg. (Lond.)*, **41**, 330.

Reagents.

1. *Patient's serum.*—1·0 ml. is required. Heat the serum in a water-bath at 56° C. for thirty min.

2. *Physiological saline.*

3. *20 per cent. guinea-pig kidney emulsion in saline.*—Take several fresh guinea-pig kidneys and, after removing any fat, cut into small pieces with scissors. Wash several times with saline to remove all the blood, and mash the tissue into a fine pulp in a mortar. To the pulp add four times its volume of saline and boil in a water-bath for one hr. Allow to cool, and add sufficient 5 per cent. phenol to give a final concentration of 0·5 per cent. Make up to original volume with distilled water.

4. *20 per cent. ox red cell suspension in saline.*—Make a 20 per cent. suspension of washed ox cells in saline and treat in exactly the same way as the 20 per cent. guinea-pig kidney emulsion described above.

(Note: Both these antigens keep well in the refrigerator.)

5. *2 per cent. suspension of sheep red cells.*—Wash the sheep cells in saline and make a 2 per cent. suspension in saline. The cells should be more than one day and less than seven days old.

The Test.

Use 3-in. $\times \frac{3}{8}$ -in. test-tubes.

In three separate test-tubes (*a*), (*b*) and (*c*) place:

- (*a*) 1·0 ml. saline.
- (*b*) 1·25 ml.¹ of guinea-pig kidney emulsion.
- (*c*) 1·25 ml.¹ of ox cell suspension.

To each tube add 0·25 ml. of heated serum. Allow to stand for one hr. at room temperature, and then centrifuge tubes (*b*) and (*c*).

Set up a rack containing three rows of 10 tubes. Into the last 9 of each row put 0·25 ml. saline. Into the first 2 tubes of the front row place 0·25 ml. of the diluted serum (*a*). From the mixture in the second tube carry over 0·25 ml. to the third tube, and continue doubling dilutions to the end of the row. Repeat this process using the supernatant fluid from (*b*) and (*c*) in the middle and back rows respectively.

To every tube add 0·1 ml. of the 2 per cent. suspension of sheep cells and mix thoroughly by shaking.

The final serum dilutions are 1 in 7, 1 in 14, 1 in 28, 1 in 56, etc.

The test is read, after the tubes have stood for twenty-four hr. at room temperature, by removing the tubes from the rack and attempting to re-suspend the cells by flicking the tubes with the finger. The end-point is the highest dilution of serum in which the cells cannot be evenly suspended. The end-point can be made more clear-cut if the tubes are centrifuged for two min. before re-suspension of the cells is attempted.

¹ The extra quantity of material in these tubes is because 1·25 ml. of a 20 per cent. emulsion or suspension contains only 1·0 ml. of fluid.



A preliminary report can be made after the test has been set up for an hour, if the tubes are centrifuged before making the reading, but a final report should be postponed until the following day.

A typical report in a case of glandular fever would be:

Heterophile agglutinin for sheep cells present in dilutions up to 1 in 448. The agglutinin is completely absorbed by ox cell suspension, but unaffected by guinea-pig kidney emulsion. Using the absorption technique, as detailed above, a titre of 1 in 28 in rows (*a*) and (*b*) is significant.

THE SHEEP CELL AGGLUTINATION TEST IN RHEUMATOID ARTHRITIS (ROSE-WAALER TEST)

Sheep red blood cells sensitised with a rabbit anti-sheep erythrocyte serum are agglutinated by the sera of 90 per cent. of patients with rheumatoid arthritis. It is thought that the serum factor concerned is a globulin which may represent an index of some inherited metabolic disturbance which predisposes the individual to rheumatoid arthritis (Kellgren & Ball, 1959).¹

REAGENTS.—*The sensitising serum* is prepared by the method on p. 340. Preserved serum containing glycerol is not satisfactory; the neat rabbit-serum is however stable in the frozen state for several months. In the test the serum is used at a dilution which just fails to agglutinate erythrocytes but which contains as high a haemolytic titre (see p. 340) as possible. Its haemagglutinating activity is determined by adding a 2 per cent. suspension of sheep red blood cells to two series of four doubling serum dilutions, one beginning at 1 in 50, and the other at 1 in 70. After incubation for 1 hr at 37° C. a reading is taken. The end point is the first tube in which cells show no agglutination and in which the cells have settled into a completely negative button.

Sensitised red cells are prepared by mixing equal volumes of the haemolytic antiserum at its determined requisite dilution and a 2 per cent. suspension of sheep cells. Sensitisation is rapid and the cells may be used within a few minutes.

The Test.—The patient's serum, previously inactivated for 30 min. at 56° C., is serially diluted in two-fold steps from 1 in 2 to 1 in 1024. Two sets of these dilutions are required; to one set is added an equal volume of sensitised cells while the second set serves as a serum control and receives an equal volume of 1.0 per cent. unsensitised sheep cells. The tubes are incubated for one hour at 37° C. and then placed in the refrigerator until the cells have completely settled. The reading is according to the pattern of sedimented cells and the end point is taken as the last tube to show definite haemagglutination.

Instead of the saline diluent some workers prefer to use 2.5 per cent. sheep serum in saline. This has the effect of increasing the sensitivity of the test without loss of specificity. The titre of positive sera is

¹ Kellgren, J. H., & Ball, J. (1959), *Brit. med. J.*, **1**, 523.

increased by this method from two- to eight-fold but the test is rather more difficult to read and end points are not so clear cut.

Serum titres of 16 or more are taken as positive and are found in rheumatoid arthritis in 90 per cent. of cases, and also in some cases of disseminated lupus erythematosus, scleroderma, and erythema multiforme.

AGGLUTINATION TESTS USED FOR THE SEROLOGICAL IDENTIFICATION OF CERTAIN ORGANISMS BY MEANS OF SPECIFIC ANTISERA

In this case the series of dilutions depends on the titre of the serum for the homologous organism. Thus, if the titre were 1 in 16,000, the following range of dilutions might be tested: 1 in 1000 to 1 32,000 in a series of doubling dilutions. In general, for identification of an unknown organism it should agglutinate in approximately as high a serum-dilution as a known homologous organism.

If the organism is a motile species and it is desired to identify both H and O antigens, formolised and alcoholised suspensions respectively are tested with H and O agglutinating antisera (*vide infra*). It should be noted that in the *Salmonella* group the H antigen may occur in two phases, one of which may have non-specific characters. The serological identification of these organisms is considered more fully on p. 589.

Special applications of the agglutination technique, e.g. in the identification of serological types, are referred to in later chapters.

SLIDE AGGLUTINATION

This method is useful where only small quantities of culture are available, as in the identification of the whooping-cough bacillus, or where agglutination is carried out with undiluted serum, e.g. in typing pneumococci or typing streptococci by Griffith's method, and it is necessary to use as small a quantity as possible. The method may be applied likewise for identifying organisms of the *Salmonella* and dysentery groups. Slide agglutination is only practicable when the clumping of organisms occurs within a minute or so; it is not suitable where the mixture of organisms and serum has to be incubated.

The procedure can be carried out quite readily on an ordinary slide, but where a number of agglutination tests have to be made it is more convenient to use a piece of $\frac{1}{4}$ -in. polished plate glass about 6 in. \times 2 in. A long horizontal line is ruled with a diamond through the middle of the glass from end to end and then a number of lines are ruled at $\frac{1}{2}$ -in. intervals at right angles to this line, thereby dividing the glass into a series of divisions.

A drop of saline is placed in one of the divisions and a small amount of culture from a solid medium emulsified in it by means of an inoculating loop. It is then examined through a hand-lens (8 or 10 \times), or

the low-power microscope (p. 254), to ascertain that the suspension is even and that the bacteria are well separated and not in visible clumps. With a small loop, $1\frac{1}{2}$ mm. diameter, made from thin platinum wire (about 32 gauge) take up a drop of the serum and place it on the slide just beside the bacterial suspension. Mix the serum and bacterial suspension and examine with the hand lens, or place on the stage of the microscope. Agglutination when it occurs is rapid and the clumps can be seen with the naked eye, but the use of some form of magnification is an advantage. For control purposes, two drops of saline can be placed in adjacent divisions and bacterial culture emulsified in both, one only being mixed with the serum. With streptococci a broth culture is used, and methods for obtaining suitable suspensions for the agglutination test are described on p. 487. Two drops of suspension are placed on the slide and a small loopful of the serum mixed with one of them and examined as described above.

After the test, the glass slide is wiped with a pedgelet of cotton-wool soaked in 3 per cent. lysol solution, washed under the tap and dried.

While the slide agglutination test is rapid and convenient, its limitations must be realised. In order to obtain rapid agglutination the serum is used undiluted or in low dilutions. In consequence, it may contain normal agglutinins which give non-specific agglutination with organisms other than that against which the serum was prepared. Thus, with regard to the *Salmonella* group particularly, slide agglutination with its high concentration of agglutinins may show low-titre reactions with organisms outside the group, e.g. paracolon bacilli, which may also have somewhat similar biochemical reactions. It is important therefore to confirm the slide test by quantitative tests in tubes, particularly when any doubt arises or where precise results from agglutination tests are desired.

PREPARATION OF AGGLUTINATING ANTISERA

The instructions given here apply particularly to organisms of the *Salmonella* and dysentery groups.

Rabbits are used for immunisation, and large healthy animals should be selected, not under 2000 g. in weight.

The purity and identity of the culture used should be carefully ascertained beforehand, and in view of variability in antigenic composition the culture selected should be such that it represents the motile "smooth" form and the specific phase of the particular species (pp. 67, 591).

The rabbits are injected intravenously (p. 419) at intervals of five to seven days with a suspension in saline of a twenty-four hr. slope culture killed by exposure for one hour at 60°C . The following series of doses may be given: 1 : 20, 1 : 10, 1 : 5, 1 : 3 and 1 : 2 culture. These doses are easily measured by emulsifying a slope culture in a given volume of saline and then injecting the appropriate fraction.

In the case of organisms of high toxicity, e.g. *Sh. shigae*, it is necessary to start with even lower doses, e.g. 1 : 100 of a culture.

Other methods for standardising dosage may be used, e.g. where the doses are stated in terms of the number of organisms, as in the administration of vaccines (p. 182), but the system indicated above is simple and sufficiently accurate for ordinary purposes.

With certain organisms, e.g. *Salmonella* group, higher titres may be obtained if living organisms are injected. To commence with, very small amounts, e.g. 0.01 ml., of a young living broth culture should be injected intravenously. As the animal becomes immune larger doses may be given until several ml. of the living culture can be tolerated.

When separate H and O agglutinating antisera are required for motile bacteria, immunisation is best carried out with selected strains known to be suitable for the purpose. For the production of the O agglutinin an alcoholised culture (p. 318) may be used as the antigen. A non-motile variant also serves well as a pure O antigen. In making tests with H and O antisera, formolised and alcoholised suspensions respectively are used.

Seven to ten days after the last injection a specimen of blood is withdrawn from an ear vein (p. 420) and the serum is tested for its agglutinating power towards the strain used for immunisation. A series of dilutions is tested and if agglutination occurs in a 1 in 1600 or higher dilution,¹ the animal is bled from the neck vessels or by cardiac puncture (p. 420), the blood is allowed to coagulate in a sterile stoppered or screw-capped bottle, placed overnight in the refrigerator, and the serum is then separated. 0.1 ml. of a 5 per cent. solution of phenol in physiological salt solution is added for each ml. of the serum—equivalent to 0.5 per cent. pure phenol. This prevents bacterial growth resulting from any accidental contamination. (Glycerol may also be used as a preservative, an equal volume being added to the serum, or 0.1 per cent. *p*-chloro-*m*-cresol.) The serum may be stored in 1-ml. or 5-ml. stoppered or screw-capped bottles, or ampoules may be used. Alternatively, it may be kept in sterile glass tubes (5–7 mm. bore), about 1 ml. in capacity, drawn out at both ends to capillary dimensions; the tubes are filled by suction, applying the mouth-piece used in pipetting (p. 316), and the ends are sealed in the Bunsen flame. The serum should be kept in the refrigerator (about 4° C.) and will retain its potency for long periods (three years). The temperature should not be allowed to fall below 0° C. if phenol is used as a preservative, as the solidification of the serum by freezing may be deleterious owing to the separation out of the phenol in the pure state. If a refrigerator at –20° C. is available, antisera frozen solid can be preserved over a long period. In this case no preservative is added to the serum.

Antisera can be preserved in the dry state by the methods described on p. 337 for the preservation of complement. The potency of antisera is retained over a considerably longer period when dried than when stored in fluid form.

¹ More powerful agglutinating sera may, of course, be obtained; 1 in 1600 is merely the *minimum* titre which should be aimed at.

AGGLUTININ-ABSORPTION TESTS

Agglutinins, like other antibodies, combine firmly with their homologous antigens, and by treating an agglutinating antiserum with the homologous bacteria and then separating the organisms by centrifuging, it is found that the agglutinin has been "absorbed" or removed by the organisms from the serum.

In certain cases, to prove the serological identity of an unknown strain with a particular species, it may be necessary to show not only that it is agglutinated by a specific antiserum to approximately its titre but also that it can absorb from the serum the agglutinins for the known organism. This becomes necessary owing to the fact that, on immunising an animal with a particular bacterium, "group antibodies" for allied organisms are developed, and in some cases these may act in relatively high titre. "Absorption" with a heterologous strain would only remove the group agglutinins without affecting the specific agglutinin. These effects are exemplified in the *Salmonella* and *Brucella* groups (pp. 573, 642). The general method of carrying out such absorption tests is to mix a dense suspension of the organism —e.g. twenty-four hours' growth on a 4-in. plate of nutrient agar, suspended in 1 ml. saline and killed at 60° C. (thirty-min.)—with an equal volume of a suitable dilution of the serum, e.g. sixty-four times the concentration of the known titre. (The bacterial growth must have been thoroughly washed with normal salt solution, i.e. by mixing with several volumes of saline, centrifuging and repeating the process 2–3 times.) Thus, if the titre is 1 in 1600, the dilution used would be 1 in 25. The mixture is incubated for three to four hr. at 37° C. and the serum is then separated from the bacteria in a high-speed centrifuge. (In some cases for complete absorption the process may require to be repeated with a similar fresh quantity of bacteria.) The dilution of the serum would now be approximately double the original dilution—in the example taken (*vide supra*) 1 in 50. From the treated serum a series of doubling dilutions is prepared as in direct agglutination tests (p. 315), so that, when an equal volume of bacterial suspension is added, the series will reach to the known titre of the serum. In the example taken above, the following series of dilutions would be tested:

1 in 50 1 in 100 1 in 200 1 in 400 1 in 800,

and after the addition of bacterial suspension these would become

1 in 100 1 in 200 1 in 400 1 in 800 1 in 1600

A control tube is also included, containing suspension but no serum, and the general technique is that employed in direct agglutination tests.

Thus, the identity or non-identity of an unknown culture (*X*) with a known (*A*) may be investigated by agglutinin-absorption as follows:

1. Absorb, as above, antiserum to *A* with a dense suspension of organism *X* = *X*-absorbed serum.

2. Test the agglutinating power of *X*-absorbed serum for *A* and *X*

(A control test would show that the antiserum to *A* after absorption with *A* agglutinates neither organism.)

Results:

(a) The absorbed serum agglutinates neither *A* nor *X*. This indicates that the organisms are identical, because *X* has absorbed agglutinins for *A*; to establish this conclusion completely an antiserum to *X* after absorption with *A* should agglutinate neither organism.

(b) The absorbed serum fails to agglutinate *X*, but still agglutinates *A*. This shows that the organisms are not identical, because *X* has not absorbed the agglutinins for *A*, though it has removed the heterologous agglutinins.

FLOCCULATION TESTS FOR SYPHILIS

The direct mixture of syphilitic sera with antigens of the type used in the Wassermann reaction results in the appearance of a flocculent deposit which is easily seen with the hand lens and which may also be visible to the naked eye. Such reactions, however, may occur in non-syphilitic infections (e.g. tuberculosis, leprosy, malaria, hepatitis, infectious mononucleosis, etc.) and the tests are therefore sensitive to many different types of infection. It is, however, possible to reduce the sensitivity of the reaction by adjusting the conditions under which the test is performed and to render the test almost specific for syphilis; many flocculation reactions give results which are closely parallel to those of the complement-fixation technique. The value of flocculation tests lies in the simplicity of the technique employed and the fact that they can be carried out in places where complement and the reagents of the haemolytic system of the Wassermann test are not available. Ideally the tests are used as a first screening investigation and any positive sera are then subjected to the full Wassermann test. Many varieties of the flocculation reaction are described and the Meinicke, Hinton, Mazzini and Kahn tests are all in common use together with the test employing the cardiolipid antigen devised by the Venereal Disease Research Laboratory. This latter test has been in use in the Bacteriology Department of Edinburgh University and can be recommended as a simple and reliable technique.

THE V.D.R.L. FLOCCULATION TEST

A rapid screening test which is simple to perform is of great value in dealing with large numbers of sera. The following method is that described in the Bulletin of W.H.O.¹ and is very satisfactory. The following reagents are required.

(1) *Antigen.* This has the following composition:

Cardiolipin	0.03 per cent.
Lecithin	0.24 per cent.
Cholesterol	0.9 per cent.

¹ Bull. World Hlth Org. (1951), 4, 151.

It may be purchased from Messrs. Burroughs Wellcome & Co., London.

(2) *Diluent.* Buffered saline prepared as follows:

Formaldehyde, neutral reagent grade	0.5 ml.
Na ₂ HPO ₄ , 12H ₂ O	0.093 g.
KH ₂ PO ₄	0.170 g.
NaCl	10.0 g.
Distilled water	1000 ml.

This solution has a pH of 6.0 ± 0.1.

(3) *Unbuffered Saline.* 1.0 per cent. sodium chloride.

(4) *Serum under test.* Prepared and inactivated as for the Wassermann test.

(5) *Antigen Emulsion.* In a stoppered bottle place 0.4 ml. of the buffered saline and add to it drop by drop from a pipette 0.5 ml. of antigen. Ensure that the antigen is added during a period of approximately six sec. and that the bottle is continuously rotated during this time. After the addition, the bottle is rotated vigorously for a further ten seconds. Now add 3.6 ml. of 1.0 per cent. unbuffered saline. Mix well and allow to stand for five min., but not longer than two hr., before use.

The Qualitative Serum Test

Use 3 × $\frac{1}{2}$ in. tubes. Transfer 0.5 ml. inactivated serum to a tube and add to it 0.5 ml. diluted antigen. Place in a rack in the Kahn shaker and shake for five minutes. The tubes are now spun at 2000 r.p.m. for ten minutes in a straight-headed centrifuge. After this the tubes are shaken again for exactly one minute and the test is read at once. Visible aggregation in a clear or very faintly turbid medium are read as positive. A slightly turbid appearance with a "silken swirl" on gentle shaking is the typical negative appearance. All borderline reactions should be reported as negative. The test can be used quantitatively with serial doubling dilutions of the patient's serum, ranging from 1 in 2 to 1 in 64. The weakest dilution giving a positive reaction is reported as the titre of the serum.

THE KAHN FLOCCULATION TEST

Apparatus required—

- (1) Small test-tubes, 3 × $\frac{1}{2}$ in., as used in the Wassermann test; these tubes should be of perfectly clear glass and thoroughly clean.
- (2) Flat-bottom glass cylinders, 1 $\frac{1}{2}$ × $\frac{1}{2}$ in., for the preparation of the diluted antigen.
- (3) 1-ml. and 0.1-ml. graduated pipettes as used in the Wassermann test.
- (4) Special pipettes: one graduated from the tip to deliver 0.0125, 0.025 and 0.05 ml. respectively; the other with one graduation to deliver 0.15 ml.
- (5) Suitable racks for the tubes.

Reagents—

- (1) Patient's serum—at least 0.5 ml. required.
- (2) Antigen¹—"Bacto" Kahn standard antigen which can be obtained commercially² is also recommended as being satisfactory in stability and sensitivity. Alternatively, it can be made from "Bacto" Beef Heart.²
- (3) 0.85 per cent. sodium chloride in distilled water.
- (4) Control sera—at least four should be included in any set of tests; these should be selected according to previous results as follows: Negative, +, ++, +++ (*vide infra*). All sera tested are heated at 55° C. for thirty minutes before testing.

Dilution of antigen—

The antigen is diluted with saline in the proportions prescribed for the preparation—usually 1: 1.1.

(1) Ascertain the total volume of diluted antigen required for the set of tests by multiplying the number of sera by 0.0875 ml. (the volume of diluted antigen required for one serum) and adding to this figure 0.3 ml. for loss in pipetting, etc. Not more than is sufficient for 40 tests should be made up at one time.

(2) Pipette *separately* into each of two small cylinders (referred to above) the volumes of normal saline and undiluted antigen required to yield in the prescribed proportions the total bulk of diluted antigen.

(3) Add the saline from one cylinder rapidly to the antigen in the other and mix by pouring from one cylinder to the other five or six times.

The diluted antigen should be used for the test not less than ten minutes and not more than thirty minutes after mixture.

The *test* for each serum is set up as follows:

Tube	1	2	3
Add <i>diluted antigen</i> . . .	0.05 ml.	0.025 ml.	0.0125 ml.
Add <i>serum</i>	0.15 ml.	0.15 ml.	0.15 ml.

The tubes are shaken by hand or preferably in a special shaking machine at 270 oscillations a minute for three min. (After shaking, incubation in a water-bath at 37° C. for fifteen min. or an incubator at 37° C. for twenty min. is advantageous.)

Then add *saline* 1.0 ml. 0.5 ml. 0.5 ml.

Readings are now made.

¹ Obtainable from Medical Research Council Venereal Diseases Reference Laboratory, Ashfield Street, Whitechapel, London, E.1. For full details of the test and the preparation of the antigen see Kahn (1928), *The Kahn Test*, Baltimore, Md.

² Obtainable from Baird & Tatlock Ltd., London.

The following *antigen control* is included in each set of tests:

Tube	1	2	3
Add <i>diluted antigen</i>	0.05 ml.	0.025 ml.	0.0125 ml.
Add <i>saline</i>	0.15 ml.	0.15 ml.	0.15 ml.
Shake tubes as above.			
Incubate as above.			
Then add <i>saline</i>	1.0 ml.	0.5 ml.	0.5 ml.

*Note.*¹—Instead of using a calibrated volumetric pipette for the antigen dilution, a dropping pipette, external diameter at the tip equivalent to a No. 55 Morse gauge hole of a Starrett plate, may be substituted. One drop from this pipette equals 0.0125 ml., two drops equal 0.025 ml., and four drops equal 0.05 ml. For patient's serum a pipette with external diameter of 2.8 mm. will deliver 0.15 ml. of serum in three drops at the rate of one drop per sec.

Reading of results.—The tubes should be held in a sloped position and the fluid viewed (if necessary with an 8× hand-lens) in a strong light against a dark background.

The following results may be observed in individual tubes:

— = the fluid remaining uniformly opalescent.

+ = minute floccules just visible to the naked eye throughout the fluid.

+++ = large floccules sedimenting completely in the tube.

++ and +++ = intermediate degrees of flocculation.

The interpretation of results is illustrated as follows:

Tube	1	2	3	Average Result	Diagnostic interpretation
Serum A	+++	+++	+++	+++	Strongly positive
" B	++	+++	+++	++	Strongly positive
" C	+++	++	++	++	Positive
" D	++	++	+++	++	Weakly positive
" E	++	+	++	+	Doubtful
" F	+	++	++	—	Negative
" G	+	+	++	—	
" H	—	±	++	—	
" I	—	—	—	—	

THE KAHN VERIFICATION TEST²

This test was introduced by Kahn with the object of ascertaining whether weak or doubtful reactions obtained by his standard flocculation test are non-specific or definitely significant of syphilitic infection. From studies of "false positive" reactions he concluded that a positive

¹ See Khairat, O. (1952), *Brit. med. J.*, **1**, 582.

² Kahn, R. L. (1941), *J. Lab. clin. Med.*, **26**, 139; and (1940), *Arch. Derm. Syph.*, **41**, 817; Beveridge, W. J. M. (1943), *Edin. med. J.*, **50**, 344.

result may in some cases be related to biological changes apart from syphilic infection but that this non-specific reaction can often be differentiated from the true syphilis reaction by the occurrence of a stronger effect at 2° C. than at 37° C. or the absence of flocculation at the higher temperature. He has called this the "general biologic type" of reaction and regards it as non-syphilitic, the reaction in the syphilitic case being usually stronger at 37° C. than at lower temperatures.

In carrying out such comparative tests at different temperatures the reagents before mixing must be adjusted to the particular temperature. Thus, for the test at 37° C., pipettes, tubes and racks are placed in a 37° C. water-bath for fifteen minutes before performance of the test, and likewise the diluted antigen, serum and saline are similarly kept at 37° C. before the mixtures are made; further, at all stages of the test the required temperature is maintained as far as possible. In carrying out the test at the low temperature an ice-water bath can be used in the same way as the 37° C. water-bath, the working temperature being about 2° C. Otherwise the test is performed as in the standard procedure (p. 330).

The various types of comparative result are illustrated in the following Table:

At 37° C.	At room temperature.	At 2° C.	Classification of result
+ + + + or + + + + +	+ + -	+ or - -	} Syphilitic
+ or - -	+ + -	+ + + + or + + + + +	} General Biologic
+ + - + + -	+ + + - -	+ + - + + -	} Inconclusive
-	-	-	Negative

It may be noted that the application of this "verification" test has shown that the sensitivity of the syphilitic reaction is increased at 37° C. as compared with room temperature, *i.e.* the temperature of the standard test.

The comparative test at 37° C. and 2° C. is of value when the usual Kahn reaction is weak or doubtful or when there is some discrepancy between the serological result and the clinical findings. It is of similar value when other syphilis serum tests also give a weak or doubtful result. Of course, it must be recognised that in a proportion of such cases even the "verification" test is inconclusive.

COMPLEMENT FIXATION METHODS

WASSERMANN SYPHILIS REACTION

This reaction depends on the "fixation" of complement by a suspension of a phosphatide lipoid (similar to lecithin and extracted from certain normal animal tissues) along with the *heated* serum of a person infected with syphilis, and constitutes an important diagnostic test.

The effect is not a true immunity reaction, though the lipoid suspension *plus* syphilitic serum fixes complement in the same way as a bacterial or other antigen *plus* its homologous antiserum (p. 68). Possibly the lipoid plays the part of a hapten (p. 58) in this reaction. Though some observers have attributed the phenomenon to a physico-chemical change in the serum, the evidence suggests that it depends on the presence of a "lipoidophile" antibody-like principle which is stable at 55° C.

For complement-fixation tests, an indicator of the presence of complement is required. The "haemolytic system" used in these tests serves this purpose (p. 339). It consists of the red corpuscles of a particular animal species "sensitised" with the corresponding haemolytic antibody, *e.g.* the red cells of the ox or sheep *plus* the serum of a rabbit which has been immunised with the red cells of the species used. The immune body in the serum is thermostable. The serum is heated at 55° C. to annul the natural complement, and stored in bottles or tubes or preserved in the dry state (*vide infra*). The heated serum is non-haemolytic by itself, but in the presence of a suitable complement brings about lysis of the homologous red corpuscles. Fixation of complement is denoted by the absence of lysis in the haemolytic system.

In its simplest form the Wassermann reaction can be represented as follows:

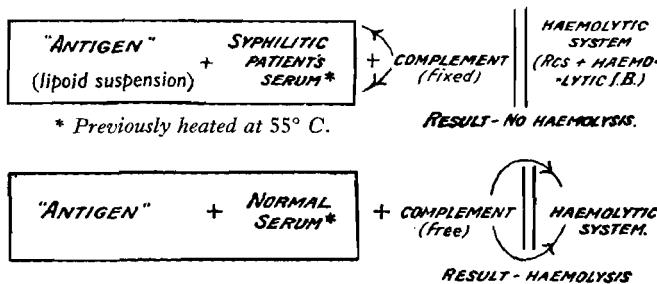


FIG. 20

It should be noted that the Wassermann test must be carried out on a quantitative basis. Not only must it indicate whether the reaction is positive, but the various degrees of the reaction have also to be determined, from a strong positive (+++) to a weak positive (+) or a

doubtful positive (\pm). Quantitative testing is most important in assessing the value of treatment or the completeness of cure.

The technical application of the reaction demands a very accurate standardisation of each reagent. Further, the amount of complement used in relation to the quantities of antigen and serum must be adjusted with such delicacy that the weakest reactions can be accepted as significant.

Several modifications of the test are employed, although the essential principles are the same. Some workers use constant amounts of antigen and of the patient's serum with varying amounts of complement. An example of this method is Harrison's technique as modified by Wyler¹ and described in previous editions. Other workers hold the amounts of antigen and complement constant and employ varying amounts of patient's serum. Two examples of the latter procedure are described. Method I (Price's Modification of the Harrison-Wyler technique)² is a convenient and rapid one-tube screening procedure in which small volumes of the reagents are added by a dropping technique.

Method II (Kolmer) employs larger volumes measured by graduated pipettes. Overnight fixation in the cold at 4° C. is used. This method has the advantage that it can be adapted readily for use with other antigens such as those prepared from the Reiter spirochaete or from other bacteria and viruses.

Method I

Antigen

Fresh ox hearts are obtained from the slaughter house and are freed of fat and connective tissue. They are cut into pieces no longer than 1 cm. and weighed. About 50 g. of the cut muscle is pounded with powdered glass in a mortar for a minute or two. This mixture is transferred to a 2 litre flask and 9 ml. absolute alcohol are added for every gram of muscle. Not more than 100 g. muscle should be treated in one flask. The flask is now tightly stoppered, shaken thoroughly, and left for five days at room temperature in a dark cupboard. The flask must be shaken well every day. The mixture is now filtered through No. 1 Whatman filter paper at room temperature and is stored in the refrigerator overnight. Filter once again through Whatman No. 1 filter paper while still cold in the refrigerator. Store the filtrate in amber glass bottles away from the light in a cool place.

For use, 6 parts of the alcoholic extract are added to 4 parts of a 1 per cent. solution of cholesterol and this mixture is then diluted with saline according to the optimal titre of the antigen. If, for example, the optimal titre is 320, 0.25 ml. of the cholesterol antigen mixture is accurately pipetted to the bottom of a 100 ml. measuring cylinder and 30 ml. saline is measured into a similar cylinder. The saline is now poured *rapidly* into the measure containing the antigen and the mixture

¹ Wyler, E. J. (1929), "The Wassermann Test". *Spec. Rep. Ser. med. Res. Coun. (Lond.)* No. 129; (1934), *J. Path. Bact.*, **39**, 521.

² Price, I. N. O. (1950), *Brit. J. vener. Dis.*, **26**, 172.

is completed by pouring from one cylinder to the other six times. The resultant opalescent mixture is the antigen to be used in the test; it should stand for twenty minutes before use and can be used throughout the working day. It should not be used after eight hours.

Human heart muscle has always been reputed to make a very sensitive Wassermann antigen but recent work indicates that it may give non-specific results.

Before use the antigen must be tested in the following three ways: (1) it must be shown to have complement-fixing ability; (2) the anti-complementary action of the antigen must be determined by itself and also in the presence of normal serum; and (3) the antigen must be titrated by the method of optimal proportion to discover the appropriate dilution to be used in the test. With commercially prepared antigens these steps have usually been carried out by the makers who supply instructions for the use of their product in the test. Full details of the method of titration of antigens are given by Price¹ but the essential steps are as follows. First, the anticomplementary activity of the antigen in the presence of normal serum is determined. To accomplish this a chess-board titration is set up with a series of antigen dilutions and a series of complement dilutions. The antigen is prepared in seven doubling dilutions from 1 in 20 to 1 in 1280 and the complement in seven dilutions ranging from 1 in 10, 1 in 20 and so on to 1 in 70. Each tube also contains normal human serum and the haemolytic system (*vide infra*). When the titration is read the complement titre for each antigen dilution is recorded and from these figures the "diagnostic doses" of complement needed in the next step is calculated by the method on p. 338.

The Optimal Proportion Titration of the Antigen.—Employs the same series of antigen dilutions that were used in determining the anti-complementary activity. They are set up in a fresh chessboard titration against a series of seven dilutions of a positive serum ranging from 1 in 10, 1 in 20 to 1 in 70. To each serum-antigen mixture the appropriate "diagnostic dose" of complement, and (after an interval for fixation) the haemolytic system is added. The known positive serum is preferably of moderate strength with a titre of about 1 in 40. The end point of the titration is that antigen dilution which reacts with the greatest dilution of the serum. It is often necessary to read the result by interpolation and since the reactivity may be spread over a zone of antigen dilutions it is permissible to take the mid-point of the zone as an end point. As a final step this antigen dilution is taken as a working suspension and retitrated to determine the amount of it to be used in the test proper. The antigen suspension is now diluted in saline in a series of seven doubling dilutions ranging from 1 in 2 to 1 in 64. It is next set up in a chessboard titration against the positive serum diluted 1 in 2, 1 in 4 and so on to 1 in 64. The volumes of reagents used and the technical procedures are those described on p. 342.

¹ Price, I. N. O. (1950), *Brit. J. vener. Dis.*, **26**, 33.

Cardiolipin (as Antigen in the Syphilis Serum Reactions)

This substance isolated from the phosphatide fraction of lipoidal extracts of heart muscle, has been extensively used in recent years in the Wassermann and flocculation tests for syphilis. It has been claimed that cardiolipin yields more specific results than those obtained with the unpurified lipoid preparations usually employed as antigen in these reactions. It is also supposed that the specific activity of these lipoid preparations with syphilitic sera depends on "cardiolipin". This substance contains phosphorus but no nitrogen; on saponification it yields fatty acids, a non-reducing carbohydrate, and phosphoric acid.

In the syphilis reactions it is used along with lecithin and cholesterol, and preparations containing an appropriate admixture of these constituents are available for diagnostic tests, e.g. the cardiolipin (Whitechapel) antigen, obtainable from Burroughs Wellcome & Co., London, which has the following composition: cardiolipin 0.05 per cent., lecithin 0.05 per cent., and cholesterol 0.5 per cent. The titre in which the antigen should be used is specified by the makers. This antigen is suitable for the Wassermann test, in substitution for the original antigen employed in this method.

Patient's Serum

A specimen of blood is obtained by vein puncture (p. 261) as for blood culture. The blood is then placed in a sterile stoppered test-tube or screw-capped bottle (p. 177) and allowed to coagulate. It is advisable to obtain about 5 ml. of blood. The serum is pipetted off after separation and heated in a water-bath at 55° C. for 30 min. Heating *eliminates the fallacy of non-specific fixation effects which may occur with normal unheated sera plus the antigen*; it also deprives the serum of its complementing property.

It is thought that the albumen fraction in unheated syphilitic serum may act as a protective colloid and that it tends to reduce precipitation in flocculation reactions. Heating the fraction eliminates this effect.

Complement

Fresh or specially preserved guinea-pig serum is used. It contains an active haemolytic complement for the red corpuscles of the ox or sheep sensitised with the homologous haemolytic antibody. When fresh serum is used, the blood is obtained twelve to eighteen hours before the test by severing the large vessels of the neck over a 6-in. funnel, from which the blood is collected in a measuring cylinder; it is allowed to coagulate and stand overnight in the refrigerator. The complement in serum too recently withdrawn is apt to be excessively "fixable", and in consequence is unsuitable for the Wassermann test.

If possible the pooled serum of several guinea-pigs should be used.

It should be noted that complement is unstable and deteriorates on keeping at ordinary temperatures. It is advisable throughout the experiment to keep the guinea-pig serum on ice.

It is now a general practice to use specially preserved serum pooled from a number of animals.

Preservation of Complement

For the preservation of complement two principles have been applied: (1) rapid drying of the serum from the frozen state *in vacuo* ("freeze-drying") and the reconstitution of the serum when required by dissolving the dried material in the appropriate amount of distilled water; this is exemplified by *Rayner's method* for the preservation of bacterial cultures and serum as described on p. 271; and this technique is also recommended for complement-serum, particularly when the complement may not be used for some time; (2) addition to the liquid serum of sodium chloride or other salts in hypertonic concentration; this is exemplified by *Richardson's method* and the *sodium acetate boric acid method*. Preservation of the complement-serum in the liquid state constitutes a simple and convenient procedure.

Richardson's Method.—Preservation of liquid complement-serum in hypertonic salt solution is effective provided the pH is adjusted to 6–6.4. A convenient method, employing borate-buffer-sorbitol for control of pH, is described here (Richardson, 1944).¹

Two stock solutions, which keep indefinitely, are used:

(A) Boric acid (H_3BO_3) 0.93 g., borax ($Na_2B_4O_7 \cdot 10H_2O$) 2.29 g., and sorbitol ($C_6H_{14}O_6 \cdot \frac{1}{2}H_2O$) 11.74 g. are dissolved in and made up to 100 ml. with saturated NaCl solution. The resulting molar concentrations are: 0.27 M boric acid, 0.12 M sodium borate, 0.6 M sorbitol in saturated sodium chloride.

(B) Borax 0.57 g. and sodium azide (NaN_3) 0.81 g. are dissolved in and made up to 100 ml. with saturated NaCl solution. The resulting molar concentrations are: 0.03 M boric acid, 0.03 M sodium borate, 0.125 M sodium azide in saturated sodium chloride.

To preserve complement-serum, mix 8 parts of serum with 1 part of solution B, followed by 1 part of solution A. This treated serum keeps very well even at room temperature. At 0°–3° C., loss of titre is not noticeable until after six to nine months. The mixture contains 0.03 M boric acid, 0.015 M sodium borate, 0.06 M sorbitol, and 0.0125 M sodium azide.

For use as 1 in 10 complement, 1 part of preserved serum is diluted with 7 parts of distilled water. Any further dilution from the 1 in 10 mixture is made with saline. Diluted serum should not be kept more than an hour or two. According to Richardson, no case of faulty behaviour in the Wassermann reaction attributable to preserved serum has come to notice.

Preservation by Sodium Acetate.—A very simple and most convenient method of preserving complement is to add to the serum an equal volume of a solution of 12 per cent. sodium acetate and 4 per cent. boric acid in distilled water.² The serum is kept in sterile screw-of the serum and the fixability of the complement in the Wassermann capped bottles at approximately 4° C. The full haemolytic activity reaction are maintained for about six months. It should be noted

¹ Richardson, G. M. (1941), *Lancet*, **2**, 696.

² Sonnenschien, C. (1930), *Z. ImmunForsch.*, **67**, 512.

in using this preserved complement that it represents a 1 in 2 dilution of the original serum.

It should be noted that traces of zinc reduce the haemolytic activity of complement. Since anaerobic preparations of sodium chloride do not at present limit the presence of zinc, it is a wise precaution to check the diluents and solutions used in preparing complement. Traces of zinc are detected by turbidity on the addition of a freshly prepared solution of 0.1 per cent. sodium di-ethyl-dithiocarbamate (Wilkinson).¹

Titration of Complement

Price's method (1949)² is recommended. A pool of six or more normal sera and another of strongly reacting positive sera are required. Prepare a series of ten dilutions of complement in saline ranging from 1 in 10, 1 in 20 to 1 in 100. Set out five rows of $3 \times \frac{1}{2}$ in. tubes as follows:

Row 1 has 10 tubes, to which are added:

- 1 volume complement at dilutions 1 in 10 to 1 in 100
- 2 volumes saline.

Row 2 has 5 tubes, to which are added:

- 1 volume complement dilutions at 1 in 10 to 1 in 50
- 1 volume saline
- 1 volume antigen diluted as in test.

Row 3 has 5 tubes, to which are added:

- 1 volume complement dilutions at 1 in 10 to 1 in 50
- 1 volume saline
- $\frac{1}{5}$ volume pooled normal serum
- 1 volume antigen.

Row 4 has 7 tubes, to which are added:

- 1 volume complement dilutions at 1 in 10 to 1 in 70
- 2 volumes saline
- $\frac{1}{5}$ volume pooled positive serum.

Row 5 has 7 tubes, to which are added:

- 1 volume complement dilutions at 1 in 10 to 1 in 70
- 2 volumes saline
- $\frac{1}{5}$ volume normal serum.

The usual volume employed in the test is 0.11 ml., as in the Harrison-Wyler technique, and the reagents are conveniently added by standard dropping pipettes attached to suitable separating funnels. Droppers of three different sizes are required (Donald's Method) (1) for *saline*, *complement* and *sensitised cells* a piece of glass tubing is drawn out, inserted into a Rawco gauge and cut squarely at a point where its outside diameter is 0.75 cm. (2) For the *antigen* suspension a dropper is cut in the same manner with an outside diameter of 0.9 cm. (3) For *undiluted human sera* the pipette dropper is inserted into the No. 56 hole in the Starrett gauge and cut as near to the surface of the gauge as possible.

¹ Wilkinson, A. E. (1950), *J. clin. Path.*, **3**, 363.

² Price, I. N. O. (1949), *Brit. J. vener. Dis.*, **25**, 157.

Pipettes (1) and (2) deliver per drop 0.11 ml. of the reagents for which they are designed. Pipette (3) delivers in 1 drop 0.022 of inactivated human sera, *i.e.* $\frac{1}{5}$ the volume of that discharged by (1) and (2). It is necessary to check the pipettes for accuracy before use.

When the reagents have been added, the rack is incubated for one hr. in a 37° C. water-bath. At the end of this period, the rack is removed from the bath and to every tube is added 1 volume of sensitised red blood cells. The rack is returned to the bath for a further thirty min. and the results are then read. The last tube in the serial dilutions to show complete sparkling haemolysis is taken as the end-point.

Two amounts of complement will be needed in the test proper: (A) *For the Serum Controls*.—This dilution of complement is the highest to show complete haemolysis in Row 4 or 5. *This is the serum control dose.*

(B) *For the Diagnostic Test*.—This dose is calculated by taking the end-point in Row 3 and multiplying it by $\frac{5}{4}$. For example, if 1 in 40 were the end-point then the complement would be used at a dilution of $\frac{1}{40} \times \frac{5}{4} = \frac{5}{160} = \frac{1}{32}$. *This is the diagnostic dose.* The 25 per cent. margin of extra complement used is sufficient to cover the occasional anti-complementary activity of normal sera.

Haemolytic System

With guinea-pig complement, a haemolytic system consisting of sheep red corpuscles sensitised with the appropriate haemolytic antibody is used.

Defibrinated blood is obtained at the abattoir (p. 206). The required quantity is thoroughly mixed with several volumes of normal saline and then centrifuged to separate the corpuscles, the supernatant fluid being pipetted off. This process has generally been designated "washing" the blood corpuscles and is repeated three or four times. The centrifuged deposit of corpuscles after the final washing is suspended in normal saline to form a 6 per cent. suspension.

Standardisation of the Red Blood Cell Suspension

Transfer exactly 1.0 ml. of the suspension to a special haematocrit tube (Price & Wilkinson, 1947).¹ After centrifuging for ten minutes at 2500 r.p.m., the height of the column of packed cells is read off. The standard packed cell volume required in the test is 0.05 ml. and the original 6 per cent. suspension is adjusted to this content by dilution by simple proportion. The following example shows the method of calculation:

$$\begin{array}{l} \text{6 per cent. suspension packed volume} = 0.057 \\ \text{Desired packed cell volume . . .} = 0.050 \\ \text{Dilution factor } \frac{0.057}{0.050} . . . = 1.14 \end{array}$$

¹ Price, I. N. O., & Wilkinson, A. E. (1947), *Brit. J. vener. Dis.* 23., 124. Obtainable from Messrs. R. B. Turner & Co. Ltd., London.

Thus, 0.14 ml. saline should be added to each 1.0 ml. of the original 6 per cent. cell suspension.

Equal volumes of this standard suspension and saline containing 12 M.H.D. of the haemolytic serum per unit volume are mixed, shaken vigorously, and incubated in the water-bath for thirty minutes at 37° C. Some workers prefer to ensure complete mixing by passing a current of air through the suspension during the period of incubation.

Preservation of Sheep Red Blood Cells

Sheep blood for complement fixation tests may be preserved at 4° C. in an equal volume of sterile modified Alsever's solution¹ consisting of:

Glucose	2.05	per cent.
Sodium chloride	0.42	per cent.
Trisodium citrate	0.8	per cent.
Citric Acid	0.055	per cent.
in Distilled Water		

Sheep cells have been satisfactory for use for a period of six weeks after collection in this solution.

Haemolytic Antiserum

The following method (Darter, 1953)² is recommended. Rabbits receive on alternate days a series of five *intracutaneous* inoculations of whole sheep blood in the following doses: 0.5, 1.0, 1.5, 2.0, 2.5 ml. These are followed on the twelfth and fifteenth days by the intravenous inoculation of 1.0 ml. of a 20 per cent. suspension of sheep red blood cells in normal saline to which has been added 0.01 per cent. magnesium sulphate. A trial bleeding is taken from the rabbit's ear on the eighteenth day and if the haemolysin content of the serum is satisfactory (the titre should be over 1 in 10,000) the rabbit is exsanguinated and the serum is separated. If the titre is not high, further intravenous injections are given. High-titre serum is usually preserved by adding to it an equal volume of sterile glycerol.

The haemolysin titre of the serum (sometimes referred to as its minimum haemolytic dose) is estimated as follows: Set out ten tubes and add to them saline according to the following tables.

	Tube No.									
	1	2	3	4	5	6	7	8	9	10
Saline solution .	None	ml. 0.5	ml. 1.0	ml. 1.5	ml. 2.0	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5	ml. 0.5

¹ Muschel, L. H., & Lowe, K. M. (1955), *J. Lab. clin. Med.*, **46**, 147.

² Darter, L. A. (1953), *J. Lab. clin. Med.*, **41**, 653.

Prepare a 1 in 1000 dilution of the haemolytic serum and add 0.5 ml. of it to the first five tubes of the titration. Then proceed as follows:

Tube No.	Process	Final haemolysin dilution
1	None	1: 1,000
2	Mix. Discard 0.5 ml.	1: 2,000
3	Mix. Transfer 0.5 ml. to tube 6. Discard 0.5 ml.	1: 3,000
4	Mix. Transfer 0.5 ml. to tube 7. Discard 1.0 ml.	1: 4,000
5	Mix. Transfer 0.5 ml. to tube 8. Discard 1.5 ml.	1: 5,000
6	Mix. Transfer 0.5 ml. to tube 9	1: 6,000
7	Mix. Transfer 0.5 ml. to tube 10	1: 8,000
8	Mix. Discard 0.5 ml.	1: 10,000
9	Mix. Discard 0.5 ml.	1: 12,000
10	Mix. Discard 0.5 ml.	1: 16,000

Now add to each tube 0.5 ml. of a 1 in 10 dilution of complement and 0.5 ml. of the 3 per cent. sensitised red blood cells prepared as above. Incubate for one hour at 37° C. in a water-bath; the last serum dilution to show complete haemolysis is taken as the end-point. The lowest titre which is acceptable for the purpose of sensitising red blood cells for the Wassermann reading is 1 in 1000.

Burroughs Wellcome haemolytic serum for *sheep* red corpuscles may conveniently be used in preparing a haemolytic system for the test. This anti-sheep haemolytic serum is obtained from the horse. It tends to exert a pronounced agglutinating effect on the homologous corpuscles, with rapid sedimentation of the cells. It is advisable therefore to add the serum to the corpuscles just before the haemolytic system is required.

The following method (as used in the U.S. Army Medical Centre, Washington, D.C.¹) can be recommended for preparing a haemolytic antiserum for sheep's corpuscles by immunisation of rabbits without shock-reactions which are liable to occur by the usual immunisation procedure.

To 1 ml. of fresh, washed, packed sheep cells add 1 ml. of undiluted fresh or reconstituted dried complement and 0.3 ml. of undiluted haemolytic antiserum having a titre of 1: 2000 or greater. Incubate in the 37° C. water-bath for fifteen min., dilute to 15 ml. with 0.85 per cent. salt solution, then centrifuge at high speed for five min. (3000 r.p.m.). The supernatant fluid is drawn off, discarded, and 15 ml. of fresh salt solution is added to the packed stroma. Mix by inverting the tube several times and centrifuge at high speed for thirty minutes, draw off the supernatant fluid and discard. About 0.5 ml. of stroma remains in the bottom of the centrifuge tube. This

¹ Sawyer, H. P., & Bourke, A. R. (1946), *J. Lab. clin. Med.*, 31, 714.

is again mixed with 15 ml. of 0.85 per cent. salt solution to remove the traces of haemolytic antiserum, complement and haemoglobin present, and then centrifuged at high speed for thirty min. The supernatant fluid is drawn off and the stroma made up to 2 ml. with 0.85 per cent. salt solution. This is used for the first dose for one rabbit. Rabbits are injected intravenously with stroma from 1 ml. of packed cells on the first day, injected with stroma from 2 ml. of packed cells on the fifth day, injected with stroma from 3 ml. of packed cells on the tenth day, and bled on the fifteenth day for final titration. If the titre is not sufficiently high, a final injection of stroma from 4 ml. of packed cells is given. The quantities of stroma injected are approximately 0.5 ml., 1 ml., 1.5 ml. and a final injection of 2 ml. when indicated. The final stock antiserum is preserved by adding an equal volume of pure neutral glycerol or by drying.

Notes—

Red cells should be used as fresh as possible.

A fresh preparation of stroma should be made for each injection.

Adult rabbits (8–12 months old) must be used.

Sensitised cells for the test are made by mixing equal volumes of the standard 6 per cent. suspension (p. 339) and the haemolytic serum diluted to contain 12 M.H.D. per unit volume. After vigorous shaking, the mixture is incubated for one hour in a 37° C. water-bath.

The Test¹

Small test-tubes 3 × $\frac{1}{2}$ in. are used and the reagents are added to them either with graduated pipettes or according to Donald's dropping method (see p. 338).

The first step, before commencing the test, is to titrate the complement using the sensitised red blood cell suspension to be used in the test (see p. 338). This is necessary because even pooled preserved complement-serum may vary in its activity against different specimens of red blood cells.

The test proper is a screening procedure and each serum has two tubes allocated to it. The reagents are added as follows:

Tube 1. Serum Control Tube:

Patient's serum	$\frac{1}{2}$ volume
Saline	2 volumes
Complement serum-control dose	1 volume.

Tube 2. Diagnostic Tube:

Patient's serum	$\frac{1}{2}$ volume
Saline	1 volume
Complement diagnostic dose	1 volume
Antigen	1 volume.

After adding the reagents the racks are shaken thoroughly and placed

¹ Price, I. N. O. (1950), *Brit. J. vener. Dis.*, 26, 172.

in a water-bath for one hr. at 37° C. At the end of this time the racks are placed on the bench and to each tube is added one volume of sensitised red blood cells. After shaking again, the racks are replaced in the water-bath for a further thirty minutes, after which the test is read.

Complete haemolysis in both tubes is read as negative reaction. No haemolysis in the diagnostic tube with complete haemolysis in the control tube is read as a positive reaction. Partial haemolysis in the serum diagnostic tube and complete haemolysis in the control tube is read as a weakly positive reaction. No result can be given if the serum control tube of any particular specimen of serum fails to show complete haemolysis. Positive and weakly positive sera are set aside to be put up for a quantitative test.

Quantitative Tests.—In this method doubling dilutions of the serum in saline from 1 in 5 to 1 in 160 are used. Seven tubes are required for each serum and they are set up as follows:

Tube 1. Serum Control Tube:

Serum diluted 1 in 5	1 volume
Saline	1 volume
Serum control dose of complement	1 volume.

Tubes 2-7. Diagnostic Quantitative Tubes:

Serum appropriate dilution (1 in 5 to 1 in 160)	1 volume
Complement diagnostic dose	1 volume
Antigen	1 volume.

The sensitised red blood cells are added in the same manner as in the test proper and the incubation times are the same. The end-point is taken as that tube which just fails to show sparkling haemolysis.

The results are reported in terms of the serum dilutions, e.g. "Positive with serum diluted 1 in 30".

Cerebrospinal Fluid.—In this test the procedure is closely similar to that used for serum. Neat cerebrospinal fluid is used in the test proper and one volume is added to both the diagnostic and control tubes instead of the one-fifth volume used for sera. The test is made quantitative by making doubling dilutions from neat cerebrospinal fluid and results are recorded as "Positive, fluid diluted 1 in 2, 1 in 4" and so on.

Each batch of tests should include known positive sera of varying degrees of reactivity and a known negative serum. Controls should be set up for each of the reagents used.

Method II (Kolmer's Method)¹

It is not possible to include the minutiae of details described by Kolmer, and workers who use this method are referred to Kolmer's own papers.

¹ Kolmer, J. A., Spaulding, E. H., & Robinson, H. W. (1952), *Approved Laboratory Technique*. London, Lewis.

The main essentials of reagents and technique are, however, given below.

Antigen.—A cardiolipin antigen containing 0.03 per cent. cardiolipin, 0.05 per cent. lecithin and 0.3 per cent. cholesterol is used. The antigen dilution is indicated on the label of the bottle; usually it is 1 in 150.

Saline.—0.85 per cent. NaCl containing 0.01 per cent. magnesium sulphate.

Sheep red blood cell suspension.—A 2 per cent. washed suspension of red cells is used. It is standardised, using the haematocrit as in Method I to contain 0.02 ml. packed cells per ml.

Haemolytic serum.—Dilute with saline to contain 4 M.H.D. per ml.

Patient's sera.—Treat as in Method I.

Complement.—Preserved complement is titrated in the presence of diluted antigen, and after one hr. in the water-bath at 37° C. the haemolysin and the sheep cell suspension are added separately.

Tube No.	Complement 1: 30	Diluted antigen	Saline Solution	37° C. water-bath for 1 hour	Haemolysin	Sheep cell suspension (2 per cent.)	37° C. water-bath for ½ hour
1	(ml.) 0.20	(ml.) 0.5	(ml.) 1.3		(ml.) 0.5	(ml.) 0.5	
2	0.25	0.5	1.3		0.5	0.5	
3	0.30	0.5	1.2		0.5	0.5	
4	0.35	0.5	1.2		0.5	0.5	
5	0.40	0.5	1.1		0.5	0.5	
6	0.45	0.5	1.1		0.5	0.5	
7	0.50	0.5	1.0		0.5	0.5	
8	None	None	2.5		None	0.5	

Remove rack from water-bath and read complement titration. The smallest amount of complement giving complete haemolysis is the exact unit. The full unit is 0.05 ml. more than the exact unit.

For the complement-fixation tests, complement is diluted so that 2 full units are contained in 1.0 ml.

Example:

		ml.
Exact unit	.	0.3
Full unit	.	0.35
Dose (2 full units)	.	0.7

Dilution of complement to be employed in the test proper may be calculated by dividing 30 by the dose, i.e. $\frac{30}{0.7} = 43$ or 1: 43 dilution of guinea-pig serum.

The Test

Two tubes are used for each serum to be treated and in each batch sera of graded reactivity from negative to strong positive are included. Additional controls are needed for the reagents used in the test, *i.e.* the antigen, the haemolytic system and the complement. The test is set up as follows:

		Diagnostic Tube	Serum Control Tube
Serum	.	0.2 ml.	0.2 ml.
Antigen	.	0.5 ml.	Nil
Saline	.	Nil	0.5 ml.

Stand at room temperature ten to thirty min.

Complement containing 2 full units per ml.	.	1.0 ml.	1.0 ml.
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Mix by thorough shaking and place overnight in the refrigerator at 6°-10° C. for 15 to 18 hr. The following day remove the racks and place them for ten min. in a 37° C. water-bath. Remove from the bath and add to all tubes except the red cell control tube, 0.5 ml. of the diluted haemolytic serum, and then add 0.5 ml. of the 2 per cent. sheep cell suspension. Mix thoroughly by shaking the tubes, and return the racks to the 37° C. water-bath. Begin to read the tubes after ten min. and watch the known sera controls and the antigen controls with care. When the known pattern of the reactions of these sera appears the final reading is made. Usually this occurs within thirty min. and should certainly be present in less than one hour. The results are interpreted in the same manner as in Method I.

An outline of the Kolmer Method for sera and cerebrospinal fluids including the amounts contained in the reagent control tubes is as follows:

Tube No.	Saline solution	Antigen	Complement 2 full units	Haemolysin 2 units	Sheep cell suspension (2 per cent.)	
Serum (ml.)	(ml.)	(ml.)	(ml.)	(ml.)	(ml.)	
1. 0.2	None	0.5	1.0	0.5	0.5	
2. 0.2	0.5	None	1.0	0.5	0.5	
Spinal fluid (ml.)						
1. 0.5	None	0.5	1.0	0.5	0.5	
2. 0.5	0.5	None	1.0	0.5	0.5	
Controls						
Antigen .	0.5	0.5	1.0	0.5	0.5	
Haemolytic system .	1.0	None	1.0	0.5	0.5	
Corpuscle .	2.5	None	None	None	0.5	
			Shake rack well. Allow to stand at room temperature for 10 to 30 minutes	Shake rack well. Primary incubation 15 hours at 6°-10° C. followed by 10 minutes in 37° C. water-bath.		Shake rack well. Secondary incubation in 37° C. water-bath.

This method is used quantitatively with serum serial doubling dilutions from 1 in 2 to 1 in 64 on all positively-reacting sera.

The Kolmer Test is readily adaptable to virological work and the Reiter Protein Test (see p. 714). In these and other circumstances, when only small amounts of antigens are available, the test is carried out with volumes one-fifth the size of those above.

The Complement-Fixation Test in Gonorrhoea

The general technique of this test is very similar to that of the Wassermann test.

Antigen.—This is made from cultures of several freshly isolated strains of gonococci. It may be prepared and titrated by Cruickshank's modification of Price's method¹ or may be purchased from Burroughs Wellcome and Co.

The Test employs the haemolytic system and complement in precisely the way described in Method I of the Wassermann test. The test proper is also carried out in this way except that one volume of inactivated patient's serum is used instead of the one-fifth volume employed in the Wassermann test.

The gonococcal complement-fixation test is of limited practical value in acute uncomplicated cases but it is a useful diagnostic aid in chronic infections especially those with closed lesions such as salpingitis, prostatitis and arthritis.

COMPLEMENT-FIXATION TESTS IN VIRUS DISEASES

Psittacosis²

The general technique of the test is similar to that of the Wassermann test, in which standard volumes of the reagents are added.

Antigen.—Various types of antigen prepared from animal or egg tissues rich in virus elementary bodies have been used. Yolk-sac antigens have proved very satisfactory. These are prepared by inoculating with psittacosis virus the yolk-sacs of 6 to 8 day chick embryos (p. 448). On the death of the embryo the yolk-sac membranes which are richest in elementary bodies are selected and an emulsion in normal saline is prepared by grinding them in a mortar with sterile sand or sintered glass powder (fine grade). The emulsion is clarified by slow centrifuging and the supernatant fluid is taken off to be centrifuged further in an angle centrifuge at 4000 r.p.m. for three hours. The deposit is re-suspended in saline to one-half or one-quarter the original volume. Finally, the antigen is placed in boiling water for twenty minutes. New antigens should be tested in dilutions of 1 : 1, 1 : 2 and 1 : 4 with known positive sera to determine the optimal dilutions for their use. 0.1 ml. of the antigen-alone in the dilution used in the test should not fix 1 M.H.D. of complement.

¹ Cruickshank, R. (1947), *Recent Advances in Clinical Pathology*. London, Churchill.

² Bedson, S. P., & Bland, J. O. W. (1949), *Brit. J. exp. Path.*, **10**, 393.

Control preparations are obtained from normal yolk-sacs and used in parallel with those made from the infected material, in each case exactly the same technique being used.

Haemolytic System.—3 per cent. suspension of sheep red cells *plus* 5 M.H.D. haemolytic antiserum.

Complement.—Prepared and titrated as for the Wassermann reaction.

Sera.—Inactivated at 56° C. for thirty minutes.

The Test.—Serial doubling dilutions of the serum to be tested from 1 in 4 to 1 in 256 are prepared, and 0.1 ml. of each dilution is used in the test. To this are added 0.1 ml. of the antigen in the required dilution (*vide supra*), and 0.1 ml. of complement in a dilution representing 2 M.H.D. 0.5 ml. of saline is now added to each tube. Controls should include known positive and negative sera, and the test serum, diluted 1 in 4, in the presence of a *normal* yolk-sac preparation; serum, complement, antigen and red cell controls are set up as in the Wassermann reaction. Fixation is allowed to proceed for two and a half hr. at room temperature, after which 0.2 ml. of the red cell suspension is added. Readings are taken after thirty min. incubation in the water-bath at 37° C. and again after standing overnight on the bench.

Interpretation.—The best evidence of infection is provided by a four-fold rise in the titre of antibodies in samples of serum taken in the acute and convalescent phases of the disease.

The isolated observation of a titre of 1 in 32 or higher is considered suggestive of an infection with a member of the psittacosis-lymphogranuloma group of viruses.

This reaction may be used with equal facility for the diagnosis of lymphogranuloma inguinale since both viruses share a common antigenic component. Sera giving positive Wassermann reactions may also react.

Influenza¹

During the early stages of their reproductive cycle the influenza viruses assume an incomplete or "soluble" form within the host cells.² At this time the young virus has the property of fixing complement in the presence of specific antibody, the antigen concerned being common to all the members of a given type of virus. Potent complement-fixing antigens may thus be prepared from young chorio-allantoic-membrane cultures of suitable viruses.

Antigen

Influenza A (P.R. 8 strain) or influenza B (Lee strain) is passed as a routine small inoculum into the allantoic cavity of ten- to eleven-day embryos. After twenty-four hours' incubation at 37° C. the allantoic fluids are harvested without pre-chilling, and used undiluted in a dose of 0.1 ml. to inoculate a batch of thirteen-day embryos.

¹ Hoyle, L. (1948), *Monthly Bull. Minist. Hlth Lab. Serv.*, **7**, 114.

² Hoyle, L. (1948), *Brit. J. exp. Path.*, **29**, 390.

After twenty-four hours' incubation the eggs are killed by chilling, and the shell over the air sac is removed. Through a cross-shaped incision in the chorio-allantoic membrane the embryo and its yolk sac can be evacuated leaving the membrane behind and lightly adherent to the shell. The membranes are then removed from the shell, rinsed free of blood and spilt yolk in three changes of 0.08 per cent. sodium azide in physiological saline, dried between filter-papers, and finally weighed in bulk.

The membranes are now suspended in 5–10 ml. azide-saline and are frozen and thawed three times by alternate immersion in a mixture of alcohol and solid carbon dioxide at -70° C . and in a water-bath at 37° C . For this purpose it is advisable to place the membrane emulsion in a plastic container. Now add sufficient azide-saline to make a 40 per cent. w/v suspension of membrane and homogenise in a blender. The suspension is now lightly centrifuged to remove the coarse debris and a few drops of chloroform are added. After standing overnight at 4° C . a precipitate of egg proteins is formed and this is removed, without significant loss of the soluble antigen, by centrifuging for one hr. in an angle centrifuge at 4000 r.p.m.

The resultant, somewhat opalescent, fluid contains the soluble antigen and is best preserved by freeze-drying small volumes of it in ampoules. Alternatively, the fluid antigen may be kept without marked deterioration for about one month at -30° C .

Before use, the antigen is tested against a positive serum of known titre. Several dilutions of antigen are titrated against a range of serum dilutions. In this way the optimum dilution for use in routine complement fixation tests is determined.

An Alternative Method of Preparation of the Antigens

Inoculate nine-day embryonated eggs with 0.4 ml. of virus infected fluids diluted to 10^{-1} . Use the intra-allantoic route and incubate at 35° C . for 40 to 44 hr. Chill the eggs and harvest the allantoic fluids. If the titre of the pooled fluids is between 640 and 1280 by the haemagglutination method, they may be used immediately or stored for an indefinite period in sealed glass ampoules in the dry icebox at -70° C .

Haemolytic System.—3 per cent. suspension of sheep red cells plus 5 M.H.D. haemolytic antiserum.

Complement.—Complement preserved by Richardson's technique (p. 337) is very satisfactory.

To titrate the complement set up a series of tubes containing 0.2 ml. of complement dilutions as follows: 1 in 10, 1 in 15, 1 in 20, 1 in 25, 1 in 30, 1 in 35, 1 in 40, 1 in 50, 1 in 60, 1 in 70, 1 in 80, 1 in 90, 1 in 100. Add to each tube 0.4 ml. saline and 0.4 ml. of sensitised red blood cells. Incubate in a water-bath for thirty min. at 37° C . A reading is made of the highest dilution showing 100 per cent. haemolysis and also of that giving 50 per cent. (made by centrifuging down the cells and matching the supernatant against a 1 in 2 dilution of a tube showing complete haemolysis). For use in the tests dilute the neat complement

to contain $2\frac{1}{2}$ M.H.D. by the 100 per cent. haemolysis reading or $4\frac{1}{2}$ M.H.D. by the 50 per cent. haemolysis reading. These two readings are usually the same, but when a discrepancy occurs the 50 per cent. haemolysis titre is the more accurate.

Sera.—Inactivated at 56° C. for thirty min.

The Test.—Serial doubling dilutions of 1 in 2 to 1 in 16 are used for normal sera and 1 in 2 to 1 in 256 for convalescent sera.

Each tube in the test contains 0.1 ml. of serum dilution, 0.1 ml. of complement diluted to contain $2\frac{1}{2}$ M.H.D., and 0.1 ml. of antigen. Serum, antigen, red cells and complement controls are set up as in the psittacosis complement-fixation reaction (*vide supra*), and known negative and positive sera are included. A fixation period of one hr. in the water-bath at 37° C. is used. 0.2 ml. of sensitised red cell suspension is then added to each tube and the racks are returned to the water bath for a further thirty min. Readings are made after the cells have settled, and 50 per cent. haemolysis or more indicates absence of complement-fixation in the particular tube.

This test may be modified to use with smaller volumes of the reagents and can be carried out in the plastic plates used in the haemagglutination inhibition technique (p. 352). Each cup allotted for a serum under test receives 0.1 ml. of serum dilution, 0.1 ml. of complement $2\frac{1}{2}$ M.H.D. and 0.1 ml. of antigen at its optimal dilution. Suitable reagent controls are included and fixation is allowed to proceed for one and a half hr. at 37° C. Overnight fixation at 4° C. is sometimes preferred and in this case the plates must be warmed for thirty min. at 37° C. after removal from the refrigerator. Each cup now receives 0.2 ml. sensitised sheep cells. The plates are incubated at 37° C. and the test is read at the end of thirty min.

With this method the following viral antigens may be employed: Influenza, A, B, C, and Sendai viruses; mumps, soluble and viral antigens; adeno-virus group antigen; psittacosis; *C. burnetii* (Q fever), and the lymphocytic chorio-meningitis viruses.

Even smaller volumes of reagents are employed in tests of this type by Fulton and Dumbell,¹ who place drops of the fluids on ruled perspex plates and incubate the mixtures in a special humidified cabinet.

Other Virus Diseases

The complement-fixation reaction can be adapted for use in many other virus infections. It is particularly valuable in the diagnosis of smallpox (p. 776), and is used in the diagnosis of herpes zoster and herpes simplex infections, mumps, lymphocytic choriomeningitis, St. Louis encephalitis and Japanese B encephalitis. For details of the technique employed the reader is referred to *Diagnostic Procedures for Virus and Rickettsial Diseases*, American Public Health Association, 1948, New York.

¹ Fulton, F., & Dumbell, K. R. (1949), *J. gen. Microbiol.*, 3, 97.

Haemagglutination-Inhibition Tests in Influenza

The value of these tests lies in their extreme specificity and the fact that they can be used to distinguish antibodies to the various substrains and variants of the influenza viruses. The reaction is of great importance also in the identification of freshly isolated strains of influenza viruses. Haemagglutination-inhibition tests thus differ from the complement-fixation reactions described on p. 346 for the latter are group specific and will do no more than distinguish the antibodies to influenza viruses A, B and C.

Saline Diluent.—Throughout the tests the buffered calcium saline is used at pH 7.2-7.4 (see p. 290). Alternatively, the veronal saline diluent with added calcium and magnesium may be used (see p. 290).

Standard Erythrocyte Suspension.—Blood is obtained from the wing vein or the heart of a fowl (see p. 423) and added to a suitable anti-coagulant. Human "Group O", or guinea-pig erythrocytes may also be used. The cells are washed thoroughly in the centrifuge and then the volume of the packed cells is determined in a graduated centrifuge tube by spinning in a straight-headed centrifuge for ten minutes at 3200 r.p.m.

The cells are then suspended to make a 0.5 per cent. suspension.

Standard antigens are prepared from high-titre pools of infected allantoic fluid. At present it is recommended that the following should be used: Type A, PR8; Type A2, Asian strain; Type B Lee, or B, England 939/59.

Titration of Virus.—Set up two rows of $3 \times \frac{1}{2}$ in. tubes, 10 tubes in each row. Place 1.0 ml. amounts of saline solution in each tube in the front row. With a 1.0 ml. pipette add 1.0 ml. of the viral antigen to the first tube of the front row. Discard the pipette. Mix well with a clean pipette and transfer 1.0 ml. to the second tube of the front row and 0.25 ml. to the first tube of the back row. Take a fresh pipette and mix the contents of the second tube of the front row and then transfer 1.0 ml. to the third tube of the front row and 0.25 ml. to the second tube of the back row. Continue through the series until all the tubes of the back row contain 0.25 ml. of doubling dilutions of the virus. *It is extremely important to take a fresh pipette to make each virus dilution.* Discard the front row of tubes. Add to each tube 0.25 ml. saline solution and 0.5 ml. of the 0.5 per cent. red cell suspension. Include an erythrocyte control tube containing 0.5 ml. saline and 0.5 ml. of the red cell suspension. Stand for one hr. at room temperature and read the results according to the haemagglutination pattern. The 0.25 ml. volume of the virus dilution which completely agglutinates the red cells is one haemagglutination unit. For the test four haemagglutinating units are required and the viral antigen is diluted to one fourth of the titre obtained.

Test Sera.—Pairs of sera obtained during the acute and convalescent phases of the illness are absolutely essential. Both sera must be examined at the same time. It is important to remove the non-specific

inhibitors of viral haemagglutination usually present in normal serum.

Preparatory Treatment of Sera for Haemagglutination-Inhibition Tests

Since the presence of non-specific virus inhibitors in many human and animal sera renders difficult the interpretation of serological findings, it is a matter of importance to eliminate this source of error. Inhibitors may be removed from human sera without affecting the titre of specific antibody by the following methods:

*Treatment with a crude filtrate of *V. cholerae**

The filtrate is prepared as follows: Semi-solid tryptic digest heart agar, spread in a thin layer in Petri dishes, is inoculated with 0.2 ml. of an eight hour broth culture of *V. cholerae* (4 Z strain). After 16 hr. incubation at 37° C. the soft agar cultures are filtered through cotton wool and paper, clarified by slow centrifugation, Seitz filtered, and neutralized. Filtrates are titrated by estimating the minimal amount which will, in a given amount of normal rabbit serum, eliminate the inhibition of virus haemagglutination.

Sera, of which 0.1 ml. are required for each virus, are treated as follows:

To 0.1 ml. serum add 0.5 ml. of an appropriate calcium saline dilution of cholera filtrate, incubate for eighteen hr. in a water-bath at 37° C., and finally heat for one hour at 56° C. to inactivate the receptor-destroying enzyme in the cholera filtrate. Add 0.2 ml. saline, and the serum now diluted 1 in 8 is ready for examination.

Treatment with Periodate

Make up a solution by dissolving 0.127 gram of potassium meta-periodate in 50 ml. distilled water. To 1 volume of serum add 2 volumes of periodate solution and store overnight at 4° C. The following morning add two volumes of 1.0 per cent. glycerol in saline and then adjust the serum dilution to 1 in 8 by adding 3 volumes of saline. The serum is now ready for use in the test.

Titration of Antibodies in Serum

For each serum to be tested set up four rows of ten $3 \times \frac{1}{2}$ in. tubes. Into all the tubes of the front row place 1.0 ml. saline solution. To the first tube of the front row add 1.0 ml. of a 1 in 8 dilution of the patient's serum. Mix well and transfer 1.0 ml. to the second tube of the front row and continue this procedure until the tenth tube. Discard the remaining serum dilution in the pipette. Now transfer 0.25 ml. of the contents of the tenth tube to the last tubes of the other three rows and using the same pipette carry out the same step with the ninth, eighth and the other tubes until all have received their appropriate serum dilutions. Discard the front row of the tubes which are not used in the test.

Now add the test viral antigens in 0.25 ml. amounts to their appropriate rows (Virus A, A₂ and B). Finally to these virus serum mixtures add 0.5 ml. of the 0.5 per cent. red cell suspension and shake thoroughly to mix the reagents. Stand at room temperature for sixty min. and then read the lists on the basis of the haemagglutination pattern. The titre of the serum is expressed as the highest initial dilution which completely inhibits viral haemagglutination. In any batch of haemagglutination-inhibition tests the viral antigens must always be retitrated and known positive and negative sera must always be included. Other controls include a serum control of 0.25 ml. 1 in 8 serum + 0.25 ml. saline + 0.5 ml. erythrocyte suspension and also a cell suspension control as used in the virus titration.

Haemagglutination-Inhibition Test in Influenza C Infections

Influenza C grows poorly in the allantoic cavity and this virus must be cultivated in the amniotic cavity of 10-day chick embryos for 26 to 30 hr. at 35° C. A suitable inoculum is 0.1 ml. of a 1 in 100 dilution of the seed virus.

Since influenza virus C elutes rapidly at room temperature, haemagglutination and haemagglutination-inhibition titrations must be carried out in the cold at 4° C.

Modification of the Haemagglutination-Inhibition Test for use with Plastic Plates

Plastic plates of the type recommended by the World Influenza Centre¹ are economical to use since the volumes of the reagents are greatly reduced and the labour of washing large numbers of tubes is eliminated. The plates, when the tests are finished, are cleaned by immersion for one hour in 2 per cent. sodium hydroxide and then for one hour in 2 per cent. hydrochloric acid. Three rinses in tap water are then required and a final soaking in ion-free distilled water for several hours follows. After shaking free of water on a towel the plates are dried in the incubator overnight.

For virus titrations the volume of antigen used is 0.25 ml. to which is added directly 0.5 ml. of 0.5 per cent. red blood cells. For the titration of serum antibodies serial dilutions of 0.25 ml. are made; this is followed by the addition of an equal volume of red blood cell suspension and 0.25 ml. of the virus antigen diluted to contain four haemagglutinating units. The reading of the test is similar to that described above.

The Measurement of Antiviral Activity in Sera by Neutralisation Tests

In only a limited number of virus diseases is there an *in vitro* test which can be relied on to give an estimate of the level of immunity in the individual and it is therefore necessary in many cases to use animal

¹ Obtained from Prestware Ltd., Raynes Park, London.

protection tests to gauge the level of antibody in the serum. Neutralisation tests are at present chiefly used in yellow fever, coxsackie virus infections and neurotropic virus infections such as poliomyelitis and the arthropod-borne encephalitides.

In one method a fixed quantity of serum is added to falling dilutions of the virus and the serum mixtures are held at room temperature for thirty min. or longer before being inoculated into groups of animals, eggs, or tissue cultures. Acute and convalescent phase sera are included in the same batch of tests and a control virus titration is set up. The neutralisation index is then determined by comparing the 50 per cent. end-point of the control virus titration with that of the virus-serum mixture.

$$\text{Logarithm of neutralisation index} = \frac{\text{Negative logarithm of control titre}}{\text{Negative logarithm of virus-serum mixture}}$$

Neutralisation index = Antilogarithm of the figure thus obtained.

Neutralisation indices below 10 are regarded as negative, and between 10 and 49 as doubtful; over 50 they are usually indicative of an infection. In a second method a fixed amount of virus, sufficient to affect 100 per cent. of the controls, is added to progressive dilutions of serum. In this case the Reed and Muench or Kärber methods (*vide infra*) are used to determine the 50 per cent. protective or neutralising end-point of both acute and convalescent phase sera. In calculating these end-points it is necessary to reverse the direction of addition of the appropriate columns dealing with the "deaths" and "survivals". By converting the logarithm of the 50 per cent. serum titre to its antilogarithm the neutralisation index is obtained. For diagnostic purposes the increase in the neutralisation index during convalescence should be at least 100.

The Measurement of Virus Infectivity

The usual procedure for estimating the potency of a suspension of living virus particles is to inoculate groups of susceptible animals with varying amounts of the preparation differing by a constant dilution factor. The end-point of the titration is taken, for accuracy, as that dilution of the virus at which 50 per cent. of the animals react. The dose which infects 50 per cent. of the animals is known as the ID₅₀ and that which kills 50 per cent. of them as the LD₅₀. In many such virus titrations it is possible to use chick embryos or tissue cultures instead of animals, but the principle remains the same.

For a reasonable degree of accuracy it would be necessary to inoculate large groups of animals with virus dilutions close to the 50 per cent. end-point. This, however, is seldom economically possible, and it is often necessary to use rather widely spaced dilutions (e.g. decimal dilutions) and groups of moderate numbers of animals. The use of the method of Reed and Muench¹ enables the 50 per cent.

¹ Reed, L. J., & Muench, H. (1938), *Amer. J. Hyg.*, 27, 493.

end-point to be determined more precisely than is possible by simple interpolation between two critical dilutions and gives an effect as if larger groups of animals had been used than were actually inoculated. There is, moreover, a tendency to equalise chance variations.

In Reed and Muench's method it is assumed that animals dying at a stated dose would also have been killed by greater amounts of the virus and conversely that those surviving would also have survived smaller doses. An accumulated value for the animals affected is obtained by adding the number dying at a certain dilution to the number killed by greater doses of the virus; a similar addition but in the reverse direction is made for the survivors (see example).

The accumulated values of the two critical dilutions between which the 50 per cent. end-point lies are now substituted in the formula and the LD₅₀ is obtained.

In making these calculations it is assumed that the doses of virus used are equally placed on the logarithmic scale, that the 50 per cent. end-point falls somewhere in the middle of the range of dilutions used, and that the same number of animals was used for each dilution. An alternative and slightly simpler method is that of Kärber.

Example:

Virus dilution	Mortality Ratio	Died	Survived	Accumulated Values				Per cent. $\frac{D}{(D+S)} \times 100$
				Died	Survived	Mortality Ratio		
10 ⁻¹	10/10	↑ 10	0	31	0	31/31	100	
10 ⁻²	10/10	10	0	21	0	21/21	100	
10 ⁻³	8/10	8	2	11	2	11/13	85	
10 ⁻⁴	3/10	3	7	3	9	3/12	25	
10 ⁻⁵	0/10	0	10 ↓	0	19	0/19	0	

The arrows indicate the direction of addition for the accumulated values.

In this titration the 50 per cent. end-point is seen to lie between 10⁻³ and 10⁻⁴. It will be located at the proportionate distance from 10⁻³.

$$\text{Proportionate distance} = \frac{\text{mortality above } 50 \text{ per cent.} - 50}{\text{mortality above } 50 \text{ per cent.} - \text{mortality below } 50 \text{ per cent.}}$$

$$= \frac{85 - 50}{85 - 25} = \frac{35}{60} = 0.58.$$

$$\begin{aligned} \text{Negative logarithm of LD50 titre} &= \text{Negative logarithm of dilution above } 50 \text{ per cent. mortality} + \text{Proportionate distance} \\ &= 3.0 + 0.58 \\ \text{LD50} &= 10^{-3.58} \end{aligned}$$

f

Procedure to calculate LD₅₀ by the method of Kärber

$$\log \text{LD}_{50} = 0.5 + \frac{\log \text{of greatest - Sum of percentage of dead animals}}{\text{titre} \quad \text{virus concentration used}}$$

For the above example:

$$\begin{aligned}\log \text{LD}_{50} \text{ titre} &= 0.5 + (-1.0) - \frac{100 + 100 + 85 + 25}{100} \\ &= 0.5 - 1.0 - 3.1 \\ &= -3.6 \\ \text{LD}_{50} \text{ titre} &= 10^{-3.6}\end{aligned}$$

CHAPTER XIV

BACTERIOLOGY OF WATER, MILK, ICE-CREAM, SHELL-FISH, OTHER FOODS, AIR

BACTERIOLOGICAL EXAMINATION OF WATER

DRINKING-WATER supplies liable to contamination with sewage or other excreted matter may cause outbreaks of intestinal infections such as typhoid fever. In safeguarding public water supplies, health authorities and water engineers rely on information obtained from the results of frequent bacteriological tests. The demonstration of pathogenic bacteria, e.g. the typhoid bacillus, would obviously constitute the most direct proof of a dangerous impurity, but these pathogens, if present, are usually so scanty that the technical difficulty of their isolation makes the test impracticable for ordinary purposes (but see 371). Instead we rely on tests which will reveal the presence of commensal bacteria of intestinal origin such as those of the coliform group, *Streptococcus faecalis* and *Clostridium welchii*. These do not themselves constitute a hazard, but they indicate that faecal matter has entered the supply and that the water is therefore liable to contamination with organisms of a more serious nature. The coliform bacilli are the most reliable indicators of faecal pollution. Although the occurrence of streptococci is strong evidence of faecal pollution, their absence does not exclude such impurity. The sporing anaerobes, on the other hand, being highly resistant, would in the absence of the other intestinal organisms indicate pollution of some remote period rather than one of recent occurrence.

Since the coliform group of bacteria may be derived from the intestines of various animals, they are likely to occur in small numbers even in water supplies far removed from the likelihood of human contamination. Water grossly polluted with human excretal matter, e.g. sewage, contains them in larger numbers. The test for their presence as an index of the degree of pollution must therefore be carried out on a quantitative basis. The coliform group of lactose-fermenting Gram-negative bacilli includes a number of different organisms (see Chapter XXVII). Those referred to as "typical" or "faecal" (e.g. *Esch. coli*) are essentially commensals of the intestine and are derived almost exclusively from this source. Others, known as "atypical" (e.g. *Kl. aerogenes*), may grow also in the soil and on vegetation, and by derivation from these sources often come to be present in waters which are not subject to excretal pollution. The typical faecal bacilli (*Esch. coli*) die in water during the course of several days or weeks after leaving the animal intestine; thus, their presence in water is an indication of recent faecal contamination, whereas the presence of the hardier atypical coliforms

is not necessarily so. In carrying out the test for coliform bacilli in water it is therefore advisable to determine whether the strains present are typical or atypical (p. 361).

A determination of the total number of viable bacteria in a water sample is a useful supplementary test, although of limited value by itself. It gives an indication of the amount and type of organic matter present in the supply. The test is carried out in duplicate at 37° C. and 20°–22° C. The bacteria which grow at 37° C. are those most likely to be associated with organic material of human or animal origin, whereas those growing at the lower temperature are mainly saprophytes which normally inhabit the water or are derived from soil and vegetation.

The routine tests generally used in bacteriological examination of water are:

1. A quantitative test for all coliform bacilli known as the *presumptive coliform count*.
2. A differential test for typical coliform bacilli (*Esch. coli*) known as the *differential coliform test*.
3. An enumeration of viable bacteria known as the *plate count*; this is done in duplicate, cultivating at 37° C. and 22° C.

Collection of Specimens¹

Specimens are taken in bottles, of approximately 230 ml. capacity, with ground-glass stoppers having an overhanging rim; they are sterilised by autoclaving, the stopper and neck of the bottle being covered by two layers of kraft paper (p. 177). Alternatively, 6-oz. screw-capped bottles can be used; the capped bottle is wrapped in kraft paper and likewise sterilised in the autoclave. The opening and closing of the bottle and its handling in the process of collecting a sample must be carried out with meticulous care to avoid any bacterial contamination from an outside source, including the hands of the person taking the sample. When water is drawn from a tap, the mouth of the tap should be flamed, e.g. with a blow-lamp or spirit lamp, and the water allowed to run for five minutes before filling the bottle. In the case of streams, rivers and lakes, the stopper should be removed carefully with one hand, and with the other the bottle held at its base should be inserted, mouth downward, a foot below the surface of the water; the bottle is then turned so that the mouth is directed to the current and water flows into the bottle without coming into contact with the hand. If there is no current, as in lakes or reservoirs, the bottle should be moved horizontally, the mouth foremost, so that water flows into it. The bottle is then brought to the surface and the stopper is replaced. Care must be taken that the stopper is not contaminated during the sampling process. This method of sampling avoids the

¹ See Report No. 71, Ministry of Health, 1956, on the Bacteriological Examination of Water Supplies.

collection of surface water, which may contain a good deal of decomposing vegetable matter.

When a sample is to be obtained from a depth, a bottle weighted with lead is used, having two cords attached—one to the neck, the other to the stopper; the bottle is lowered to the required depth, and is filled by jerking out the stopper by means of the attached cord; the bottle is then quickly raised to the surface and re-stoppered.

When a certain length of time (three hours or more) must elapse before the laboratory examination can be carried out, the bottles should be kept on ice. Special insulated boxes for the purpose can be obtained and are essential where specimens have to be transported some distance.

Neutralisation of Chlorine.—If a sample is taken from a chlorinated water supply it is important that any traces of free chlorine should be neutralised immediately as otherwise killing of bacteria may proceed during the time that elapses before the specimen is examined in the laboratory, and the result would not be representative of the original condition of the water. A crystal of sodium thiosulphate introduced into the sampling bottle prior to sterilisation serves to effect neutralisation.

Immediately before testing, the water sample should be mixed by inverting the bottle twenty-five times. Thereafter some of the contents are poured off, the stopper is replaced and the bottle is shaken vigorously twenty-five times by an up-and-down movement.

Presumptive Coliform Count (Multiple Tube Technique)

An estimation of the number of coliform bacilli in a water supply is usually made by adding varying quantities of the water (from 0.1 ml. to 50 ml.) to bile salt lactose peptone water (with an indicator of acidity) contained in bottles with Durham tubes to show the formation of gas; acid and gas formation (a "positive" result) indicates the growth of coliform bacilli. In this way it is possible to state the smallest quantity of water containing a coliform bacillus and thus to express the degree of contamination with this group of organisms. Further, by isolating the coliform bacillus so demonstrated and determining its characters, the smallest amount of water containing a *typical coliform bacillus* can also be stated.

This method, however, involves "random sampling", and it is not sufficient to test one sample only of each quantity in the series, for in this way misleading results are obtained. Thus, if two samples of a given quantity were tested, one might be positive, the other negative, and a certain quantity might give a positive result while a larger volume was negative. The difficulty can be got over to some extent by duplicating the samples of each quantity in the series and only reporting that a certain volume contains a coliform bacillus if both samples show it and if all the larger volumes tested yield a positive result. Greater accuracy

can only be obtained by increasing the number of samples of each quantity so that an average result can be stated. It has been shown that if one 50 ml., five 10 ml. and five 1 ml. volumes, or five 10 ml., five 1 ml. and five 0.1 ml. volumes are tested, the probable number of coliform bacilli in 100 ml. can be computed according to the various combinations of positive and negative results, tables compiled by McCrady being used for the purpose (p. 360). This is the method recommended for routine use.

5

Media required:

- (1) 2 per cent. peptone water containing 0.5 per cent. sodium taurocholate, 1 per cent. lactose, and bromo-cresol purple as indicator (modified MacConkey's fluid medium).
- (2) Same constituents as (1), but in double concentration.

Measured amounts (*vide infra*) of these media are placed in sterile bottles stoppered with cotton-wool plugs or with screw caps, and containing a Durham tube for indicating gas production. The size of the bottle varies with the quantity of medium and water to be added to it. The medium, after bottling, is sterilised at 100° C. for twenty minutes in the steam steriliser on three successive days.

With sterile graduated pipettes the following amounts of water are added:

One 50 ml. quantity to 50 ml. double strength medium	~
Five 10 ml. quantities each to 10 ml. double strength medium	
Five 1 ml. "	5 ml. single "
Five 0.1 ml. "	5 ml. "

This range of quantities may be altered according to the likely condition of the water examined; thus, the 50 ml. quantity is included when testing filtered or chlorinated water, and in this case it is unnecessary to examine 0.1 ml. volumes. This amount, *i.e.* 1 ml. of the sample diluted 1 in 10 (for dilution technique, see p. 375), is tested only when the water supply is suspected of being highly contaminated.

The bottles are incubated at 37° C. and examined after eighteen to twenty-four hours. Those that show acid and sufficient gas to fill the concavity at the top of the Durham tube are considered to be "presumptive positive" as a result of the growth of coliform bacilli. Any remaining negative bottles are reincubated for another twenty-four hours, and if acid and gas develop they too are regarded as being positive. In reporting the results of the presumptive test reference is now made to McCrady's probability tables (one of which is quoted on p. 360). According to the various combinations of positive and negative results obtained the probable number of coliform bacilli in 100 ml. of the water can be read from the appropriate table.

Probability Table (according to McCrady)

QUANTITY OF WATER	50 ml.	10 ml.	1 ml.	
No. of samples of each quantity tested	1	5	5	
	0	0	0	0
	0	0	1	1
	0	0	2	2
	0	1	0	1
	0	1	1	2
	0	1	2	3
	0	2	0	2
	0	2	1	3
	0	2	2	4
	0	3	0	3
	0	3	1	5
	0	4	0	5
	1	0	0	1
	1	0	1	3
	1	0	2	4
	1	0	3	6
	1	1	0	3
	1	1	1	5
	1	1	2	7
	1	1	3	9
	1	1	0	5
	1	2	1	7
	1	2	2	10
	1	2	3	12
	1	3	0	8
	1	3	1	11
	1	3	2	14
	1	3	3	18
	1	3	4	20
	1	4	0	13
	1	4	1	17
	1	4	2	20
	1	4	3	30
	1	4	4	35
	1	4	5	40
	1	5	0	25
	1	5	1	35
	1	5	2	50
	1	5	3	90
	1	5	4	160
	1	5	5	180+

Number giving positive reaction (acid and gas).

Probable number of coliform bacilli in 100 ml. of water.

ml. being 100 divided by the figure in the last column; thus, if the number of coliform bacilli in 100 ml. is 5, the result can be stated as "coliform bacillus present in 20 ml."

Differential Coliform Test

To ascertain whether the coliform bacilli detected in the presumptive test are *Esch. coli*, the Eijkman test is usually employed. This depends on the ability of *Esch. coli* to produce gas when growing in bile-salt lactose peptone water at 44° C., and the inability of atypical coliform bacilli to do this. The following procedure may be adopted: After the usual presumptive test, subcultures are made from all the bottles showing acid and gas into fresh tubes of the same medium (single strength as used for the original test). It is advisable to heat the tubes to 37° C. in a water-bath before inoculating them. They are then incubated at 44° C. and examined after six to twenty-four hours. Those yielding gas may be regarded as containing *Esch. coli* and a computation of the number in 100 ml. of water can be made as before.

An alternative medium for use in the Eijkman test is *brilliant green bile broth* (p. 217). This was found by Mackenzie *et al.*¹ to be superior to MacConkey's fluid medium for detecting typical coliform bacilli, since the brilliant green tends to suppress the growth of anaerobic lactose-fermenting organisms such as *Cl. welchii*, which otherwise might give false positive reactions at 44° C.

Positive results in this medium are indicated by gas production and turbidity. There is no colour change.

Two types of atypical coliform bacilli are known to give rise to gas production at 44° C. They are Irregular Type II and Irregular Type VI as classified by Wilson *et al.*² Unlike typical coliform bacilli these types are unable to produce indole at 44° C. Although they rarely occur in water supplies in this country, it is advisable when carrying out Eijkman tests to inoculate at the same time tubes of peptone water from the positive "presumptive" tubes. All tubes are incubated at 44° C.

The following interpretation of results is given by Mackenzie *et al.*¹

Gas in brilliant green bile broth at 44° C.	Indole production at 44° C.	
+	+	Typical coliform bacilli
+	-	Irregular Type II
-	+	Irregular Type VI
		Other coliform

than 0.5° C. from 44° C. An incubator is not considered satisfactory for maintaining cultures at this temperature.

For fuller differentiation of the coliform group by means of the methyl-red, Voges-Proskauer, citrate-utilisation and sugar fermentation reactions, reference should be made to Chapter XXVII.

The Plate Count

With a sterile graduated pipette place 1 ml. water in a sterile Petri dish (4 in. diameter) and add 10 ml. nutrient agar (standardised to pH 7.2), melted and cooled to 50° C.; mix thoroughly and allow to solidify. The agar should be as transparent as possible.

If the water is suspected of contamination, plate out a smaller quantity, e.g. 0.1 ml., and in dealing with specimens which may be highly polluted it is advisable to make a series of plate cultures with further decreasing quantities of the water. Serial dilutions may be made from the sample, e.g. 1 in 10, 1 in 100, etc., using sterile quarter-strength Ringer's solution as a diluent. In preparing the 1 in 10 dilution, 10 ml. of the well-mixed sample is added to 90 ml. of Ringer's solution contained in a screw-capped bottle of 120 ml. capacity. After thorough mixing, further tenfold dilutions can be prepared by transferring 10 ml. of the 1 in 10 dilution to a second bottle of 90 ml. Ringer's solution and so on. One ml. quantities of each dilution is then plated.

Prepare duplicate plates from each volume or dilution, and incubate one at 37° C. for one day and the other at 20°–22° C. for three days. Those organisms which grow rapidly at 37° C. are mainly parasitic and are derived from excremental contamination, while those growing best at 20°–22° C. are the natural saprophytes of water and soil. It is customary in some laboratories to extend the incubation period at 37° C. to forty-eight hours. This is not recommended since after two days certain saprophytic bacteria capable of growing more slowly at 37° C. may have developed into visible colonies.

After incubation, the colonies that have developed in the medium are counted using a hand lens if necessary to detect small colonies. Each colony may be taken to represent one viable bacterium in the original specimen.

To facilitate the counting of colonies, to prevent eye-strain and to minimise inaccuracies, it is desirable that a special illuminated counting box and a magnifying glass should be used. A suitable apparatus for this purpose is one devised by Engineering Developments (England) and known as a "colony illuminator" (EDM205). It consists

red from the inside by two fluorescent tubes arranged	1 standard-size Petri dish which rests
1 the black plastic top, supported on a	1 and surrounded by a translucent opal

It diffuses evenly. Five inches according to the older system of notation, meter and magnification 2× certatively as "coliform bacillus present" stic screen to prevent direct

daylight from reaching the Petri dish. A mechanical hand tally counter should be used and as each colony is recorded it should be "spotted" by pen and ink on the under surface of the plate. If there are large numbers of colonies present divide the plate. If there are large lightly on the under surface with a grease pencil and count the colonies in each section. If the plates prepared and count the colonies show between 30 and 300 colonies, these from the undiluted water are more than 300 colonies and the sample should be counted. If there are plates giving counts between 30 and 300, has been diluted, then the others discarded. If all plates show 300 colonies should be selected and the result should be reported as more than 300 colonies, then the dilution of the highest dilution used; e.g. if 300 multiplied by the reciprocal of the sample was diluted in 1 in 100, the result would be given as "the sample was diluted in 1 in 100, provided no more than 500 colonies than 30,000". Alternatively, 300 colonies may be made as accurate as possible, a count of more than 300 colonies may be made as accurate as possible and the result given as an approximate one. Since only as possible and the result given present in the water are capable a proportion of the bacteria originally present in the water are capable a proportion of the bacteria originally the test, the total colony count developing under the conditions of ml. of the sample, which represents the number of organisms per (i.e. 37° C. or 22° C.) in the specimen at the specified temperature. The result is expressed briefly as the plate count per ml. at 37° C. and 22° C.

Interpretation . Results

It must be realised that it is not possible to lay down rigid bacteriological standards to which all drinking water supplies should conform. The bacteriological flora of water varies widely according to the nature of the supply, i.e. whether it is derived from a well, river, lake or ground. The aim of the authorities prevailing in the gathering knowledge of the topography, ities should be to obtain a thorough this establish a standard for the catchment area and in the light of frequent bacteriological examinations. Any later deviation from that standard should be viewed with suspicion.

Generally speaking, with suspicion. of little value in estimating results of plate counts are by themselves though where regulating the hygienic quality of a water supply, high count on a particular occasion may draw attention to a fault well which is horizon. A slight rise in the count of water from a deep defect has occurred very pure may be the earliest indication that a pollution from in the structure of the well and is allowing its efficacy of water-side. The plate count is also of value in judging the particular water treatment processes and in indicating whether a drink, which supply is suitable for use in the preparation of food and

The presence of a high bacterial content may lead to food spoilage. quality of coliform bacilli is of much greater value in assessing the nature of a water supply. In interpreting the results, however, the supply must still be taken into account.

test, an estimate of the probable number of *Cl. welchii* in 100 ml. of water can be made.

Although in recently contaminated water *Cl. welchii* occurs in much smaller numbers than *Esch. coli*, it is able to survive for much longer periods than the non-sporing bacteria of faecal origin. The chief value of the test therefore is in detecting pollution of some previous time or to confirm the faecal origin of atypical coliform bacilli in the absence of *Esch. coli*.

Bacteriological Examination of Sewage and Sewage Effluents

The bacteriological examination of sewage may be carried out to determine the purity of an effluent from a sewage purification process. The procedure is the same as in water examination; an estimation of the viable bacteria present is made by plating and counting colonies, and the test for coliform bacilli is carried out as with a specimen of water; much smaller amounts, however, are tested than in the case of water, depending on the likely extent of dilution of the effluent. The numbers of bacteria per ml. in crude sewage vary greatly, e.g. from 1 million to 100 million. Sewage may also be examined by the membrane filter technique (p. 368).

For isolation of typhoid-paratyphoid bacilli from communal sewage see p. 371.

Bacteriological Control of Swimming Baths

Public swimming pools and indoor swimming baths may become infected with pathogenic organisms derived either from contaminated water entering the pool or from the skin, mucous surfaces and excretions of bathers. Unless adequate means of purifying the water are provided, this contamination may lead to outbreaks of diseases, such as gastro-enteritis, infections of the respiratory tract, otitis media, infections of the conjunctiva and the skin.

Most modern swimming pools are operated on a system which provides a continuous circulation of the water from the bath at the deep end through a purification plant where it undergoes filtration, clarification and chlorination before entering the pool again at the shallow end. The amount of chlorine introduced into the water is accurately measured and controlled so that the free residual amount present in the bath is maintained between 0.2 and 0.5 p.p.m.¹

The results of bacteriological examinations of samples of water taken from the inlet and outlet of the bath give an indication of the effectiveness of the treatment in maintaining the water free from undesirable contaminants. The methods usually employed are those used for

¹ Rep. Minist. Hlth (1951): "The Purification of the Water of Swimming Baths." H.M.S.O., London.

testing samples of drinking-water, viz. an estimation of the number of viable bacteria by the plate counts at 37° C. and 22° C. and the presumptive test for coliform bacilli followed by the differential tests for *Esch. coli* (p. 361). The bacteriological quality of the water should approximate to that of high purity drinking-water.

Owing to the fact that the water in swimming pools tends to become contaminated by the organisms associated with the skin and mucous membranes of the bathers, some authorities believe that the presence of such organisms as *Neisseria catarrhalis*, staphylococci and streptococci are of more significance than the coliform group for estimating the quality of the water. It appears from the results of an investigation carried out by the Water Sub-committee of the Public Health Laboratory Service¹ that staphylococci, both coagulase-positive and negative strains, are more resistant to chlorination than are the coliform bacilli and may withstand as much as 0.5 p.p.m. of free residual chlorine. Streptococci of the β -type were rarely encountered even when the chlorine level was low whereas α -streptococci, like the staphylococci, were resistant to high concentrations of free chlorine. *Neisseria catarrhalis* may be isolated by inoculating the surface of a series of heart muscle infusion agar plates containing phenol red (0.0025 per cent.) and glucose (1 per cent.) with 0.5 ml. of the water and also with the deposits obtained from centrifuging 5 ml. and 50 ml. amounts. The organism produces red colonies on this medium, and its presence may be confirmed by staining films of the colonies and examining them microscopically.

The water sub-committee were of the opinion that there was little advantage in using the test for *Neisseria catarrhalis* rather than the test for coliform bacilli. Their findings showed that the best indication of the purity of the water was afforded by the plate count at 37° C.

The following standards for water purity were recommended:

"No samples examined from a bath should contain any coliform organisms in 100 ml. of water; and in 75 per cent. of the samples examined from the bath the plate count at 37° C. from 1 ml. of water should not exceed 10 colonies and in the remainder should not exceed 100 colonies."

Amies² maintains that "before a swimming pool can be pronounced as satisfactory, the surface water should be examined bacteriologically as well as the main body of the water". He presents evidence to show that oral or nasal bacteria collect in the surface film of fatty substances derived from the skin and hair of the bathers, and are protected by it from the action of the chlorine. He considers that this surface film may be a contributory factor in the spread of bacterial and viral diseases by swimming pools. For the method by which a sample of the surface film may easily be removed for bacteriological examination reference should be made to the original paper.

¹ *Monthly Bull. Minist. Hlth Lab. Serv.* (1953), **12**, 254.
Amies, C. R., *Canad. J. Pub. Hlth*, **47**, 93.

THE MEMBRANE FILTER TECHNIQUE FOR THE BACTERIOLOGICAL EXAMINATION OF WATER AND SEWAGE

This method is based on the use of a highly porous cellulose membrane, the pore structure of which enables fairly large volumes of water or aqueous solutions to pass through rapidly under pressure, but prevents the passage of any bacteria present in the sample. These are retained on the surface of the membrane which is then brought into contact with suitable liquid nutrients. These diffuse upwards through the pores thereby inducing the organisms to grow, producing visible surface colonies which can then be counted.

The technique was first described in this country by Windle Taylor *et al.* in 1953¹ and, after certain modifications had been devised, the test was applied to standard methods at present in use in this country.^{2,3}

Membrane filters may also be used for the isolation of pathogens from water and sewage and for the demonstration of tubercle bacilli in cerebrospinal fluid and other fluid specimens including sputa from cases of tuberculosis.⁴

Apparatus

Various types of filtering apparatus suitable for water and sewage examination are now available. A German-made funnel has been used extensively by the Metropolitan Water Board. It may be obtained in England from Hudes Merchandising Corporation.⁵ It is known as the "Coli 5" model and consists of a funnel of nickel-plated brass of 500 ml. capacity attached by means of a bayonet-locking device to the base of the apparatus which contains a disk of sintered glass on which the membrane is supported. The outlet is provided with a tap and fits into the rubber stopper of a suction jar. Some of the other types of funnel do not have this outlet tap and are therefore not so convenient to handle. It is also an added advantage to have the inside of the funnel provided with graduation marks for easy measurement of the water sample.

The special "Coli 5" German-manufactured membranes for use with the apparatus described above are paper-thin, porous disks, 5 centimetres in diameter, composed of a cellulose derivative which forms the framework for a thin gelatinous layer of suitable porosity. They are quite tough and elastic even when dry, white in colour and opaque with a glazed surface. They may be obtained marked with a grid to simplify the counting of the colonies. English-made membranes manufactured by Courtaulds Ltd., and marketed by Oxo Ltd., have

¹ Windle Taylor, E., Burman, N. P., & Oliver, C. W. (1953), *J. appl. Chem.*, 3, 233.

² Windle Taylor, E., Burman, N. P., & Oliver, C. W. (1955), *J. Inst. Water Engrs.*, 9, 248.

³ Windle Taylor, E. (1955-56), *37th Ann. Rep. Dir. Water Exam. Met. Water Bd.*, London.

⁴ Haley, L. D., & Rosty, A. (1957), *Amer. J. clin. Path.*, 27, No. 1.

⁵ Hudes Merchandising Corporation Ltd. 52 Gloucester Place, London, W.1.

been found in some ways more satisfactory for the culture of the bacteria, though they tend to be brittle and more readily damaged than the German ones. Membranes that have been used for the coliform count may be washed in running water, dried between blotting-paper and sterilised for further use. This may be done up to three or four times, but damaged membranes should always be discarded.

Sterilisation

The filtering apparatus is assembled without the membrane, wrapped in kraft paper and sterilised by autoclaving at 15 lb. per sq. in., for fifteen minutes. Thereafter, between each test, it is sufficient to apply a jet of live steam, or alternatively a pad of cotton-wool attached to a metal rod, dipped in alcohol and ignited. Both the inner and outer surfaces of the funnel as well as its base and the sintered glass disk require to be sterilised in this way. The routine examination of large numbers of water samples is facilitated by the use of several funnels for each piece of apparatus. While one sample is being filtered, the spare funnels for subsequent samples can be sterilised and cooled.

Membrane filters may be sterilised by either of two methods.

(1) Gentle boiling in prefiltered distilled water on two occasions each of twenty minutes duration. Vigorous boiling tends to make the membranes buckle. This method not only sterilises but washes out residual solvents and air present in the pores.

(2) Autoclaving at 10 lb. per sq. in. for ten minutes. For this purpose bundles of 10 membrane filters are interleaved with disks of good quality absorbent paper (subsequently to be used for holding the liquid medium). The bundles are secured between two pieces of thin card held in position by adhesive tape and the whole is wrapped in kraft paper and sterilized.

Media

1. *For Coliform Bacilli Estimations.* Endo's sodium sulphite medium is used widely on the continent of Europe and in America, for the detection of typical coliform bacilli in water supplies; MacConkey's bile-salt-lactose broth is the medium of choice in this country but, in order to apply it satisfactorily to the membrane filter technique, it has been necessary to modify the original composition of the broth. The following formula was found to give the most satisfactory results, but it is recommended that the optimum proportion of bile salts should be determined for each new batch obtained.¹

Peptone	10 g.
Lactose	30 g.
Bile salts	4 g.
Sodium chloride	5 g.
Brom-cresol purple (1 per cent. alcoholic solution)								12 ml.
Water (pH 7.4)	1 l.

¹ Burman, N. P. (1955), *Proc. Soc. Water Treatment and Examination*, 4, 10.
2A

2. *For the Enumeration of Viable Bacteria.* In order to obtain results comparable with those of the agar plate count method for all types of water, it is necessary to use nutrient broth having the following composition:

Yeastrel (see p. 197)	6 g.
Peptone	40 g.
Distilled water	1	to 1 l.
pH	7.4

It is understood that Oxo Ltd. are preparing media suitable for use with membrane filters. Their yeastrel broth and modified MacConkey broth in sealed ampoules (one ampoule being sufficient for one test) have both been proved satisfactory by the Metropolitan Water Board (see footnote, p. 368).

Method of Filtration

After sterilisation, the filtering apparatus is inserted into the suction jar attached to the vacuum pump, the outlet tap being closed. The funnel is removed, and with sterile forceps one of the membranes is laid, grid-side up, on the top of the sintered glass disk. By turning on the pressure and opening the tap carefully, the membrane is sucked down and comes to lie quite flat against the disk. (It has been suggested that in order to protect the membrane and hasten filtration, a disk of filter paper should be inserted between the supporting glass disk and the membrane.) After closing the tap and releasing the pressure, the funnel is screwed into place and a suitable amount of the water to be examined is poured into it. The actual amount depends on the likely degree of pollution. If there is doubt about this, two or more different volumes of the sample should be filtered. The following amounts are recommended:

Purified tap water	250-500 ml.
Well-water	10 and 100 ml.
River-water	1 and 10 ml.

Water which is highly contaminated should be diluted to 1 in 10, 1 in 100 and 1 in 1000 and each dilution filtered. Quantities smaller than 20 ml. should be made up to that amount with sterile distilled water before being passed through the filter.

When the water has been filtered and a small amount of sterile distilled water allowed to pass through as a final rinse the funnel is removed and the vacuum released. The membrane is then transferred with sterile flat-bladed forceps to a 2-in. Petri dish containing a sterile absorbent pad¹ saturated with the appropriate liquid medium (approximately 2 millilitres should be sufficient). The membrane should be placed on the moist pad in such a way as to exclude any air bubbles. The Petri dish is then inverted with the pad and membrane adhering to the base and incubated in a moist atmosphere at 37° C.

¹ Whatman's No. 17 pads (5 or 6 cm. sizes) have proved satisfactory.

After eighteen to twenty-four hours the colonies have developed sufficiently on the surface of the membrane to be counted. The incubation period should not be prolonged over twenty-four hours or the colonies may run together and make their counting difficult. The number of colonies per ml. of undiluted water which appear on the membrane in contact with the nutrient medium may then be calculated.

The yellow colonies which develop on the membrane in contact with the modified MacConkey's medium merely represent lactose fermenting organisms which may or may not be gas producers. Their identity is presumed to be that of coliform bacilli for the purpose of routine water examination, but this can be confirmed by the usual tests (see p. 361) if required.

Windle Taylor states that it is possible to obtain directly an accurate count of typical coliform bacilli by first incubating the membrane at 37° C. in contact with a pad soaked in concentrated yeastrel broth (*vide supra*). After two hours at that temperature the membrane is transferred to a pad soaked in the special bile-salt medium and incubated for eighteen hours at 44° C. The preliminary incubation is thought to encourage the growth of organisms which may have become attenuated as a result of the inhibitory action of inorganic salts present in the water supply.¹

This technique of "resuscitation" may also be applied to the presumptive coliform estimation, by incubating the membrane after filtration in contact with concentrated yeastrel broth for one hour before transferring it to modified MacConkey's medium for an additional eighteen hours at 37° C.

For recent comparative studies on the Membrane Filter Technique and the Multiple Tube Test for coliform bacilli and *Esch. coli* estimations reference should be made to the report of the Metropolitan Water Board.²

Isolation of Pathogenic Organisms from Water and Sewage

(a) **By Membrane Filter Technique.**—Relatively large amounts of the fluid to be tested, *i.e.* 500 ml. or more, depending on the amount of suspended matter present, can be passed through the membrane filter fairly rapidly. If pathogenic organisms are present, even in small numbers, they will be retained on the surface of the membrane, and by transferring it to a suitable differential medium there is a reasonable chance of isolating them.

For isolating typhoid and paratyphoid bacilli Wilson and Blair's bismuth sulphite medium (p. 220) has been reported as giving

¹ Allen, L. A., Pasley, S. M., & Pierce, M. S. F. (1952), *J. gen. Microbiol.*, 7, 257.

² Windle Taylor, E. (1957-58), *38th Ann. Rep. Dir. Water Exam. Met. Water Bd.*, London.

satisfactory results by this method. The membrane may be placed directly on the agar medium in a small Petri dish, the proportion of agar having been reduced to 1.5 per cent., but Kabler and Clark¹ recommend the use of absorbent pads impregnated with double-strength liquid medium (agar omitted). Characteristic colonies appear within thirty hours at 37° C. They are convex, black with a paler periphery and, if discrete, each one is surrounded by a halo, showing a metallic sheen. It had been observed that should the growth be too dense for the colonies to be distinguished, blackening of the medium underneath the membrane is suggestive of typhoid colonies. In such a case, it is necessary to transfer the membrane to a tube or bottle of liquid medium such as tetrathionate broth or selenite enrichment medium (p. 221) subculturing from this after eighteen to twenty-four hours' incubation at 37° C. on MacConkey's agar or desoxycholate-citrate agar.

In order to isolate intestinal pathogens, other than typhoid and paratyphoid bacilli, Kabler and Clark recommend a preliminary incubation of the membrane in contact with an enrichment medium before transferring it to a differential medium. Single-strength tetrathionate broth without chalk was found to give satisfactory results. Incubation of the membrane for three hours on this medium tends to inhibit coliform bacilli and enhances the growth of salmonellae other than *Salmonella typhi*. The membrane is then transferred to a suitable differential medium and incubated for a further eighteen hours, after which time characteristic colonies may be recognised and subcultured in order that the usual confirmatory tests may be carried out.

(b) **By a Concentration Technique, using a membrane filter apparatus with pulverised diatomaceous earth.**—The Metropolitan Water Board have reported satisfactory results from the use of a modification of the method of Hammerström and Ljutov^{2,3}, for isolating pathogenic intestinal organisms from water and sewage. The technique is so simple and quickly carried out that its use is to be recommended rather than that mentioned above.

A layer of diatomaceous earth (Hyflo Supercel) is substituted for the membrane at the bottom of the filter funnel, supported on a disk of stainless steel wire micro-mesh. After setting up the filtering apparatus with the micro-mesh in place, a small amount of sterile distilled water is poured into the funnel and to this is added a quantity of 1 per cent. sterile Hyflo Supercel suspension to give the required thickness. The pressure is turned on and the measured sample poured in before all the sterile water has gone through. (Up to 100 litres of water can be filtered in thirty minutes, sewage from 3 to 10 ml. suitably diluted, and effluent 50–500 ml.). After all the fluid has been sucked through, the wire-mesh support is lifted off with sterile forceps and the paste tipped into 100 c.c. selenite medium in a screw-capped bottle (4 in. in depth

¹ Kabler, P. W., & Clark, H. F. (1952), *Amer. J. publ. Hlth*, **42** (1), 390.

² Hammerström, E., & Ljutov, V. (1954), *Acta path. microbiol. scand.*, **35**, 365.

³ Ljutov, V. (1954), *ibid.*, 370.

by 2 in. in diameter). This is incubated at 42° C. After eighteen hours subcultures are made on selective media.¹

BACTERIOLOGICAL EXAMINATION OF MILK

In hygiene work the bacteriological examination of milk generally consists in:

- (1) An enumeration of viable bacteria present in a given quantity.
- (2) A quantitative estimation of contamination by coliform bacilli.
- (3) The determination of the presence of specific pathogenic organisms, e.g. *Myco. tuberculosis*.

Since 1936 the *methylene blue reduction test* has been used as a standard official method in England for gauging milk purity, *i.e.* as a substitute for the bacterial count. It depends on the reduction and decolorisation of the dye by the bacteria in the milk, and the rate of reduction affords a measure of the degree of bacterial contamination.

As a check on the pasteurisation of milk, the *phosphatase test* is now a standard procedure; it determines the inactivation by heat of the enzyme phosphatase, which is normally present in cow's milk. Activity of this enzyme implies that the milk has not been adequately heated for the destruction of pathogenic organisms present.

The *turbidity test* has recently been introduced as an official test for "sterilised" milk, *i.e.* milk which has been heated to 212° F. or over for a certain length of time (according to the Scottish regulations 220°-235° F. for a period not exceeding thirty minutes). The test depends on the fact that by heating to the degree necessary for sterilisation the heat-coagulable proteins are precipitated, so that if ammonium sulphate is added and the mixture filtered and boiled for five minutes, no turbidity results. The test also distinguishes between pasteurised and "sterilised" milk.

Under the Milk (Special Designation) (Raw Milk) Regulations, 1949 and 1950, and the Milk (SPECIAL DESIGNATION) (Pasteurised and Sterilised Milk) Regulations, 1949 and 1950, of England, and the Milk (Special Designations) (Scotland) Order, 1951, standard methods for testing milk have been prescribed in official memoranda. These should be consulted for full details of the methods recommended.

Bacteriological Standards

England and Wales

The following standard has been laid down under the Milk (Special Designation) (Raw Milk) Regulations, 1949 and 1950:

"Tuberculin Tested" milk when tested by the prescribed method must not decolorise methylene blue within four and a half hours when the sample is taken from 1st May to 31st October, and within five and a half hours when the sample is taken from 1st November to 30th April.

¹ See also Metropolitan Water Board, 37th Report cited in footnote, p. 368.

Under the Milk (Special Designation) (Pasteurised and Sterilised Milk) Regulations, 1949 and 1950, "*Pasteurised*" milk must satisfy the phosphatase test (*vide infra*), i.e. when tested under the prescribed conditions the milk must give a reading of 2.3 "Lovibond blue units" or less. A sample of *pasteurised* milk taken on the day of delivery to the consumer must also satisfy the prescribed methylene-blue test, i.e. it must not reduce methylene blue within thirty minutes after being kept in the laboratory at atmospheric shade temperature not exceeding 65° F. till between 9 and 10 a.m. on the day following that on which it is taken (*vide infra*).

"*Sterilised*" milk must satisfy the turbidity test described on p. 379

Scotland

The special designations which may be used in Scotland are "*Certified*", "*Tuberculin Tested*", "*Pasteurised*" and "*Sterilised*". The following standards have been laid down under the Milk (Special Designations) (Scotland) Order, 1951.

"*Certified*" milk must contain not more than 30,000 bacteria per millilitre and no coliform bacillus in 0.1 millilitre.

"*Tuberculin Tested*" milk must contain not more than 200,000 bacteria per millilitre and no coliform bacillus in 0.01 millilitre.

"*Pasteurised*" milk must contain no coliform bacillus in 0.01 millilitre, and must satisfy the phosphatase test as defined on p. 378.

"*Sterilised*" milk must satisfy the turbidity test referred to on p. 379.

Sampling.—If the milk is contained in retail bottles, one unopened bottle delivered to the laboratory would constitute the sample. When the milk is in churns it must be carefully mixed before a specimen is taken. This can be done by means of a sterile plunger which is moved up and down several times in the milk. The specimen is then obtained with a sterile dipper and placed in a sterile 4-oz. stoppered or screw-capped bottle. Samples taken before the milk leaves the custody of the producer should be maintained at atmospheric shade temperature for a period of nine to eleven hours after the time of milking in the case of morning milk before being examined. Milk produced in the evening should be held at atmospheric temperature and tested as soon as practicable on the following day. Other samples should be tested immediately after arrival in the laboratory. If there should be any unavoidable delay in testing, the samples should be kept at a temperature of 0°–5° C.; no sample shall be kept for longer than twenty-four hours.

Technique of Estimating the Number of Viable Bacteria.—The medium recommended in the Scottish regulations is yeast extract milk agar (see p. 197).

A series of dilutions of the milk sample is made up in sterile stoppered bottles with sterile tap water as follows:

- | | | |
|-----------------|--------------|---------------------------------|
| 1 in 10 . . . | 90 ml. water | plus 10 ml. milk |
| 1 in 100 . . . | 90 ml. " | 10 ml. of the 1 in 10 dilution |
| 1 in 1000 . . . | 90 ml. " | 10 ml. of the 1 in 100 dilution |

Before making these dilutions, the specimen should be carefully mixed

by inverting the sample bottle twenty-five times. The dilutions must also be mixed but without vigorous shaking. The pipettes used should be straight-sided and appropriately graduated. For each dilution a separate sterile pipette should be used.

For testing *certified* milk under the Scottish regulations 1 ml. of the 1 in 100 dilution is plated, duplicate or preferably triplicate plates being made; in examining *tuberculin tested* milk 1 ml. of the 1 in 1000 dilution is plated as above. The diluted milk is placed with a sterile pipette in a sterile Petri dish (4 in. diameter) and 10 ml. of melted agar cooled to 50° C. are added and mixed with the milk by rotating the plate carefully first to the right, then to the left, so that the organisms are uniformly distributed throughout the agar.

The time between the preparation of the dilutions and the mixing with medium should not exceed fifteen minutes.

After the medium has solidified, the plates are incubated in the inverted position for two days at 37° C.

The number of colonies is counted in each plate and the mean calculated; this multiplied by the dilution is reported as the "number of viable bacteria per millilitre". The count is made as described on p. 362. If the number of colonies in a plate is over 300, a count may be made of those in a given part of the plate and the total is then calculated; but it is advisable in examining a milk of unknown quality to plate 1 ml. of each dilution (*vide supra*) and use for the count those showing 30 to 300 colonies.

Under the most favourable conditions a specimen of raw milk may contain at least 500 bacteria per ml.; but under bad conditions the numbers may reach even several million per ml. The standards given on p. 373 indicate the degree of bacterial contamination allowable in the case of the designated milks.

Test for Coliform Bacilli.—Varying amounts of milk are added to tubes or bottles of bile-salt-lactose medium (p. 359). The range of amounts that require to be tested depends on the likely degree of contamination. In the case of milk of unknown quality the following series is suggested:

1.0 ml. of a 1 in 10	dilution of the milk
" " 1 in 100	" "
" " 1 in 1000	" "
" " 1 in 10,000	" "

The decimal dilutions are prepared in series (*vide supra*).

The smallest amount which yields acid and gas is ascertained.

Under the Scottish regulations, for *certified* milk three tubes or bottles containing 10 millilitres of the above medium are inoculated (by means of a sterile pipette) each with 1 millilitre of the 1 in 10 dilution of the sample and incubated at 37° C. for forty-eight hours. For the other designated milks, three tubes are inoculated each with 1 millilitre of the 1 in 100 dilution. The tubes are examined for acid and gas production; the milk is taken to have passed the test if acid and gas are absent from two of the three tubes.

Methylene-blue Reduction Test.—Under the English regulations this test is used instead of the plate count and the test for coliform bacilli in the case of milk of all designations (except *sterilised* milk). Standard methylene-blue tablets must be used. (The names of manufacturers who supply such tablets are furnished by the Ministry of Health.) A standard solution is prepared as follows: one tablet is dissolved in 200 ml. cold sterile glass-distilled water in a sterile flask with a rubber stopper. The solution is then made up to 800 ml. with distilled water and stored in a cool, dark place. This solution gives a final concentration of methylene blue of approximately 1/300,000, and should not be used after two months.

Test-tubes conforming to the British Standards Specification 152/16 ($6 \times \frac{5}{8}$ in.) with an internal diameter of 13.5 mm. (approx.) and a mark indicating 10 ml. are used. They are stoppered with cotton-wool or aluminium caps and sterilised in a hot-air oven (160° C.—two hours). Rubber stoppers to fit the tubes are also required. These are sterilised in boiling water before use.

A thermostatically controlled covered water-bath with rack to hold the tubes immersed in the water is required; the water should be at 37°–38° C.

1-ml. straight-sided pipettes are used for measuring the methylene-blue solution (these should conform to a prescribed specification). They are sterilised in the hot-air oven.

The sample is mixed thoroughly, as prior to making the bacterial count (*vide supra*).

The milk is poured, with the usual aseptic precautions, into a test-tube up to the 10 ml. mark, and 1 ml. of methylene-blue solution is carefully added. The tube is closed with a sterile rubber stopper which should be inserted with sterile forceps. It is then inverted slowly once or twice and placed in the water-bath.

The following controls should be put up: (1) 10 ml. mixed milk *plus* 1 ml. methylene-blue solution, (2) 10 ml. mixed milk *plus* 1 ml. tap water. These control tubes are placed for three minutes in boiling water to destroy the natural reducing system of the milk. Comparison with (1) indicates when decolorisation is beginning and with (2) when it is complete.

The tubes are examined every half-hour, and if no change has occurred on each occasion they are inverted once.

Decolorisation is considered complete when the whole column of milk is decolourised or decolourised-up to within 5 mm. of the surface. The time of complete decolorisation is recorded if within the prescribed period (standards given on p. 374).

Rationale of the Various Bacteriological Tests used for the Examination of Milk

Whichever test is adopted for the routine examination of milk it should be capable of indicating the degree of bacterial contamination

and thereby of showing whether the conditions under which the milk is produced and handled are hygienically satisfactory.

The advantage of the plate count for this purpose is that it gives a direct assessment of the number of viable bacteria in the supply. The results are readily understood by the dairyman, and since it is as suitable for milk of low bacterial content as for grossly contaminated supplies, it will indicate any changes in conditions of production leading to either an improvement or deterioration in quality. On the other hand, the plate count test is costly in time and material, the results are not available for forty-eight hours and owing to the fact that a very small amount of milk is tested and that some of the bacteria are distributed in small clumps and chains, the error of sampling is high.

The coliform test is usually carried out in conjunction with the plate count. It indicates mainly the degree of contamination by coliform organisms arising from dust or unsterile utensils. Since adequate pasteurisation destroys the majority of coliform bacilli, the presence of these in milk which passes the phosphatase test is an indication of contamination after pasteurisation.

The methylene-blue test is simple to carry out and requires a minimum of equipment. In general, the greater the number of bacteria in the milk, the greater their metabolic activity and consequently the shorter the reduction time. According to Chalmers,¹ milk supplies may be classified as follows on the basis of the reduction times and the approximate bacterial counts which correspond to them:

<i>Class of Milk</i>	<i>Reduction Time</i>	<i>Approximate Number of Organisms per ml.</i>
Class 1. Good	over $4\frac{1}{2}$ hours	200,000 (or less)
Class 2. Average	$2\frac{1}{2}$ to $4\frac{1}{2}$ hours	200,000–2,000,000
Class 3. Poor	less than $2\frac{1}{2}$ hours	2,000,000–10,000,000

However, milk heavily contaminated with inert bacteria may give a long reduction time, while short reduction times may be the result of non-bacterial reducing systems which are sometimes present in freshly produced milk, milk obtained late in the lactation period and milk containing leucocytes or other cells.

The time required to reduce the dye is also dependent on the temperature at which the milk is held prior to testing; thus, in the winter, milk gives a longer reduction time than it would in the summer, with the same bacterial content. Allowance is made for this in the higher standard required in the winter time by the English regulations.

It is difficult to compare high-quality milks by means of the standard methylene-blue test, since the reduction time is very long for all of them, but the test will readily detect milk of poor hygienic quality. With *pasteurised* milk, provided the milk is kept for at least twenty-four hours at a temperature not exceeding 65° F. before being tested, the

¹ Chalmers, C. H. (1955), *Bacteria in Relation to the Milk Supply*. London, Arnold.

correlation between the reduction time at 37° C. and the degree of bacterial contamination is fairly good. The test is of no value for freshly pasteurised milk.

Phosphatase Test for Pasteurised Milk¹

This test determines inactivation of the enzyme phosphatase, normally present in cow's milk, by such degree and time of heating as to destroy non-sporing pathogenic organisms, e.g. 145° F. (62.8°C.) for thirty minutes or 161° F. (71.6° C.) for fifteen seconds, as in the recognised methods of pasteurisation. The standard method for detecting the presence of the enzyme is based on its ability to liberate phenol from disodium phenyl-phosphate, the phenol being estimated colorimetrically with Folin and Ciocalteu's reagent which yields a blue coloration, and the result is expressed in arbitrary units; deep blue i.e. over 6 units, indicates a large amount of phenol; pale blue, i.e. under 2.3 units, a small amount.

Reagents Required:

(1) Buffer substrate tablets (disodium phenyl-phosphate and sodium barbitone)¹: dissolve one tablet in 50 ml. boiling distilled water, boil for one minute and cool rapidly. This solution must be freshly prepared.

(2) Folin and Ciocalteu's phenol reagent.² This solution is diluted with twice its volume of sodium hexametaphosphate solution 5 per cent. w/v.

(3) Fourteen per cent. aqueous solution (w/v) of pure anhydrous sodium carbonate (Analar).

(a) *Method.*—To 10 ml. of the buffer substrate solution in a 25-ml. stoppered test-tube marked at 10 ml. (e.g. as used for methylene-blue reduction tests), add 0.5 ml. of the well-mixed milk and mix thoroughly. Add 3 drops of chloroform, stopper the tube, and incubate for 24±2 hours at 37°–38° C. At the end of this time, cool, add 4.5 ml. of the diluted Folin-Ciocalteu reagent, mix, allow to stand for three minutes, and filter, using a Whatman filter-paper No. 40 (or No. 30). To 10 ml. of the filtrate add 2 ml. of the sodium carbonate solution, mix, place the tube in a boiling water-bath (kept boiling) for two minutes. Compare the colour in a Lovibond comparator with the series of colour standards (on the appropriate "phosphatase" disk), each of which represents a given unitage: 6, 2.3, 1.5 and 0.5.

(b) *Controls.*—The test on each milk sample should be made in duplicate, and the following control must be included along with each set of tests: a "blank" in which the reagents only, without milk, are tested as above. Milk samples are also kept in the refrigerator

¹ Kay, H. D., Aschaffenburg, R., & Neave, F. K. (1939), "The phosphatase test for control of efficiency of pasteurisation." *Imperial Bureau of Dairy Science, Technical Communication, No. 1.*

² Obtainable from British Drug Houses, Ltd.

for twenty-four hours after the tests have been put up, and if a sample has given a reading of over 2·3 units (*vide infra*) then the test is repeated but *omitting incubation*.

(c) *Interpretation.*—The "blank" should give a reading of not more than 0·5 Lovibond blue units, and the control test omitting incubation not more than 1·5 units. With such control results, milks which give readings of 2·3 Lovibond blue units or less are classified as "properly pasteurised", or "giving a negative phosphatase test"; those giving readings between 2·4 and 6·0 units are classified as "improperly pasteurised"; while those milks with a reading of more than 6·0 units should be reported as "grossly underpasteurised".

Where possible, the milk should be tested within eighteen hours of pasteurisation. The test is extremely sensitive, and great care is necessary in the cleaning of all glassware, which should be placed in glass-cleaning solution and kept apart from phenol-containing substances, *e.g.* lysol and coal-tar soaps. The rubber stoppers must be tested for phenolic impurities before use. Care must be taken not to contaminate pipettes with saliva, and a separate pipette must be used for each sample.

The Turbidity Test for Sterilised Milk.—Add 20 ml. of the well-mixed milk to a 50-ml. conical flask containing 4 gm. ammonium sulphate (AR). Shake thoroughly for three minutes to dissolve all the ammonium sulphate. Allow to stand for five minutes and then filter into a test-tube through a Whatman No. 12 folded filter paper (12·5 cm. in diameter). Collect at least 5 ml. of clear filtrate and place the tube in a beaker of boiling water for five minutes. Cool in cold water and examine the tube for turbidity, holding it in front of an electric light suitably shaded from the eyes. It is advisable to compare it with a tube of milk heated in a boiling water bath for twenty minutes and then treated with ammonium sulphate in the same way.

An absence of turbidity indicates that the milk has been heated to at least 212° C. for a period of at least five minutes, which denatures the soluble proteins in the milk so that they can no longer be precipitated by ammonium sulphate. The test will detect the presence of 0·6 to 0·8 per cent. raw milk, but owing to the heat resistance of some sporing organisms it gives no indication of the probable keeping quality of the "Sterilised" milk.

Examination for Tubercle Bacillus.—The sample is thoroughly mixed and a quantity of 100 ml. is divided into 50 ml. amounts and centrifuged for half an hour at a minimum speed of 3000 revolutions per minute. The sediment in each tube is suspended in 2·5 ml. of sterile saline solution. It is advisable to add some of the cream to this inoculum. Two guinea-pigs are injected subcutaneously on the inner side of one thigh with the suspended sediment and kept under observation to ascertain whether tuberculous lesions result (p. 549). One guinea-pig is killed at the end of four weeks and an autopsy carried out; if it shows no tuberculous lesions, the other animal is kept for eight weeks, when it is killed and examined.

Lesions should be examined microscopically for the tubercle bacillus to confirm their tuberculous nature. (It has been shown that *Br. abortus*, which may occur in cow's milk, produces tubercle-like lesions in guinea-pigs.) It is advisable to inoculate at least two animals from one specimen, as inoculated guinea-pigs may die sometimes from infection with other organisms present in the milk, e.g. sporng anaerobic bacilli or some intercurrent disease—e.g. pneumonia, enteritis, etc.—may also cause death before tuberculous lesions have developed and so nullify the test if only one animal is injected.

A direct microscopic examination of the deposit of centrifuged milk for tubercle bacilli may be made. Before staining the dried film should be treated for thirty seconds with equal parts of ether and absolute alcohol to remove the fat. With the low power objective the presence or absence of cell groups (so-called epithelioid cells) should be noted. In addition to being characteristic of tuberculous milk these cell groups are of importance in that tubercle bacilli are frequently found within or around them.

The absence of tubercle bacilli in films, however, does not exclude their presence in the specimen. On the other hand acid fast bacilli other than the tubercle bacilli may be revealed. The microscopic test, therefore, is not a valid method of demonstrating tubercle bacilli.

The method of *direct cultivation* described on p. 547 can very suitably be applied to unmixed milk taken directly from the cow, but the animal inoculation test is the standard procedure for demonstrating tubercle bacilli in milk samples generally.

Other Pathogens in Milk.—The methods for demonstrating typhoid-paratyphoid bacilli and the diphtheria bacillus correspond to those used for the isolation of these organisms. For the former, the sediment, after centrifuging, should be plated out on the surface of desoxycholate-citrate medium or Wilson and Blair's medium, and at the same time tubes of tetrathionate or selenite F medium are inoculated as in isolating the organism from faeces. For the diphtheria bacillus, plates of tellurite medium are inoculated with the sediment.

Br. abortus may be demonstrated in milk by inoculating two guinea-pigs as in the test for the tubercle bacillus. The animals are killed and examined after four and eight weeks respectively, cultures are obtained from the spleen by the appropriate method and the organism is then identified. An agglutination test with the serum of the inoculated guinea-pig and a suspension of *Br. abortus* affords evidence of infection from the milk without the necessity of isolating the organism. (See also p. 647.)

Brucella Ring Test.—This test, which is used to detect infected milk from cows suffering from brucellosis, depends on the fact that brucella agglutinins present in the milk are adsorbed on the fat globules. On the addition of a concentrated suspension of *Br. abortus* stained with haematoxylin, the antigen-antibody rises with the fat globules and the cream layer becomes deeply blue. For the technique of this test see p. 648. Care must be taken in interpreting a positive result

since milk of herds immunised against brucellosis within two years of the test being carried out may give a blue ring reaction.

Whey Agglutination Test.—This is a convenient method of diagnosing brucella infections in animals. Milk from the four quarter of the udder is mixed and the whey separated by the addition of rennin. The whey is then tested for brucella antibodies. For further details of the technique see p. 648.

BACTERIOLOGICAL EXAMINATION OF ICE-CREAM

The general principles and methods applicable to milk can be adopted for the bacteriological examination of ice-cream. This subject has been reported on by the Public Health Laboratory Service Staff of the Medical Research Council.¹

In a circular issued by the Ministry of Health with reference to the Ice-Cream (Heat Treatment, etc.) Regulations of 1947, attention is drawn to a form of the methylene-blue reduction test (p. 376) for grading ice-cream from the hygienic standpoint. An outline of the test is given here, but full details can be obtained from the published report.

The test should be commenced at 5 p.m. on the day on which the sample is taken. With a graduated pipette, 7 ml. of one-quarter strength of Ringer's solution are added to the reduction tube, as used for milk (p. 376), and 1 ml. of standard methylene-blue solution; the sample is then added up to the 10 ml. mark (*i.e.* 2 ml. and constituting a 1 in 5 dilution). With precautions to avoid bacterial contamination the tube is closed with a sterile rubber stopper and inverted once. The tube is placed in a water-bath at 20° C. until 10 a.m. on the following day. It is then placed in a water-bath at 37° C. and inverted once every half-hour until decolorisation is complete, as compared with the control, the time for decolorisation being recorded. This control consists of a tube to which are added 8 ml. one-quarter strength Ringer's solution, and ice-cream to the 10 ml. mark, and incubated at 20° C. and 37° C. as in the actual test. A methylene-blue control should also be included as in milk-testing (*q.v.*).

According to the time taken at 37° C. for complete decolorisation the sample is graded provisionally as follows:

- Grade 1. over 4 hours
- Grade 2. 2½ to 4 hours
- Grade 3. ½ to 2 hours
- Grade 4. Decolorised at time of removal from the 20° C. bath.

In the Ministry of Health Circular it is suggested that if ice-cream consistently fails to reach grades 1 and 2, it would be reasonable to regard this as indicating defects of manufacture or handling which call for investigation.

¹ *Monthly Bull. Minist. Hlth Lab. Serv.* (1947), 6, 60; (1948) 7, 84; (1949) 8, 155; (1950) 9, 231.

The quality of a manufacturer's ice-cream should not be judged on one sample alone, but on a series of samples taken throughout the year. As in assessing the quality of a water supply, it is suggested that in any one year, 50 per cent. of samples should fall into Grade 1, 80 per cent. should not fall below Grade 2, and the remainder should not fall below Grade 3. If a manufacturer's samples repeatedly fall into Grade 2 further examination should be made to identify the predominating organism, since it is possible that certain heat-resisting bacteria are sometimes responsible for a lower grading than might be expected from the conditions of manufacture.

The Department of Health for Scotland in a Circular No. 43/1948 advises the use of the plate count and the test for coliform bacilli in the bacteriological examination of ice-cream. Although no test is considered to be sufficiently valid as a statutory test, it is suggested that a bacterial count of more than 100,000 per g. and the presence of coliform bacilli in 0.01 g. would indicate faults in the manufacture and handling of ice-cream.

BACTERIOLOGICAL EXAMINATION OF SHELLFISH

The method used by Bigger¹ for examining mussels is recommended with a few slight modifications. It may also be adapted to the examination of all types of shellfish.

Bigger's procedure for preparing the mussel emulsion is as follows:

- (1) Ten mussels of average size are selected.
- (2) These are washed with running tap water, using a boiled nail brush.
- (3) One is grasped with sterile ovum forceps, rinsed under the tap and then with sterile water.
- (4) It is placed on a piece of sterile parchment paper in which it is grasped with the left hand. The shell is held with the flat edge towards the body, the anterior (pointed) end to the left and the left valve of the shell upwards.
- (5) A small portion of the shell at the broad (posterior) end is nibbled away with a sterile nibbling forceps, and through the opening the blade of a sterile scalpel is inserted. With this the posterior adductor muscle and the other attachments of the mussel to the left valve are cut, and, holding them with the paper interposed between them and the hands, the two valves of the shell are separated and the left one removed.
- (6) All the fluid in the shell is poured off and, with the help of the scalpel, the body is transferred to a small beaker provided with a graduation at the 25 ml. level.
- (7) The body of the mussel in the beaker is thoroughly minced with a sterile pair of scissors. Sterile saline is added up to the 25 ml.

¹ Bigger, J. W. (1934), *J. Hyg. (Lond.)*, 34, 172.

mark and mixed thoroughly with the minced body, the scissors being used for this purpose.

It has been found advisable to include the shell fluid in the test and to make up the volume to 25 ml. with sterile water instead of saline in order to counteract the high salt content already present.

Varying amounts, viz. 0.5, 0.1 and 0.02 ml. of the minced mussel emulsion are then added to tubes of bile-salt-lactose medium (p. 359). The following technique is suggested:

Add 25 ml. sterile water to the beaker containing the 25 ml. minced mussel emulsion, thus making a dilution of 1 in 2. With a sterile pipette, 1 ml. of this dilution is added to a tube of bile-salt-lactose medium; 2 ml. of the dilution are next transferred with the same pipette to 8 ml. sterile water in a test-tube giving a second dilution of 1 in 10. Using a fresh sterile pipette, 1 ml. of the 1 in 10 dilution is added to a second tube of bile-salt-lactose medium and 0.2 ml. of the same dilution to a third tube of the medium. For greater accuracy duplicate tubes of bile-salt-lactose medium are recommended for each dilution. This procedure is repeated with each mussel and the cultures are incubated at 37° C. for twenty-four hours when they are examined for acid and gas production. An additional reading is made after a further twenty-four hours. When two tubes of medium are employed for each dilution, results are reported as positive only when acid and gas are produced in both tubes.

It has been found advantageous to use Eijkman's test (p. 361) to confirm the presence of typical or "faecal" coliform bacilli. Subcultures are made from each "positive" tube into fresh tubes of bile-salt-lactose medium and are incubated at 44° C. The development of gas at this temperature within twenty-four hours is considered to be evidence of the presence of typical coliform bacilli.

Interpretation of Results.—According to Bigger's suggested standard, a batch of mussels should be considered undesirably contaminated if more than seven out of the ten tested have coliform bacilli in 0.5 ml. of minced mussel emulsion or more than three in 0.1 ml., or more than one in 0.02 ml.

Consignments of shellfish should not be condemned on the result of one examination alone, but only on a series of results combined with what is known of the condition of the source of the supply and the methods of treatment and handling after harvesting.

BACTERIOLOGY OF CANNED FOOD

Deleterious changes in canned food known as "spoilage" may be brought about through the development of micro-organisms. These may be present in the food either as a result of their resistance to the heating process or through being introduced after processing through defects in the structure of the can.

Heat-resistant organisms and the types of spoilage caused by them

vary according to the nature of the food they infect. Foods preserved by canning can be divided broadly into two groups: (a) medium, low and non-acid foods with pH above 4.5, including meat, fish, vegetables, soup, milk and starch foods; (b) high acid foods with pH of less than 4.5. The border-line of pH 4.5 has been chosen because spores of the most heat-resistant of the food poisoning organism, viz. *Clostridium botulinum*, will not germinate in conditions of acidity higher than this. For this reason it is not usual to heat the foods in group (b) above 100° C., which is sufficient to destroy all vegetative forms. Pressure heating on the other hand is necessary to render safe all foods with pH above 4.5. The actual amount of heating employed varies with the food to be processed and is determined by careful laboratory tests carried out by specialists in the canning industry, the main considerations being that although it should be sufficient to destroy spores of pathogenic organisms in the centre of the contents it should not be so great as to alter the appearance and palatability of the food in question.

The minimum degree of heat necessary to destroy the spores of *Clostridium botulinum* may not be adequate to sterilise completely the food and where extremely heat-resistant spores remain, and the temperature of storage is such that germination and growth of the organisms can take place, spoilage will occur.

Organisms which bring about spoilage of food as a result of their heat-resistance are aerobic and anaerobic spore formers of the genera *Bacillus* and *Clostridium*. Many are thermophilic, having an optimum temperature of 55° C. but with the ability to grow slowly at temperatures considerably below this. The type of spoilage gives an indication of the organisms responsible. The following are the main types of spoilage which may occur in group (a) foods.

A. *Saccharolytic Spoilage*

1. Acid without gas, known as "flat sour" spoilage produced by certain species of the genus *Bacillus*.
2. Acid with gas production sufficient to cause "swelling" of the can, "Hard Swell", due to saccharolytic species of the genus *Clostridium*.
3. Slight acid production with hydrogen sulphide. No "swelling" occurs since the gas is soluble, but the contents become dark in colour. The organism usually responsible is *Clostridium nigrificans*.

B. *Putrefactive Spoilage*

Digestion of the food with gas production results from the growth of putrefactive species of the genus *Clostridium*, e.g. *Clostridium botulinum* and *Clostridium sporogenes*.

Spoilage of acid foods of group (b) is brought about by acid tolerant bacteria and occasionally by yeasts and moulds, which survive the short periods of heating at temperatures below 100° C. The bacteria responsible include sporing aerobes and anaerobes as well as non-sporing species, all capable of developing in high concentrations of acid.

Gas may or may not be produced. Examples are *Bacillus thermacidurans*, *Clostridium pasteurianum*, *Lactobacillus lycopersici* and *Leuconostoc*.

Contamination after Processing

Micro-organisms may enter leaking cans and infect the food after processing. They may include a variety of sporing and non-sporing bacteria, often derived from the water used for cooling the cans. If pathogenic organisms gain entrance in this way, cases of food poisoning will result. Staphylococci and organisms of the *Salmonella* group have occasionally been incriminated in food poisoning outbreaks due to canned food, probably contaminated in this way.

Technique for examining Canned Food

To test the sterility of canned food, and where spoilage has occurred or the food is suspected of causing food poisoning, the following procedure, based on that of Tanner,¹ is recommended for the isolation of the organisms responsible.

Unless the can shows visible signs of spoilage through "swelling" it is advisable to stimulate the multiplication of heat-resistant organisms which may be present in only small numbers and probably in a "dormant" condition by incubating it before opening at 37° C. for at least one week for mesophilic and 55° C. for thermophilic organisms. Acid foods should be incubated at 25° C. for ten days.

Before being opened the can is carefully examined for physical defects, particularly round the seams. Any signs of "swelling" are noted, and where rustiness or dents have occurred these are scrutinised for pin holes. After examination the can is scrubbed with soap and water and rinsed with alcohol or ether to remove the grease. The area where an opening is to be made is then sterilised by flaming or by treatment with 70 per cent. alcohol. If heat is applied it should be carefully distributed in such a way as to avoid overheating the contents, which may then spurt out when the can is opened. If the can is swollen, it is advisable not to sterilise by heating but rather by the use of alcohol. The point of a sterile opener is then inserted into the sterilised area and an opening is cut sufficiently large to enable a portion of the food to be withdrawn in the following aseptic manner:

Liquid food is withdrawn with a sterile pipette or an untapered glass tube and inoculated directly into the culture medium. Fifteen to twenty ml. should be tested in this way. Solid food is sampled with a modified cork borer, 10 inches long and three-quarters of an inch in diameter, having a rod inserted to expel the contents. The sample should include food from the centre of the contents, where heat-resistant organisms are likely to occur, and from the surface, where contamination through leakage may have taken place. The solid food

¹ Tanner, F. W. (1944), *Microbiology of Foods*, 2nd ed. Champaign, Ill.; Garrard Press.

should then be thoroughly emulsified in sterile water by grinding with a sterile pestle and mortar or shaking in a screw-capped bottle with pieces of broken glass (Baumgartner).¹

Technique for Culture

Tubes containing 10 ml. amounts of suitable fluid media are inoculated with 1 ml. of the liquid food and incubated at 37° C. and 55° C., both aerobically and anaerobically. Cooked meat medium is recommended for culturing anaerobic bacilli, and glucose broth is suitable for aerobic mesophilic and thermophilic organisms. In order to culture spoilage organisms from acid foods, tomato-glucose broth is recommended by Tanner. This consists of tomato juice and nutrient broth in equal parts with the addition of 1 per cent. glucose. The tubes are examined after twenty-four and forty-eight hours, and where growth has occurred the organisms may be identified by microscopic examination of stained films and by further culture tests. If the food is suspected of causing food poisoning through infection with organisms of the *Salmonella* and *Staphylococcus* groups, media selective for these organisms should also be inoculated with portions of the food. For *salmonellae*, tetrathionate broth and selenite medium are suitable; for *staphylococci*, cooked meat medium to which 10 per cent. sodium chloride has been added is recommended.

Organisms developing in these selective media should be further examined by the methods described in the appropriate sections dealing with them.

It is advisable to make direct films of the food for microscopic examination although no significance should be attached to organisms seen unless the cultures confirm that they are viable.

After removing samples for culture, the food is turned out of the can and examined carefully for any abnormalities in appearance and smell. The inside of the can too should be inspected for defects in its manufacture.

The Bacteriological Examination of Milk Bottles

To test the adequacy of the cleansing and sterilisation of milk bottles at farms and creameries, the following technique, based on the recommendations of the Ministry of Agriculture and Fisheries² is advocated.

At least four bottles should be picked at random immediately after washing. They should be capped or fitted with a sterile rubber bung and sent immediately to the laboratory so that testing may be begun within four hours of sampling.

To each bottle, irrespective of its size, 20 ml. of sterile quarter-strength Ringer solution are added and the cap or bung replaced. The

¹ Baumgartner, J. G. (1945), *Canned Foods*. London, Churchill.

² Min. of Agriculture and Fisheries (*National Milk Testing and Advisory Scheme*), Technique No. B 743/T.P.B., 1947.

bottle is then laid horizontally on the bench and rotated by rolling so that the whole of the internal surface is rinsed with the solution. This process is repeated at intervals over a period of half an hour, the bottle being kept on its side during that time.

Five ml. of the solution are then plated in duplicate using 20 ml. "Yeastrel" agar (p. 197), this large amount being necessary to produce solidification. One plate is incubated at 37° C. for forty-eight hours and the other at 22° C. for three days. (For greater accuracy duplicate plates may be prepared for both temperatures.) The results are reported as the colony count per bottle, *i.e.* the individual plate count multiplied by 4.

Based on the 37° C. count, the following scheme of classification was suggested:

Average Colony Count per bottle	Classification
Not more than 200	Satisfactory
Over 200 to 600	Fairly satisfactory
Over 600	Unsatisfactory

In addition, a test for the presence of coliform bacilli should be carried out by inoculating each of two bottles containing 10 ml. double-strength MacConkey's broth with 5 ml. rinse solution. These are examined for acid and gas production after forty-eight hours' incubation at 37° C. If adequate methods are employed in cleansing and sterilising the bottles, no coliform bacilli should be present.

The Bacteriological Examination of Washed Crockery and Cutlery

The adequacy of washing-up methods employed in the kitchens of catering establishments, schools and other institutions may be tested by bacterial examinations of swabs taken from freshly washed crockery and cutlery. The technique consists of swabbing the significant surfaces of the utensils and then immersing the swabs in quarter-strength Ringer's solution. This is thoroughly shaken to disintegrate the swab and dislodge the bacteria. Finally, the solution is examined by the plate count and coliform tests and the number of organisms derived from each utensil is calculated.

Preparation of Swabs.—Absorbent cotton-wool swabs $\frac{3}{4}$ in. long, as used for clinical purposes (p. 435) may be employed. It is more convenient to have them on wooden applicator sticks $6\frac{1}{2}$ in. long than on wires, so that after the specimen has been collected the swab may be broken off above the cotton-wool and allowed to drop down into the container of Ringer's solution. The swabs are inserted into test-tubes 5 in. by $\frac{1}{2}$ in. plugged with cotton-wool and sterilised by autoclaving for fifteen minutes at 10 lb.

Higgins¹ obtained a greater recovery of organisms by using swabs made of calcium alginate wool instead of cotton-wool, the advantage being that the alginate swabs may be completely dissolved in Ringer's solution containing sodium hexametaphosphate. In this way all the bacteria contained in the swab are liberated into the solution. Not more than 50 mg. of wool should be used for each swab to ensure complete solution even in cold weather, when larger amounts tend to form crystals. It is important that the calcium alginate wool should be declared by the manufacturer to be free from bactericidal substances such as "Fixanol C", a quaternary ammonium compound which was originally impregnated into the wool for use in the manufacture of certain textiles.

Method of Swabbing.—One swab is used for five similar articles. It is first moistened by dipping in sterile quarter-strength Ringer's solution, the surplus liquid being squeezed out against the inside of the screw-capped container. The swab is then rubbed thoroughly over the whole of the appropriate areas, which are as follows:

- the inner surfaces of plates and bowls that come in contact with food;
- the inner and outer surfaces of cups, mugs and glasses to a depth of 3 cm. below the rim;
- bowls and the backs of spoons and the back and front surfaces of forks and between the prongs.

After swabbing five similar articles in this way, the swab is returned to the test-tube and sent to the laboratory without delay.

Method of Testing

(a) If cotton-wool swabs have been used, the swab is broken off the wooden stick with sterile forceps and allowed to drop into a screw-capped bottle of 1 oz. capacity containing 10 ml. of sterile quarter-strength Ringer's solution. If delay in transporting the sample to the laboratory is unavoidable, this should be done by the person taking the sample. In the laboratory the bottle is shaken vigorously to disintegrate the swab and liberate as many as possible of the bacteria contained in it. 1 ml. quantities of the test solution are plated out in duplicate in "Yeastrel" agar (p. 197), one plate of each being incubated at 37° C. for forty-eight hours and the other at 22° C. for three days. The results are reported as the bacterial counts per utensil for each temperature (*i.e.* count per ml. $\times 2$). (b) If calcium alginate swabs are used, it is recommended by Higgins and Hobbs² that two swabs should be employed for each test, one being moistened in Ringer's solution before use and the other used dry. The surface of five articles are rubbed over, first with the moistened swab and then with the dry one.

¹ Higgins, M. (1950), *Monthly Bull. Minist. Hlth Lab. Serv.*, 9, 50.

² Higgins, M., & Hobbs, B. (1950), *Monthly Bull. Minist. Hlth Lab. Serv.*, 9, 38.

Both swabs are then broken off the sticks with sterile forceps and allowed to drop into 9 ml. quarter-strength Ringer's solution. 1 ml. of 10 per cent. sodium hexametaphosphate solution (sterilised by autoclaving) is then added and the bottle shaken until both swabs have dissolved. The solution is plated out either by the method described under (a) or by the Miles and Misra technique, using blood agar plates on which the test solution is inoculated in the form of drops of 0.02 ml. volume delivered from a calibrated Pasteur pipette. This method was found to yield higher colony counts than those obtained by the pour-plate method with "Yeastrel" agar. The higher recovery rate may result from the predominance on washed crockery of organisms such as streptococci which grow poorly on "Yeastrel" agar. The blood medium may also serve to neutralise the effect of traces of bactericidal substances, such as quaternary ammonium compounds used in the washing-up process. The technique has the added advantage of indicating the nature of the organisms present, as well as their number.

Standards

There is no standard officially recognised in Great Britain, but attention is drawn to the United States Public Health Standard¹ for washed crockery. Based on the swabbing technique followed by the standard plate count test it allows a maximum of 100 colonies per utensil examined. If the Miles and Misra method is used, specimens giving 2 colonies or less per 6 drops of undiluted test suspension on blood agar plates may be considered to conform to the American standard.

INVESTIGATION OF FOOD POISONING OUTBREAKS —LABORATORY AND FIELD TECHNIQUES

Bacterial Food Poisoning (acute gastro-enteritis) results from the consumption of food infected with certain pathogenic organisms which are capable of proliferating in food if conditions are favourable. They fall into two categories depending on the manner in which they produce their harmful effects, viz: (a) those which infect the body (notably the salmonella group), and (b) those which produce a toxin during their growth in the food (these include some coagulase-positive staphylococci and certain types of *Clostridium welchii* characterised by the heat resistance of their spores). Before attempting the laboratory diagnosis of an outbreak of suspected food poisoning, it is advantageous to know the nature of the symptoms and the time of their onset. The following table indicates the differences in the effects produced by the three main types of food-poisoning bacteria:

¹ Tiedman, W. D., et al. (1944), *Amer. J. publ. Hlth*, 34, 255.

Type of Food Poisoning	Infection (Salmonella)	Intoxication	
		(Staphylococcus)	(<i>Cl. welchii</i>)
Incubation . .	12-24 hr	2-6 hr	8-22 hr
Duration . .	1-14 days	6-24 hr	12-24 hr
Diarrhoea . .	Very common	Common	Extremely common
Vomiting . .	Not common	Extremely common	Very rare
Abdominal pain . .	Present	Present	Present
Pyrexia . .	Common	Absent	Absent
Prostration . .	Rare in early stages	Common	Common

It should be borne in mind, however, that in addition to outbreaks resulting from specific food poisoning organisms, cases of acute gastro-enteritis occur for which no specific organisms can be proved responsible. It is thought that if food becomes contaminated by normally harmless bacteria such as those of the proteus group, paracolon bacilli and *Streptococcus viridans*, and the conditions of storage permit of their rapid proliferation, the ingestion of such food may give rise to symptoms of food poisoning. The appearance and taste of the food may remain unaffected and whether the symptoms result from toxic substances produced by the organisms or from infection, is not yet clear.

Specimens for Bacteriological Examination

The following material should be sent to the diagnostic laboratory for examination immediately after notification of a suspected food poisoning outbreak.

- (a) Specimens of faeces and vomit from the patients.
- (b) Samples of foodstuffs of a likely nature eaten within twenty-four hours prior to the onset of symptoms. Meat preparations (ham, brawn, sausages, etc.), and made-up dishes such as sandwiches prepared by hand, have been responsible for outbreaks of salmonella and staphylococcal food poisoning, while pre-cooked meat, stews, beefsteak pies, etc., prepared the day before serving, frequently cause outbreaks of *Cl. welchii* food poisoning. Eggs used in the preparation of uncooked or only partially cooked foods, have caused salmonella infections, while unpasteurised milk and milk products have in the past been responsible for both staphylococcal and salmonella food poisoning. Any of the above foodstuffs should be viewed with suspicion if they have been eaten by the patients shortly before the commencement of symptoms, and samples should be examined bacteriologically for the three main food poisoning organisms.

For the methods of isolation and identification of the various specific

bacteria, reference should be made to the appropriate sections in Part III.

Examination of Foodstuffs in Outbreaks of Food Poisoning

Non-specific Contamination.—The examination of food samples should commence with a careful inspection to determine if there are any abnormalities in appearance or smell. This is followed by a microscopic examination of stained preparations which will indicate any gross contamination. It is also useful to determine the total count of viable bacteria per gram of food at 37° C. and 22° C., by means of the *pour-plate method* (see p. 362) applied to a suspension of 1 gm. of the food in 10 ml. of sterile Ringer's solution. This test should be combined with an examination for coliform bacilli as in the examination of milk samples (see p. 375). These preliminary tests will indicate whether or not the food has been subjected to contamination of a non-specific nature arising from poor standards of kitchen hygiene.

Salmonella Infection.—In testing foodstuffs for organisms of the salmonella group, both direct culture and enrichment techniques should be carried out (see p. 579). When examining liquid foods, an amount of double-strength fluid medium (selenite medium or tetrathionate broth) equal to that of the food should be used. It may be advantageous to incubate at different temperatures (*e.g.* 37° C. and 42° C.) and to subculture after twenty-four, forty-eight and seventy-two hours.

Staphylococcal Intoxication.—There is no reliable laboratory method for identifying the enterotoxin of *Staphylococcus aureus*, and diagnosis of this type of food poisoning can only be made if coagulase-positive staphylococci are isolated from the food. By the Miles and Misra counting technique (see p. 304), using 10 per cent. salt milk agar (p. 227) as a selective medium, it is possible to determine the approximate number of staphylococci per gm. of suspected food. Although it is not known how many staphylococci are necessary to cause food poisoning, large numbers growing in direct culture from suspected food would be highly suggestive of it being the cause. In order to isolate *Staphylococcus aureus* from foodstuffs heavily contaminated with other organisms, the use of Ludlam's medium (see p. 227) may be advantageous. Any coagulase-positive staphylococci isolated by the above methods should be typed by bacteriophage or serological methods. In order to determine the source of the infection, all persons engaged in the preparation and handling of the food should be examined to determine whether they are harbouring the same organism in their noses or in skin lesions.

***Cl. welchii* Intoxication.**—This form of food poisoning which has been increasingly recognised in recent years is caused by a variant of Type A *Cl. welchii* which is characterised by being non-haemolytic, feebly toxicogenic and having spores which can survive boiling for several hours.

In order to diagnose food poisoning due to heat-resistant *Cl. welchii*

it is necessary to isolate the organism both from the faeces of the patient and from the suspected food. The following method is recommended¹:

1. FAECES.—Direct culture on blood agar is not usually satisfactory for isolating *Cl. welchii*, since coliform bacilli tend to predominate even in anaerobic culture. Since, however, the organism appears to spore readily in the intestine, heat-resistant strains may be isolated by inoculating a small portion of the stool into a tube of Robertson's meat broth and heating at 100° C. for one hour. The tube is then incubated at 37° C. overnight to allow the spores to germinate. Subcultures are then made on blood agar plates and incubated anaerobically for a further eighteen to twenty-four hours, after which time the non-haemolytic colonies characteristic of heat-resistant *Cl. welchii* will have developed.

2. Food.—It is not easy to isolate heat-resistant *Cl. welchii* directly from suspected pre-cooked food by the above method, since at that stage the organisms are probably in the vegetative form and will not survive the heat treatment in broth. However, anaerobic culture on blood agar plates inoculated by the Miles and Misra technique will indicate the degree of contamination by anaerobic or facultative anaerobic organisms. Unless they grow in relatively pure culture it may not be easy to distinguish the colonies of non-haemolytic *Cl. welchii* from these of non-sporing organisms. They may be more readily isolated by inoculating small portions of the food into an enrichment medium such as cooked-meat medium and incubating for eighteen hours before culturing on blood agar, but it should be borne in mind that such indirect culture does not have the same significance as direct culture. Use may be made of the selective medium of Willis & Hobbs (pp. 232 and 698). The identity of any possible *Cl. welchii* colonies isolated by the above methods should be further confirmed. Their resistance to heat may be determined by first growing them for eighteen hours in Ellner's medium (p. 233), which tends to promote spore formation. The culture is then steamed for periods of up to one hour and subcultured into cooked-meat medium at intervals during the steaming.

Finally, serological tests should be applied to determine whether the strains isolated from the food and from the patient are identical. This is done by immunising two rabbits with formalised suspensions of the individual strains (see p. 318). At seven days after the final intravenous injections, the antisera are examined by agglutination and agglutinin-absorption tests with the homologous and heterologous strains. Only if the two are shown by these tests to be identical can their aetiological significance be considered as positive.

***Cl. botulinum* Intoxication.**—Botulism, a rare type of food poisoning has an incubation period which varies from less than 24 hours to 72 hours. The highly potent exo-toxin formed during the proliferation of *Cl. botulinum* in the food is absorbed through the gastric mucosa and affects the nervous system rather than the gastro-intestinal tract. This subject will be dealt with more fully on p. 70.

¹ Hobbs, B. C., et al. (1953), *J. Hyg. (Lond.)*, **51**, 75.

Foods which have been incriminated include improperly processed canned and preserved meat, meat and game pastes, and vegetables that are eaten uncooked or only partially cooked (the toxin is destroyed by heating to 90° C.). The spores of *Cl. botulinum* may survive boiling for several hours but are destroyed within 15 minutes by a temperature of 120° C. They fail to germinate if the pH of the food is less than 4.5 (see p. 383; Bacteriology of Canned Food).

BACTERIOLOGICAL EXAMINATION OF AIR

In the past the procedure frequently adopted for determining the relative number and species of micro-organisms present in air has been to expose open plates of culture medium for given periods of time, e.g. $\frac{1}{2}$ or 1 hour. A count of the colonies after incubation of the plates yields a relative estimate of the number of organisms present, and if blood agar is used, the occurrence in the air of haemolytic streptococci can be determined. This method has proved valuable in demonstrating the presence of pathogenic staphylococci and streptococci in the air and dust of hospital wards in which these organisms are being spread. Such findings have also thrown light on cross-infection in hospitals.

It is recognised, however, that this simple method of exposing plates has certain limitations as a means of studying the bacteriology of air; for example, it is not a satisfactory method of detecting bacteria in very small suspended particles such as droplet-nuclei. More elaborate procedures have therefore been adopted. A technique introduced by Bourdillon, Lidwell and Thomas¹ involves the use of a special instrument, the "slit sampler", by which a known volume of air is directed on to a plate through a slit 0.25 mm. wide, the plate being mechanically rotated so that the organisms are evenly distributed over it. One cubic foot of air per minute is allowed to pass through the slit, and samples of 1 to 10 cubic feet, or more, may be tested. More advanced models of the slit sampler have a timing arrangement which allows the number of colonies on each sector of the plate to be related to the number of bacteria-carrying particles sampled in a particular part of the sampling period. These instruments can be obtained commercially.²

The slit sampler and other air samplers have been used in examining the amount of bacterial contamination in the air of hospitals, schools, factories and other places, with a view to determining the danger of air-borne infection and the factors which increase and decrease numbers of air-borne bacteria.³ Observations have been made in surgical operation rooms in relation to the efficacy of different ventilation systems in minimising aerial contamination, and in schools, hospitals and other places in tests of the efficacy of ultraviolet irradiation and chemical vapours for air disinfection.

¹ Bourdillon, R. B., Lidwell, O. M., & Thomas, J. C. (1941), *J. Hyg. (Lond.)*, **41**, 197.

² C. F. Casella & Co. Ltd., Fitzroy Square, London, W.1.

³ Bourdillon, R. B., et al. "Studies in Air Hygiene." *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 262, 1948.

CHAPTER XV

ANTIMICROBIAL AGENTS

SUBSTANCES which are used against micro-organisms may be described as *cidal* if they kill cells quickly, or *static* if their effect is predominantly one of inhibiting growth. Most substances, however, which are static are cidal if their concentration is raised, or if exposure is sufficiently prolonged. *Disinfectants* are substances which are strongly bactericidal in the concentrations usually used and sometimes they also destroy bacterial spores. They are generally used to eradicate micro-organisms from inanimate material. *Antiseptics* are less irritant substances which destroy or inhibit the growth of micro-organisms and which may be applied locally against pathogenic organisms growing in living tissues. They are, in most cases however, general protoplasmic poisons and therefore tend to be toxic to tissues as well as to bacterial cells. Clearly the difference between disinfectants and antiseptics is one of degree only, and some disinfectants may be used as antiseptics. The terms *germicide* and *bactericide* are used to describe both disinfectants and antiseptics.

Antimicrobial drugs are chemotherapeutic substances which destroy or inhibit the growth of micro-organisms in living tissue. They usually differ from antiseptics and disinfectants in possessing a high *therapeutic index*, i.e. they are much more toxic to microbes than to tissues, and a more selective range of antimicrobial action. They may be given parenterally or orally against deep-seated or systemic infections. *Antibiotics* are substances produced by some living organisms which kill or inhibit the growth of other organisms and some are sufficiently non-toxic to be used as antimicrobial drugs.

DISINFECTANTS AND ANTISEPTICS

A great variety of compounds can act as disinfectants and antiseptics. Those most commonly used include the following: *acids and alkalis*; *metallic salts*—e.g. mercuric salts; *organic metallic compounds*—e.g. merthiolate; *halogens*—e.g. chlorine and iodine; *alcohols, ethers and aldehydes*—e.g. ethanol and formaldehyde; *phenols*—e.g. phenol and cresols; *oxidising and reducing agents*—e.g. hypochlorites, hydrogen peroxide and sulphurous acid; *organic dyes*—e.g. brilliant green, crystal violet and proflavine; *soaps and synthetic detergents*—e.g. quarternary ammonium salts. These substances are usually general protoplasmic poisons and act relatively non-specifically on bacteria (with the exception of the organic dyes, which are more selective and are used in connection with selective methods of cultivation). They will also kill mammalian cells such as phagocytes, often at concentrations

lower than those required to kill bacteria. The non-specific action of these substances is due to their activity in destroying or denaturing compounds or structures common to all living organisms. Thus mercuric salts react with the -SH groups of proteins, rendering them biologically inactive, while phenols, soaps and detergents probably act by destroying the semi-permeable membrane at the surface of all cells.

A variety of factors will affect the activity of a disinfectant or antiseptic and these should be borne in mind during use. The most important are:

(1) *Concentration*.—The higher the concentration of the germicide the greater will be the rate of killing. This is particularly important with the phenolic group of compounds, whose activity falls off very rapidly with dilution.

(2) *Time and temperature*.—In general, germicidal activity is increased with time and a sufficient exposure is imperative for efficient disinfection. An increase of temperature will also raise the rate of killing.

(3) *Organic matter*.—Most germicides are reduced in activity by the presence of organic matter and particularly by the presence of proteins such as those in body fluids.

(4) *Number of organisms*.—The larger the number of organisms, the greater will be the time required for disinfection.

(5) *The presence of spores*.—Spores are exceptionally resistant to the great majority of disinfectants.

Testing of Disinfectants

A simple way to test the effect of a particular antiseptic is to incorporate a series of different concentrations of the antiseptic in 10 ml. amounts of nutrient agar, pour the medium in plates and then make a stroke inoculation from a bacterial suspension. This should be prepared in sterile distilled water from a young culture on solid medium and should show just a faint turbidity to the naked eye. Different organisms can be tested at the same time by making stroke inoculations on each of the various plates. The plates are incubated for forty-eight hours, when observations can be made.

Instead of solid medium, tubes of broth or serum (sterile ox serum previously heated at 56° C.) may be substituted. To a series of such tubes varying concentrations of the antiseptic are added and then each is inoculated with a standard amount of bacterial suspension. The effect is observed after incubation at 37° C. for forty-eight hours, the presence or absence of living organisms being decided by subculturing; one stroke is made from a loopful of each mixture. In this way one agar plate will serve for subcultures from a series of tubes.

In the case of slowly acting bactericidal substances, such as the flavine and other antiseptic dyes, it is often found that concentrations which have entirely inhibited growth, as shown by their complete transparency, still contain a few living organisms as tested by subculture. The lowest concentration of antiseptic which produces this

result (inhibitory concentration) yields a satisfactory index of the potency.

The efficiency of disinfectants is most frequently assessed by measuring the rate of kill against a selected range of organisms under specified circumstances. The majority of methods employ phenol as a standard reference so that a *phenol coefficient* is frequently quoted for disinfectants. It may be said to express the bactericidal power of a particular substance as compared with pure phenol. The principle techniques are the Rideal-Walker, Chick-Martin and United States (F.D.A.) test.

Their chief application is in comparing disinfectants composed of coal-tar derivatives which are water-soluble or water-miscible. They are of no use in assessing the relative merits of different classes of compounds—e.g. coal-tar and quaternary ammonium compounds.

Rideal-Walker Test¹

Materials required:

(1) Standard loop of 28 S.W.G. wire, 4 mm. internal diameter, bent almost at a right angle to the wire, so that in the subsequent manipulations the plane of the loop is horizontal.

(2) Culture of *S. typhi*. It is of the utmost importance to use always a standard culture.²

Subcultures should be made in the standard broth at twenty-four hour intervals, three times before the test is carried out, and a twenty-four hour broth culture used for the test proper.

Method of Testing:

(1) Determine beforehand the inhibition concentration of the particular germicide for the standard strain of *S. typhi* and make up a series of five graded concentrations in distilled water, the lowest being slightly greater than the inhibition concentration.

(2) Make up 100 ml. of a 5 per cent. stock solution of the pure phenol in sterile distilled water, and from it prepare the following dilutions of phenol: 1 in 95, 1 in 100, 1 in 105, 1 in 110 and 1 in 115.

(3) To 5 ml. (in stoppered sterile test-tubes) of each of the solutions prepared from the germicide to be tested, add with a sterile pipette 0.2 ml. of the twenty-four hour broth culture of *S. typhi* and shake the mixtures. The tubes containing the solutions should be kept during the test in a water-bath at 18° C.

(4) At intervals of 2½ minutes up to 10 minutes remove a large loopful from each mixture, using the standard wire loop and transfer to tubes of 5 ml. standard broth. The bacterial suspension in (3) should be

¹ For full details of technique, see *Technique for Determining the Rideal-Walker Coefficient of Disinfectants*, British Standards Specification, No. 541, 1934, with amendments dated 1943 and 1951; obtainable from British Standards Institution, 28 Victoria Street, London, S.W.1.

² Obtained from The National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London, N.W.9; the purpose for which the culture is required should be stated.

added to the tubes of antiseptic in succession at definite intervals, e.g. 30 seconds. The loop-transfers to broth from each tube, after $2\frac{1}{2}$, 5, $7\frac{1}{2}$ and 10 minutes respectively, can then be accurately timed.

(5) and (6) Carry out with the phenol solutions the same procedure as in (3) and (4).

(7) Incubate the broth tubes for forty-eight hours and note those in which growth has occurred.

(8) The coefficient is calculated by dividing the figures indicating the degree of dilution of the disinfectant which shows life in $2\frac{1}{2}$ and 5 minutes but no life thereafter by that figure indicating the degree of dilution of phenol which shows life in $2\frac{1}{2}$ and 5 minutes but no life thereafter.

The following results illustrate the test:

Dilution	Time in Minutes			
	$2\frac{1}{2}$	5	$7\frac{1}{2}$	10
Unknown Germicide	{ 1 : 400 . . .	-	-	-
	{ 1 : 500 . . .	-	-	-
	{ 1 : 600 . . .	+	-	-
	{ 1 : 700 . . .	+	+	-
	{ 1 : 800 . . .	+	+	+
Phenol	{ 1 : 95 . . .	+	-	-
	{ 1 : 100 . . .	+	+	-
	{ 1 : 105 . . .	+	+	+
	{ 1 : 110 . . .	+	+	+
	{ 1 : 115 . . .	+	+	+

(+ = growth, - = no growth)

$$\text{Phenol coefficient} = \frac{700}{100} = 7.0.$$

The Rideal-Walker test compares the action of the antiseptic with that of phenol on *S. typhi* in distilled water only and does not necessarily give any indication of the disinfecting action under practical conditions where much organic matter is usually present. Accordingly, therefore, the Chick-Martin test has been advocated in which the disinfectant is tested in the presence of organic material, which is the quantity of solid matter present when heat-sterilised liquid faeces containing 10 per cent. of solids is mixed with twice its volume of disinfectant. The use of faeces in this test is open to several objections, and Garrod has devised a modification of the Chick-Martin test with yeast instead of faeces.¹ The yeast is made up in suspension in distilled water equivalent to 5 per cent. of dry yeast, and for the test 48 ml. are added to 2 ml. of the *S. typhi* broth culture. 2.5 ml. of this mixture are added to 2.5 ml. of dilutions, varying by 10 per cent., of the disinfectant and of phenol.

¹ Modified Technique of the Chick-Martin Test for Disinfectants, British Standards Specification, No. 808, 1938; see footnote, p. 396.

After thirty minutes, samples are taken exactly as in the Rideal-Walker test. The phenol coefficient is calculated by dividing the mean of the highest concentration of phenol permitting growth and the lowest concentration producing sterility with the corresponding mean of the disinfectant. Thus, supposing there was no growth with 2.0 per cent. phenol, but growth with 1.8 per cent., the mean is 1.9. Similarly, suppose there was no growth with 0.457 per cent., but growth with 0.411 per cent. of the disinfectant, the mean is 0.434. The result is expressed in the following form:

$$\text{Phenol coefficient} = \frac{1.9}{0.434} = 4.4.$$

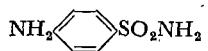
The rapidly expanding use of disinfectants and antiseptics for removing micro-organisms from surfaces has focussed attention on tests which allow the antimicrobial agent to act upon the micro-organism on the surfaces of objects. Recent techniques utilise glass cylinders (Mallman and Hanes, 1945)¹ and squares of test material such as cloth (Stedman, Kravitz and Bell, 1954),² and the original papers should be consulted for details.

ANTIMICROBIAL DRUGS

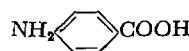
Antimicrobial drugs must have a selective action against micro-organisms as compared to mammalian tissues, *i.e.* they must possess a high therapeutic index. This specificity may depend upon the target mechanism being peculiar to the microbe, or being more accessible, or more immediately vital to the microbe than to the mammalian tissue. The basis for such activity is probably often the specific inhibition of particular enzymes.

The mode of action of the sulphonamides— competitive inhibition

The sulphonamide series of drugs are all based upon the molecule of sulphanilamide, usually with various organic groupings substituted on the $-\text{SONH}_2$ grouping. The first clue to their mode of action came in the finding that natural extracts (*e.g.* yeast extract) would reverse the inhibitory effect of the sulphonamides on bacterial growth; the substance responsible for this action proved to be *p*-aminobenzoic acid (PABA).



Sulphanilamide



p-aminobenzoic acid

PABA was found to be a growth factor for many micro-organisms, while a conjugated form related to folic acid was of universal occurrence in living organisms, being concerned as a coenzyme in certain enzyme

¹ Mallman, W. L., & Hanes, M. (1945), *J. Bact.*, **49**, 526.

² Stedman, R. L., Kravitz, E., & Bell, N. (1954), *Appl. Microbiol.*, **2**, 119.

systems essential for growth. Sulphanilamide was shown to inhibit an enzyme concerned in the transformation of PABA to this coenzyme form. The inhibition was of the competitive type, PABA and the sulphonamide competing for the active site on the surface of the enzyme. This is because of their similar structure and therefore the ability of both to "fit" into the enzyme surface. It should be noted that the inhibitory effect on bacterial growth is observed only when the ratio sulphonamide/PABA in the environment exceeds a certain value, irrespective of the absolute concentration of the two substances. Thus the inhibitory effect of the sulphonamides carried over from body fluids to culture media may be reversed by the addition of PABA (see p. 205).

Competitive inhibition of this kind may be the mechanism of action of many other antimicrobial drugs.

Antibiotics¹

Most antimicrobial drugs used today are antibiotics. These substances are produced mainly by micro-organisms living in the soil where they may play a part in overcoming competing species in their natural habitat. The most important antibiotic-forming group of micro-organisms are the Actinomycetes, although some fungi and aerobic spore-forming bacilli are also active. Although most antibiotics are too toxic to the host tissues to be used as antimicrobial drugs, or are rapidly inactivated in the animal body, a large number of therapeutically useful agents have been isolated and manufactured recently. Those most commonly used include the following (the name of the antibiotic-producing organism is given in brackets): penicillin (*Penicillium notatum* or *P. chrysogenum*); streptomycin (*Streptomyces griseus*); chlortetracycline or aureomycin (*Streptomyces aureofaciens*); oxytetracycline or terramycin (*Streptomyces rimosus*); chloramphenicol or chloromycetin (*Streptomyces venezuelae*); erythromycin (*Streptomyces erythreus*); neomycin (*Streptomyces fradiae*); polymyxin or aerosporin (*Bacillus polymyxa*); bacitracin (*Bacillus subtilis*).

Many antibiotics will inhibit the growth of a wide range of bacteria and these are usually called *broad spectrum antibiotics*, e.g. the tetracyclines; others have a more restricted antibacterial spectrum, e.g. penicillin is active against Gram-positive bacteria, neisseriae and spirochaetes, whereas streptomycin is most active against Gram-negative and acid-fast bacteria. The chemical nature of these antibiotics is very varied and this variety is reflected in their mode of action. One of the most characteristic structures in bacteria is the cell wall, which is quite different from those of higher organisms. It is probable that many antibiotics owe their specificity to an inhibition of bacterial cell-wall synthesis (e.g. penicillin and bacitracin). Although chloromycetin is probably active by inhibiting protein synthesis and streptomycin by inhibiting carbohydrate metabolism, the initial site of action of these drugs has not yet been determined.

¹ For further general details on antibiotics, see *Brit. med. Bull.*, 16, pt. 1.

Drug Resistance in Bacteria

In species which are normally susceptible to a particular drug, some strains or variants may occur which are specifically resistant to it. The choice of a drug to be used for treatment may therefore have to be based not only on a species identification of the causal organism isolated from the patient but also on the results of *in vitro* sensitivity tests made with this particular strain. The likelihood of encountering resistant strains, and thus the importance of sensitivity testing, varies in the different bacterial species. For example, it may be assumed for practical purposes that strains of *Strept. pyogenes*, pneumococcus or meningococcus will be sensitive to penicillin, but that strains of *Staph. aureus* will include many which are resistant to this antibiotic.

Drug resistant infections originate in two distinct ways of very different epidemiological significance. (1) *Drug resistance variation due to genetic mutation* (p. 39) may occur in a sensitive strain in a patient undergoing treatment. After a favourable initial response to the drug, associated with destruction of the sensitive parent-type organisms, the infection relapses due to proliferation of the drug-resistant mutants. Variation of this kind is particularly liable to occur during treatment with streptomycin, e.g. in the tubercle bacillus and many kinds of Gram-negative bacilli. Tubercle bacilli are also liable to mutations conferring resistance to isoniazid and *p*-aminosalicylic acid. However, it is found that simultaneous treatment with two of these drugs generally prevents the emergence of resistant variants. Apparently this is due to the almost negligible chance of a given cell undergoing simultaneously the two mutations required to confer the two kinds of resistance.

(2) *Infection may occur with an already resistant strain derived from an exogenous source.* Some bacterial species rarely or never undergo resistance mutation to a particular drug in patients receiving treatment, yet include many naturally resistant strains which are disseminated throughout the host community. Thus the *Staph. aureus* strains found in patients and healthy carriers include a proportion that are naturally resistant to penicillin by virtue of their capacity for producing penicillinase, an enzyme which destroys penicillin. Strains resistant to a given drug occur most frequently in communities where the drug is widely used, e.g. in hospitals, since their survival and spread is selectively favoured as against that of sensitive strains. Patients undergoing drug treatment in hospital are thus very liable to suffer cross-infection with a "hospital strain" which is resistant to a multiplicity of drugs and in some cases may be highly virulent from frequent passage in infected tissues. The skin and nostrils of hospital staff are subject to frequent contamination with traces of spilt antibiotics, and this may result in a high proportion of healthy carriers harbouring drug-resistant strains.

TESTING THE SENSITIVITY OF BACTERIA TO ANTIMICROBIAL AGENTS

Specific antimicrobial therapy may be instituted if the species of infecting micro-organism is one whose drug-resistant variants are known not to assume clinical importance, and to this end a working knowledge of the antimicrobial spectra of each of the antimicrobial drugs in general use is of advantage.

However, it must be emphasised that strains within many species of micro-organism are not alike in their antibiotic susceptibility so that the only reliable guide to the therapeutic use of antimicrobial agents is an *in vitro* sensitivity test coupled to clinical experience. In most infections the sensitivity of the causative organism should be determined before specific therapy has begun and if a specimen is available the most vital information concerning bacteriological diagnosis and antibiotic susceptibility can usually be available within twenty-four hours. This requires speed in the preliminary bacteriological investigations, and antibiotic sensitivity tests must be considered as part of these preliminary investigations. Diffusion tests on solid media have been adopted by most laboratories to cope with the demand for a simple, expeditious and reliable *in vitro* technique.

Diffusion Tests.—In these the antimicrobial agent is held in a reservoir from which it diffuses through agar medium to form a diffusion gradient to which the micro-organisms, growing in or on the agar, are exposed. Diffusion of the antimicrobial agent takes place continuously from the reservoir outwards through the surrounding agar so that the concentration gradient is continuously changing. Zones of inhibition of growth are formed when the organism is susceptible, and these are frequently complex showing the effect of different concentrations on the micro-organisms during growth. The size of these zones depends upon factors which influence the diffusion of the antimicrobial agent such as pH, depth, hydration and concentration of the agar, and nutrients and other substances in the gel, as well as the rate of growth of the organism. However, since the rate of growth of the majority of the pathogens encountered in the clinical laboratory is similar, and the experimental conditions can be standardised, diffusion tests can give results of a high standard of reproducibility as well as a reasonable degree of accuracy.

Replicate tests are easily set up and a number of antimicrobial agents can be tested on a single plate.

The reservoir may be a hole or gutter cut out of the medium and filled with antimicrobial agent in solution or mixed with agar. Alternatively, the antimicrobial agent may be placed in a cylinder of glass, porcelain or steel resting on the surface of the medium, or in absorbent paper disks.

A simple technique originally used by Fleming is as follows. A strip of agar about $\frac{1}{2}$ in. wide is cut from the centre of a plate of suitable culture medium (nutrient or blood agar) and discarded. An appropriate amount of antimicrobial agent (sulphonamide 200 μg . per ml.; penicillin 5–10 units per ml.; streptomycin 100 μg . per ml.; tetracycline 50 μg . per ml.; chloramphenicol 50 μg . per ml.) is added to 5 ml. of molten agar and pipetted into the gutter in the medium. The surface of the agar is inoculated by stroking loopfuls of cultures to be tested at right angles to the gutter and it is desirable to control the test by including a known sensitive and resistant strains.

During incubation growth is inhibited for a distance varying with the sensitivity of the strain to the antimicrobial agent under examination. The size of the inoculum is relatively unimportant in testing the susceptibility of most species to the antibiotic agents though generally speaking the larger the inoculum the more resistant the organism appears. The apparent sensitivity of penicillinase producing strains of staphylococci is markedly affected by inoculum size. With the sulphonamides the size of the inoculum may have a marked effect and must therefore be carefully controlled.

Alternatively, culture plates may be inoculated first with control and test organisms and strips of filter paper or blotting-paper, about 3 in. long and $\frac{1}{2}$ in. broad, soaked in the solutions of the antimicrobial agents are laid on the surface of the medium at right angles to the inoculation streaks.

Tests for sensitivity to sulphonamides may be unsatisfactory because of the presence of sulphonamide antagonisers such as para-aminobenzoic acid in the medium. The use of lysed horse blood overcomes this difficulty (Harper and Cawston, 1945).¹

The following medium is useful in carrying out diffusion sensitivity tests to sulphonamides:²

Peptone (Evans)	5 g.
Lab.-Lemco	3 g.
Agar Powder (New Zealand)	15 g.
Distilled water	1,000 ml.

Dissolve the ingredients in the distilled water, adjust the pH to 7.2–7.4, filter through paper pulp, bottle in 100 ml. amounts and autoclave at 15 lb. for twenty minutes. Before use, melt the medium, cool to 50°–60° C. and add 0.5 per cent. oxalated horse blood. Mix thoroughly, pour plates and store in the refrigerator for at least twelve hours before use to allow complete neutralisation of the antagonist.

¹ Harper, G. J., & Cawston, W. C. (1945), *J. Path. Bact.*, **57**, 59.

² Jewell, P., & Pearmain, G. E. G. (1954), *J. clin. Path.*, **7**, 308.

THE USE OF IMPREGNATED FILTER-PAPER DISKS FOR DETERMINING THE SENSITIVITY OF BACTERIA TO ANTIBIOTICS¹

This constitutes a simple and reliable technique specially applicable in routine bacteriological work. It consists in impregnating small disks of a standard filter paper with given amounts of an antibiotic, placing them on plates of culture medium inoculated with the organism to be tested, and after incubation determining the degree of sensitivity by measuring the easily visible areas of inhibition of growth produced by the diffusion of antibiotic from the disks into the surrounding medium.

These disks are 6.25 mm. in diameter punched from No. 1 Whatman filter paper and sterilised by dry heat at 150° C. for one hour in batches of 100 in screw-capped bottles. The required antibiotic solutions are prepared quantitatively in sterile distilled water from the preparations issued for therapeutic use; 1 ml. of the solution is added to each bottle of 100 disks, and as the whole of this volume is absorbed, it can be assumed that each disk contains approximately 0.01 ml. The solutions are therefore made up so that 1 ml. contains 100 times the required amount of antibiotic (*vide infra*).

The disks are used in the wet condition. They can be stored at 4° C. and will retain their moisture and potency for at least three months in screw-capped bottles (the caps being carefully screwed down so that the bottles are air-tight). The disks are placed on culture medium with sterile fine-pointed forceps.

Standard graphs for each antibiotic are prepared by testing disks containing varying amounts with a standard organism of known sensitivity, e.g. *Staph. aureus*, Oxford "H" strain N.C.T.C. 6571. The organism is grown for eighteen hours in broth and diluted to approximately 100 million bacterial cells per ml. by comparison with opacity standards (p. 305). Agar or blood-agar plates are inoculated uniformly from this broth culture (a sterile capillary pipette being used) by flooding the surfaces and then removing the excess. The open plates are then allowed to dry in the inverted position in an incubator for thirty minutes or open on the bench for one hour. Finally, the disks are placed on the medium suitably spaced apart and the plates are incubated overnight at 37° C. For each antibiotic the diameters of the circular areas of inhibition are plotted against the logarithms of the antibiotic concentrations and the resultant graph is approximately a straight line. It should be noted that the areas of inhibition measured include that of the disk as well as the surrounding zone.

Quantitative Interpretation of the Disk Diffusion Test

The zones of inhibition of growth may be related to the amounts of antimicrobial agent required for inhibition of growth in the following manner.

¹ Gould, J. C., & Bowie, J. H. (1952), *Edinb. med. J.*, 59, 178.

The sensitivity (Minimum Inhibitory Concentration) of any test organism may be calculated from the prepared graphs by reference to the amount of antibiotic required to inhibit the standard organism to the same degree (diameter). The sensitivity of the standard organism (*Staph. aureus*, N.C.T.C. 6571) is known; therefore the calculation of the sensitivity of the test organism is as follows:

$$\text{Sensitivity} = \frac{\text{Amount per disk required to inhibit test organism}}{\text{Amount per disk required to inhibit standard organism to same degree}} \times \text{sensitivity of standard organism.}$$

For all routine tests, disks containing the same amount of antimicrobial agent are used, therefore the amount used to inhibit the test organism is constant. The amount required to inhibit the standard organism is obtained from the prepared graphs as in the following example for penicillin. Suppose the diameter of the zone of inhibition of a test organism using a 1 unit penicillin disk is 13 mm. The graph for penicillin (Fig. 21) indicates that a disk containing 0.05 unit

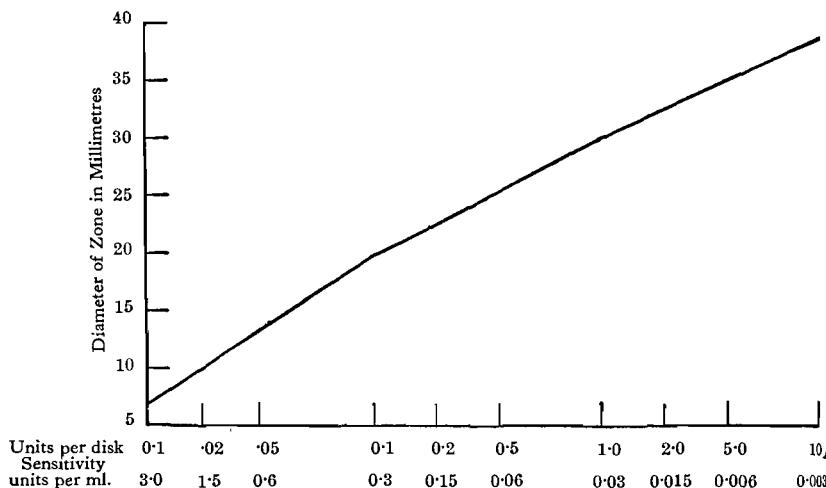


FIG. 21

Standard Graph for Penicillin

(Reproduced by permission of the *Edinburgh Medical Journal*)

penicillin will inhibit the standard organism to 13 mm., therefore the sensitivity of the test organism is $\frac{1}{0.05} \times 0.03 = 0.6$ units per ml.

In this way the sensitivity in units or $\mu\text{g.}$ per ml. is denoted along the abscissae of the graphs (see graph for penicillin).

Disks containing the following amounts of antibiotic are used in routine tests: penicillin 1 unit; streptomycin 10 $\mu\text{g.}$; chloramphenicol 25 $\mu\text{g.}$; chlortetracycline 50 $\mu\text{g.}$; oxytetracycline 10 $\mu\text{g.}$; erythro-

mycin 10 µg.; after overnight incubation the areas of inhibition are measured and from the graphs the sensitivity is recorded. The amounts of each antibiotic recommended above have been found to give with sensitive organisms, zones of inhibition which are easily measurable.

PRIMARY SENSITIVITY TEST

In testing organisms of unknown sensitivity a result adequate for clinical purposes can be obtained from a *primary sensitivity test* in the majority of cases.

The area of initial inoculation (reservoir) of the specimen on the diagnostic culture plate is made so that it covers a sufficient area of the medium and the material is spread as uniformly as possible. Disks containing antimicrobial agents are applied within this area, their centres separated by two or more centimetres. After incubation the zone of inhibition, if any, is measured. These may be compared with the results of subculture tests if desired. There will be a number of primary tests in which the inoculum is so sparse that the result cannot be assessed properly, and in such cases recourse to a subculture test is necessary.

SUBCULTURE SENSITIVITY TEST

Subcultures of the test organisms are made in broth and incubated for six to eighteen hours. Any density of inoculum may be used, but in most cases the surfaces of culture plates may be sown as already described with broth containing approximately 100 million cells per ml. After drying, the surface disks are applied, the plates incubated and zones of inhibition measured.

Colouring of Disks

The disks may be coloured for identification using "cotton" dyes which are "fast" to the paper and do not interfere with the activity of the antimicrobial agents nor exert any antibacterial activity themselves in the concentrations recommended.¹

Penicillin, Chloramphenicol, Oxytetracycline, Chlortetracycline, Erythromycin.—Solutions or suspensions of these antibiotics, twice the final potency per ml. required to prepare the disks, are made by adding sterile water to commercial preparations, *i.e.* penicillin 200 units/ml.; chloramphenicol 5000 µg./ml.; oxytetracycline 2000 µg./ml.; chlortetracycline 10000 µg./ml.; and erythromycin 2000 µg./ml.

Solutions of the following dyes are made in distilled water and autoclaved:

" Clayton "	aniline tolamine pink	.	.	.	RED
" Durazol "	yellow I.C.I. GR 200	.	.	.	YELLOW
" Chlorazol "	sky blue I.C.I. FF 200	.	.	.	BLUE
" Durazol "	fast orange I.C.I. R 150	.	.	.	ORANGE
" Durazol "	scarlet I.C.I. 4B, 150	.	.	.	SCARLET

¹ Bowie, J. H., & Gould, J. C. (1952), *J. clin. Path.*, 5, 356.

and the following colours used to identify the antibiotics:

<i>Antimicrobial Agent</i>	<i>Dye</i>	<i>Colour</i>
Penicillin . .	RED	RED
Chloramphenicol .	3 : 1 mixture of YELLOW and BLUE	GREEN
Chlortetracycline .	YELLOW	YELLOW
Oxytetracycline .	1 : 1 mixture of ORANGE and SCARLET	TERRA-COTTA
Erythromycin . .	1 : 1 : 8 mixture of ORANGE, BLUE and RED	PUCE
Polymyxin . .	2 : 1 mixture of RED and BLUE	PURPLE
Bacitracin . .	2 : 2 : 1 mixture of SCARLET, ORANGE and BLUE	BROWN
Sulphonamide . .	BLUE	BLUE

Equal parts of the antibiotic and dye solutions are mixed and 1 ml. of the colour mixture added to 100 disks.

For Streptomycin, Polymyxin, Bacitracin and Sulphonamides.

—These antibiotics precipitate the dyes in solution, and although this does not interfere with the activity of the antibiotic the colouring of the disks is not satisfactory. Uncoloured disks are used for streptomycin; the disks for polymyxin and bacitracin are first coloured with the dye, dried and then impregnated with antibiotic solution. Solutions of these antibiotics are made up therefore as follows: streptomycin 1000 µg./ml.; polymyxin 10,000 units/ml.; and bacitracin 1000 units/ml. 1 ml. of these solutions are added to each bottle of 100 disks.

Disks for sulphonamides are dyed blue and then impregnated with solutions of the sodium salts of sulphonamides containing 12.5 mg. per ml.

Commercial disks are available, impregnated with antibiotic and marked to indicate the nature of the drug. Some of these are of absorbent paper, others consist of a vehicle with antibiotic and binding agent compressed into a tablet. The amount of antibiotic in these preparations has been found to vary.¹ and prolonged storage under variable conditions may result in a falling off in potency. The substances used as vehicles or binding agents in the tablets may be biologically active and under test conditions become antibacterial or interfere with the action of the antibiotic. For these reasons the standardisation of commercial disks for antibiotic-sensitivity tests is desirable.² If commercial disks are used it is recommended that they be compared with fresh disks of known potency prepared in the laboratory.

¹ Branch A., Starkey, D. H., & Power, E. E. (1957), *Antibiotics Annual 1957-1958*, New York, Medical Encyclopaedia Inc; Sleigh, J. D. (1958), *Scot. med. J.*, 3, 454.

² Greenberg, L., Fitzpatrick, K. M., & Branch, A. (1957), *Canad. med. Ass. J.*, 76, 194.

Replica Plate Method to show Bacteriostatic and Bactericidal Action

A zone of inhibition of growth around a disk in the preceding test may indicate that the antimicrobial agent is either bactericidal or merely bacteriostatic. The presence or absence of living organisms within the zones of apparent complete inhibition of growth on antibiotic diffusion plates may be shown by the replica plate method.¹ Bacteria are transferred from the surface of the sensitivity test plate to a second (replica) plate containing no antibiotic, without disturbing their spatial relationships. This is done by means of a "stamp". Cylindrical wooden blocks, 3 cm. in height and of a diameter slightly less than the Petri dish used, are made from seasoned, close-grained wood. The surfaces should be as even and smooth as possible. An even layer of latex adhesive is brushed over one face and a piece of good-quality furnishing velour of about 2.5 mm. thickness, with a close pile, is smoothed over the surface with the pile facing away from the wood. Trim the edge of the velvet to the block. These "stamps" are autoclaved and stored in pairs with their faces in contact. They may be used repeatedly until the velvet becomes matted or uneven, when the fabric must be replaced.

The velvet surface of a sterilised "stamp" is pressed evenly and firmly on the surface of a sensitivity plate, avoiding lateral movement. The stamp is then lifted off and pressed firmly on the replica plate. This plate is incubated and examined for growth within the areas corresponding to the zones of inhibition on the sensitivity plates. Approximately 1 per cent. of the organisms are transferred from plate to plate by this technique.

Serial Dilution Tube Technique

Serial dilution tests in which the concentration gradient (Fig. 1) is discontinuous are frequently used to determine the antibiotic sensitivity of strains of bacteria and to assay the antibiotic activity of body fluids during treatment.

To each of a series of sterile stoppered test-tubes a standard volume of medium which will support the growth of the test organism is added. A solution of the antimicrobial agent is prepared in broth and a series of doubling dilutions prepared (p. 315) with sterile pipettes. The range of concentrations should extend from at least twice the highest concentration likely to be found in the tissues during treatment to half that which inhibits the growth of the most sensitive member of the species being tested. A control tube containing no antimicrobial agent is included. The inoculum, consisting of a suitable dilution of an overnight broth culture of the test or standard control organism, is added, one loopful to each tube.

¹ Elek, S. D., & Hilson, G. R. F. (1954), *J. clin. Path.*, 7, 38.

The tubes are incubated at 37° C. for eighteen to twenty-four hours and examined for turbidity. The tube with the highest dilution showing no visible turbidity is the minimum inhibitory concentration (bacteriostatic concentration). To measure the bactericidal concentration it is necessary to subculture from the tubes showing no visible growth on to agar or into broth free of antimicrobial agent. The highest dilution yielding no growth is the bactericidal concentration. Where available, a specific neutraliser of the antimicrobial agent should be used in the subculture medium, e.g. penicillinase for penicillin. Wherever possible, replicate tests should be set up and control titrations with control organisms of known sensitivities carried out.

TESTS FOR ANTIBIOTIC SYNERGISM AND ANTAGONISM

Mixtures of antibiotics may show greater or lesser bactericidal effects than equivalent concentrations of the individual components alone. Methods to determine these effects are used to help in the selection of antimicrobial drugs in the treatment of some infections such as cases of sub-acute bacterial endocarditis in which a single drug does not control the growth of the infecting microbe. The exact concentrations of component antimicrobial drugs in a mixture having the greatest bactericidal effect may be ascertained only by setting up large series of tubes containing combinations of different concentrations of each drug. However, for most clinical purposes either of the following tests gives an adequate result.

Tube Test to measure Combined Antibiotic Action¹

This comparatively simple test uses a limited number of tubes and a single concentration of each drug to be tested in combination, the concentration being that most likely to be attained in the tissues during therapy.

Antibiotics in their appropriate concentrations are added to tubes containing serum or digest broth. The combinations which appear at top of p. 409 have been found useful.

The inoculum is a drop of 1: 100 dilution of an overnight broth culture of the test organism added to each tube. Immediately after mixing, a loopful from each tube is spread over a segment of a culture plate. The tubes and plates are incubated for eighteen to twenty-four hours at 37° C. and another loopful is plated from each tube on to a second series of culture plates. After the second set of plates have been incubated, the two sets are compared and any increased or decreased killing effect of the combinations used, measured by counting the number of colonies.

¹ Chabbert, Y. (1953), *Ann. Inst. Pasteur*, **84**, 545.

<i>Tube 1</i>	<i>Tube 2</i>	<i>Tube 3</i>	<i>Tube 4</i>
Penicillin	Penicillin +	Penicillin +	Penicillin +
	Streptomycin <i>Tube 5</i>	Chloramphenicol <i>Tube 6</i>	Tetracycline <i>Tube 7</i>
	Streptomycin	Streptomycin +	Streptomycin +
		Chloramphenicol <i>Tube 8</i>	Tetracycline <i>Tube 9</i>
		Chloramphenicol	Chloramphenicol +
			Tetracycline <i>Tube 10</i>
			Tetracycline
	<i>Tube 11</i>		
	Control		
	No Antibiotic		

A duplicate set of tubes may be set up, but with a more dilute inoculum, e.g. 1:10,000 dilution of an overnight broth culture, since increased bactericidal effects, if any, may be more easily observed under these conditions.

The Disk Test to measure Combined Antibiotic Action

Some information on the combined effect of two or more antibiotics *in vitro* can be obtained by using the disk technique after the manner of Lamanna and Shapiro.¹ The disks are placed with their centres at a suitable distance apart so that the respective antibiotics will diffuse into one another to produce a continuous range of concentrations in the early hours of incubation. This is best done after subculture tests have determined the sensitivity of the test micro-organism to individual antibiotics, e.g. "A" and "B", and the position of the disks containing these antibiotics can be predetermined to effect the desired concentration mixtures; for example, disks A and B can be placed x mm. apart when $x=a+b$, a being the radius of the zone of inhibition with A and b the zone with B. After incubation, the two zones of inhibition will make contact and there will be a thin wedge of growth on either side of the point of contact of the zones. When there is appreciable additive or synergistic effect by individually sub-inhibitory concentrations of A and B, the growth in the area of these wedges is inhibited. On other occasions resistant growth within the zone of inhibition produced by one drug may be inhibited by relatively small concentrations of the other which has diffused into the area. The concentration of the individual components producing these inhibitory effects can be inferred from the standard graphs in the usual way by measuring the distances from the individual disks to the area of inhibition.

Mutual interference (antagonism) between antibiotics is similarly shown by decrease in the size of the zones of inhibition.

¹ Lamanna, C., & Shapiro, I. M. (1943), *J. Bact.*, **45**, 385.

METHODS FOR TESTING THE SENSITIVITY OF MYCOBACTERIUM TUBERCULOSIS TO ANTIMICROBIAL DRUGS

Several antimicrobial drugs such as streptomycin, para-amino-salicylic acid (PAS) and iso-nicotinic acid hydrazide (isoniazid) have a beneficial effect in cases of tuberculosis infection if the organism is sensitive to the drug. Unfortunately, tubercle bacilli may become resistant to these drugs in a few months, especially when they are given alone, so that it is customary to administer two or more drugs simultaneously. It is essential therefore to test strains before and at intervals during treatment, to determine that the organism is and remains sensitive to the drugs used.

The criteria for interpreting resistance from the tests to be described have been determined by closely correlating the results of sensitivity tests with clinical findings.¹

*Sensitivity to Streptomycin*².—The testing of strains of tubercle bacilli for sensitivity to streptomycin is carried out on Löwenstein-Jensen medium.³ The antibiotic concentrations used are 1 µg. per ml. by twofold increments to 64 µg. per ml. These are the actual concentrations in the medium before inspissation. A control without the drug is included. Dispense the medium in 2-ml. amounts into $\frac{1}{4}$ -oz. screw-capped bottles. Inspissate for one hour at 75°–80° C. and store in the refrigerator at 4° C. They may be kept for at least one month without loss of potency of the antibiotic.

To prepare the inoculum, sterilise $\frac{1}{4}$ -oz. screw-capped bottles containing 0.3 ml. water and two $\frac{1}{4}$ -in. glass beads. Make a suspension of the culture to be tested by shaking a loopful of the growth in the 0.3 ml. water on a mechanical shaker for fifteen minutes. Use an inoculating loop of 3 mm. diameter and streak a loopful of the suspension up the centre of the control and each antibiotic-containing L.J. slope. In each batch of tests include a control test using the standard drug-sensitive strain of *Myco. tuberculosis* H37 Rv. Read at the end of twenty-eight days' incubation at 37° C.

The end-point is the lowest concentration of the antibiotic which inhibits growth. Growth is considered to be inhibited if fewer than 20 colonies appear on the slope. The result is expressed as a *resistance ratio* by comparison with the control as follows:

$$\text{Resistance ratio} = \frac{\text{Lowest concentration of the antibiotic which inhibits patient's strain of } Myco. \text{ tuberculosis}}{\text{Lowest concentration of the antibiotic which inhibits the standard drug-sensitive strain, H37 Rv}}$$

¹ Dr. A. T. Wallace, personal communication.

² Stewart, S. M. (1955), *J. clin. Path.*, **8**, 237.

³ Holt, H. D., & Cruickshank, R. (1949), *Monthly Bull. Minist. Hlth Lab. Serv.*, **8**, 103.

For example, if the patient's strain is inhibited by 16 µg. per ml., and the standard, drug-sensitive strain by 4 µg. per ml., then the resistance ratio is $\frac{16}{4} = 4$.

Strains are considered resistant to streptomycin if the resistance ratio is 8 or more. A ratio of 4 is suggestive of resistance, but not conclusive. In such a case, other cultures from the patient's strain should be tested and the previous chemotherapy considered.

Sensitivity to Para-aminosalicylic Acid (PAS).—Sensitivity tests to PAS are performed in the same way as for streptomycin using twofold differences of concentration of PAS ranging from 0.25–16 µg. per ml. The result is again expressed as a resistance ratio.

Strains are considered resistant to PAS if the resistance ratio is 8 or more. A ratio of 4 is suggestive, but not conclusive. In such a case, other cultures from the patient's strain should be tested and the previous chemotherapy considered.

Sensitivity to Isoniazid.—The following method is that recommended by the Medical Research Council.¹ The medium, method of inoculation and period of incubation are the same as for testing streptomycin sensitivity and the concentrations of isoniazid 0.2; 1; 5 and 50 µg. per ml. The end-point is the lowest concentration inhibiting growth to 20 colonies or less. Strains are resistant to isoniazid if growth occurs on 1 µg. per ml. or more. Growth on 0.2 µg. per ml. is suggestive of resistance, but is not conclusive. In such a case other cultures from the patients strain should be tested and previous chemotherapy considered.

Alternatively, a closer range of isoniazid concentrations (from 0.025 to 10 µg. per ml.) may be used and the results reported as the resistance ratio. A ratio of 4 indicates resistance.

Sensitivity tests for other Antitubercular Drugs.—Tests for viomycin, cycloserine and oxytetracycline may be carried out in a manner similar to that for testing streptomycin.

TITRATION OF ANTIMICROBIAL AGENTS IN BLOOD AND OTHER BODY FLUIDS²

It may be desirable to estimate the amount of antibiotic or other antimicrobial agent in body fluids during treatment to ensure that an adequate dosage is being administered. The methods employed are similar to those used to determine the sensitivity of bacteria to antibiotics. A simple method suitable for most clinical purposes is as follows.

The fluid to be examined is collected aseptically. If it is likely to be

¹ Med. Res. Coun. (1953), *Lancet*, 2, 213.

² For micro-methods of estimating penicillin in serum and other body fluids, the reader should consult the following papers: Fleming, A. (1944), *Lancet*, 2, 620; and Fleming, A., Young, M. Y., Suchet, J., & Rowe, A. J. E. (1944), *Lancet*, 2, 621; Fleming, A., & Smith, C. (1947), *Lancet*, 1, 401.

contaminated with micro-organisms, these must be removed, e.g. by filtration. Blood is withdrawn by venepuncture and the serum separated, being centrifuged if necessary to free it completely from suspended red cells. The test should be carried out as soon as possible after collection of the fluid.

Suitable dilutions of the fluid under examination are prepared in broth and inoculated with a standard organism of known sensitivity such as the Oxford strain of *Staph. aureus* which is sensitive to concentrations of all the commonly used antimicrobial agents which can be easily attained in the tissues. A control tube containing the medium alone is included. The tubes are incubated for eighteen to twenty-four hours and examined to find the tube with the highest dilution which has no turbidity.

A control test is set up in parallel using a fresh sample of the same body fluid known to contain no antimicrobial agent and to which a known amount of the agent being assayed has been added. By comparing the dilutions which inhibit the growth of the standard organism an exact estimation of the amount of antimicrobial agent can be made, taking into account the bacteriostatic action of the fluid under test, e.g. if the unknown fluid inhibits growth at a dilution of 1: 60 and the control fluid containing 5 µg./ml. at a dilution of 1: 120, the unknown contains $\frac{60}{120} \times 5 = 2.5 \mu\text{g./ml.}$

There are many detailed techniques, both chemical and biological for assaying individual antibiotics, and for these appropriate textbooks should be consulted (e.g. D. C. Grove & W. A. Randall, *Assay Methods of Antibiotics, A Laboratory Manual*, Med. Encyclop. Inc., Antibiotics Monographs No. 2, New York).

ESTIMATION OF STREPTOMYCIN IN SERUM, CEREBROSPINAL FLUID AND OTHER BODY FLUIDS

Method

This method,¹ recommended by an M.R.C. Sub-Committee, can be used when a rapid clinical assay is required. When a more precise determination is necessary, the method of Mitchison and Spicer should be employed.²

Test Organism.—A strain of *Pneumobacillus (Klebsiella pneumoniae)*, N.C.T.C.3.

Indicator Medium

Horse serum	1 part
10 per cent. glucose in distilled water	1 part
Saturated solution of phenol red, in sterile distilled water	2 parts

¹ Medical Research Council Sub-Committee Report (1948), *Lancet*, 2, 862.

² Mitchison, D. A., & Spicer, C. C. (1949), *J. gen. Microbiol.*, 3, 184.

Indicator System.—Boil 5 ml. of the indicator medium in a test-tube. Cool. Add 0.1 ml. of a twenty-four hours' broth culture of the pneumobacillus.

Serum Dilutions.—Set up specimens of patients' serum and a standard serum containing a known concentration of streptomycin (16 µg. per ml.) in serial two-fold dilutions by the drop method on sterile waxed microscope slides.

The procedure for each serum (test and standard) is the same and is as follows:

First heat the sera in a water-bath at 56° C. for thirty minutes.

Flame the surfaces of two waxed slides and place nine separate drops of sterile normal saline in positions 2, 3, 4, 5, 6, 7, 8, 9, 10 on the slides.

1	2	3
4	5	
6	7	8
9	10	

Place one volume of serum in position 1; add one volume of serum to the saline in position 2; mix and transfer one volume to the saline in position 3; and so on. After mixing in position 9, discard one volume; 10 is used as a control. Add one volume of indicator medium to the serum in position 1, to the serum dilutions, and to the saline control. The final dilutions of serum are thus 1:2 . . . 1:512, and the final concentrations of streptomycin in the dilutions of standard serum are 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0 µg.

Draw up the mixtures of serum dilution and indicator system into capillary tubes. Place the tubes horizontally in plasticine, leaving the ends unsealed. Incubate at 37° C. for twenty-four hours.

End-point of Titration.—Where full growth of the test organism has occurred, the column of fluid in the capillary tube shows opaque clotted serum, coloured uniformly yellow; where no growth has occurred, the fluid remains red and transparent. The highest dilution showing no growth is taken as the end-point.

Compare the reading obtained in the case of the test serum with that of the standard serum, and the concentration of streptomycin in the test serum is calculated from the formula $\frac{\text{T.E.}}{\text{S.E.}} \times 16$, where T.E. is the reciprocal of the test serum end-point and S.E. the reciprocal of the standard serum end-point.

Example.—T.E. = 16; S.E. = 64.

$$\frac{16}{64} \times 16 = 4.$$

Therefore the unknown serum contains 4 µg. of streptomycin per m

PRODUCTION OF PENICILLINASE

Penicillin is rapidly destroyed by the products of growth of many organisms, and such material has been termed "penicillinase". Some strains of *B. subtilis* produce it in large quantities. Penicillinase is useful when it is desirable to destroy penicillin present in body fluids from which cultivation tests are carried out: for example, blood culture, when ascertaining the survival times of organisms subjected to the action of penicillin or when testing preparations of penicillin for sterility.

B. subtilis (strain N.C.T.C. 6346) is grown in broth, the culture is filtered and the resultant fluid constitutes "penicillinase." The filtrate is tested for potency, with penicillin and the standard Oxford strain of staphylococcus (p. 402).

Preparation of Penicillinase

Distribute digest broth in shallow layers in 20- or 32-oz. flat bottles. Inoculate the broth with a culture of *B. subtilis* (strain 6346).

Incubate the bottles horizontally for fourteen days at 26° C. A pellicle forms which is shaken down each day.

At the end of incubation, filter the culture through a Seitz disk. The filtrate contains 100,000 units or more of penicillinase per ml.

Tests for Potency

Make up a solution of penicillin, 1000 units per ml.

Prepare in tubes or bottles a series of 10 ml. amounts of broth containing 1000 units of penicillin, and add varying amounts of penicillinase.

Inoculate each tube or bottle with a loopful of an overnight culture of the standard strain of staphylococcus, and incubate for forty-eight hours.

The end-point is the least amount of penicillinase which allows growth. This amount neutralises 1000 units of penicillin and thus contains 1000 units of penicillinase. For example, if the least amount of filtrate permitting growth is 0.005 ml., then the penicillinase content is 200,000 units per ml.

TESTING OF SURGICAL CATGUT FOR STERILITY

The following method was prescribed in the earlier Therapeutic Substances Regulations (Therapeutic Substances Act) and has been extensively used.

"... The sample shall, when practicable, be the contents of at least one whole container or packet . . .

(a) the container or packet shall be opened and the sample removed with aseptic precautions;

(b) after all the adherent fluid has been drained off as completely as possible, the sample shall be placed entire in a test-tube at least 3.5 cm.

in diameter and 17.5 cm. in length and containing 50 ml. of sterile distilled water. This tube shall then be closed by some method which will preclude the access of bacteria, and be placed in an incubator at 37° C. for twenty-four hours;

(c) after this incubation, the sample shall be aseptically transferred to a similar tube containing a solution of 1 per cent. of sodium thiosulphate and 1 per cent. of crystallised sodium carbonate in distilled water, the tube and solution having been previously sterilised in the autoclave. In this solution the sample shall again be incubated for twenty-four hours at 37° C.;

(d) after the second incubation the sample shall again be removed aseptically and, without further washing, shall be examined for the presence of living bacteria and their spores.

The sterility tests shall be carried out . . . by placing the sample in a tube at least 3.5 cm. in diameter and 17.5 cm. in length, containing not less than 50 ml. of a culture medium prepared by dissolving 0.2 per cent. of prepared agar-agar in a nutrient bacteriological broth . . . the broth may preferably be made by the digestion of meat with trypsin (Douglas's broth or Hartley's modification thereof) . . . the mixture being sterilised in the autoclave; . . .

(e) The tubes of culture medium containing the sample shall be incubated at 37° C. for twelve days and examined daily for the growth of bacteria;

(f) if no such growth is detected during this period, the batch from which the sample was drawn shall be treated as free from living bacteria and their spores, and as having passed the test; . . ."

An *alternative culture medium* which has been used is the cooked-meat medium (described on p. 233) with 30 ml. of broth added to each tube (these being 6×1 in.). Before transferring the catgut to the medium from the thiosulphate solution it is placed in a tube of sterile distilled water for a few hours. Meat medium has been found to be particularly valuable for obtaining growths of sporing anaerobic bacilli from catgut, and also serves very well for the detection of other organisms which may occur as contaminants in catgut.

It should be emphasised that in all the manipulations of the catgut sample in the above specified processes, care is required to exclude extraneous contamination and it is advisable to carry out these manipulations under an inoculating hood, or with the aid of a similar apparatus, to prevent aerial contamination (p. 251).

For details of present requirements in the application of sterility tests to Surgical Catgut reference should be made to the latest Therapeutic Substances Regulations (1957).

CHAPTER XVI

ANIMAL INOCULATION AND AUTOPSY

IN Great Britain animal experiments may be performed only under a licence granted by the Home Secretary. In addition to the licence various certificates have also to be obtained, depending on the nature of the experiments and the animals used.

The usual animals employed for microbiological experiments are the guinea-pig, rabbit, mouse, white rat, hamster and fowl, and the commonest method of inoculation is by means of a hypodermic needle and syringe. According to the method of inoculation and the size of the animal, the amount of injected material varies, and a number of syringes of different sizes are used. A convenient "battery" of syringes is the following: 1-ml. "tuberculin" syringe graduated to 0.01 ml.; 2-ml. syringe graduated to 0.1 ml.; 5-ml. graduated to 0.25 ml.; 10-ml. graduated to 0.5 ml. A 20-ml. syringe is used only occasionally. Syringes should preferably be of the all-glass "Luer" type.¹ A selection of needles is required, of which the following are useful: fine-bore, No. 25 gauge; medium-bore for general use, Nos. 21 and 22 gauge; large-bore, Nos. 17-18 gauge, for inoculating thick suspensions or emulsions of tissue. The needles should be made from stainless steel.

Sterile syringes should always be used: a full account of the sterilisation of syringes and their care is given on p. 178.

MATERIAL INOCULATED

Urine, cerebro-spinal fluid, blood and serous fluids are easily inoculated with a medium-bore needle. Tenacious material such as *pus* and *sputum* is injected through a wide-bore needle.

Cultures.—Fluid cultures are easily drawn through a medium-bore needle. It may be found advantageous first to pour the culture into a small (2-in.) Petri dish, or a wide-mouthed 1-oz. screw-capped bottle. Growths on solid media may be scraped off and suspended in broth or saline, or the diluting fluid may be poured on the culture which is then emulsified with a wire loop.

Tissues.—Small fragments of soft tissues such as brain, liver, spleen and kidney are readily homogenised by crushing them with a suitable diluent in a Ten Broeck grinder. If larger volumes of tissue suspensions are needed or if firmer tissues such as muscle or lung have to be used, an electrically powered blender of the Waring type is recommended. Tough and fibrous tissues such as skin or chronically inflamed lymph

¹ For a specification of all-glass syringes see p. 178.

glands should be cut into small pieces in a sterile porcelain mortar by means of scissors sterilised by boiling. Some clean coarse sand, previously washed with acid to remove carbonates, or fine powdered sintered glass, contained in a stoppered bottle and sterilised by hot air, is then added to the mortar and the whole thoroughly ground with the pestle. When the tissue has been well ground up, saline is added and the mixture further triturated. On standing for a short time, the sand and tissue rapidly settle to the bottom of the mortar and the supernatant fluid can be drawn into the syringe. When intravenous inoculation of tissue suspension has to be employed, care must be taken that no large particles are injected. To avoid this, the suspension must be centrifuged at low speed and only the supernatant fluid used.

Source of Experimental Animals

It is essential that experimental animals should be obtained from a reliable breeder, where the condition of the stock is known, and whose premises are satisfactory. The Laboratory Animals Centre, M.S.C. Laboratories, Woodmansterne Road, Carshalton, Surrey, has established a register of Accredited Breeders, and users of animals in Great Britain should apply to the Director for details.

GUINEA-PIGS

These animals vary in size, and weigh from 200 grams (small) to 1000 grams (large). A good average weight for general purposes is 400 grams.

Subcutaneous Inoculation.—An assistant holds the animal during the operation, and the injection is made under the skin of the flank. The animal is grasped across the shoulders in one hand, with the thumb curved round the animal's neck so that it rests on the lower jaw. The hind legs are secured between the first and second, and second and third fingers of the other hand, the knuckles being uppermost, and the animal is held so that the flank is presented for inoculation. The skin may be disinfected with tincture of iodine. The operator picks up a fold of skin and introduces the point of the needle into the base of the fold so that it lies in the subcutaneous tissue. Amounts up to 5 ml. can be introduced. A 2-ml. or a 5-ml. syringe is convenient for the purpose.

Some workers inoculate by picking up a fold of skin about the mid-abdomen. The needle is introduced into the base of the fold and passed down in the subcutaneous tissue until it reaches the groin, where the injection is made. This method obviates superficial ulceration when tuberculous material is injected.

Intracutaneous Inoculation.—This method is used chiefly in testing cultures of the diphtheria bacillus for virulence (p. 518). The hair is removed from the flanks of the animal by plucking or alternatively by means of a fresh 5 per cent. solution of sodium sulphide or a depilating

powder. White guinea-pigs (300-400 grams weight) are used, as the skin is unpigmented and the results of the test can easily be read.

The depilating powder is made as follows:

Barium sulphide, commercial powder	7 parts
White household flour	7 parts
Talcum powder	7 parts
Castile soap powder	1 part

Remove the hair from the flanks as closely as possible with hair clippers. Make up the depilating powder into a smooth paste with water, and rub into the animal's hair with a wooden spatula or toothbrush. Allow the paste to act for one minute and renew the application. After two minutes remove the paste with the spatula or handle of the toothbrush. Now wash the animal's skin and surrounding hair with warm water and dry with a cloth. The depilated surface should be quite smooth and white. It is advisable not to leave the paste on too long as the skin becomes red and excoriated in patches, making the subsequent observation of reactions very difficult. The depilating powder should be used at least one hour before the intracutaneous injection is carried out.

For the test a 1-ml. all-glass tuberculin syringe, fitted with a short needle of No. 25 or 26 Gauge (exactly as used for Schick and Dick tests), is employed. The skin of the animal is pinched up between the thumb and forefinger, and the point of the needle is inserted at the top of the fold so that the bevel of the needle is towards the surface of the skin. The needle passes only into the dermis, as near the surface as possible, *and not into the subcutaneous tissue*. 0.2 ml. is the amount usually used, and when several tests are to be made the injections should be about one inch apart and not too near the middle line of the abdomen. No more than ten injections should be made on one animal. The results are read twenty-four to forty-eight hours later.

Intraperitoneal Inoculation.—The animal is held in a similar manner. The inoculation is made in the mid-line in the lower half of the abdomen. The assistant holds the animal with its head downwards, so that the intestines fall towards the diaphragm. The skin is pinched up, the point of the needle is first passed into the subcutaneous tissue and then downwards through the abdominal wall into the peritoneal cavity. There is no risk of damage to the intestines. Not more than 5 ml. can safely be inoculated intraperitoneally.

Collection of Blood.—Small amounts, up to 0.5 ml., may be taken by simple incision of the marginal ear-vein. Cardiac puncture is, however, the only satisfactory way of obtaining larger volumes. The animal is lightly anaesthetised with ether and then laid on its back with its front limbs drawn forwards. An area over the fourth and fifth left intercostal spaces is plucked and the skin painted with tincture of iodine. The position of the apex beat of the heart is defined by digital palpation and at this point a needle (No. 20 Gauge mounted on a syringe) is inserted between the ribs. A sharp downward movement inclined towards the mid-line then takes the needle point through the ventricular wall. As

much as 15 ml. of blood may be obtained from a 350-400 g. animal, though smaller amounts are advisable if the guinea-pig is to survive. Very sharp needles are essential for the success of this operation.

The normal rectal temperature of the guinea-pig is $100.8 \pm 1.2^{\circ}$ F. To ascertain the animal's temperature, a clinical thermometer, with a small round bulb which is smeared with vaseline, is gently inserted into the rectum of the animal.

RABBITS

These animals are often unsatisfactory for experimental purposes owing to their liability to parasitic and intercurrent infections. The animals used should be free from snuffles (a chronic nasal inflammation), subcutaneous abscesses and mange. They should be plump, their fur should be in good condition and they should not be suffering from diarrhoea. If the animal is in poor condition it is probably affected with coccidiosis or intestinal worms. Rabbits are very prone to die from septicaemia (due to *Pasteurella multocida*) and from pneumonia. The animals should be obtained from an accredited breeder, where the condition of the stock is known, and not purchased casually and indiscriminately from a dealer.

The chief use of the rabbit lies not so much in diagnostic work as in its value for experimental purposes. It is extensively used for the production of immune sera, such as agglutinating and haemolytic sera, which are frequently employed for routine laboratory diagnosis.

Under Certificate "A" of the Home Office the animal may be inoculated intravenously, intraperitoneally, subcutaneously or by scarification, without the use of an anaesthetic. Certificate "B" is required if an anaesthetic is used and the animal is allowed to recover.

Scarification.—The hair is removed from the flank of the animal by first clipping and then shaving, or by means of the depilating mixture described on p. 418. The skin is cleansed with alcohol, which is allowed to evaporate. A number of parallel scratches are made with a sharp sterile scalpel, just sufficiently deep to draw blood. The infective material is rubbed into the scarified area with the side of the scalpel. This method is mainly used for the propagation of vaccinia virus.

Subcutaneous inoculation may be made either into the abdominal wall or into the loose tissue about the flank or at the back of the neck. The hair is clipped, the skin is sterilised with iodine and then pinched up, and the needle is inserted. The technique is the same as that for the guinea-pig.

Intravenous inoculation is employed when material has to be introduced directly into the circulation. The marginal vein of the ear is the most convenient site. The rabbit may be held by an assistant or placed in a special box so that only its head protrudes. The hair over the vein should be dry-shaved with a sharp razor. The vein may be distended for ease of inoculation either by vigorous rubbing with a piece of cotton-wool or by holding the ear over an electric-light bulb, when the heat

causes a dilatation of the blood vessels. According to the amount of material to be injected, a suitable sterile syringe is selected. The operator faces the animal and the ear is held horizontally by means of the left hand. The needle is kept as nearly parallel as possible to the vein and the point inserted towards the head of the animal. When the injection is completed, the needle is withdrawn and a small piece of cotton-wool placed on the vein, which is then compressed between the thumb and finger.

Intraperitoneal inoculation is carried out as in the case of the guinea-pig.

Intracerebral Inoculation.—The animal is anaesthetised with ether, the hair over the head shaved, and the skin disinfected with alcohol and tincture of iodine. A short incision is made through the scalp at a point situated 2 mm. lateral to the sagittal suture and 1.5 mm. anterior to the lambdoidal suture. The skull is then perforated with a trephine or a mechanical drill, and the needle, which is cut down to $\frac{5}{16}$ in. long, introduced through the opening. About 0.45 ml. of material can be inoculated into the occipital lobe of a large rabbit. After injection the needle is rapidly withdrawn, the skin sutured and the area covered with collodion solution.

Rabbits may also be inoculated in the frontal lobe, at a point situated 2 mm. lateral to the median plane on a line joining the two external canthi of the eyes.

Collection of Blood.—From the ear vein of a large rabbit 20–30 ml. of blood may be obtained easily and without causing any distress to the animal. The ear is shaved and sterilised with sterile gauze soaked in 70 per cent. alcohol. Meanwhile a small vessel containing vaseline has been heated over the Bunsen to render it sterile, and when cool, but still fluid, the vaseline is painted over the vein, and on the margin and under-side of the ear. The ear is held forward and the vein is made prominent by means of a small spring clip at the base of the ear, and then incised with a small sharp sterile scalpel. The blood flows over the vaseline, and is allowed to drop into a sterile flask containing glass beads or a suitable anticoagulant according to requirements. The vessels of the ear can be dilated by holding an electric bulb below it or by rubbing the part not covered by vaseline with a plect of wool moistened with xylol. When sufficient blood has been obtained the clip is removed and a piece of cotton-wool pressed firmly over the cut in the vein. The xylol is removed from the ear with alcohol, and some vaseline then lightly smeared on.—Water should always be provided in the cage of the animal after bleeding.

Larger amounts of blood can be obtained by *cardiac puncture*. The animal is fastened to a board and the fur clipped over the left side of the chest; the area is shaved and then sterilised with alcohol and ether. A 100-ml. bulb pipette (*vide* diagram) is cut down at both ends to 9 in. in length, one end being slightly tapered and the other end stoppered with cotton-wool. It is wrapped in kraft paper and sterilised in the hot-air oven. A wide-bore transfusion needle is fitted into a

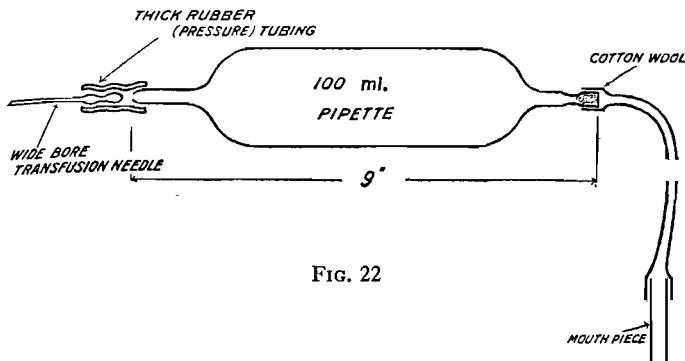


FIG. 22

short length ($1\frac{1}{2}$ in.) of thick rubber tubing and sterilised by boiling. When the animal is anaesthetised, the rubber tubing is attached to the tapered end of the pipette and to the other end is fitted a mouth-piece such as that used in pipetting (p. 316). The needle is inserted into the left side of the chest and suction applied. The needle should lie in the right ventricle of the heart, and blood rapidly flows into the pipette. About 50 ml. of blood per kg. of body-weight can be obtained. The blood is then transferred to a sterile 500-ml. flask or bottle containing glass beads for defibrillation.

The average normal rectal temperature of the rabbit is 102.4° F., but the variations are great. No temperature under 104° F. should be considered pathological.

The leucocyte count of the rabbit is also subject to great normal variation.

RATS

Care must be exercised in handling these animals as the sharp incisor teeth are capable of inflicting a severe wound. They should be held by the loose tissue at the nape of the neck with a pair of crucible tongs or artery forceps, and the animal is kept taut by pulling on the tail. *Intraperitoneal* and *subcutaneous inoculations* are made in a manner similar to that used for the guinea-pig. *Intravenous inoculation* may be made into the vein at the root of the tail. The vein should be dilated by immersing the tail in warm water.

Hamsters

The golden hamster (*Mesocricetus auratus*) is susceptible to many bacterial and viral infections. The animals should be handled gently and carefully, for they can inflict a deep biting wound if they are suddenly disturbed. *Intraperitoneal* and *subcutaneous inoculation* may be carried out in the manner used for rats or guinea-pigs.

MICE

Subcutaneous Inoculation.—An assistant grasps the loose skin at the nape of the neck in one hand and the tail in the other. In this manner the animal is held in a fixed position while the needle is introduced under the skin near the root of the tail. Amounts up to 1 ml. may be injected.

Intraperitoneal Inoculation may be carried out if the animal is similarly held and then turned over. For steadiness, the assistant's arms should rest on the table. The injection is made to one side of the middle line in the lower half of the abdomen and amounts up to 2 ml. can be introduced.

Intraperitoneal inoculation may also be done without an assistant. The animal is held at the nape of the neck with the left hand, and kept extended by holding the tail with the right hand. The left hand is turned over so that the mouse lies on its back in the upturned palm. The tail is then fixed by the little finger of the left hand. The mouse is now firmly held, and the right hand is free to pick up the syringe and make the injection.

Intravenous Inoculation may be made into a vein at the root of the tail if a fine needle be used and the vein dilated by placing the tail of the animal in water at about 45° C. The maximum amount which can be injected is 0.5 ml. for a mouse of 20 grams. A small cylindrical cage made of perforated zinc, and just large enough to hold the mouse with its tail protruding, is useful for this procedure.

Intracerebral Inoculation.—The skin over the head is cleansed with 70 per cent. alcohol and the animal is lightly anaesthetised with ether for the inoculation. A fine-bore needle attached to a 1-ml. syringe (as used for intracutaneous inoculation) is employed and easily penetrates the skull. The site of injection is midway between the outer canthus of the eye and the point of attachment of the pinna of the ear at about 3 mm. from the mid-line. The point of the needle is carried through the skull for $\frac{1}{8}$ in. to $\frac{1}{4}$ in. Approximately 0.03 ml. of fluid can be injected with safety.

Intra-nasal Inoculation.—This should if possible be carried out in an inoculating chamber designed for the purpose for there is a risk that the operator may himself inhale infective material. Failing this, a mask should be worn. The mouse is lightly anaesthetised with ether and as soon as its breathing has become deep and automatic 0.1 ml. of the inoculum is introduced into the anterior nares on one side.

Collection of Blood.—Under deep anaesthesia the animal is pinned out and the skin over the thorax and abdomen is reflected as for autopsy (*vide infra*). The great vessels in the axilla are incised and the blood which wells out is taken up in a sterile Pasteur pipette. An alternative and less time-consuming method is to displace the globe of the eye forwards and to puncture the retro-orbital plexus of veins with the tip of a fine Pasteur pipette which is then used to take up the free-flowing blood. About 1.0 ml. may be obtained and the mouse may be permitted to recover from the anaesthetic and to survive.

Inoculation of Infant Mice.—Suckling mice no older than forty-eight hours are used for the isolation of the herpes simplex and enteric viruses. Great care and cleanliness is required in handling the litters if cannibalism by the mothers is to be avoided. The following injections can be used: 0.03 ml. subcutaneously, 0.05 ml. intraperitoneally, 0.03 intracerebrally. Sometimes the intraperitoneal and the intracerebral routes are used together in the same animals.

FOWLS

The red blood cells, serum and plasma of the domestic fowl are frequently required in virological work and it is often necessary to keep a number of cockerels in the animal house. For certain types of work hens' blood is unsuitable. Turkeys, ducks, geese and pigeons are occasionally used and the day-old domestic chicken is a valuable experimental animal in some types of infectivity studies. The use of the chicken embryo is described on p. 445 *et seq.*

Subcutaneous inoculation is carried out in the pectoral or thigh regions.

Intraperitoneal inoculation is carried out in the mid-line between the vent and the posterior end of the sternum.

Insufflation.—Infective material is dropped from a Pasteur pipette into the nostril.

Anaesthesia.—It should be noted that *ether anaesthesia is seldom satisfactory* in birds and that the injection of sodium nembutal in a dose of 25 mg. per kilo body-weight intramuscularly in the thigh region gives better results. The anaesthetic should be given about half an hour in advance.

Collection of Blood.—If clear plasma is required, the bird should be deprived of food for about eight hours before bleeding.

(a) *From the Wing Vein.*—A 10- or 20-ml. syringe with an No. 21 or 22 Gauge needle is required and it is usually necessary to treat it and the tubes into which the blood is to be placed with a solution of heparin (25 mg. per cent., see p. 460). The bird is placed on its side and the upper wing is fanned out to expose its under-surface. Feathers are then plucked from an area over the "elbow" and the large brachial vein can be seen running over the bone and just beneath the skin. The area is cleaned with 70 per cent. alcohol, and when dry the vein is gently pierced with the needle. Great care must be taken to avoid passing the needle too deep and on through the far side of the vein for if this happens a large haematoma results and it becomes impossible to obtain any blood.

(b) *Cardiac Puncture: Method (1).*—The bird is anaesthetised and placed on its right side. The needle is inserted over the heart between the second and third ribs at a point close to where the edge of the breast muscle can be felt. At a depth of $1\frac{1}{2}$ in. the needle enters the ventricle. This method carries a risk of lung puncture and requires experience before it can be used with confidence. *Method (2)* is perhaps easier. Place the bird on its back, ventral side uppermost, and stretch its neck

over the edge of the table. Pluck the base of the neck and clean the skin with alcohol as before. A 20-ml. syringe with a No. 17 or 18 Gauge needle 2 in. long is inserted horizontally just deep to the sternum; it should be tilted very slightly downwards and will enter the heart at a depth of $1\frac{1}{2}$ in.

AUTOPSY

All experimental animals, whatever the cause of death, should be examined *post mortem* as a routine. When a virulent organism such as the bacillus of plague or of anthrax has been used, special care must be taken, otherwise the infection may be disseminated, with danger to the operator and other workers.

Details will be given of the procedure in conducting an autopsy in the usual manner, and also the method used when dealing with highly infectious organisms.

As a primary reason for the autopsy is to recover organisms previously injected into the animal, the examination must be conducted with strict aseptic precautions.

Materials required:

A suitable animal board or table, on which the carcase can be fixed in the supine position.

Instruments.—Three scalpels; scissors, ordinary size, four pairs; mouse-toothed forceps, four pairs; small bone forceps if the skull is to be opened; a searing iron—a 4-oz. soldering bolt is suitable for the purpose; sterile capillary pipettes; sterile Petri dishes; sterile test-tubes, and tubes, bottles or plates of media.

The knives are sterilised in strong lysol (about 20 per cent.) and then placed in a weaker solution (2 per cent.), and the metal instruments by boiling in a sterilising bath, e.g. an enamelled "fish-kettle". When ready for use, the tray of instruments is lifted out of the steriliser and laid on a spread towel which has previously been soaked in 1: 1000 solution of perchloride of mercury.

It is a useful practice, where cultures have to be made, first to immerse the animal completely in weak lysol solution (3 per cent.) for a few moments. This not only destroys most of the surface organisms, but prevents the dust in the fur from getting into the air and contaminating other materials. The animal is now fixed to the board and towels moistened with antiseptic are placed over the head and lower extremities.

The instruments are removed from the steriliser. A long median incision through the skin of the abdomen and chest is now made and the skin widely dissected, exposing the abdominal and chest muscles. With another set of instruments the peritoneal cavity is opened and the abdominal wall is reflected to each side. With fresh instruments the spleen is removed and placed in a sterile Petri dish. Other organs such as the liver and kidneys may be similarly removed. The ensiform cartilage is now tightly gripped with a pair of strong forceps, and by

means of a sterile pair of strong scissors a cut is made on either side of the chest through the costal cartilages. The sternum is raised and pulled towards the head. The heart is now exposed. A sterile capillary pipette, furnished with a teat, is passed through the heart wall. Blood can thus be withdrawn and inoculated into various media. If the autopsy has been properly performed, it is not necessary to sear the surface of the heart. The lungs are then removed with fresh instruments by cutting each organ free at the hilum. Care must be taken not to open into the oesophagus if the lungs are to be used for cultivation.

After the organs to be used for culture have been removed and placed in separate Petri dishes, the autopsy can be completed.

While the instruments are again being boiled the naked-eye appearances of the organs should be studied. For culture the spleen gives the best results, but the other solid viscera may similarly be used. The organ is cut with sterile instruments and a small portion is taken up with a stiff wire and smeared on the surface of solid media. Liquid media are inoculated with a small fragment of the tissue.

In conducting *post-mortem* examinations, various animal diseases, such as worm infestation, coccidiosis, pseudo-tuberculosis, etc., may be noticed, and the worker should be familiar with their appearances.

When the animal is infected with highly pathogenic organisms the worker *must* wear rubber gloves. The carcase is soaked in antiseptic solution as before and nailed to a rough piece of board of the appropriate size. This board is then placed in a large enamelled iron tray. The autopsy is carefully performed in the usual way. The carcase is finally covered with 10 per cent. lysol, which flows over the board and into the tray. The whole contents of the tray—board and carcase—are then destroyed in a furnace or incinerator. The rubber gloves, instruments and tray are thoroughly sterilised. When performing animal autopsies we strongly advise the wearing of a large overall made of waterproof material, and in addition, the use of some form of glasses or goggles to protect the eyes.

CARE OF ANIMALS¹

General Directions

Litter for all animals may be sawdust, wood chips or peat moss.

All cages and runs should be cleaned out twice weekly. The daily duties of the animal-keeper should be written out and posted up in the animal-house. Feeding-troughs should be of earthenware, about four inches in diameter. They must be cleaned daily, and in the case of experimental animals it is important that the troughs are returned to their respective cages.

¹ See *A System of Bacteriology*, Medical Research Council, 1931, vol. ix., chap. 17, from which certain of the data in this section are quoted by permission of the Controller, H.M. Stationery Office. See especially UFAW Handbook on the Care and Management of Laboratory Animals, 2nd Edition, 1957, London; and *Notes on Communicable Diseases of Laboratory Animals*, by H. J. Parish, 1950, E. and S. Livingstone, Edinburgh.

Separate barrows should be used for foodstuffs and soiled bedding. The runs and cages should be regularly inspected for sick animals, which should be removed and kept separate. It is very important that the temperature of the animal-house is kept as even as possible. Draughts should be avoided. Guinea-pigs particularly are susceptible to changes of temperature, and deaths are frequent amongst inoculated animals if the temperature is allowed to vary.

Guinea-Pigs

Food in pelleted form is recommended in preference to mashes. Pellets are placed in a hopper in the animal cage and provide a continuous source of food. This system eliminates waste, prevents contamination with urine and faeces, and is simple and labour saving in operation. Diet 18 of Bruce and Parkes (1947)¹ contains balanced proportions of protein, fat and carbohydrate with added vitamins, salt and trace elements. This diet is recommended and to it must be added 2 oz. of cabbage or kale and 2 oz. hay daily for each animal. In winter, carrots, swedes or mangos may be substituted for the green food. Fresh water must be provided from a bottle attached to the cage.

If, however, a mash is to be used instead of pellets, the following simple formula may be of value:

Crushed oats	2 parts
Broad bran	1 part

It may be fed dry or slightly moistened with water. This mash lacks sufficient protein to maintain reproduction and the growth of young animals. It must be supplemented with cabbage and hay as above and it may be necessary to add protein concentrates such as fish or meat meal.

Cages.—Stock runs should be about 3 ft. square, with sides 1 ft. 8 in. high.

For experimental animals, galvanised-iron cages are best, as they are more easily cleaned and sterilised after use. A convenient size is 14×9×8 in., fitting in a tray 1½ in. deep.

Breeding.—Period of gestation seventy days; animals may be used for breeding after six months; three litters yearly, average litter three; young weaned at fourteen days.

Common Diseases

Pseudo-tuberculosis (p. 639).—May be either acute or, more commonly, chronic. In the acute type the animal dies in a few days. In the chronic type the liver, spleen and mesenteric glands show very numerous yellowish-white areas scattered through them, somewhat suggestive of tuberculosis. Often a whole stock becomes infected, and experimental animals frequently die before the experiment is completed.

¹ Bruce, H. M., & Parkes, A. S. (1947), *J. Hyg. (Lond.)*, 45, 70.

Abscesses in lymphatic glands, due to haemolytic streptococci of Group C, are not uncommon. (These organisms may also produce septicaemia.).

Respiratory Tract Infections.—Guinea-pigs are liable to pneumonia and pleurisy, often haemorrhage and septicaemia due to such organisms as the pneumococcus, pneumobacillus, haemolytic streptococci, *Pasteurella* group, etc.

Intestinal Infections.—Organisms of the *Salmonella* group, e.g. *S. typhimurium*, are the cause of the most lethal of all guinea-pig diseases. Explosive epizootics may occur in which practically the whole of a colony is destroyed.

Virus diseases, such as guinea-pig paralysis and pneumonia, may be met with.

Rabbits

The pelleted food recommended for guinea-pigs may be used for rabbits. Hay, green food and fresh water should be added *ad lib.* Alternatively, the following diet, per animal, may be used:

Morning

Oats, 1 part	:	:	:	:	:	2½ oz. (32 g.)
Bran, 3 parts	:	:	:	:	:	
Water.						

Afternoon

Hay	4 oz. (113 g.)
Cabbage, kale or other green food supplemented by roots	3 oz. (85 g.)
Water.	

Cages.—Galvanised iron or zinc should be used. A convenient size is $19\frac{1}{2} \times 14\frac{1}{2} \times 11\frac{1}{2}$ in., with a door in front, resting in a loose tray $20\frac{1}{2} \times 15\frac{1}{2} \times 2$ in. Young rabbits up to three months of age may be housed together, after that time the sexes should be separated.

Breeding.—Period of gestation thirty-one days; animals may be used for breeding after six months; four litters yearly; average litter four; young weaned at six weeks. The mother should be disturbed as little as possible for the first ten days after parturition.

Common Diseases

Pseudo-tuberculosis (p. 639).—This is a chronic disease, in which the animal loses weight and eventually dies. The liver and spleen particularly show numerous well-defined yellow areas like miliary tubercles.

Respiratory Tract Infections—of which *Snuffles*, due to *Pasteurella multocida* (p. 639) is the most common.—Infected animals should be destroyed at once.

Intestinal Infections.—Diarrhoea may be due to organisms of the *Salmonella* group, e.g. *S. typhimurium*. Recently mucoid enteritis, a condition of obscure origin, has been the cause of epizootics; it has

a mortality rate of about 33 per cent. in adults and up to 100 per cent. in young rabbits.

Coccidiosis.—This is a common disease of rabbits. The intestine and liver are involved and at autopsy show yellowish-white nodules. Diarrhoea and progressive loss of condition result. Oocysts of the parasite can be seen microscopically in the faeces.

Parasitic Mange.—This is due to a mite and usually affects the ears. It is best treated by liquid paraffin containing 1 per cent. of phenol.

Worms.—The cysticercus stage of the dog tape-worm, *Taenia pisiformis*, is the commonest type of infestation and is characterised by numerous cysts in the omentum and sometimes in the liver.

Ulcerative lesions of the genitals due to *Treponema cuniculi* (which is very similar morphologically to *Treponema pallidum*, q.v. are frequently seen.

Rats

The use of rat feeding cubes has almost entirely replaced the use of mashes. Pellets of diet 86 of Howie (1952)¹ comprise a balanced mixture of ground cereals, meat, fish and milk proteins with yeast and cod liver oil; they support growth, reproduction and lactation without any need for supplements. The cubes are fed to the animals from a hopper suspended in the cage in the same way as for guinea-pigs and rabbits.

Drinking-water from a special glass container should always be available.

Cages.—Galvanised-iron cages with narrow mesh are used for experimental animals.

For stock animals large wooden boxes with zinc bottoms and perforated zinc tops, 36×18×15 in., are suitable.

Breeding.—Period of gestation three weeks; three to four litters yearly; average litter six; young are weaned at six weeks and the female rested a further two weeks.

Common Diseases

Rats suffer infrequently from intercurrent infections, except mange.

Respiratory tract infections, e.g. pneumonia, may sometimes occur.

Intestinal Infections.—*Salmonella* infections, e.g. *S. enteritidis*, may cause fatal epizootics.

Mange appears especially at the root of the tail and ears as a grey warty condition. The animals should be examined twice weekly, and if infected the parts should be smeared with an ointment composed of flowers of sulphur, 2 parts; sodium carbonate (anhyd.), 1 part; lard or vaseline, 16 parts.

Hamsters

Hamsters should be kept at an even temperature in conditions similar to those suitable for mice.

¹ Howie, J. W. (1952), *J. Anim. Tech. Ass.*, 2.

Feeding.—Commercially available cubed diets for mice, rats or guinea-pigs are satisfactory for the basic diet but better health results when daily supplements of fresh green foods are given. Milk added to a bran and oats mash is valuable as an additional supplement for pregnant or lactating females. Fresh drinking-water must always be available.

Cages.—Galvanised-iron cages in sheet metal or mesh are satisfactory. A convenient size is 17 x 7 x 9 in. Cages of aluminium, zinc or wood are not suitable as hamsters may gnaw through them.

Breeding.—Period of gestation sixteen days; animals may be used for breeding after two to three months; three to four litters yearly; young weaned at twenty to twenty-five days. The mating of animals is best carried out under observation for the female after coitus may often attack the male and injure him severely.

Diseases.—Golden hamsters are remarkably free from spontaneous disease. They may, however, acquire *Salmonella* infections and mange in the animal-house.

Mice

The room in which mice are kept should be held at an even temperature between 65° and 75° F. Large fluctuations of temperature must be avoided and draughts of cold air excluded. The humidity of the room should be between 40 and 60 per cent., and if it can be kept close to 50 per cent. cannibalism of newly born infant mice by their mothers can largely be avoided.

A cubed diet should be fed in the same way as for rats. Both diet 86 (see p. 428) and Diet 41 of Bruce¹ are satisfactory.

Drinking water is essential and should be provided from a special glass container.

Cages.—Metal cages made of aluminium alloys are preferred. Many types are available, and for further information the reader is referred to the U.F.A.W. Handbook (see p. 425).

Breeding.—Mice are easily bred. Place one male and two to five females in the box. Remove male after fourteen days and the females will litter together. Period of gestation eighteen to twenty-one days; four litters yearly; average litter four. Young are weaned at one month; female is rested a further two weeks. It is advisable to replace breeding stock after three litters.

Common Diseases

Intestinal infections due to organisms of the *Salmonella* group (*S. enteritidis* and *S. typhimurium*), and termed "Mouse typhoid", may produce severe epizootics. Existing stock should be destroyed, cages disinfected and fresh stock obtained.

Infectious Ectromelia (p. 783).—A virus disease occurring in either an acute or chronic form. In the acute disease there is necrosis in the

¹ Bruce, H. M. (1950), *J. Hyg. (Lond.)*, **48**, 171.

liver and spleen. In the chronic form there is enlargement of one foot (usually hind), due to oedema, followed by an exudation of serous fluid and scab formation, after which gangrene either of a digit or whole foot may supervene.

Miscellaneous Virus Infections.—Among the many viruses affecting mice the more important are the mouse hepatitis virus, the "P.V.M." or pneumonia virus of mice, Niggs pneumonitis virus, Theiler's encephalomyelitis virus and related strains such as G.D. VII, and the virus of lymphocytic choriomeningitis.

Epizootic infection by *Streptobacillus moniliformis* (p. 666) may sometimes occur in mouse stocks.

Ringworm is met with, and also favus (p. 888).

Worms may occasionally cause ill-health or death.

Tumours are not uncommon, particularly mammary carcinoma.

Fowls

Feeding.—One of the pellet diets ("breeding" or "laying" diets) available commercially can be used; it should be properly balanced and contain vitamins and trace elements. Green foods may be fed two or three times a week and grit and fresh water must be provided *ad lib.*

Cages.—Galvanised-iron wire cages about 24 in. tall and 20×20 in. are suitable for individual birds. For further information on the breeding and care of poultry the reader is referred to Ministry of Agriculture Bulletins Nos. 54 and 56.

Common Diseases

Intestinal infections due to Salmonellae of various types (e.g. *S. typhimurium*) are not uncommon. *S. pullorum* is the cause of bacillary white diarrhoea of young pullets and *S. gallinarum* causes outbreaks of "Fowl typhoid" (p. 590). Coccidiosis is another cause of acute enteritis in young chicks. *Tuberculosis* due to the avian type of *M. tuberculosis* is now relatively uncommon.

Parasitic infections due to lice and red mites may be controlled by the use of an aerosol spray of 2.0 per cent. piperonyl hydroxide + 0.4 per cent. pyrethrum. Two applications spaced seven to ten days apart should be used.

Avian leucosis of three different types, lymphoid leucosis, myeloid leucosis and erythroleucosis, is a very common cause of loss in poultry.

Virus diseases include infectious laryngotracheitis, fowl pest due in Great Britain to the Newcastle virus and also, elsewhere, to the fowl plague virus, and fowl pox (see p. 784).

Monkeys

As an experimental animal the monkey is now mainly used in testing the virulence of strains of the poliomyelitis virus and in the safety

testing of vaccines made with this virus. Extensive use is made of excised monkey tissues, especially the kidney, for tissue cultures for virus cultivation (see p. 455). Special cages, skill and experience are needed in handling and caring for monkeys. For further information the reader should consult the U.F.A.W. Handbook (see p. 425).

CHAPTER XVII

COLLECTION OF SPECIMENS FOR LABORATORY EXAMINATION

A. BACTERIOLOGICAL SPECIMENS

WHEN specimens are forwarded to a laboratory for bacteriological examination they must be placed in appropriate sterile containers to prevent contamination or leakage. Containers and outfits for transmission of specimens are supplied from the laboratory. The material should be sent to the laboratory as soon as possible after being obtained.

Full particulars should accompany the specimen as this is of great assistance to the bacteriologist when making his report. Printed forms are usually issued along with the containers and it is essential that these should be completely and accurately filled in. The container in which the specimen is placed should bear the name of the patient and other relevant details.

Inflammatory Exudates, Pus, Blood for Wassermann and Widal tests, Cerebro-spinal fluid.—Strong glass test-tubes, 5 in. x $\frac{3}{8}$ in., with rubber bungs may be used. The glass tubes are sterilised in the hot-air oven; the rubber bungs are boiled for five minutes, picked out of the water with forceps, flamed and inserted into the sterile tube.

We recommend the use of a screw-capped bottle known as a *Universal container*, which consists of a strong moulded glass bottle with a flat base and wide mouth, and having a screw cap. Its size is $3\frac{1}{4}$ in. high and $1\frac{1}{8}$ in. diameter, and the full capacity is 28 ml. The aluminium screw cap is furnished with a black rubber washer 3 mm. thick. These bottles are supplied already cleaned and capped in 1-gross boxes. They are sterilised by autoclaving, with the caps loosely screwed on; after sterilisation the caps are tightened. They cannot be sterilised in the hot-air oven, as the rubber washers will not withstand the temperature. After use, the bottles are washed and fitted with new screw caps; the old caps should be discarded, as it is difficult and uneconomical to wash them satisfactorily.

The screw-capped Universal container has many advantages over the glass tube and rubber bung. It is stronger, cheaper and more readily and effectively sterilised. The screw cap keeps the mouth of the container always sterile, whereas with the tube and bung, dust tends to accumulate at the rim of the tube. The contents cannot leak or become contaminated. The Universal container stands quite stable on its base, which is of particular convenience when specimens are taken at the bedside. It can also be used for sputum, faeces, small pieces of tissue, etc.

Serous Fluids.—Pleural fluid may coagulate on standing, and the fibrin clot entangles the cells and renders a cell count or centrifuging difficult. The sodium citrate "Biochemical bottle" described on p. 434 is very suitable for preventing coagulation. The exudate when withdrawn is added directly to the bottle. Clotting does not take place, while the very small quantity of citrate solution present does not affect the accuracy of the cell count.

Faeces.—A small squat bottle of about 2-oz. capacity, or a glass specimen tube 2 in. \times 1 in., fitted with a bark cork in which a small metal spoon is fixed, is sometimes used. Such containers have the disadvantage that any fermentation of the faeces tends to blow out the cork and cause leakage of the contents. The corks have to be discarded after use. The shoulder on the bottle makes cleaning difficult.

For small quantities of faeces the Universal container is suitable. A small "spoon" made of tin plate 3 $\frac{3}{8}$ in. \times $\frac{3}{8}$ in. with one end bent in a small U is employed. These spoons are wrapped in kraft paper and sterilised. For use, the spoon is unwrapped, a portion of faeces taken up in the U-shaped end, the whole dropped into the container and the cap screwed on. Alternatively, a wide-mouth 2-oz. screw-capped jar, known as a "pomade pot", is used, and the faeces taken up in small cardboard spoons (such as are used for ice-cream cartons).

When there is likely to be a delay of some hours before laboratory cultivation can be carried out, two volumes of 30 per cent. neutral glycerol in 0.6 per cent. sodium chloride should be added to the faeces and the whole thoroughly mixed (p. 579). The solution is buffered to make it alkaline and phenol red is added as an indicator. The fluid should not be used if it becomes acid, which is indicated by a yellow colour. The specimen of faeces is added directly to the container.

The solution is prepared as follows. Make up 30 per cent. glycerol in 0.6 per cent. saline. Add 1 per cent. of anhydrous disodium hydrogen phosphate and steam to dissolve. Then add 0.02 per cent. phenol red solution (p. 277) until a purple-pink colour is obtained (about 15 ml. phenol red solution per litre). The colour should be judged by pouring a small quantity of the solution into a Universal container. Distribute in 6 ml. amounts in Universal containers and sterilise at 5 lb. pressure.

Urine.—For small quantities of urine—e.g. from cases of enteric fever, cystitis, etc.—the Universal container is used. For catheter specimens a sterile 8-oz. wide-mouth screw-capped bottle is very convenient. The end of the catheter can be placed in the bottle and the urine taken off directly. For larger quantities, e.g. twenty-four hour specimens, 20-oz. screw-capped bottles are convenient.

Sputum.—Bottles of 2-oz. capacity or Universal containers are used. Where the sputum is not sent by post, screw-capped waxed cardboard cartons of 2-oz. capacity (such as are used for cream and ice-cream) are suitable. The patient expectorates directly into the carton, the wide mouth of which prevents any fouling of the outside, the cap is screwed on, and the name of the patient, etc., written on with an ordinary pencil or grease pencil. After the specimen is examined in the laboratory

the carton and contents are burned. Owing to the postal regulations (*vide infra*) the waxed carton cannot be sent through the post. For transmission by post the 2-oz. screw-capped "pomade pot" (p. 186) may be used.

Blood for Culture.—Blood can be placed in a sodium citrate "biochemical bottle" or preferably added directly to the medium in the special blood-culture bottle described on p. 262.

Biochemical Bottles.¹—For biochemical examinations of blood it is convenient to place the specimen in a 1-oz. screw-capped bottle (p. 185) containing the necessary preservative or anticoagulant and fitted with a perforated cap similar to a blood-culture bottle. The anticoagulant varies with the examination required and the following is a useful range.

Anticoagulant	Amount	Distinguishing mark	Use
Sodium fluoride and thymol }	90 mg. 10 mg.	Red label	For blood sugar (venous blood), inorganic phosphorus, uric acid and non-protein nitrogen tests
Neutral potassium oxalate	40 mg.	Blue label	For blood urea, and the majority of blood tests
Sodium citrate .	0·3 ml. of 20 per cent. solution (about 60 mg.)	Mauve label	For some blood tests, including animal inoculation; also used in submitting specimens of serous effusions and cyst fluids for general laboratory examination
Sodium fluoride and potassium oxalate }	20 mg. 15 mg.	Yellow label	Majority of blood tests

The sodium fluoride and thymol mixture is added to the already sterilised bottle. The potassium oxalate is added in the form of 0·2 ml. of a 20 per cent. solution and the fluid is rolled round the bottle to distribute as much as possible on the side; the bottle (uncapped) is then placed in the hot-air oven for a short time (about ten minutes) to evaporate the solution, the object being to have as much potassium oxalate on the side of the bottle and in a thin layer for easy solution on addition of the blood; the sterile cap is then fitted. The sodium citrate solution is placed in the bottle and sterilised by autoclaving (with the cap fitted). The fluoride-oxalate mixture is first made up in solution: sodium fluoride 4 per cent., potassium oxalate 3 per cent.; 0·5 ml. of the solution is added to each bottle and evaporated as described above.

The cap is covered with a No. 2 transparent viskap.

¹ McCartney, J. E., & Ayling, T. H. (1935), *Lancet*, 1, 1388.

For use, the viskap is not removed, but the top wiped with a little alcohol. Blood is taken from a vein with needle and syringe, and the needle inserted through viskap and washer, and the blood added to the bottle. Not less than 10 ml. of blood should be taken. Gentle but thorough shaking for three minutes is necessary to ensure solution of the anticoagulant; this is especially necessary in the case of the sodium fluoride.

When small amounts of blood are required it is convenient to use the $\frac{1}{4}$ -oz. (bijou) bottles. The following table shows the amount of anticoagulant necessary for 2 ml. of blood.

Anticoagulant	Amount	Distinguishing mark
Sodium fluoride 10 mg. Thymol 1 mg. : } .	11 mg.	Red label
Neutral potassium oxalate .	10 mg.	Blue label
Sodium citrate 15 mg. : } .	1 drop of 20 per cent. solution	Mauve label
Sodium fluoride 5 mg. Potassium oxalate 4 mg. : } .	9 mg.	Yellow label

No viskaps are applied to these small bottles.

Swabs.—A swab consists usually of a piece of aluminium or tinned iron wire, 15 gauge and 6 in. long. One end is made rough for about $\frac{1}{2}$ in. by squeezing it in a small metal vice or cutting edge of pliers. Around this end a thin plegget of absorbent cotton-wool is tightly wrapped for about $\frac{3}{4}$ in. The wire is placed in a narrow thick-walled test-tube, 5 in. $\times \frac{1}{2}$ in., and the top of the tube plugged with cotton-wool. Alternatively, and where swabs have to be sent by post, the wire should be $4\frac{1}{2}$ in. long and the top inserted into a bark cork which stoppers the tube. The tube with swab should be sterilised in the autoclave and not in the hot-air oven, as in the latter the wool may char and give rise to tar-like products which may be inimical to bacteria on the swab (p. 150). These swabs are very useful for taking specimens from:

(a) Throat: in cases of suspected diphtheria, tonsillitis, pulmonary tuberculosis, etc.

(b) Wounds, or surgical conditions, e.g. fistula, sinus, etc. Some of the purulent material is taken up on the cotton wool.

(c) Post-nasal or naso-pharyngeal space: for this purpose the terminal $\frac{3}{4}$ in. is bent through an angle of 45 degrees, and in use is inserted behind the soft palate. This procedure is useful for suspected meningo-coccal carriers and for the early diagnosis of whooping-cough (p. 657).

For the diagnosis of whooping-cough a "pernasal" swab may be conveniently used; this is made from 7 in. of flexible copper wire or

nichrome S.W.G.25 (0.51 mm. diameter), the terminal $\frac{1}{4}$ in. being bent back to take the pledge of cotton-wool, a very thin layer of which is wound firmly round it. The swab is contained in a $6 \times \frac{1}{2}$ in. test-tube plugged with cotton-wool. The swab is passed gently back from one nostril along the floor of the nasal cavity until it reaches the posterior wall of the naso-pharynx, and then withdrawn.¹

(d) Rectum: rectal swabs are very useful in bacillary dysentery cases, especially in young children.

(e) Cervix uteri: in gonorrhoea and puerperal infections. (A longer wire, 9 in., is preferable for these specimens.)

Instead of wire, swabs may be prepared from thin wooden sticks $6\frac{1}{2}$ in. long, which are specially made for the purpose and are known as "Peerless" wooden applicators. A cotton-wool pledge is wrapped round one end as above, and the tube is plugged with cotton-wool. They may be used as above under (a), (b), (d) and (e), but of course cannot be bent and cannot be used conveniently with a bark cork.

Where some time may elapse before the swab is examined and especially where delicate pathogens are concerned, e.g. meningococcus or *B. pertussis*, it is advantageous to place about $\frac{1}{4}$ in. of saline agar (2 per cent. of agar in 0.85 per cent. sodium chloride solution) at the bottom of the swab tube. This is done before the swab is sterilised. A bark cork is used as a stopper and the wire pushed through the cork so that the cotton-wool is clear of the agar. After the specimen has been taken, the swab is inserted into the tube and the wire pushed down until the cotton-wool pledge is in contact with the saline agar.

A special method for preserving the viability of the gonococcus in swabs is described on p. 242.

The problem of bacterial survival is not confined to the *Neisseria*, since slow-drying is known to be lethal to most bacterial species. Rubbo and Benjamin (1951),² as a result of their comparative findings with many different pathogens, recommend the use of a serum-coated cotton-wool swab to prolong viability. The swabs are prepared by dipping the cotton-wool swab on a wooden applicator into undiluted ox serum for ten to thirty seconds, spreading them out on sheets of blotting-paper, drying in an incubator at 37° C. for about half an hour, and finally sterilising in the autoclave at 15 lb. per sq. in. for twenty minutes. The finished product is a compact honey-coloured pledge, 3–5 mm. in diameter, in which the cotton-wool fibres are firmly bound to each other and to the applicator. For a more detailed account of swabs and swabbing methods see Cruickshank (1953).³

Tissue.—Small pieces of tissue may be sent in the Universal container. Larger pieces of tissue, e.g. from *post-mortem* examinations, should be sent in sterile 2-oz., 8-oz., or 1-lb. screw-capped jars according to size.

¹ Cockburn, W. C., & Holt, H. D. (1948), *Monthly Bull. Minist. Hlth Lab. Serv.* 7, 156.

² Rubbo, S. D., & Benjamin, M. (1951), *Brit. med. J.*, 1, 983.

³ Cruickshank, R. (1953), *ibid.*, 2, 1095.

Material in formol-saline or other fixative for sections should be sent in any of the above screw-capped containers.

B. VIROLOGICAL SPECIMENS

General Instructions

It is essential to exercise great care in the collection and preparation of all types of specimens for virus work and to submit the maximum amount of information with them to the laboratory. Neglect of detail will usually make it impossible to perform the tests properly.

The isolation of viruses is time-consuming, expensive and requires special laboratory resources. Attempts to isolate viruses are often futile unless the materials are collected in the very early stages of the illness, promptly preserved, properly frozen, and sent to the laboratory by a very rapid method.

Virus Isolation

Specimens for virus isolation should be placed at temperatures below 4° C. without delay after collection, and they must be kept at these temperatures until the tests are carried out. It is often convenient in hospital practice to store the specimen in the ice trays of a domestic refrigerator. For transport to the laboratory the specimens in their containers may be surrounded by ice or preferably an ice-salt mixture in a suitable jar or tin. *Do not send such specimens by post.* The best way of transmitting frozen specimens to a laboratory at some distance is to pack them in a special insulated box or thermos flask surrounded by "dry ice" (*i.e.* solid carbon dioxide). Such containers are sometimes available on demand at the laboratory.

Serological Diagnosis

Serological tests for complement-fixing or neutralising antibodies often give valuable diagnostic information; they form the usual routine means of laboratory investigation. Since small traces of antibody may persist long after recovery and are frequently demonstrable in normal healthy individuals the examination of a single sample of serum seldom yields information of any value. To this statement an exception may be made in the case of infections with the psittacosis and lymphogranuloma venereum viruses where the single observation of a high antibody titre may be significant. In all other infections the results of serological tests are only of diagnostic significance if it has been shown that there has been at least a fourfold rise of antibody titre during the period between the onset of the illness and convalescence.

It is therefore essential to send at least two samples of serum to the laboratory; the first taken as soon as possible after the onset of the disease, and the second after about three weeks. Details of the optimum times for the collection of sera are given in the table, on p. 441.

For the test 1-2 ml. of clear serum showing no trace of haemolysis are required. The serum should be removed from the clot within twenty-four hours of collection and then kept at 4° C. or lower. When both acute and convalescent samples have been collected they may be sent to the laboratory by post. Whole blood is unsuitable for transmission by post and should not be sent.

Special Instructions for Particular Virus Diseases

Enteric Viruses (Poliomyelitis, ECHO, and Coxsackie Viruses).—The most reliable source of these viruses is faecal material. A sample should be sent as early as possible in the course of the infection and it is valuable to send a second two or three days later. If faeces are not available, a rectal swab is a reasonably satisfactory alternative. These viruses may also be recovered from throat swabs or oropharyngeal washings although, in the case of the poliovirus, with considerably less frequency. Cerebro-spinal fluid from cases of abacterial meningitis may often contain ECHO or Coxsackie viruses, but seldom, if ever, the polio viruses. These materials may be held for up to twenty-four hours at 0°-4° C., in a domestic refrigerator, but for longer periods they should be preserved frozen at -30° C. or in 50 per cent. glycerol saline.

Influenza.—The three known types of influenza virus A, B and C may be isolated from throat washings by the inoculation of developing chick embryos or ferrets.

During the first forty-eight hours of the acute phase the patient is instructed to cough vigorously and then to gargle immediately with 5-10 ml. of physiological saline. The contents of the mouth are then expectorated into a 1 oz. wide-necked screw-capped bottle containing 2.0 ml. nutrient broth. In the case of young children who cannot gargle, a satisfactory specimen may be obtained from a well-taken post-nasal swab. The specimen is at once placed in the freezing coils of the refrigerator and conveyed as soon as possible to the laboratory with the precautions mentioned above. The first sample of serum is conveniently collected at the same time. It is improbable that the virus will be isolated from the patient after the second day of the illness.

In fatal cases the autopsy should be performed with a minimal delay and portions of lung tissue involved in any areas of consolidation should be frozen at the earliest opportunity.

Variola: Vaccinia: Cowpox: Varicella.—The tests used for these infections are both rapid and sensitive, so that within forty-eight hours of collection of the material the laboratory can make at least a provisional diagnosis of variola. It is of the utmost importance to employ these tests when there is even the slightest possibility of smallpox and they give valuable information in many atypical or unusually severe cases of chicken pox. They may also be used in the investigation of vaccinia lesions and in Kaposi's varicelliform dermatitis.

An essential precaution for the worker who collects or handles

material from suspect cases of smallpox is that he should have been satisfactorily vaccinated during the preceding twelve months. Specimens must be taken from the patient at as early a stage of the disease as possible and for this purpose the following apparatus is required. 6 well-cleaned, grease-free slides, a scalpel or needle, forceps, 3-4 capillary tubes, throat swabs, a screw-capped vial, ether, a spirit lamp, and suitable wooden boxes in which to transmit the specimens by post. The variola-vaccinia group of viruses are stable at atmospheric temperatures especially in dried material protected from sunlight, and are thus an exception to the rule that precludes the despatch of such material by post. The following specimens are required:

1. Pre-eruptive or viraemic stage. A sample of 5-8 ml. of whole blood should be taken at the first indication of illness in all persons known to have been in contact with a case of smallpox.

2. Maculo-papular stage. Clean the site of the lesion with ether especially if any ointment has been applied. Scrape 6 or more of lesions and transfer the material to the slides making a minimum of 6 reasonably thick films. Dry the films in air; *do not heat them*.

3. Vesicular stage. Collect vesicle fluid from several intact vesicles into capillary tubes and seal the ends of the tubes in a flame. If this is not possible take up material from the lesions on throat swabs and also make smears on slides from scrapings from the bases of the lesions.

4. Pustular or crusted stage. Remove 4-6 crusts with forceps and place them in the screw-capped bottle.

Send all the specimens collected together with the needle or scalpel blade used without delay to the Laboratory.

The tests used (see Table and p. 777) will differentiate variola from vaccinia and cowpox, and all three from varicella, and herpes simplex. The distinction between variola and vaccinia is, however, a matter of some difficulty and depends largely on the appearance of the lesions produced by the two viruses on the allantoic membrane.

Serological tests for antibodies seldom give information in time to be of use in the early diagnosis of smallpox; their main value is in establishing the nature of the infection in retrospect. When, however, the hypersensitive state has been established by vaccination in the past, infection with the variola virus occasions an accelerated outpouring of antibodies. The demonstration of a rapid increase in the amount of antibody in these circumstances may give valuable diagnostic information, in cases of suspected smallpox where a small number of lesions has escaped observation and no material has been taken for virus identification, or in cases of *variola sine eruptione*. If the patient has been recently vaccinated it will, however, not be possible to demonstrate a significant rise in antibody titre. Paired samples of sera are required together with full information of the vaccination history of the patient.

Postal Regulations.—The Postmaster-General has laid down the following instructions for sending pathological material through the post and these should be rigorously observed.

"Articles sent for Medical Examination or Analysis.—Deleterious liquids or substances, though otherwise prohibited from transmission by post, may be sent for medical examination or analysis to a recognised Medical Laboratory or Institute, whether or not belonging to a Public Health Authority or to a qualified Medical Practitioner or Veterinary Surgeon within the United Kingdom, by *letter post*, and on no account by *parcel post*, under the following condition:

"Any such liquid or substance must be enclosed in a receptacle, hermetically sealed or otherwise securely closed, which receptacle must itself be placed in a strong wooden, leather or metal case in such a way that it cannot shift about, and with a sufficient quantity of some absorbent material (such as saw-dust or cotton-wool) so packed about the receptacle as absolutely to prevent any possible leakage from the package in the event of damage to the receptacle. The packet so made up must be conspicuously marked 'Fragile with care' and bear the words 'Pathological Specimen'.

"Any packet of the kind found in the parcel post, or found in the letter post not packed and marked as directed, will be at once stopped and destroyed with all its wrappings and enclosures. Further, any person who sends by post a deleterious liquid or substance for medical examination or analysis otherwise than as provided by these regulations is liable to prosecution.

"If receptacles are supplied by a Laboratory or Institute, they should be submitted to the Secretary, General Post Office, in order to ascertain whether they are regarded as complying with the regulations."

The following receptacles have been approved by the Postmaster-General:

For Universal containers, media bottles (Fig. p. 185) and 2-oz. pots, a leatherboard box, internal size $4\frac{3}{8}$ in. \times $2\frac{1}{8}$ in. \times $1\frac{7}{8}$ in. deep with metal-bound edges and full-depth lid, is used. The glass container is wrapped in a piece of cellulose tissue, 19 in. \times $4\frac{1}{2}$ in., and then fits securely in the box which is placed in a shaped gummed envelope having a tag for the postage stamps.

Swabs or cultures in tubes are wrapped in cellulose tissue and placed in hinged metal boxes having rounded corners, size $6\frac{1}{4}$ in. long, $2\frac{1}{2}$ in. wide and 1 in. deep. Leatherboard boxes with metal-bound edges of the same size are also permitted. These are placed in stout manilla envelopes which have a tag at the end for the postage stamps.

For the 8-oz. pots and the 1-lb. jars, a larger piece of cellulose tissue is required, while the leatherboard box is similar in construction to the one mentioned above and large enough to take these receptacles.

Gummed labels printed with the name and address of the laboratory and the information required by the Post Office Regulations are often issued by laboratories when sending out the postal materials.

THE LABORATORY DIAGNOSIS OF VIRUS INFECTIONS OF MAN¹

Disease	Detection of Virus			Detection of Serum Antibodies	
	Material Required	Transport to Laboratory	Tests Used	Dates for Collection of Sera	Tests Used
Variola; ² Vaccinia; Cowpox.	1. Serum in pre-eruptive phase. 2. Films on slides made from scrapings of macules and papules. 3. Vesicle fluid in capillary tubes. 4. Crusts in screw capped bottles.	By hand or by post.	Microscopy for elementary bodies. Cultures on allantoic membrane. Complement fixation; for viral antigen.	Before 5th and after 15th day.	Comp. fixation; Neutralisation.
Varicella; Herpes Zoster.	Vesicle fluid.	By hand or by post.	Microscopy for elementary bodies. Complement fixation for viral antigen. Tissue culture. Egg or animal inoculation.	Before 5th and after 15th day.	Comp. fixation.
Herpes simplex.	1. Vesicle fluid. 2. Crusts or scrapings from bases of lesions on slides. 3. Skin or brain from autopsy.	Frozen. Frozen.	In glycerol saline and frozen.	Before 5th and after 15th day.	Comp. fixation; Neutralisation.
Influenza ³	1. Throat washings in first 48 hours. 2. Throat swab (children). 3. Lung tissue at autopsy.	Frozen. Frozen.	Egg or ferret inoculation.	Before 3rd and after 15th day.	Comp. fixation; Haemagglutination-inhibition.

THE LABORATORY DIAGNOSIS OF VIRUS INFECTIONS OF MAN¹—continued.

Disease	Detection of Virus			Detection of Serum Antibodies	
	Material Required	Transport to Laboratory	Tests Used	Dates for Collection of Sera	Tests Used
Acute Respiratory Disease due to: (a) Haemadsorption Viruses, Croup Associated viruses, etc. (b) Adeno Viruses.	Throat washings or throat swabs. 1. Throat washings or throat swab. 2. Conjunctival swab. 3. Sputum. Throat and oral swab.	Frozen. By hand or by post.	Tissue culture inoculation. Tissue culture inoculation.	Before 3rd and after 15th day. Before 3rd day and after 15th day.	-Neutralisation; Comp. fixation. Comp. fixation (group); Neutralisation (type).
Measles.		Immediate.	Tissue culture inoculation. Egg inoculation.	Before 3rd day and after 15th day. During first 6 days and after 21st day.	Neutralisation; Comp. fixation; Haemagglutination-inhibition.
Mumps.	1. Saliva during first 3 days. 2. Cerebrospinal fluid in first 3 days of encephalitis.	Frozen.	"	During first 6 days and after 21st day.	Comp. fixation;
Psittacosis.	1. Sputum 2. Citrated blood. 3. Lung and spleen from autopsy.	Frozen. acute phase.	Egg or animal inoculation. "	After 21st day and if possible before 6th day.	Comp. fixation.

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Lymphogranuloma venereum.	1. Pus from bubo.	Frozen.	Animal or egg inoculation.	After 21st day, and if possible before 10th day.	Comp. fixation.
	2. Biopsy material.	One portion of biopsy material to be frozen another fixed in Zenker's fluid.	Histological pic- ture. Frei's test positive after 2 weeks.		
Primary atypical pneumonia.	Sputum in first four days.	Frozen.	Animal or egg in- oculation.	Before 7th and after 14th day.	Comp. fixation for O. fever, pneumococci, in- fluenza; Strep- tococcus M.G. agglutination; Cold agglu- tins.
Trachoma; Inclu- sion conjuncti- vitis.	Smears of scrapings from tarsal conjunctiva.	By hand or post.	Microscopy for elementary bodies and inclusions.	Before 6th day. After 21st day.	Weil-Felix, ag- glutination and Comp. fixation.
Rickettsial infec- tions.	Whole blood.	Immediate.	Animal inocula- tion.		
Rabies.	1. Saliva. 2. Animal brain. One half in glycerol saline. One half in Zenker's solution.	Frozen. By hand or post. " " "	Animal inocula- tion. " Examination for Negri bodies. Animal inocula- tion.	Before 5th and after 14th day.	Neutralisation. Haemagglutina- tion; Neutral- isation; Comp. fixation.
Encephalitis (Her- pes; Louping- ill, and Arthro- pod borne virus infections.	1. C.S.F. in first four days. 2. Brain from autopsy in glycerol saline.	Frozen.			

THE LABORATORY DIAGNOSIS OF VIRUS INFECTIONS OF MAN¹—continued.

Disease	Detection of Virus			Detection of Serum Antibodies	
	Material Required	Transport to Laboratory	Tests Used	Dates for Collection of Sera	Tests Used
Lymphocytic choriomeningitis. ²	1. C.S.F. 2. Citrated blood. } in first 4 days.	Frozen.	Animal inoculation.	Before 10th day, after 21st day and again after 50 days.	Comp. fixation.
Poliomyelitis. ³	1. Whole blood during prodromal period. 2. Pharyngeal swab during first week. 3. Faeces during first week. 4. Brain or spinal cord from autopsy.	By hand or frozen. , By hand or post. In glycerol saline by hand or post. By hand or frozen.	Inoculation of animal or tissue cultures.	Before 3rd and after 14th day.	Neutralisation; —Comp. fixation.
ECHO Virus infections; Coxsackie Virus Infections; Bornholm Disease; Herpangina; Aseptic Meningitis.	1. Faeces or rectal swabs 2. Swabs from oral lesions 3. C.S.F.		Suckling mouse inoculation. Tissue culture. Inoculation.	Before 5th and after 21st day.	Neutralisation; Comp. fixation.

Note.—At present there are no diagnostic tests available in infective hepatitis, homologous serum jaundice, or rubella.

Routine tests for all the virus infections listed may not be available. In special emergencies the laboratory should always be consulted.

¹ This table has been modified from that in *Virus and Rickettsial Diseases*, Edward Arnold and Company, London, 1950.

² See special instructions.

CHAPTER XVIII

THE CULTIVATION OF VIRUSES

VIRUSES multiply only within living cells and therefore cannot be grown on inanimate artificial culture media. Setting aside bacteriophages, which grow inside bacterial cells (p. 874), the viruses with which we are concerned may be propagated in three ways. They will grow in the body of a living animal, in the membranes and tissues of the chick embryo and in the cells of tissue cultures. Each of these host systems has its own special value, but no single one can be used successfully for all viruses. Since they are both more convenient and less expensive, chick embryo and tissue cultures have in recent years largely replaced the methods of animal inoculation. Animals still, however, provide the only susceptible hosts for a number of viruses; for example, the detection of the rabies virus in routine diagnostic work depends on the results of mouse inoculation experiments, and Coxsackie group A viruses can only be demonstrated by the lesions they produce in suckling mice. The methods recommended for the infection of animals or their tissues with particular viruses are given under their appropriate headings. The techniques for the various routes of inoculation and instructions on the care of experimental animals are described in Chapter XIV.

Cultivation of Viruses in the Embryonated Egg

Fertile hen eggs are used, preferably with light-coloured shells, *e.g.* from white Leghorn birds. The shells should be perfectly clean. Incubation of the eggs must begin not later than ten days, and if possible within five days, after being laid, and in the interval they should have been kept at a temperature between 4·5° and 20° C. Incubation is carried out in a commercial incubator at 37° C. and the eggs are turned twice daily. Before inoculation, *e.g.* after seven to twelve days' incubation, the eggs are examined by trans-illumination ("candled"); for this purpose a 100-watt lamp enclosed in a box with an oval opening is used, and the candling is best done in a dark-room. The embryo which is seen as a dark shadow must be alive, as demonstrated by its spontaneous movements and by the well-defined shadows of the blood vessels in the chorio-allantois. The air sac can be seen at the rounded pole of the egg and can be outlined, for later guidance, by pencil marking on the shell. For various methods of inoculation, the shell is cut through with a rotating disk operated by a dental drill. A vulcanite carborundum disk can be recommended. Special care must be taken to avoid damage to the underlying membranes and consequent bleeding. Mounted dissecting needles, forceps,

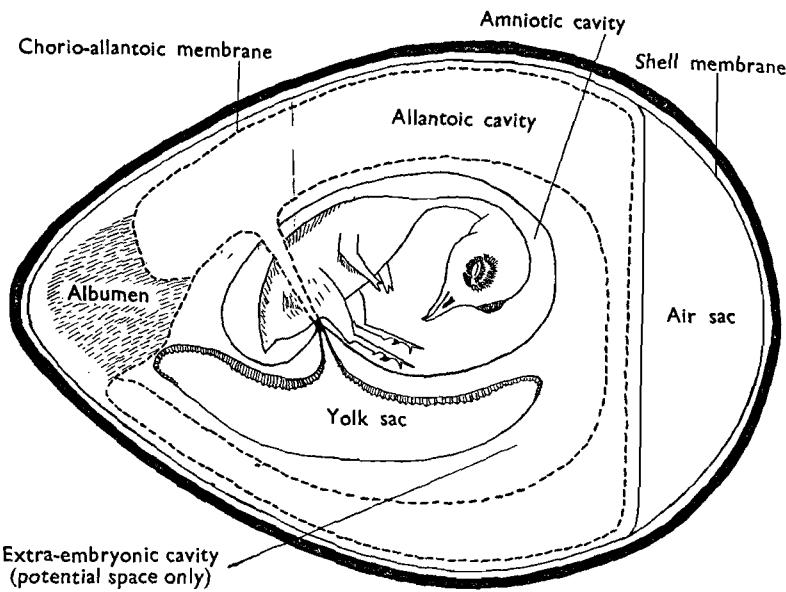


FIG. 23.

Diagram of embryonated hen's egg after eleven days' incubation, to illustrate the various sites of inoculation with viruses (R.H.A.S.).

scissors, syringes and capillary pipettes are required for the manipulations involved in inoculating the egg. Inoculation must be carried out by an aseptic technique and with sterilised instruments. In carrying out various manipulations it is found convenient to lay the egg on a triangular wooden stand ($4 \times 4 \times 2$ in.) which supports it at three points, with the area to be opened uppermost. After inoculation the eggs are incubated in a bacteriological incubator at $35^{\circ}\text{--}36^{\circ}$ C. They must be kept stationary, resting on special stands or trays.

Inoculation of the Chorio-allantoic Membrane.—The eggs are incubated usually for twelve days. In candling, a 12 mm. equilateral triangle is pencilled over the area of densest opacity, i.e. where the chorio-allantois is best developed and opposite the area in which the chorio-allantois fails to line the inside of the egg. The triangular area should not overlie any large blood vessel. The shell is drilled with the rotating disk along the sides of the triangle so marked out, and is also drilled over the centre of the air sac so as to make a small perforation through the shell membrane into the sac. The drilled triangle of shell is then carefully removed by inserting a dissecting needle at one angle and gently raising the cut piece of shell without damaging the shell membrane. A drop of normal saline is now deposited on the exposed shell membrane, and the membrane under the drop is split along the line of the fibres by means of a dissecting needle. Suction with a rubber teat is next applied to the opening in the air sac, and as a result the chorio-allantois recedes

from the shell membrane, the saline being aspirated into the underlying space between chorio-allantois and shell membrane. The opening in the shell membrane is then enlarged and with a capillary pipette held vertically the inoculum (in an amount of 0·05 ml.) is dropped into the space, *i.e.* on to the chorio-allantoic membrane. The opening is finally sealed with commercial transparent adhesive tape ("Scotch tape") 1 in. wide. The inoculated egg is incubated for two to four days. After this the egg is placed on a cotton-wool pad (moistened with antiseptic solution) in an open Petri dish; the seal is removed and the edge of the opening flamed with a Bunsen burner. The shell is broken off down to the level of the displaced chorio-allantois, which is then separated by cutting with scissors and carefully removed, spread out in buffered saline in a Petri dish and examined for pocks against a black background.

Allantoic Inoculation.—For this purpose eggs are incubated for ten to eleven days. The outline of the air sac is pencilled in the process of candling and a point is marked on the shell where the chorio-allantois is well developed but without large vessels. A small groove (about 3×1 mm.) is drilled at this point of the shell and the egg is placed on the triangular stand with the groove uppermost. The inoculation is made by injecting 0·1–0·2 ml. through the groove with a tuberculin syringe. The opening is sealed with melted paraffin or nail varnish. Incubation is carried out for two days and the egg is then refrigerated for two to four hours to kill the embryo and obviate bleeding in later manipulations. For withdrawal of the allantoic fluid, the shell over the air sac is drilled and removed; the egg being supported in the upright position, *e.g.* in an egg-cup, the shell membrane and chorio-allantois in the floor of the air sac are cut away with scissors and forceps. The fluid of the underlying allantoic sac can then be aspirated with a capillary pipette.

Amniotic Inoculation.—Eggs are used after thirteen to fourteen days' incubation. On candling, the densest part of the embryo is marked and a rectangular area ($2 \times 1\cdot5$ cm.) in the long axis of the egg is drilled at this point. Two further cuts of 1 cm. are made within the rectangle to form a triangle with the side of the rectangle towards the rounded pole of the egg, as the base. The triangular piece of shell is then removed, an opening is also made in the air sac and the same procedure as in chorio-allantoic inoculation is adopted to produce recession of that membrane. The remainder of the drilled rectangular area is finally removed and the underlying shell membrane is cut away. A part of the chorio-allantois which is free from large vessels is picked up with forceps and opened with scissors, avoiding any damage to the amnion. This membrane is now picked up with forceps through the opening in the chorio-allantois and pierced with a tuberculin syringe containing 0·05–0·25 ml. of inoculum and about 0·1 ml. of air; a little air is first introduced to ascertain if a bubble forms under the amnion, and if so the inoculation is completed. The amnion is allowed to fall back into position, and the shell opening is sealed with transparent adhesive tape.

In an alternative method a circle of shell 3 cm. in diameter is removed with its adherent shell membrane from over the air sac. Sterile liquid paraffin is applied to the inner layer of the shell membrane and through the resulting clear area a pair of forceps is inserted through the chorio-allantoic membrane and the amniotic sac is pulled upwards. The inoculation is then made in the manner described above. For this method the eggs must be incubated with the rounded end uppermost both before and after inoculation. The egg is incubated for three to five days at 36° C. The shell is then removed down to the level of the receded chorio-allantois and the latter is cut away with scissors. The allantoic fluid is drained off and the amnion is picked up with forceps, and amniotic fluid for investigation aspirated with a capillary pipette.

Yolk-Sac Inoculation.—Eggs are incubated for five to nine days. At the rounded pole of the egg over the air sac a small groove is drilled, and the yolk sac is inoculated with a syringe and 12 to 14 gauge needle, 3 to 3·5 cm. long. The needle is passed into the egg through the shell opening and in the long axis of the egg to a depth of about 3 cm., i.e. to just beyond the centre of the egg. 0·1 to 1·0 ml. of inoculum is introduced and the opening is sealed. Incubation is then carried out and the egg is candled daily. The yolk sac is removed when the embryo dies. The shell is drilled and separated over the air sac, and the shell membrane and chorio-allantois in the floor of the sac are cut away. The contents of the egg can then be turned out into a Petri dish; and since the sac membrane itself contains a large amount of virus, this is retained for further investigation.

For further details of the techniques of egg inoculation the reader is referred to a report by Beveridge and Burnet.¹

Tissue Culture

The simplification and refinement of tissue culture techniques in recent years has enabled many laboratories to adopt these methods and to apply them in a great variety of fields in microbiology. Among the advances are the isolation of pure lines of cells which can be propagated at will in defined culture media and the use of enzymes to disperse the cells of tissues and whole organs such as the monkey kidney.

Cultures of living cells provide an experimental host which possesses many advantages over the intact animal. It is possible to use pure clones derived from a single cell, to count the cells in a culture and to study the metabolic and morphological changes which result from infection. The cell surfaces are directly accessible to invasion and they are free from the immune defence mechanisms and the hormonal influences of the whole animal.

¹ Beveridge, W. I. B., & Burnet, F. M. (1946). The cultivation of Viruses and Rickettsiae in the Chick Embryo, *Spec. Rep. Ser. Med. Res. Counc. (Lond.)*, No. 256.

Apparatus

Glassware of borosilicate glass made by Pyrex is generally preferred for all tissue culture work, but soda glass as used in medical prescription bottles is also satisfactory. It may be mentioned that new glassware, even after very careful cleaning, may at first give a poor growth of cells. After it has been used once or twice, however, the difficulty disappears and satisfactory results are obtained. No explanation of this has yet been found. Tissue culture glassware requires to be cleaned by special methods and to be rinsed very thoroughly by ion-free distilled water (see p. 288).

Stoppers for Tissue Culture Vessels.—Red or black rubber bungs and the rubber liners of screw-capped bottles contain substances which are toxic to cells in tissue culture. The surface impurities on these stoppers can to some extent be removed by boiling in weak alkali (*e.g.* 5 per cent. sodium carbonate), but even with this precaution it is always necessary to ensure that the rubber never touches any culture medium. This hazard can be overcome by using stoppers made of silicone rubber or grey virgin rubber, which are practically non-toxic for cells; they are greatly to be preferred.

Rubber tubing suffers from the same disadvantages and in general is best avoided. Silicone rubber tubing is recommended and is easily sterilised by heat.

Instruments must be kept scrupulously clean. They may be sterilised by dry heat or by boiling. New scalpel blades or razor blades are often covered by a protective layer of grease which is toxic to cells. They should be cleaned by wiping them with a cloth soaked in carbon tetrachloride before they are washed and sterilised.

Filters.—Before use, Seitz filter pads should be washed free of alkali and detachable asbestos fibres by the passage of considerable amounts of sterile demineralised water. Sintered glass filters should be cleaned after use by treatment with strong acid. Concentrated sulphuric acid to which a few crystals of sodium nitrate and sodium chlorate have been added is allowed to seep through the filter. Afterwards the filter must be rinsed thoroughly with a very large volume of de-ionised water.

Media for the Growth and Maintenance of Cells.—Most media employ for their base a balanced salt solution containing a number of essential inorganic chemicals. Since cells form acid as they grow, buffers are added to stabilise the pH and sodium bicarbonate is also included for this purpose. Glucose is added as a source of carbon and energy, and phenol red is used as a pH indicator.

Balanced Salt Solutions.—Double glass distilled water or demineralised water (see p. 288) should always be used in making up balanced salt solutions. The salts used should be of the highest analytical purity and the solutions should be kept in polythene bottles set aside specially for the purpose. Phenol red is added to the solutions as a pH indicator and is non-toxic to cells in concentrations up to 0.005 per cent. In preparing balanced salt solutions, care is required to avoid the formation of calcium and magnesium carbonate and phosphate.

In order to achieve this, the calcium salt is dissolved separately and added slowly to the main solution in its final dilute stage. Hanks' solution is one of the most useful in tissue culture work and details are given of its preparation as an example of the procedure needed. The same procedure is adopted for Earle's and Gey's solutions.

Hanks' Solution.—Stock solutions of the salt mixtures are convenient and are made up with ten times the concentration of all the components except for the bicarbonate solution, which is made up separately.

Phenol Red Indicator (0·4 per cent.).—Dissolve 1·0 g. phenol red in the minimum volume of 0·05 N NaOH and then bring the volume to 250 ml. by the addition of distilled water.

Stock Solution A:

(1) NaCl	160·0 g.
KCl	8·0 g.
MgSO ₄ .7H ₂ O	2·0 g.
MgCl ₂ .6H ₂ O	2·0 g.
H ₂ O	800·0 ml.
(2) CaCl ₂	2·8 g.
H ₂ O	100·0 ml.

Mix these two solutions slowly and adjust the volume to 1000 ml. with water. Add 2·0 ml. chloroform and store in a polythene bottle at 4° C.

Stock Solution B:

Na ₂ HPO ₄ .12H ₂ O	3·04 g.
KH ₂ PO ₄	1·2 g.
Glucose	20·0 g.
Water	800·0 ml.

When dissolved add

Phenol red solution 100 ml.
Water to 1000 ml.

Add 2 ml. chloroform and store as with Solution A.

Sodium Bicarbonate Solution:

NaHCO ₃	1·4 g.
Water	100 ml.

Sterilise by autoclaving in a container with a tightly closed screw cap for 10 min. at 9 lb. pressure.

Hanks' Solution is made by adding 1 volume of stock solution A and 1 volume of stock solution B to 18 volumes of distilled water. It is sterilised by steaming for 1½ hr. Immediately before use add 0·5 ml. of sterile 1·4 per cent. NaHCO₃ solution to each 20 ml. of Hanks'

solution. Hanks' solution is used when considerable fluctuations in pH are not anticipated and where a large buffering capacity is not required.

Gey's Solution (1936):

NaCl	7.00 g.
KCl	0.37 g.
CaCl ₂	0.17 g.
MgSO ₄ .7H ₂ O	0.07 g.
MgCl ₂ .6H ₂ O	0.21 g.
Na ₂ HPO ₄ .2H ₂ O	0.15 g.
KH ₂ PO ₄	0.03 g.
Glucose	1.00 g.
NaHCO ₃	2.27 g.
Water	1000 ml.

Earle's Solution.—As in Hanks' solution the calcium salt should be dissolved separately and added slowly.

NaCl	6.80 g.
KCl	0.40 g.
CaCl ₂	0.20 g.
MgSO ₄ .7H ₂ O	0.10 g.
NaH ₂ PO ₄ .H ₂ O	0.125 g.
Glucose	1.00 g.
Phenol red solution	12.5 ml.
NaHCO ₃	2.20 g.
Water to make	1000 ml.

Gey's and Earle's solutions contain greater amounts of sodium bicarbonate; they are useful when cultures produce much acid or have a large cell population. When used for cultures with small numbers of cells they may lose their CO₂ and become alkaline; such cultures need to be "gassed" with 5–10 per cent. CO₂ in air after the medium is changed and before stoppering.

Nutrient Media.—Many defined nutrient media have been devised incorporating amino acids, vitamins, enzymes, accessory growth factors, glucose and inorganic salts in varying proportions. They are used without supplementation to maintain established cultures for periods of three or four days when cell multiplication is not required. With serum or tissue extracts added they are used to promote the active growth of cells.

The most widely used defined medium is Morgan, Morton and Parker's Medium 199¹ or a modification of it; it is particularly useful in the maintenance of cells for virus production in vaccine manufacture and in diagnostic work with the entero-viruses.

¹ Morgan, J. F., Morton, H. J., & Parker, R. C. (1950). *Proc. Soc. exp. Biol. (N.Y.)*, 73, 1.

Medium 199 (*Morgan, Campbell and Morton Modification M150*)¹

Solution		Mg. per 100 ml.	Solution		Mg. per 1000 ml.
1	<i>Amino Acids</i>		4	<i>Fat Soluble Group:</i>	
	L-Arginine	70.0		Cholesterol	0.20
	L-Histidine	20.0		Menadione (Vit. K)	0.10
	L-Lysine	70.0		Calciferol	0.10
	DL-Tryptophane	20.0		Vitamin A	0.10
	DL-Phenylalanine	50.0		Tween 80	20.0
	DL-Serine	50.0	5	<i>Vitamin C Mixture:</i>	
	DL-Valine	50.0		L-Cysteine HCl	0.1
	DL-Alanine	50.0		Glutathione	0.05
	Glycine	50.0		Ascorbic acid	0.05
	DL-Methionine	60.0	6	Disodium tocopherol	
	DL-Threonine	60.0		Phosphoric acid (Vit. E)	0.01
	DL-Aspartic acid	60.0	7	Folic acid	0.01
	DL-Leucine	120.0		Biotin	0.01
	DL-Isoleucine	40.0	8	Adenine sulphate	10.0
	L-Proline	40.0			
	DL-Glutamic acid	150.0	10	<i>Purine and Pyrimidine mixture:</i>	
	L-Hydroxyproline	10.0		Guanine HCl	0.30
	Sodium acetate (anhydrous)	50.0		Xanthine (monosodium)	0.30
				Hypoxanthine	0.30
				Thymine	0.30
				Uracil	0.30
2	L-Tyrosine	40.0	11	Adenosine triphosphate (disodium)	10.8
	L-Cystine	20.0			
3	<i>Vitamin B Group:</i>		12	Fe(NO ₃) ₃ .9H ₂ O	0.10
	Niacin	0.025			
	Niacinamide	0.025	13	Adenylic acid	0.20
	Pyridoxine	0.025			
	Pyridoxal	0.025	14	D-Ribose	0.50
	Thiamine	0.010		Deoxyribose	0.50
	Riboflavin	0.010		Glutamine	100.0
	Ca-Pantothenate	0.01			
	i-Inositol	0.05			
	p-Aminobenzoic acid	0.05			
	Choline chloride	0.50			

Medium 199 is made up in Earle's balanced salt solution from the fourteen stock solutions given in the table. The glucose and the sodium bicarbonate of the Hanks' solution together with a solution of L-glutamine to give a final concentration of 100 mg. per l. are added immediately before sterilisation by passage through sintered glass

¹ Morgan, J. F., Campbell, M.E., & Morton, H. J. (1955), *J. nat. Cancer Inst.*, **16**, 557.

filters. For the details of preparation of the stock solutions reference should be made to Morgan, Campbell and Morton's paper.

Many attempts have been made to simplify defined media of the 199 type and the best of these is that devised by Eagle. This medium contains only those ingredients which are essential for cell growth rather than those needed for optimal growth. It is unable to support growth in the total absence of added biological fluid. Although favoured by some workers, it has not found general acceptance, for, although simpler than 199, it is still complex enough to require much time in its preparation.

For details of Eagle's medium and other defined media the reader is referred to *Cell and Tissue Culture* by J. Paul.¹

Antibiotics—Nearly all the culture media in general use contain antibiotics to control chance contamination during the handling of cell cultures. For this purpose, stock solutions of penicillin and streptomycin are held available in the refrigerator and are added to the media immediately before use. Final concentrations of 50 units of sodium penicillin G and 50 µg. of streptomycin per ml. of the medium are sufficient for the purpose and are not toxic to the cells. Fungal contamination may be held in check by mycostatin (nystatin Squibb) added to give a concentration of 20-50 µg. per ml. but it must be remembered that this antibiotic is unstable at 37° C. and that after twenty-four hours its influence is lost. Neomycin at a concentration of 20 µg. per ml. is also useful to control bacterial contamination and is particularly valuable against pleuro-pneumonia organisms which are often chronic contaminants of cell lines.

Antibiotics in high concentrations are valuable in elimination of bacteria from heavily contaminated tissues or from faecal material to be examined for the presence of viruses. Treatment of these materials for a short period with a solution containing 1000 units penicillin, 1000 µg. streptomycin is highly effective and neomycin 500 µg. per ml. is also valuable. The presence of antibiotic-resistant organisms contaminating material of this type can best be overcome by determination of sensitivity of the organisms to a range of antibiotics and subsequent choice of the most suitable agent. Occasionally in faecal emulsions, antibiotic-resistant bacteria can be removed by prolonged centrifugation at speeds of over 10,000 r.p.m. When attempting to isolate viruses of the psittacosis lymphogranuloma group sulphonamides, penicillin and tetracyclines must be avoided, but streptomycin in concentrations up to 2000 µg. per ml. will control most bacterial contaminants without harming the chick embryo.

The Preparation of Cultures of Cells from Fresh Tissues

Human Amnion Cell Culture—A placenta with its attached membranes is obtained following a normal delivery or preferably at a Caesarean section. It is important that the amnion should not be badly

¹ Paul, J. (1959), *Cell and Tissue Culture*. Edinburgh, Livingstone.

lacerated, that it should have been delivered with a minimum of handling, and that it should not have been in contact with any disinfectant. If possible the placenta should be received directly into a wide-mouthed, screw-capped sterile jar containing 200 ml. Hanks' solution with added antibiotics. It is convenient to use placentae delivered in the early hours of the morning; they should not be used after eight hours and on no account should they be placed in a refrigerator.

On receipt at the laboratory the placenta is suspended by the cord held by Spencer Wells forceps in a retort clamp over a large sterile bowl. If the placenta has many infarcts or if the amnion is badly lacerated, soft or oedematous, it should be rejected. Otherwise, dissection is begun at the point of entry of the cord and the amnion is stripped gently downwards from the underlying chorion. The whole amnion is separated and transferred to a sterile flask containing about 250 ml. Hanks' solution with added antibiotics. After agitation the membrane is rinsed in two changes of the solution and is then transferred to a sterile 6 in. Petri dish in which it is cut into inch wide strips with sterile scissors. Adherent blood and mucus are scraped off the surface of the strips using the edges of sterile microscope slides and the pieces of membrane are then placed in fresh Hanks' solution.

When this stage is completed the strips are transferred to a sterile flask containing 150 ml. of 0·25 per cent. trypsin (Difco 1:250) in Hanks' solution warmed to 37° C. After thirty minutes the trypsin solution is removed and discarded and replaced with an equal volume of fresh solution. A sterile glass-covered magnet is now introduced into the flask which is placed on a magnetic stirrer in the 37° C. incubator. Stirring proceeds at the slowest possible rate and the tryptic digestion is continued for two and a half hours. At half-hourly intervals the cloudy supernatant fluid which contains the detached cells is removed and replaced with fresh trypsin solution. The portions removed are bulked together in a flask and kept at 4° C. in the refrigerator. It is very important that the cells should not be damaged by exposure to trypsin at 37° C. for longer than is absolutely necessary.

When the last portion of cell suspension has been added to the pool the whole is spun for five minutes in a straight-headed centrifuge at 600 r.p.m. The supernatant trypsin solution is now discarded and the cells are resuspended in 10 ml. of fresh Hanks' solution. Next, a count of the number of cells in the suspension is made in a haemocytometer chamber. For this purpose, 0·5 ml. of the suspension is added to 1·0 ml. of a 0·1 per cent. solution of crystal violet in 0·1 M citric acid. In making the count clumps of several cells adherent to each other are to be recorded as single cells. Cell counts may range from 50 to 80 million per ml. The suspension is now diluted with the culture medium to be used so that it contains 400,000 cells per ml.

The final step is to dispense the solution in 1·0 ml. amounts into tissue culture tubes. This is carried out while the cells are kept in continuous agitation by the magnetic stirrer and a syringe of the

Cornwall¹ type for automatically pipetting fixed volumes is recommended for the purpose. Amounts of 50 ml. may also be placed in suitable bottles. Tubes and bottles are placed horizontally in the 37° C. incubator and are held stationary without further disturbance for three days.

The culture medium is changed twice weekly and it is recommended, though not essential, that, after the first change of medium on the third day, the tubes are transferred to a roller drum. By seven to ten days a sheet of cells has usually grown out and the cultures are ready for use. Some workers recommend the washing of the cell sheets twice with fresh medium before inoculation, but this step is not essential.

The medium recommended to promote amnion cell growth is Hanks' solution with 0.5 per cent. lactalbumin hydrolysate and 20 per cent. horse serum added. After the cells have been infected with virus they are maintained in a medium comprising Earle's solution which contains 0.5 per cent. lactalbumin hydrolysate, and 0.5 per cent. yeast extract (Yeastolate Difco). If the cells are to be maintained for longer than seven days, the medium should also contain 5 per cent. horse serum.

Bottle cultures of amnion cells are treated in the same way as tube cultures. It is sometimes convenient to use a confluent sheet of cells in a bottle to set up secondary tube cultures. For this purpose the cells are detached from the glass of the bottle by exposure for thirty minutes to trypsin at 37° C. The suspension is then counted and transferred to tubes in the way described above for the primary cultures.

Monkey Kidney Cell Culture.—A kidney is removed from a rhesus or cynomolgous monkey under Nembutal anaesthesia, using full aseptic technique. One kidney is sufficient to provide over 1000 tissue culture tubes and it is a common practice to allow the animal to survive for about three weeks before killing it to obtain the other kidney. After this interval, the second kidney is often hypertrophied and provides an increased cell yield.

Once removed, the kidneys are immediately decapsulated with sterile instruments and the pelvis and calices are dissected away. With sterile long-handled scissors the kidney tissue is cut into 2–3 mm. fragments which are rinsed twice in Hanks' solution with added antibiotics. Next the fragments are transferred to 100 ml. of trypsin solution in Hanks' solution warmed to 37° C. After gentle agitation for twenty minutes on a magnetic stirrer, the trypsin is discarded and replaced with fresh solution. At twenty-minute intervals on three or four occasions, the turbid solution with its suspended cells is removed, stored at 4° C. and replaced with fresh solution. The pooled cell suspensions are now gently centrifuged and the cells are washed twice with Hanks' solution. The cell deposit is next resuspended in 10 ml. of the culture medium to be used and the cells are counted in a haemocytometer. The cell suspension is now diluted with culture medium so that it contains 300,000 cells per ml. The final step is to dispense the cell suspension

¹ Made by Becton, Dickinson & Co. Obtainable from R. B. Turner & Co., London.

in 1·0 ml. volumes into tissue culture tubes, in 15 ml. amounts in 16-oz. medicine bottles, or in 80 ml. amounts in Roux bottles according to requirements.

During active growth the cells are cultured in Hanks' solution containing 0·5 per cent. lactalbumin hydrolysate and 20 per cent. horse or human serum. (Human serum may, of course, contain antibodies which are able to inhibit virus growth.) During the first forty-eight hours the culture tubes are held stationary in a horizontal but slightly tilted position in racks or drums. Care must be taken that the culture medium does not come into contact with the corks of the tubes. Once the cells are adherent to the glass it is recommended, but not essential, that they are rotated in roller drums. When the cell growth has formed a confluent sheet, usually some four to six days later, the medium is changed to one of the maintenance type such as medium 199 and the tubes can then be inoculated with virus. If the cells are to be observed for more than three days, 2 per cent. calf serum should be added to medium 199.

Cell Strains.—The cells which are obtained from disaggregated tissues or from fragments growing in plasma clots can usually be carried in subculture for only a few transfers. After this the rate of multiplication declines and the cells die out. In some cases, however, after a variable static period there is a sudden outgrowth of new cells and thereafter proliferation is rapid. It is then possible to carry the strain in repeated serial transfers. After such a transformation occurs and when some dozen or more successful subcultures have been made it becomes apparent that the cells can be propagated indefinitely. In these circumstances the cells can be considered to constitute a strain or line.

Many cell strains have been derived from such normal human tissues as liver, kidney, intestines, embryonic skin and muscle (fibroblasts), and amnion. Other cell lines have been obtained from monkey kidney and sarcomatous tissue in the mouse. Two cell strains of great value in medical virology have originated in human malignant tissue. The HeLa strain of epithelial cells was obtained by Gey from a squamous cell carcinoma of the cervix uteri and the KB strain by Eagle from a carcinoma of the nasopharynx. Every cell strain has its own characteristic pattern of susceptibility to infection by viruses. HeLa cells can be infected with an obvious cytopathic effect by the three types of poliovirus, the whole group of adenoviruses, the pox viruses, and by the herpes simplex virus; in general they are not susceptible to infection by the Coxsackie viruses of groups A or B or by the ECHO viruses.

The Cultivation of Cell Strains.—Cell strains grow uniformly as suspensions or as sheets on the surface of glass. Often it is important to be able to examine the cells during their growth and for this purpose they are manipulated so that a layer one cell thick (a "monolayer") is formed on the side of test-tubes ($\frac{1}{2}$ or $\frac{5}{8}$ in. in diameter) which can be viewed with $\frac{2}{3}$ in. objective of the microscope. For other purposes they can be cultivated on the flat surfaces of Carrel flasks or Petri dishes and

when required in great numbers they are propagated on the sides of ordinary prescription bottles or Roux bottles.

The Detachment of Cells from a Culture on Glass.—Cells can sometimes be brought into suspension simply by gentle scraping with a glass rod covered with silicone rubber tubing. This method cannot, however, be relied upon and it is usually necessary to detach the cells by one of the following two methods.

In the first method a chelating agent, "Versene", is used to bind divalent ions such as calcium and magnesium with the effect that the cells round up and leave the glass. "Versene" is diaminooctane-tetra-acetic acid and its disodium salt is employed in the following solution:

Disodium versenate	0·2 g.
NaCl	8·0 g.
KCl	0·2 g.
Na ₂ HPO ₄	1·15 g.
KH ₂ PO ₄	0·2 g.
Glucose	0·2 g.
Water	1000 ml.

To suspend the cells remove the growth medium and wash the surface of the cell sheet with balanced salt solution (Ca and Mg free) warmed to 37° C. Replace the salt solution with the versene solution and incubate the culture for ten to fifteen minutes at 37° C. At the end of this time the cells will be free in the versene solution and should be removed without delay by centrifugation for five minutes at 1000 r.p.m. They are resuspended in growth medium and dispersed by gentle pipetting. The cells in the suspension are then counted in a haemocytometer and diluted to give a concentration of the order of 100,000 per ml. With delicate or slow-growing cells, concentrations as high as 500,000 ml. may be required. This method is valuable for HeLa cells.

Proteolytic enzymes may be used to release the cells from the surface of the glass. A stock solution of 5 per cent. trypsin (Difco 1:250) in Hanks' solution is convenient for this purpose. Before use, the stock solution is diluted 1 in 10 (0·5 per cent.) with Hanks' solution and warmed to 37° C. The culture medium is removed from the monolayer culture and replaced by an equal volume of trypsin. After about ten minutes incubation at 37° C., and as soon as the cells leave the vessel wall, they are spun down in a sterile tube for a few minutes at 1000 r.p.m. and the supernatant is replaced by culture medium. After the cells have been counted the suspension is diluted to give the required concentration in culture medium.

The second method is most commonly used. Some workers consider a lower concentration of trypsin (*e.g.* 0·025 per cent.) is desirable. During trypsinisation a careful watch must be kept on the pH. Below pH 7·0 trypsin is practically inactive and above pH 8·0 the cells are badly damaged. In centrifuging the cells, high speeds may cause damage and death of the cells, so that the slowest effective speed of the centrifuge should be used.

Maintenance and Feeding of Cells.—When the cell suspensions have been placed in the culture tubes, and vessels, they are incubated for forty-eight hours at 37° C. in the horizontal position until the cells are adherent to the glass. Thereafter tube cultures may be incubated in a rotating drum. The drum consists of sheet metal, or a plastic such as Perspex, pierced with holes of the appropriate size to hold the culture tubes and mounted on a horizontal shaft. The drum is tilted at an angle of 5 degrees in order to prevent the nutrient medium touching the stoppers of the tubes; it is rotated at a speed of 12–20 revolutions per hour by an electric motor fitted with a reduction gear.

The feeding of cell strains consists simply of pipetting off the old culture medium and replacing it with new. Medium may conveniently be removed by means of a pipette attached to a suction pump. A flask acting as a trap should be inserted between the pipette and the pump. A mechanical dispenser (*e.g.* an automatic syringe, see p. 455) greatly reduces the labour of adding medium to the tubes.

The medium used for growth of the cells varies with the cell strains but the following is in common use:—

Hanks' solution to which have been added 0·5 per cent. lactalbumin hydrolysate and 0·1 per cent. yeast extract. To this mixture 20 per cent. serum is usually added. Horse serum is perhaps the best for most purposes, but human, calf, and rabbit serum may also be used either separately or in combination (*e.g.* 10 per cent. horse serum + 10 per cent. rabbit serum).

For maintenance of the cells medium 199 alone will suffice to keep the cells in good condition for periods of three to six days, but if maintenance for longer periods is needed, 2–5 per cent. horse serum should be added. If it is desired to slow the rate of cell multiplication, Earle's solution is used in making the medium and the concentration of serum is reduced by 50–75 per cent. Some workers also increase the sodium bicarbonate content of the medium. In the routine maintenance of cell strains it is convenient to handle them twice a week. On the first occasion they are fed and on the second they are detached from the glass and transferred to new vessels.

Plaque Technique.—When a very dilute suspension of virus is applied to the surface of a monolayer of growing cells each elementary body initiates a focus of infection and as the neighbouring cells become involved necrotic areas or plaques are formed.¹ Different viruses form plaques of different sizes and shapes and so the method is valuable in distinguishing and characterising these agents.² The method also offers a precise means of titrating the infectivity of viruses and enables pure clones to be obtained.

Technically the method requires great care in the production of even monolayers of growing healthy cells in Petri dishes or on the sides of

¹ Dulbecco, R., & Vogt, M. (1954), *J. exp. Med.*, **99**, 167.

² Hsuing, G. D., & Melnick, J. L. (1957), *J. Immunol.*, **78**, 128.

prescription bottles. If human serum has been used in the culture medium the cell sheet must be washed with balanced salt solution to remove it. The cells are inoculated by adding 0·5 ml. of the appropriate virus dilutions and allowing the virus to disperse over the surface of the cell sheet. One hour at 37° C. is allowed for the virus to infect the cells and the sheet is then covered with an overlay of molten agar prepared as follows.

Melt a 3 per cent. mixture of agar (Difco Nobel agar) in balanced salt solution, cool to 43° C. and add it to an equal volume of culture medium at the same temperature (Hanks' solution containing 20 per cent. horse serum or a maintenance medium). Some workers add neutral red to give a final concentration of 0·002 per cent.; this vital dye stains the living but not the dead cells and thus renders the plaques more easily visible. Quickly cover the cell layer with the molten agar and set aside to solidify. Invert the bottles and incubate at 37° C. Examine the bottles daily in an oblique light for plaque formation.

Cooper¹ has described another method for plaque production, in which a cell-virus suspension is incorporated into molten agar. A suspension comprising about 15 million cells per ml. in a culture medium made up in Earle's solution is mixed with a dilute virus preparation and added to an equal volume of molten 1·8 per cent. agar at 44° C. The mixture is poured into 10 cm. Petri dishes and incubated, as soon as it has set, in an atmosphere containing 10 per cent. CO₂. Replacement of the bicarbonate buffer in the medium with 0·3 per cent. "tris buffer" (2-amino-2(hydroxy-methyl) 1:3-propanediol) enables the use of the CO₂ containing atmosphere during the incubation period to be dispensed with.

Explantation in Plasma Clots.—A wide variety of human or animal tissues may be used to provide host cells for virus growth in tissue cultures. It is important that tissues removed at operation or at autopsy should be placed in a refrigerator at 4° C. without delay. As soon as possible the material should be sliced with very sharp instruments into small pieces about 5 mm. thick and then immersed in Hanks' solution containing penicillin and streptomycin. The cells in such slices remain viable at 4° C. for between eighteen and thirty-six hours. Mincing of the tissues into fragments of 1 mm. diameter is required for some types of cultures and may be undertaken with pieces of new razor blades held in a handle or by a fine pair of Spencer-Wells forceps. The fragments so made are rinsed several times in Hanks' solution to free them from amorphous debris and are then set up in cultures as follows.

A drop of chicken plasma is placed on the side of a test-tube and is spread out to cover the lower half of its length. Three or four tissue fragments are next placed at intervals on the plasma-coated tube. A drop of chick embryo extract is then flooded over the fragments and the plasma is allowed to clot during the next five to ten minutes. Next 0·5-1·0 ml. culture medium, which is similar in composition to that

¹ Cooper, P. D. (1955). *Virology*, **1**, 397.

used for HeLa cells, is added to the tubes, which are then tightly stoppered and incubated at 37° C. in a rotating drum. The cultures are fed when the medium begins to become acid—usually every third day. After a few days, the growing cells, which may be epithelial or fibroblastic, migrate into the plasma and begin to grow. In three days the cells have often produced enough trypsin to digest the plasma clot and to form a large hole in it. This may be overcome by patching the hole with a drop of fresh plasma which is again made to clot by chick embryo extract. Alternatively the action of the trypsin can be entirely checked if the culture medium contains 0·05 mg. per ml. of a pure crystalline soya bean trypsin inhibitor.¹

Biological Fluids and Extracts

Serum is a valuable ingredient of the great majority of tissue culture media. Human, horse, calf and chicken sera are those most often used; they are obtained from whole blood after it has clotted. Serum must be tested for bacterial sterility before use and is best stored in a deep freeze cabinet at -30° C. Some sera are markedly toxic for growing cells and it is a wise precaution to test each fresh batch of serum in a number of replicate cultures of HeLa cells. Human placental cord serum is regarded as being particularly good for promoting the growth of cells and is relatively free from toxic properties.

Ascitic and pleural fluids are useful culture media and are prepared and tested in the same way as sera.

Chicken Plasma.—Blood is taken from the wing vein into a sterile syringe containing sufficient heparin solution to give a strength of 5 units of heparin per ml. of blood. Greater strengths of heparin should be avoided as the plasma thus obtained may not clot. The blood is discharged into a clean dry test-tube and the plasma is separated by centrifuging at 1000 r.p.m. for five minutes. Store in small quantities at 4° C.

A convenient heparin solution is made by adding 1 ml. of heparin (Roche Liquemin, 1 ml.=1000 international units) to 9 ml. Hanks' solution; it can be sterilised by autoclaving at 10 lb. pressure for ten minutes and penicillin and streptomycin sulphate should be added to concentrations of 500 units and 500 µg. 0·5 ml. of this solution is sufficient for 10 ml. of blood.

Bovine Amniotic Fluid.—This provides a valuable means of promoting the growth of tissue cultures without the interfering action of specific antibodies present in human serum.

An intact cow's uterus, some three months gravid is obtained from the slaughter house. The contained embryo should be between 8 and 12 inches long. The uterine surface is flamed and carefully incised at some dependent point away from the embryo. The amniotic fluid is allowed to escape into a sterile one litre measuring cylinder. Penicillin, 100 per ml., streptomycin 100µg. per ml. and mycostatin 20 units per

¹ Available commercially from V. & A. Howe, Pembridge Road, London, W.11.

ml. are added together with sufficient stock phenol red solution (0·4 per cent.) to give a final concentration of 0·002 per cent. Using aseptic methods the fluid is filtered through gauze and then spun at 2000 r.p.m. in a straight headed centrifuge for thirty minutes. The resulting supernatant fluid is filtered through a Seitz E.K. filter, dispensed in small volumes in hard glass containers, stoppered, and stored at -30° C.

Chick Embryo Extract.—After chilling for two hours at 4° C. nine-day-old embryos are removed from the eggs and washed once in Hanks' solution. For some purposes it is necessary to remove the eyes of the embryos. Place the washed embryos in a homogenising flask and add 1·5 ml. per embryo of Hanks' solution. Add sufficient penicillin and streptomycin sulphate to give a final concentration of 100 units and 100 µg. per ml. Homogenise at medium speed for ten minutes. Centrifuge the suspension at 3000 r.p.m. for ten minutes, transfer the supernatant to suitable tubes, stopper, and store at -25° C. Chick embryo extract is best used fresh but may be used for a week or more after its preparation.

Bovine Embryo Extract.—An embryo 4-6 in. long is obtained, cut into small pieces and placed in a Waring blender with an equal volume of Hanks' solution. It is homogenised at high speed for two to five minutes and the resulting pulp is transferred to centrifuge bottles and spun down. The resultant somewhat turbid supernatant is used as the extract in culture media. Some workers prefer to purify it further by ultra-centrifugation and as a final step to pass it through a fine sintered glass filter.

Test for the Activity of Trypsin.—It is necessary to check from time to time the activity of the trypsin used in solutions to disaggregate the cells of tissues such as monkey kidney. This can be carried out by preparing a series of about 10 doubling dilutions from 1 in 10 onwards of the trypsin solution in Hanks' balanced salt solution. One drop of each dilution is placed on a strip of X-ray film together with one drop of Hanks' solution as a control. The strip is then placed on moist blotting-paper in a Petri dish, covered, and incubated for thirty minutes at 37° C. Remove the strip and allow to cool to room temperature or run cold water over the reverse side (no film) until the gelatin sets firmly. Now flood the whole film gently with cold water. Wherever trypsin was present the gelatin will have been digested to water-soluble products and a punched-out hole will appear in the film. The control area (Hanks' solution) is not dissolved at all.

PART III

**PATHOGENIC AND COMMENSAL MICRO-
ORGANISMS**

CHAPTER XIX

STAPHYLOCOCCI

GRAM-POSITIVE cocci which grow in clusters are ubiquitous and can be isolated from air, dust, water and oil, and human and animal sources. These cocci which grow on solid nutrient media to give relatively large opaque, white or coloured colonies with a smooth surface and which ferment glucose aerobically and anaerobically and may liquefy gelatin belong to the genus *Staphylococcus*. Some of them are saprophytic and may be useful in agricultural processes because of their fermentative activity. Others have become adapted to live as parasites and are found as commensals or pathogens in human beings and animals.

The staphylococci are classified primarily on the basis of coagulase-production (p. 469). Coagulase-positive strains, most of which also produce α and β haemolysins, are called *Staphylococcus aureus*; an alternative name preferred by some workers is *Staphylococcus pyogenes*. Coagulase-negative strains are called *Staphylococcus epidermidis*, since they commonly occur on the skin; these strains do not produce haemolysins.

Most strains of coagulase-positive staphylococci grow as colonies with a yellow or orange pigment; hence the name *Staph. aureus*. Colour, however, is subject to variation and a significant minority of pathogenic staphylococci grow as white or creamy colonies; these should be described as white varieties of *Staph. aureus* but are sometimes called *Staph. pyogenes* var. *albus*. Occasionally colonies of coagulase-positive staphylococci have a lemon colour (*Staph. pyogenes* var. *citreus*). Most strains of *Staph. epidermidis* grow as white colonies and in this book *Staph. albus* will be used as an alternative name for these commensals. Other coagulase-negative staphylococci have growths coloured lemon (*citreus*), golden (*aurantiacus*) and red (*roseus*).

The great majority of strains of staphylococci isolated from lesions in man and animals are coagulase-positive. It is doubtful if coagulase-negative strains can themselves be responsible for initiating infection. The greater part of the surface of the healthy human skin yields large numbers of *Staph. epidermidis*, and this organism appears to grow and multiply in the sebaceous and sweat glands and hair follicles. Certain restricted sites on the skin are colonised by *Staph. aureus* in a large minority of healthy

persons who are, therefore, carriers of the organism. The commonest of these sites is the nasal vestibule (anterior nares). The perineum, groin, axilla and, to a lesser extent, the umbilicus may also be colonised.

In animals staphylococci occur as commensals of the body surfaces in many species that have been examined, and lesions, though probably less frequent than in man, are also similar in character.

The great majority of lesions caused by *Staph. aureus* are superficial, e.g. boils, styes and wound infections. Less frequently a more extensive infection involving deeper tissues may develop, e.g. broncho-pneumonia, osteomyelitis; and occasionally septicaemia and death may result from widespread dissemination of the organism from a focus.

Generally speaking, the staphylococci show a marked degree of variation in their biological characters. This is reflected in the variable reaction of many strains to antibiotics and chemotherapeutic agents so that the antibiotic resistance of strains of *Staph. aureus* has become of great epidemiological and therapeutic importance.

STAPHYLOCOCCUS AUREUS

Morphology and Staining.—Gram-positive spherical cocci arranged in irregular clusters, the individual cells being approximately $1\ \mu$ in diameter. Single forms and pairs may also be noted. In films made from cultures a certain amount of breaking-up of the clusters occurs and a few short chains may be noted. Long chains are never found.

Cultural Characters.—Aerobe and facultative anaerobe. Temperature range for growth 10° – 42° C.; optimum 35° – 37° C. Growth occurs in ordinary nutrient media. A uniform turbidity forms in broth cultures. Colonies are circular disks, relatively large after twenty-four-hour growth with a diameter of 2–4 mm. They are opaque and convex with a shining surface and may be pigmented white (var. *albus*), yellow, golden-yellow or golden (var. *aureus*). Confluent growth appears like "oil-paint".

Staphylococci will grow in the presence of 10–15 per cent. sodium chloride. The salt may be incorporated in media, making them highly selective for *Staph. aureus*, e.g. 10 per cent. salt broth or 7 per cent. salt-milk agar (p. 226).

On blood agar the colonies are similar to those on agar but somewhat larger. Marked zones of haemolysis appear on sheep or rabbit blood agar, particularly in an atmosphere containing carbon dioxide, but are small or absent on horse blood agar.

On milk agar (p. 226) the colonies are similar to those on nutrient agar, but the pigment is more rapidly formed, more intense, and different shades of colour are easily recognised. Areas of clearing around the colonies may occur which represent digestion of heat coagulated casein by staphylococcal proteases.

On MacConkey's medium colonies are small but show a characteristic colour due to the yellow pigment being tinged pink by the acid change of the neutral red indicator.

Gelatin is liquefied quickly and coagulated serum slowly.

Colonies of staphylococci growing on media containing a number of biological fluids such as milk or egg-yolk, may be surrounded by zones of opacity or clearing. Many of these are the result of the action of enzymes, e.g. proteinases or lipases. As these enzymic activities are usually associated with the coagulase-positive staphylococci, they have been adapted by certain workers in selective media for the isolation of pathogenic staphylococci.

*Opacity in Medium containing Egg-yolk.*¹—When coagulase-positive staphylococci are grown in glucose-yolk broth a dense opacity due to the formation of tiny droplets of fat results. This effect can be neutralised by staphylococcal antiserum. The reaction is given by a high proportion of human strains but only by a few animal strains. Coagulase-negative strains do not give this reaction.

Opacity in media containing plasma or fibrinogen may be due to coagulase, and also other factors such as the β -lysin. Frequently coagulase-negative strains produce opacity. The test may be made more specific for coagulase by incorporating fibrinogen in the medium as follows²: Make up a 1.5 per cent. solution of bovine fibrinogen in saline (Messrs. Armour, Hampden Park, Eastbourne, Sussex). Clarify the solution through a filter paper and add a trace of soya-bean inhibitor before filtering through a membrane filter (a Seitz filter removes much of the fibrinogen). Add 4–5 ml. of the filtered, sterile fibrinogen solution to 100 ml. of nutrient agar at 50° C. together with 2–3 ml. of sterile human plasma. Store the plates at 4° C. for up to ten days. Dry plates before use. Colonies of coagulase-positive staphylococci are easily identified by the white halo or ring of opacity which they produce. The soya-bean trypsin inhibitor prevents fibrinolysin from digesting this opacity and producing a zone of clearing.

Viability.—The thermal death point is about 62° C. for half an hour, but some strains are more resistant to heat, withstanding

¹ Gillespie, W. A., & Alder, V. G. (1952), *J. Path. Bact.*, **64**, 187.

² Duthie, E. S., & Lorenz, L. L. (1952), *J. gen. Microbiol.*, **6**, 95.

70° C. for a short time. Laboratory cultures survive for months, and in some cases for years. Freeze-dried cultures in broth or serum survive for many years. *Staph. aureus* is resistant to slow drying and will survive for many months in dust in the absence of direct sunlight.

Staphylococci are readily killed by most antiseptics and disinfectants at the appropriate concentrations in the absence of serum, pus or albuminous material, e.g. it is killed in a few minutes by 2 per cent. phenol. A concentration of 1: 500,000 crystal violet is inhibitory to staphylococci, and when incorporated in solid culture media, e.g. blood agar, acts as a selective medium for the isolation of streptococci and pneumococci in mixed wound and middle ear infections.

Low concentrations of brilliant green (1 in 10,000,000) and other organic dyes are bactericidal. Proflavine and acriflavine maintain their effect in the presence of serum and pus at concentrations of 1 in 100,000, and 1 in 1,000 solutions may be safely used on wounds.

Antibiotics.—As a general rule *Staph. aureus* strains are highly sensitive to most of the antibiotics used in therapy. Growth of these strains is prevented by 0·02–0·05 µg./ml. of penicillin, 0·5 µg./ml. of streptomycin, 1·5–10 µg./ml. of chloramphenicol, 0·1–1·0 µg./ml. of tetracyclines and 0·25 µg./ml. of erythromycin, dependent to a certain extent on the method of test.

In the hospital environment, and to a lesser extent in the general community, the proportion of strains isolated from carriers and lesions which are resistant to antibiotics used in therapy has increased, roughly in proportion to the amount of these substances used. Thus in many hospitals today over 80 per cent. of strains isolated are penicillin-resistant; 50 per cent. are resistant to streptomycin; 25–50 per cent. are tetracycline-resistant; and 5–15 per cent. are resistant to chloramphenicol. Among the general community about 20–30 per cent. of strains are penicillin-resistant, and much smaller proportions are resistant to the other antibiotics.

The penicillin-resistant strains of *Staph. aureus* found among cases and carriers are resistant because they produce the enzyme penicillinase. Penicillinase-producing cells arise by spontaneous mutation in a limited number of staphylococcal populations and are subsequently selected by environmental factors which include the antibiotic. Staphylococci resistant to other antibiotics probably also arise by mutation and their growth and spread is favoured by the continued use of these antibiotics. Practically all strains that are resistant to streptomycin, the tetracyclines,

erythromycin and chloramphenicol are also penicillinase producers. A minority of strains are isolated which are resistant to all of these antibiotics, and drug treatment depends on the use of more recently developed antibiotics such as novobiocin, Vancomycin and Ristocetin, against which resistant mutants have not yet appeared in large proportions.

Most strains are sensitive to polymyxins, neomycin, xanthocillin and a number of other antibiotics which are frequently used in topical therapeutic preparations and creams to suppress nasal carriage of *Staph. aureus* (p. 476). Some strains are susceptible to the sulphonamides.

Biochemical Reactions.—Various carbohydrates are fermented with acid but no gas production, e.g. glucose, lactose, sucrose and mannitol. Most strains from human sources ferment mannitol, and as *Staph. epidermidis* and other coagulase-negative strains rarely ferment this carbohydrate, mannitol fermentation has been used as a test to indicate probable pathogenicity. There is no correlation between mannitol fermentation and coagulase production by animal strains.

Nitrites and methylene blue are reduced. Urea is hydrolysed and catalase is produced.

Coagulase.—The production of coagulase is characteristic of *Staph. aureus*; this substance is almost certainly an enzyme and a precursor of a thrombin-like substance which coagulates blood plasma. A simple and reliable way to test for its production is as follows. Dilute citrated, oxalated or heparinised human or rabbit plasma 1 in 10 with isotonic saline or other suitable diluent. Place 0.5 ml. of diluted plasma in each of 2 small test-tubes; to one tube add 5 drops of an overnight broth culture or agar culture suspension. Incubate both tubes at 37° C. and examine after one hour and at intervals up to twenty-four hours. A clot after forming may be lysed at a variable rate so that care must be taken to avoid a false negative reading.

Clotting usually occurs within a few hours and indicates that the strain is coagulase-positive. The second tube serves as a control and should show no coagulation. It is advisable to include tubes containing a known coagulase-positive and coagulase-negative strain as controls in any batch of tests.

Slide Coagulase Test.—An alternative technique is to carry out the test on a microscope slide as follows. Divide the slide into two sections with a grease pencil. Place a drop of normal saline on to each area; emulsify a small amount, e.g. one or two colonies from an agar plate, of the test strain in each of the two drops to make a smooth suspension. Add a drop of undiluted human or

rabbit plasma to one of the drops and stir gently with a wire. Clumping of the organism results if the strain is coagulase-positive because fibrinogen precipitates on the cell surfaces, causing them to stick together. The factor causing this is the "clumping factor" or "bound" coagulase which is attached to the cell and acts directly on the fibrinogen.¹ (The tube test measures "free" coagulase which requires an accessory factor present in the plasma.) Coagulase-negative strains remain unchanged. The second drop is a control to show spontaneous granularity of the strain which if it occurs invalidates the test.

This slide test is fairly reliable, though a number of false positives occur. Doubtful results are best confirmed by a tube test. A small number of strains give a positive slide test and negative tube test due to the production of "bound" coagulase alone which is not detected by the tube test.

Phosphatase Test.—It has been found that there is a certain degree of correlation between phosphatase and coagulase production by staphylococci. The detection of phosphatase in direct plate cultures has been suggested for the exclusion of non-virulent strains and as a substitute for the coagulase test, e.g. in cultures obtained from possible carriers of pathogenic (coagulase-positive) staphylococci. For this purpose an agar medium incorporating phenolphthalein diphosphate is used. Organisms producing phosphatase liberate free phenolphthalein which can then be detected by exposing the plate culture to ammonia vapour, when the growths become bright pink.²

Antigenic Characters.—There are protein antigens common to both pathogenic and non-pathogenic staphylococci, but sera prepared against pathogenic strains do not agglutinate non-pathogenic strains, and the converse is also true.

Agglutination tests using sera prepared against a selection of coagulase-positive strains can be used to distinguish a number of groups or types of *Staph. aureus*. Using simple slide agglutination and agglutinin-absorbed sera, Cowan³ recognised three types (I, II and III). Six further types were added by Christie and Keogh.⁴ Other systems of classification on the basis of agglutination tests have been described by Hobbs,⁵ Oeding,⁶ and Elek and Stern.⁷

The typing of strains by these agglutination techniques has

¹ Duthie, E. S. (1954), *J. gen. Microbiol.*, **10**, 427, 437; *ibid.* (1955), **13**, 383.

² Barber, M., & Kuper, S. W. A. (1951), *J. Path. Bact.*, **63**, 65.

³ Cowan, S. T. (1939), *J. Path. Bact.*, **48**, 169.

⁴ Christie, R., & Keogh, E. V. (1940), *J. Path. Bact.*, **51**, 189.

⁵ Hobbs, B. C. (1948), *J. Hyg. (Lond.)*, **46**, 222.

⁶ Oeding, P. (1952), *Acta path. microbiol. scand.*, **31**, 145.

⁷ Elek, S. D., & Stern, H. (1957), *J. Path. Bact.*, **73**, 473.

been used in epidemiological studies of outbreaks of staphylococcal infection, but on the whole the information has not been as satisfactory as that obtained by phage typing (*vide* below).

Precipitin reactions show that at least two serologically distinct carbohydrates occur in staphylococci, one characteristic of pathogenic strains and the other of non-pathogenic strains.

The diffusible toxins of different strains of *Staph. aureus* appear to be serologically identical with the exception of coagulases of which at least three distinct antigenic types have been described.

Phage Typing of Staphylococci.—Strains of staphylococci may carry a phage which is lytic to other strains (see p. 880); potent preparations of these phages can be prepared and used to type strains of coagulase-positive staphylococci, and this has proved to be of practical value in investigations for tracing the source and spread of this organism in the community.

Staphylococcal phages are not specific for individual strains of staphylococci, but by using selected phages a "pattern" of lysis can be observed. These patterns are reproducible within reasonably narrow limits and determine the "phage type" of the particular strain.

Phages used by most laboratories have been numbered according to an internationally recognised scheme. There are four main groups of these phages, and member phages within each group are antigenically related to one another. Patterns of lysis most frequently involve lysis with phages of one group and less frequently are phages of different groups found to lyse one strain of staphylococcus. This is particularly true if the phages are used relatively dilute (e.g. at routine test dilution, R.T.D., see p. 881). Patterns tend to be wider when undiluted phages are used. There is a correlation between the main serological types and phage groups.

The phages in each of the groups are shown in the table.¹

Phage Group	Individual Phages	Common Patterns
I	29; 42B; 52; 52A; 80 and 81.	29; 52/52A; 52/80/81; 80.
II	3A; 3B; 3C; 51; 55; 71; 171.	3A/3B/3C; 3C/51/55, etc.
III	6; 7; 42C; 42E; 47; 47A; 53; 54; 75; 76; 77; and 79.	Complex
IV	42D.	

¹ For details of method of phage typing, see Williams, R. E. O., & Rippon, J. E. (1952), *J. Hyg. (Lond.)*, 50, 320.

Virulence.—Most, if not all, strains of *Staph. aureus* are potentially pathogenic to man or animals. Strains of *Staph. aureus* isolated from human lesions are actively pathogenic to rabbits; thus a small quantity of culture injected subcutaneously produces a localised abscess, and intravenous inoculation leads to either septicaemia or pyæmnia with multiple abscesses in the kidneys, lungs, myocardium or other organs. Mice and guinea-pigs can also be successfully infected, but are less susceptible than rabbits.

Many strains of *Staph. aureus* are strongly toxigenic; they are haemolytic (when mixed with suspensions of red cells), kill and lyse leucocytes (when added to a preparation of leucocytes), produce necrosis of tissue (when injected into the skin), and exert a rapidly lethal effect on intravenous injection. Thus *haemolysin*, *leucocidin*, *leucolysin*, *necrotoxin* and *lethal* toxin activity can be demonstrated in cultures. In addition, certain strains produce *fibrinolysin* and *hyaluronidase* which are distinct from those produced by streptococci (p. 481). As yet the part played by these toxic factors in natural infections has not been elucidated. The digestion of the clot formed by coagulase is due to a fibrinolytic agent, staphylokinase, which activates a protease, plasmin, present in human and animal plasma.¹

Different types of staphylococcal haemolysin can be recognised; the α -lysins produce rapid lysis of rabbit and sheep red cells at 37° C.; the β -lysins lyses sheep cells, the effect being progressive at room temperature (e.g. after the test mixtures of blood suspension and culture filtrate have been removed from the incubator and allowed to stand overnight at room temperature); and δ -haemolysins have also been defined and can be demonstrated by testing with human or horse red cells. The α , β and δ lysins are antigenically distinct. The α -haemolysin may be responsible for the necrotic and lethal effects of filtrates referred to above. The α and δ haemolysins seem to be characteristic of strains of human origin and are generally associated with coagulase production. Coagulase-negative strains do not produce them. β -lysins are produced by strains of animal origin, many of which also produce α and δ lysins.

A special medium (Walburn's) has been found very suitable for the preparation of staphylococcal toxin; this consists of a meat extract prepared from ox heart to which are added 0.5 per cent. Witte peptone, 0.2 per cent. potassium dihydrogen phosphate and 0.03 per cent. magnesium sulphate; the pH is adjusted to 6.8.

¹ See "Staphylococcus pyogenes and Its Relation to Disease". S. D. Elik, E. & S. Livingstone, 1959, for fuller consideration of virulence.

The culture is grown in an atmosphere of 20–25 per cent. carbon dioxide. Another method is to grow the organism on 0·8 per cent. nutrient agar for forty-eight hours in an atmosphere of 25 per cent. carbon dioxide, and then add to the culture a buffered broth (sugar-free broth containing 4 per cent. peptone with an equal volume of 0·067 M potassium dihydrogen phosphate solution); the culture is finally incubated for three days in 25 per cent. carbon dioxide as before.

Certain strains of *Staph. aureus* produce an *enterotoxin* which is different from the other staphylococcal toxins. It is thermostable and can resist boiling for a short period. When ingested this toxin produces a gastritis or gastro-enteritis in man and is a common cause of *food-poisoning*. Unfortunately there is no satisfactory *in vitro* or animal test for demonstrating this toxin.

Occurrence.—*Staphylococcus aureus* is a common commensal of the skin, particularly those areas with apocrine glands, the nasal vestibule, groin, perineum and axilla. The organisms may be isolated in large numbers from the anterior nares of 30–40 per cent. of the healthy adult population, from the groin and perineum of 10 per cent. and less frequently from other areas such as the axilla. It may also colonise the nasopharynx, though this is more common in babies and young children. In a minority of persons quite large numbers of *Staph. aureus* may be isolated from the faeces.

In hospitals the proportion of members of staff whose anterior nares are colonised with *Staph. aureus* is usually higher (50–60 per cent.) than in the general population, and patients in hospital tend to be colonised depending on the length of their stay. Young babies are extremely susceptible to colonisation by the organism, and 80–100 per cent. of infants born in hospital yield large numbers of *Staph. aureus* from the anterior nares and, less frequently, from the throat, umbilicus and faeces on discharge eight to ten days after birth.

Numerous staphylococci are found in the air and dust and on clothing and fomites. A variable proportion of these will be *Staph. aureus*, depending upon the site. Thus the relative and absolute number of this organism is greater in the hospital environment than in the home, and the environment reflects the rate of dissemination from human sources of the organism.

Staph. aureus is also a commensal of a number of animals such as dogs, cats, sheep and horses.

Staph. aureus occurs commonly in pyogenic lesions in the human subject. The great majority of these infections are superficial inflammatory lesions with pus formation such as skin

pustules, boils, carbuncles, blepharitis, styes, impetigo,¹ and pemphigus neonatorum and "sticky eye" in babies. It is a common cause of wound suppuration and of mastitis in lactating mothers. More serious and deep-seated infections are osteomyelitis, renal carbuncle, peri-renal abscess, bronchopneumonia and localised abscesses. In a minority of cases pyaemia, septicaemia and malignant endocarditis may result from spread from a primary focus.

Cases of food-poisoning are frequently due to the *enterotoxin* of staphylococci growing in certain articles of food, such as cooked meats, milk and milk-products (cream-cakes, custard, ice-cream), fish and gravies. These foods are most frequently contaminated by food-handlers, although when milk and milk-products are responsible the staphylococci may have been derived from the milk itself, e.g. from an udder lesion in the cow. The production of the toxin depends upon suitable conditions (time, temperature, moisture) for the growth of the organism in the food.

Inflammatory and suppurative lesions similar to those found in the human subject are found in animals, though on the whole they are less frequent, e.g. mastitis in most species of domestic animals; pyaemia associated with tick infection in lambs; and septicaemia and arthritis in poultry. In the condition of "botryomycosis" in horses (*Staphylococcus ascoformans*) the cocci are frequently capsulated in the tissues and, especially in chronic lesions, occur in zoogaea-like masses or clusters. These aggregates may resemble in naked-eye appearance actinomycotic "granules" (p. 662). In culture, capsules are not seen and the organism resembles *Staph. aureus* in its general characters. A similar staphylococcal infection, sometimes pathologically resembling actinomycosis, may occur in the udders of cattle and pigs.

Epidemiology.—The sources of *Staphylococcus aureus* are human beings who are carriers or suffering from lesions. Less important sources are animals and inanimate material in which the organism is capable of growth, e.g. contaminated foodstuffs under suitable growth conditions.

Healthy carriers or persons with lesions disseminate the organism over their skin surfaces and impregnate their clothes. The environment is contaminated by direct contact, e.g. by direct contact via the hands, or by the distribution of contaminated

¹ *Staph. aureus* isolated from impetigo lesions mostly belong to one phage type ("71"), are penicillin-resistant and have an inhibitory action on *C. diphtheriae* (Parker, M. T., Tomlinson, A. J. H., & Williams, R. E. O. (1950), *J. Hyg. (Lond.)*, 53, 458).

particles from the skin and clothing which occurs during movement, and particularly by use of the handkerchief. New hosts are infected by direct contact, or indirectly from the contaminated environment.

A proportion of staphylococcal lesions are due to autogenous infection as occurs when a carrier contracts a lesion due to the same staphylococcus "type" as is present on anterior nares or other carrier site. In other cases the source of the organism is exogenous and cross-infection takes place. Autogenous infections are more common among the general population; the phage "types" of *Staph. aureus* producing these infections are diverse and most of the strains are sensitive to penicillin and other antibiotics. Cross-infection is more common in hospitals; a limited number of "types" are involved in any one hospital and the majority of these strains are resistant to penicillin and frequently to one or more of the other antibiotics.¹

Laboratory Diagnosis

Examination of Material from Lesions.—This is most conveniently carried out by sampling the pus or exudate with a sterile swab, or if there is sufficient material, collecting in a capillary or test-tube. Blood-agar and milk-agar plates are inoculated and a Gram-stained film prepared. After overnight incubation the plate cultures are examined for colonies morphologically resembling *Staph. aureus*. Representative colonies are examined for coagulase production (and haemolysins if desired).

Subculture of representative colonies is made in nutrient broth and these may be used to measure antibiotic sensitivity and determine the phage type.

Recognition of Carriers.—Swabs are taken from the anterior nares, perineum or other suspect site and plated out on suitable medium such as milk agar. Occasional colonies of *Staph. aureus* probably indicate transient contamination of the surface that has been sampled. A large number of colonies, sometimes confluent growth, indicate true colonisation characteristic of the carrier. A persistent carrier state may be confirmed by examining three swabs taken at weekly intervals, as the presence of *Staph. aureus* may be intermittent in some persons.

Serological Diagnosis.—Normal serum frequently agglutinates staphylococci in low titre. In staphylococcal infections the titres may be quite high, but the results are too variable to be of

¹ For fuller discussions on staphylococcal infections in hospitals, see *Min. of Hlth Report* (1959) and Williams, R. E. O. (1959), *Lancet*, 1, 190.

diagnostic value. Various other antibody titres can be demonstrated in the sera of animals immunised with *Staph. aureus*, but their relationship to disease is not clear and little of clinical value can be learnt from their presence in the sera of patients.

Assistance in the diagnosis of suspect cases of deep-seated staphylococcal infections such as bone or kidney disease may be obtained by estimating the serum titre of anti-a haemolysin. Normal titres range from 0-2 units. A titre of 4 units or more, and especially a rising titre is more significant, but the absence of demonstrable antibody does not exclude staphylococcal disease.

Chemotherapy.—Owing to the variable susceptibility of strains of *Staph. aureus* to antibiotics it is advisable to carry out antibiotic sensitivity tests on the causative organism whenever possible. Guidance as to the best antibiotic to use can be obtained most quickly by the use of the primary sensitivity test (p. 404). Penicillin is effective in the majority of staphylococcal infections in the general population which require antibiotic therapy. Few infections occurring in hospital are amenable to penicillin therapy. Combinations of antibiotics are recommended by several workers for the treatment of patients infected with strains resistant to multiple antibiotics. The best combinations can be determined by laboratory tests.

Prophylaxis.—The prevention of staphylococcal infection depends on (a) preventing the dissemination of the organisms from open lesions and dangerous carriers, (b) reducing the numbers of the organism in reservoirs in the environment and (c) preventing access to susceptible hosts.

(a) Staphylococcal lesions must be carefully treated and covered with impervious dressings. In hospital, strict aseptic techniques in dressing lesions and isolation nursing should be carried out wherever possible.

Staphylococci growing at carrier sites, e.g. the anterior nares, may be suppressed by applying antibacterial creams containing antibiotics such as neomycin or bacitracin, or antiseptics such as chlorhexidine or hexachlorophene. This eliminates or reduces contamination of the carrier's skin, handkerchief and clothing and reduces dissemination to the environment. Frequent washing of the hands with antiseptic soaps reduces spread by contact.

(b) The number of staphylococci in the environmental reservoirs may be controlled by means designed to reduce dust—oiling of floors and blankets with spindle oil, use of cotton blankets which can be boiled, damp dusting and sweeping or use of vacuum

cleaners, cleaning of surfaces with disinfectants. Surgical theatres can be equipped with efficient air filtration and positive-pressure ventilation systems. Ultra-violet light may be used to reduce bacterial counts around the operation site.

(c) Patients known to be susceptible to infection with *Staph. aureus* (influenza, chronic bronchitis) should be nursed away from likely sources of the organism. The administration of antibiotics frequently renders patients more susceptible to colonisation or infection with antibiotic-resistant strains. The use of antitoxin is generally thought to be of little value.

Immunisation.—For the treatment of chronic or recurrent staphylococcal infections, stock and autogenous vaccines have been extensively applied in the past, but with variable success. Staphylococcal toxoid has also been advocated for immunisation in such cases, again with variable results: it has proved useful in the treatment of pustular acne.

STAPHYLOCOCCUS EPIDERMIDIS

(*Staphylococcus albus*)

Morphologically and culturally this organism is similar to *Staphylococcus aureus*. Colonies are porcelain white or creamy in colour. It does not produce coagulase and is best distinguished from *Staph. pyogenes* var. *albus* in this way.

This organism is much less active than *Staph. aureus* in its liquefaction of gelatin and fermentation of sugars; on blood-agar colonies may or may not show zones of haemolysis. Most strains fail to produce any haemolysin.

It occurs as part of the normal flora of the skin. Whilst regarded as non-pathogenic, it has been reported in lesions such as acne pustules and "stitch" abscesses, and rarely in more serious lesions, e.g. subacute bacterial endocarditis.

Staphylococcus citreus.—A relatively uncommon and mainly saprophytic type of organism; does not liquefy gelatin, is coagulase-negative and is distinguished by its lemon-yellow colour.

OTHER GRAM-POSITIVE COCCI

A great variety of these occur as saprophytes in nature and may be met with often as contaminants of plate-cultures, being derived from air and dust. Some resemble staphylococci in morphology, others appear in the form of tetrads or packets of eight (*Sarcinae*).

Colonies of growth resemble those of the staphylococci and some species are chromogenic, producing yellow, orange or red growths. They do not produce coagulase and are non-toxigenic. Examples of this group are *Sarcina lutea*, characterised by its yellow growths, and *Micrococcus ureae* which is found as a contaminant in urine and converts urea to ammonium carbonate; it is non-chromogenic. For the classification of staphylococci and related organisms see Shaw *et al.*¹

GAFFKYA TETRAGENA

(*Micrococcus tetragenus*)

Morphology and Staining.—Gram-positive spherical coccii in tetrads, each cell being about $1\text{ }\mu$ in diameter, and capsulated when in tissues.

Cultural Characters.—Aerobe and facultative anaerobe. Optimum temperature $37^\circ\text{ C}.$; grows well on ordinary media and colonies resemble those of *Staph. epidermidis*. Gelatin is not liquefied.

Occurrence.—It is a commensal of the mucosa of the upper respiratory tract and can be isolated from suppurative lesions of the mouth, neck and respiratory tract, e.g. dental abscesses, cervical adenitis, pulmonary abscess and rarely endocarditis.

Cultures of *Gaffkya tetragena* are often pathogenic to the mouse producing a generalised infection. Thus it may sometimes be isolated from mixed cultures by injecting this animal. Rabbits and guinea-pigs show only localised lesions.

¹ Shaw, C., Stitt, J. M., & Cowan, S. T. (1951), *J. gen. Microbiol.*, 5, 1010.

CHAPTER XX

STREPTOCOCCI: PNEUMOCOCCI

BACTERIOLOGICAL DIAGNOSIS OF PYOGENIC INFECTIONS

STREPTOCOCCI are Gram-positive, spherical or oval cells arranged in pairs or chains of varying length; each cell is approximately $1\text{ }\mu$ in diameter, non-motile, non-sporing and may be capsulate.

Classification of the *Streptococcus* family presents difficulties. The majority are aerobes or facultative anaerobes, but there are species which are anaerobic or micro-aerophilic. The aerobes may first be divided into those which produce a soluble haemolysin and those that do not. The first of these groups usually produces a clear zone of haemolysis on fresh blood-agar—*beta* (β) *haemolytic*—and includes most of the species associated with primary streptococcal infections in man and animals. They can be subdivided into broad groups according to the chemical nature of the carbohydrate (or C antigen) contained in the body of the organism (Lancefield groups). Strains which belong to Lancefield's group A are responsible for over 90 per cent. of human streptococcal infections and they can be further divided into Griffith types according to their surface protein antigens (M, T and R). The non-haemolytic varieties may be divided into two broad categories according to their effect on blood-agar or heated blood-agar. Those that produce a greenish pigmentation with a narrow zone of partial haemolysis are called *alpha* (α) *haemolytic* or *Streptococcus viridans* and it must be understood that these strains do not produce a soluble haemolysin. Those without effect on the blood-containing medium may be called *an-haemolytic* or *gamma* (γ) type streptococci and include the faecal streptococci (*Streptococcus faecalis*). While most of the Lancefield group A streptococci (*Streptococcus pyogenes*) produce β -*haemolysis*, some variants are non-haemolytic. Conversely, a variant of *Strept. faecalis* (Lancefield group D) may be actively haemolytic on blood-agar, although it does not produce a soluble haemolysin.

STREPTOCOCCUS PYOGENES (GROUP A STREPTOCOCCUS)

Morphology and Staining.—As above.

Cultural Characters.—Aerobe and facultative anaerobe;

temperature range, 22°–42° C.; optimum 37° C.; grows on ordinary media but better on serum or blood-containing media.

Blood agar—colonies are 0·5–1 mm. in diameter after twenty-four hours' incubation, circular, discrete, semi-transparent, low-convex disks, showing β -haemolysis on fresh blood-agar plates. Virulent strains isolated from lesions give a matt type of colony, whereas avirulent strains produce glossy colonies; a mucoid colony type is also encountered and corresponds in virulence to the matt type.

Viability.—The thermal death-point is about 54° C. for half an hour. The organism can survive for days in dust, particularly if protected from daylight; laboratory cultures do not survive for long unless stored at refrigerator temperatures, preferably in blood broth or cooked-meat medium. Like staphylococci, *Strept. pyogenes* is highly sensitive to the antiseptic dyes, e.g. proflavine, but is more resistant than staphylococci to crystal violet; it is sensitive to sulphonamides and to a wide range of antibiotics. It is more sensitive to bacitracin than other haemolytic streptococci and this phenomenon can be used for preliminary grouping (p. 485).

Biochemical Reactions.—These have, in the past, been extensively used for the differentiation of β -haemolytic streptococci but have largely been replaced by serological procedures; certain reactions can be employed to recognise species within a particular serological group (p. 489).

Antigenic Characters.— β -haemolytic streptococci can be allocated to one of 15 serological groups (A–Q; no groups designated I or J) based on the group-specific carbohydrate antigens, present as structural components of the cell wall; these can be extracted in soluble form and identified by precipitation reactions with the corresponding antisera. The groups are, in general, related to various animal hosts (p. 489); the majority of strains from man belong to group A.

Group A streptococci also possess one or more additional antigens (M, T, R) of a protein nature. M antigens are type-specific, located at or near the cell-surface whence they can be removed with trypsin without destroying the organism; they resist heating at low pH, e.g. pH 2 for thirty minutes at 100° C. M antigens occur in organisms producing matt or mucoid colonies but are absent from glossy, avirulent colonies.

T antigens, so-called because they were originally considered to be type-specific, frequently occur along with M antigens, but they are distributed independently of the latter and are not type-specific. Unlike M antigens, the T antigens resist digestion by proteolytic enzymes and are destroyed by heating at an acid pH;

they occur in avirulent as well as in virulent strains and can be detected in intact streptococci by means of agglutinating antisera.

R antigen was long regarded as a unique, type-specific M protein characterising type-28 strains; but a similar, serologically distinct, R antigen occurs in serotype-3 strains; the importance of R antigens lies in their liability to confuse type identification.

The serological techniques involved in group and type identification of *Strept. pyogenes* are dealt with on p. 495.

Virulence.—Recently isolated cultures are usually virulent to rabbits, mice and guinea-pigs, producing local inflammatory and suppurative lesions on subcutaneous inoculation; intravenous injection usually results in septicaemia with the formation of multiple pyaemic abscesses if the animal survives. The pathogenicity of group A streptococci is directly related to the M protein content rather than to specific exotoxins (*vide infra*), since the latter cannot be effective until the organism has successfully established itself in the host tissues.

Serum containing M antibodies has a protective effect against infection with the homologous serotype; T and R antigens have no known relationship to virulence and their antibodies have no protective influence in experimental infections.

The virulence of group C streptococci for mice is related to the production of capsules composed of hyaluronic acid; the injection of hyaluronidase protects mice against 1,000–100,000 M.L.D. of such strains, whereas it has very little protective value against experimental infection with group A streptococci. Yet the hyaluronic acid of the capsules of strains belonging to groups A and C is chemically identical, and equally large capsules, composed of this polysaccharide, are formed by streptococci of both groups.

In addition to the cellular constituents mentioned above, *Strept. pyogenes* produces several exotoxins. Two distinct haemolysins can be recognised; O-streptolysin which is oxygen-labile but can be reactivated by reducing agents and is produced in serum-free broth; and S-streptolysin, which is not oxygen sensitive and not produced in serum-free broth. These two streptolysins are antigenically distinct and both are toxic to animals.

Leucocidal activity can be demonstrated *in vitro* with culture filtrates of *Strept. pyogenes*; streptococcal leucocidin may be identical with the O-streptolysin.

Fibrinolysin (streptokinase) is present in culture filtrates of recently isolated strains and causes rapid lysis of human fibrin *in vitro*.

Hyaluronidase is produced by many strains; this product gives
2H

increased permeability of tissues by hydrolysing hyaluronic acid, which forms the cement substance in tissues.

Erythrogenic toxin is so designated because it produces an erythema when injected intradermally in susceptible persons or animals. The role of this toxin in scarlet fever is dealt with below.

Occurrence.—*Strept. pyogenes* is the principal aetiologic agent in tonsillitis and scarlet fever and the organisms can be isolated in large numbers from the primary site; on recovery, the patient may continue to harbour the organisms for varying periods. A feature of streptococcal infection is its tendency to spread locally to neighbouring tissues, e.g. from the throat to the middle ear, or by the lymphatics to regional lymph glands. *Strept. pyogenes* also causes primary skin infections (impetigo, erysipelas) and occurs in infected wounds (cellulitis) and burns, puerperal sepsis, localised abscesses and in suppurative adenitis, otitis, mastoiditis, arthritis, etc. Healthy individuals may act as carriers of *Strept. pyogenes*, the site of carriage commonly being the throat and less frequently the nose; from these sites the carrier suffers extensive contamination of his body and clothing particularly if he is a nasal carrier.

Strept. pyogenes is also causally related to acute rheumatism and acute glomerulo-nephritis.

Scarlet Fever (Scarlatina)

Group A streptococci of any serotype may cause this disease provided that the strain produces erythrogenic toxin and the host lacks immunity to the latter. The site of infection is usually in the upper respiratory tract, most frequently on the tonsils; the terms puerperal and surgical scarlet fever are employed when the primary lesion is in the puerperal uterus or in a wound or burn.

The *Dick Test* is a biological test which determines the immune status of an individual to erythrogenic toxin; it is performed by injecting intradermally, 0·2 ml. of a standardised preparation of erythrogenic toxin (*Dick Test Toxin*) in the flexor surface of one forearm and, as a control, a similar volume of the same material previously heated to destroy the erythrogenic toxin (*Dick Control Fluid*) may be injected into the skin of the other forearm.

In a *positive* (susceptible) reaction, an erythematous area at least 1 cm. in diameter appears at the site of the test toxin injection within six to sixteen hours and begins to fade in the next twenty-four hours; the control fluid injection site shows no response.

Dick testing was employed particularly to test the immune

status of nursing and medical attendants in scarlet fever units as a preliminary to active immunisation of positive reactors with erythrogenic toxin. This latter procedure is no longer undertaken and Dick testing is rarely performed (p. 489).

Schultz-Charlton Reaction.—This reaction is occasionally employed clinically to assist diagnosis in doubtful cases of scarlet fever. It was originally performed with serum from convalescent cases of scarlet fever; such serum, containing erythrogenic antitoxin, when injected intradermally in a patient with a scarlatinal rash causes a local blanching or extinction of the rash within six to eighteen hours. In practice, the test is now performed with erythrogenic antitoxin obtained from animals which have been actively immunised with erythrogenic toxin.

Acute Rheumatism

The aetiologic relationship of group A streptococcal infections to acute rheumatism was first postulated on *clinical* grounds more than sixty years ago. Early attempts to isolate such strains from the blood stream and affected tissues did not succeed, but confirmation of the relationship was obtained from *epidemiologic* studies; further evidence implicating *Strept. pyogenes* has accrued from recent bacteriologic, serologic and chemotherapeutic investigations.

The use of appropriate antibiotics for the prevention of recurrences in rheumatic subjects and the elimination of acute rheumatism as a sequel to streptococcal infections in non-rheumatic individuals has afforded the most convincing evidence of the part played by *Strept. pyogenes* in inciting acute rheumatism (p. 489).

Acute Glomerulo-nephritis

Unlike other diseases caused by group A streptococci, *nephritis* is associated with only a few specific serotypes, namely types 12, 25 and 4; type-12 strains are the commonest of these nephritogenic types, both in sporadic cases and in epidemics of nephritis. Experimental studies indicate that the nephritogenic agent produced by type-12 strains is of a polypeptide nature and when administered intravenously in low dosage to rabbits produces the clinical picture of nephritis; histologic examination of kidney tissue from these animals shows lesions characteristic of the disease.^{1, 2}

Epidemiology.—The factors influencing the spread of infection

¹ Reed, R. W., & Matheson, B. H. (1954), *J. infect. Dis.*, **95**, 191.

² Matheson, B. H., & Reed, R. W. (1959), *J. infect. Dis.*, **104**, 213.

caused by *Strept. pyogenes* are numerous; certain bacterial factors which determine the virulence of a strain have already been mentioned (p. 481).

Sources of infection are cases of any one of the recognised clinical illnesses caused by *Strept. pyogenes* and also carriers; the modes of spread are similar to those of other organisms discharged principally from the upper respiratory tract, namely, *direct contact* which includes spread by large droplets projected directly from one individual to another as well as by more intimate associations, *airborne* spread by dust and indirect spread by fomites (books, toys, etc.).

Carriers are not all equally dangerous to the community; the factors determining the importance of a carrier are: *Age*—children are more likely to transmit infection than are adults, probably because they have greater opportunities to infect susceptible contacts at school, etc.; *duration of carriage*—chronic carriers are less commonly the source of new infections than are convalescent carriers; perhaps increasing duration of carriage is associated with reduced M protein production and consequently virulence of the strain; *location of the organisms*—although nasal carriers of *Strept. pyogenes* are less common than throat carriers, they are much more dangerous because of the large numbers of streptococci they disseminate in the environment.

Factors affecting host susceptibility are:

Age.—School-age children experience a high incidence of infection, probably due to increased exposure but perhaps also because they are more susceptible.

Pre-existing Disease.—Patients suffering from virus respiratory infections (measles, influenza, etc.) are particularly liable to secondary streptococcal infections, including scarlet fever.

Familial Susceptibility.—Acute rheumatism is not infrequently seen in particular families; whether this is due to environmental and/or hereditary influences is still not clear. It has been shown that, in comparison with a rheumatic-free group of children a similar group with rheumatic fever contained a significantly higher proportion of Lewis^a secretors. Prospective studies should reveal whether such individuals, i.e. non-secretors of A. B. H. blood-group substances are more prone to acute rheumatism.

Previous Infection with Group A Streptococci.—Recovery from such infection is accompanied by the formation of M antibodies which are type-specific and long-lasting; re-infection with a strain of identical serotype is unusual.

Laboratory Diagnosis.—In taking a throat swab from a suspect case of tonsillitis it is essential that a spatula be employed so that

after inspection the affected area can be swabbed accurately and without unnecessary contamination from the buccal cavity; swabbing should not be undertaken within six hours of gargling with antiseptics.

Serum-coated swabs are distinctly advantageous in ensuring the survival of *Strept. pyogenes*¹; the swab should be transmitted to the laboratory promptly, and if a delay of twelve or more hours is expected, the swab should be stabbed into a tube of modified Pike medium (p. 228).

Gram-stained films of throat swabs are of no value in diagnosis, since the commensal streptococcal flora is indistinguishable from *Strept. pyogenes* in such preparations; on the other hand, a direct smear from swabs of burns, pus, etc., is worth making, provided that any conclusions resulting from its examination are regarded as tentative.

As well as attempting isolation of *Strept. pyogenes* from the throat swab, media designed to selectively isolate *C. diphtheriae* may be used and also the preparation and staining of a film to exclude Vincent's angina.

The medium recommended for the isolation of *Strept. pyogenes* is crystal-violet blood agar (C.V.B.A.) (blood agar containing a 1 in 500,000 or 1 in 1,000,000 concentration of crystal violet added from a 1 in 10,000 stock solution); plates of this medium should be incubated anaerobically as well as aerobically. A disk impregnated with bacitracin should be placed in the well-inoculum of the plates before incubation to ensure rapid recognition of group A strains.²

If group identification of β -haemolytic colonies on the C.V.B.A. is not contemplated, then Gram-stained film preparations must be examined to ensure that the organisms are Gram-positive cocci; not dissimilar colonies are given by haemolytic haemophili and certain corynebacteria.

Serologic Identification of β -haemolytic Streptococci. Lancefield Grouping.—Extraction of group-specific polysaccharide can be undertaken by one of the following methods:

(1) *Acid extraction (Lancefield)*

The centrifuged deposit from 50 ml. of overnight culture in Todd-Hewitt broth (p. 192) is harvested in a $3 \times \frac{1}{2}$ in. test-tube. The deposit is thoroughly re-suspended in 0·4 ml. of 0·2 N HCl. Place tube in a boiling water-bath. After ten minutes' exposure, remove tubes from bath and allow to cool. Add 1 drop of 0·02 per cent. phenol red.

¹ Rubbo, S. D., & Benjamin, M. (1951), *Brit. med. J.*, 1, 982.

² Maxted, W. R. (1953), *J. clin. Path.*, 6, 224.

Neutralise carefully with 0·5 N and 0·2 N NaOH. The clear supernatant obtained by centrifugation is the extract.

(2) *Formamide extraction* (Fuller)

The centrifuged deposit from 5 ml. of overnight culture in Todd-Hewitt broth is re-suspended in 0·1 ml. of formamide in a $3 \times \frac{1}{2}$ in. test-tube. Place tube in oil-bath at 160° C. for fifteen minutes; centrifuge and discard any deposit. Mix supernatant with 0·25 ml. of acid-alcohol and centrifuge. 0·5 ml. of acetone is added to the new supernatant and the precipitate obtained by further centrifugation is dissolved in 0·4 ml. of saline. Add 1 drop of phenol red and neutralise with 0·2 N NaOH.

(3) *Enzyme extraction* (Maxted)

Suspend a loopful of growth from an eighteen-hour blood-agar culture in 0·25 ml. enzyme solution (see below) contained in a flocculation tube. Place tube in 50° C. water-bath. Inspect tube at one, one and a half and two hour periods, and when contents are clear, use as extract.

The enzyme is produced from a *Streptomyces albus*¹ growing in the following medium:^{2, 3}

NaCl	5 g.
K ₂ HPO ₄	2 g.
MgSO ₄ , 7H ₂ O	1 g.
CaCl ₂	0·04 g.
FeSO ₄ , 7H ₂ O	0·02 g.
ZnSO ₄ , 7H ₂ O	0·01 g.
Yeastrel	5 g.
Agar powder	11 g.
Distilled water	1 l.

Place suitable amounts (75–100 ml.) in Roux bottles, sterilise and add aseptically glucose and casamino acids, each in a final concentration of 0·5 per cent. pH should be 7·0–7·4.

(1) Inoculate the surface of above medium by flooding with *Streptomyces albus* glucose-broth culture.

(2) Incubate at 30–37° C. for four to five days.

(3) Place Roux bottles in a –10° C. refrigerator and then allow to thaw out; the fluid expressed on thawing is the enzyme solution.

(4) Adjust pH to 7·5 by adding 1 N HCl; filter through a Seitz disk.

(5) Test for potency of the filtrate by adding 0·1 ml. of a heavy suspension of heat-killed group A streptococci to 0·4 ml. of the enzyme preparation. Place in a 50° C. water-bath along with a tube containing a control mixture in which the enzyme has been destroyed by heating. An active preparation will lyse the streptococcal suspension in one-half to one hour.

¹ Obtainable from N. C.T.C., Colindale Avenue, London.

² Maxted, W. R. (1948), *Lancet*, ii, 255.

³ McCarty, M. (1952), *J. exp. Med.*, 96, 555.

(6) Stored in the cold with 0·5 per cent. phenol as preservative, the preparation keeps well and is active over a pH range of 5·6–9·6.

The enzyme extraction method is reliable for streptococci in groups A, C or G; for other groups the acid or formamide methods are preferred, the latter being less likely to give minor cross-reactions occasionally encountered with acid extracts. Group O polysaccharide is sensitive to formamide so that such extracts do not react with O antiserum. Acid extracts can also be used for identification of type-specific M antigens.

*Precipitation Test for Grouping of *Strept. pyogenes*.*—This may be performed in the narrowing neck of small Pasteur pipettes. A small volume of group A antiserum is placed in the pipette and the antigenic extract carefully superimposed. If the extract contains polysaccharide specific for group A, then precipitation will be observed at the interface with the serum within five minutes; reactions appearing after this time should not be regarded as positive. Extracts should also be tested with antisera for groups C and G routinely, and if necessary with other group sera.

In order to conserve serum, tests may be performed in capillary tubes; a $\frac{1}{2}$ -in. column of serum is run into the tube, the exterior of which is carefully wiped before an equivalent volume of antigen extract is introduced. The contents are allowed to run well up the tube and the upper end is then occluded with the forefinger until the tube has been placed in a plasticine block. Macroscopic precipitation should be evident within the time limits stated above if the reaction is positive.

Type Identification of Group A Strains.—All strains should be tested for type both by agglutinating (T) and precipitating (M) antisera, since a smaller percentage of strains will thus be regarded as untypable than when either method is employed alone.¹

Slide agglutination test.—The strain is grown in 5 ml. of Todd-Hewitt broth (p. 192) at 28° C. for eighteen to twenty-four hours and the centrifuged deposit thoroughly resuspended in 0·5 ml. of supernatant broth. Provided that the suspension is not granular, 6 loopfuls are placed on a clean glass slide and each then mixed with a small (1 mm.) loopful of pooled antisera and the slide rocked to and fro for one minute. Agglutination may be noted with one of the pool antisera and fresh loopfuls of suspension should then similarly be tested with all the specific sera comprising that particular pool. Strains may react in more than one type-specific serum, but the pattern of such reactions is epidemiologically significant.

¹ Williams, R. E. O., & Maxted, W. R. (1953), *Congr. int. Microbiol. 6th Congr. Rome*, 1, 46.

Granular suspensions and those that react with many sera should be treated as follows: Add 1 drop of B.D.H. Universal Indicator and 2 drops of pancreatic extract (p. 192) to the suspension; adjust the pH to 8-8·5 with 0·2 N NaOH and place in 37° C. water-bath for one hour, shaking the tubes every fifteen minutes. On re-testing with pooled and specific antisera as above, many such strains will react normally; if results are still unsatisfactory, a further period of fifteen minutes in a 50° C. bath may be tried.

Precipitation test.—Acid extracted antigen (p. 485) prepared for group determination is used. Using the results of slide agglutination as a guide, the extract is tested against the relevant antisera by the capillary tube method (p. 485). The mixtures are incubated for two hours at 37° C. and results noted; after overnight refrigeration the tubes are again examined.

β -haemolytic streptococci of groups other than group A are only occasionally incriminated as human pathogens; such strains belong almost invariably to groups C, G, B and, in the case of urinary tract infections, to group D.

Group C.—Predominantly animal parasites; 4 biochemical types are recognised (see table, p. 491) and that designated *Strept. equisimilis* is most commonly associated with human disease. It has been found most often in puerperal infection, but has been isolated from cases of cellulitis, tonsillitis, wounds and scarlet fever.¹

Group G.—The majority of strains have been found as commensals in the human subject; its pathogenic role is virtually restricted to puerperal infections; has been responsible for epidemics of canine tonsillitis.

Group B.—Colonies on blood agar do not produce such marked β -haemolysis as do group A strains. Some strains give α - or γ -haemolysis. Most often associated with bovine mastitis (p. 491), but is encountered as a commensal in the human vagina and throat. Only rarely pathogenic to the human subject, but has been recorded in a few cases of puerperal infection, including cases of ulcerative endocarditis. Group B streptococci correspond to the organism designated *Strept. agalactiae*.

Group D.—Originally described as β -haemolytic. Such strains were isolated from human faeces and the vagina, and their relationship to the enterococcus was recognised. The group includes strains which are devoid of haemolytic activity; they may be classified according to their biochemical activities as shown in the following table.

¹ Hutchinson, R. I. (1946), *Brit. med. J.*, 2, 575.

Biochemical "types" of Group D streptococci

Type	Sorbitol	Arabinose	Gelatin Liquefaction	Growth at pH 9·6	Haemolysis on Horse-blood Agar
<i>Strept. faecalis</i> var. <i>faecalis</i>	A	—	—	+	—
var. <i>liquefaciens</i>	A	—	+	+	—
var. <i>zymogenes</i>	A	—	+	+	β
<i>Strept. faecium</i> .	—	A	—	+	α
<i>Strept. durans</i> .	—	—	—	—	α or β
<i>Strept. bovis</i> .	—	A	—	—	α

Key: Fermentation Reaction: A = acid produced; — = no fermentation.

Prophylaxis.—The spread of *Strept. pyogenes* from cases of disease can be limited by early penicillin therapy. In semi-closed communities measures aimed at reducing the streptococcal population of the environment, e.g. oiling of floors, bed-linen and patients' bed-wear, are successful, but only in certain circumstances is this reduction accompanied by a similar fall in morbidity rates.¹

In the past, persons at special risk, e.g. the staff of infectious disease units who were Dick-positive reactors were actively immunised with erythrogenic toxin; this is no longer advocated, not only because of the multiple injections required and the frequency of their side-effects but also since the protection thus afforded was antitoxic and did not reduce the incidence of infection with *Strept. pyogenes*. Prompt eradication of *Strept. pyogenes* from cases being treated in hospital is the most effective way of controlling infection in nurses and other susceptible contacts.²

Chemoprophylaxis with sulphonamides was practised during the Second World War, but the emergence of sulphonamide-resistant strains discourages such a practice.

Tonsillectomy has no prophylactic value either in reducing *Strept. pyogenes* infections in the upper respiratory tract or in protecting rheumatic subjects against recurrences.

Prevention of Acute Rheumatism.—The prompt and effective treatment of streptococcal sore throat with penicillin eliminates the risk of acute rheumatic sequelae. Therapy should be instituted as soon as the diagnosis has been confirmed and preferably within

¹ Wright, J., Cruickshank, R., & Gunn, W. (1944), *Brit. med. J.*, **1**, 611.

² Jersild, T. (1959), *Rheumatic Fever*, p. 58. Oxford, Blackwell.

seven days of onset; penicillin therapy should, however, still be undertaken even if a case does not come under medical care until this period has elapsed.¹ Sulphonamides should *not* be used in treatment of streptococcal infections, since they are bacteriostatic and the organisms are not eradicated. It is essential that adequate blood-levels of penicillin should be maintained for seven to ten days, even although the patient has fully recovered from the infection.

It is recommended that persons with a previous history of acute rheumatism should be protected against streptococcal infection by continuous prophylaxis.² Provided that the person is initially free of *Strept. pyogenes*, sulphonamides may be employed in small daily doses. Penicillin, preferably a long-acting preparation given intramuscularly, is a better agent for long-term prophylaxis in such cases. Not only is there 100 per cent. reduction in recurrence rate of acute rheumatism compared with an 85 per cent. reduction in those receiving sulphonamides, but monthly intramuscular administration ensures regular medical supervision and eliminates the possibility of interrupted prophylaxis associated with oral administration. Alternatively, daily doses of oral penicillin (200,000 units) may be given, but control of medication is less certain and a break in prophylaxis need only be brief for the subject to be again at risk.

Community control of streptococcal infection is being undertaken in some areas, with a view to preventing first attacks of rheumatic fever. So far such projects have been restricted to the U.S.A.^{3,4}

Prevention of Acute Glomerulo-nephritis.—From published evidence it would appear that the prophylactic value of treating the primary streptococcal illness with penicillin is not so dramatic as in the case of rheumatic sequelae; even with adequate dosage of penicillin given in the primary infection the incidence of acute nephritis was reduced by only 60 per cent.⁵

The Occurrence of *Streptococci* in Diseases of Domesticated Animals

Streptococci are relatively infrequent in suppurative lesions of sheep and swine, but are not uncommonly associated with mastitis

¹ Catanzaro, F. J., et al. (1954), *Amer. J. Med.*, **17**, 749.

² Report. *World Hlth Org. techn. Rep. Ser.* (1957), 126.

³ Bunn, W. H., & Bennett, H. N. (1955), *J. Amer. med. Ass.*, **157**, 986.

⁴ Phibbs, B., et al. (1958), *J. Amer. med. Ass.*, **166**, 1113, 1120.

⁵ Rammelkamp, C. H. (1955), *Ann. intern. Med.*, **43**, 511.

of cattle and with strangles and contagious pleuropneumonia of horses.

Bovine Mastitis

Streptococci are frequently found as the sole organism, especially in chronic mastitis (*Strept. agalactiae*); such streptococci vary in their action on blood agar and may exhibit α - or β -haemolysis or none. They belong to Lancefield group B and identification with group-specific antiserum has replaced fairly extensive biochemical testing.

Another streptococcus occurring in bovine mastitis is designated *Strept. dysgalactiae* and is non-haemolytic; serologically it belongs to group C; biochemical differentiation of *Strept. dysgalactiae* from group C strains associated with other hosts may be undertaken as in the table.

	Lactose	Trehalose	Sorbitol
<i>Strept. dysgalactiae</i> . . .	+	+	v
<i>Strept. equi</i> (horses) . . .	-	-	-
<i>Strept. equisimilis</i> (human) . .	v	+	-
<i>Strept. zooepidemicus</i> (animals) .	+	-	+

Key: + = acid produced; v = acid production variable; - = no reaction.

Laboratory Diagnosis of Bovine Mastitis.—Centrifuged deposits from milk samples are plated out on a medium comprising 2 ml. 0.1 per cent. crystal violet, 1 g. aesculin and 50 ml. defibrinated ox-blood in 1 l. of Lemco agar (pH 7.4). Many of the other organisms present in milk are inhibited by the crystal-violet and these which are dye-resistant usually produce black colonies in the presence of aesculin and can be readily differentiated from streptococci. Pure cultures of any streptococci isolated are obtained and their serological group determined; if the strain belongs to group C it can be biochemically identified as in the table.

Streptococcus viridans

Morphology and Staining.—Similar to *Strept. pyogenes*; no significance should be attached to length of chains in differentiation.

Cultural Characters.—Essentially similar to *Strept. pyogenes* in cultural requirements.

Blood agar.—Colonies tend to be smaller and more convex than those of *Strept. pyogenes* and produce distinctive changes in blood

agar; surrounding the colonies there is a zone of partial haemolysis and greenish discolouration with, often, a thin outer rim of complete lysis, especially in cultures stored in the refrigerator after incubation.

Viability.—Thermal death point is approximately 55° C. for half an hour. Survival in nature and in laboratory cultures is similar to that of *Strept. pyogenes*.

Biochemical Reactions.—These have been studied in an endeavour to differentiate species within the group but with little success. From the medical viewpoint the value of such reactions lies in the differentiation of *Strept. viridans* from pneumococci; the relative rarity with which *Strept. viridans* ferments inulin has been used for such purposes but has been superseded by tests of bile solubility and optochin sensitivity (p. 495).

Antigenic Characters.—Apart from the fact that *Strept. viridans* do not possess carbohydrate group antigens like β -haemolytic streptococci, little is known of their antigenic structure. Several distinct serotypes have been recognised in strains isolated from healthy mouths and from cases of subacute bacterial endocarditis.¹

Occurrence.—*Strept. viridans* occurs in the throat and mouth secretions of virtually all persons and there leads a commensal existence; in individuals with predisposing cardiac lesions, e.g. rheumatic endocarditis and congenital defects, *Strept. viridans* is incriminated as the commonest cause of subacute bacterial endocarditis. This is an endogenous infection, the organisms gaining entry to the blood stream in subjects with poor dental hygiene, particularly during dental therapy even of a conservative nature. In the otherwise healthy individual the streptococci are rapidly eliminated from the blood stream, but in those with heart lesions of a congenital or rheumatic nature the organisms may settle in the defective valves.

Laboratory Diagnosis.—*Strept. viridans* is almost constantly present on blood-agar media inoculated from throat swabs and sputum; in such cases it is important to differentiate it from pneumococci (p. 495). In the diagnosis of subacute bacterial endocarditis, repeated blood culture (p. 260) should be undertaken; venepuncture should preferably be performed during pyrexial episodes and the blood inoculated into a good substrate broth medium which may contain saponin (p. 262) to prevent clotting. Growth appears after a few days' incubation; usually as small compact colonies on the surface of the blood layer, provided that the culture-bottle is left undisturbed.

Chemotherapy.—Penicillin remains the drug of choice in sub-

¹ Solowey, M. (1942), *J. exp. Med.*, **76**, 109.

acute bacterial endocarditis; provided that treatment is instituted early and continued for four to eight weeks, case fatality rates of 10–15 per cent. are obtainable in comparison with the invariably fatal outcome before penicillin was available.

Prophylaxis.—Individuals with congenital or other valvular cardiac defects should be given penicillin before having any dental attention and such protection continued for at least two days thereafter.

Streptococcus faecalis (Enterococcus)

Morphology and Staining.—Usually oval in shape and occur in pairs or short chains.

Cultural Characters.—Colonies are similar to those of *Strept. pyogenes*, but rarely is any change noted on blood-agar media.

MacConkey medium.—Colonies are minute, 0·5–1 mm., and magenta-coloured.

Viability.—Withstand exposure to 60° C. for thirty minutes, which kills other streptococci; similarly, *Strept. faecalis* has a much wider growth range, 10°–45° C., and has considerable viability in culture.

Biochemical Reactions.—In contrast to other aerobic streptococci, *Strept. faecalis* is capable of growing on media containing bile-salts (e.g. MacConkey's) and in the presence of 6·5 per cent. NaCl; fermentation of mannitol with gas production also differentiates enterococci from other streptococci.

Antigenic Characters.—Characteristically belong to sero-group D and biochemical types within the group can be recognised (p. 489).

Occurrence.—Lead an essentially commensal existence in the human and animal intestine but are not infrequently incriminated in urinary tract infections, sometimes alone but more often in association with *Esch. coli*, etc. They are also rarely causative organisms in subacute bacterial endocarditis.

Laboratory Diagnosis.—Routine plating of urine specimens on MacConkey's as well as blood-agar medium allows ready recognition of *Strept. faecalis*.

Anaerobic Streptococci

Streptococci which can only grow as obligate anaerobes have been recognised for several decades and the pathogenicity of some of these for man is undoubtedly; nevertheless, they have attracted relatively little attention and *Peptostreptococcus putridus* is the only well-documented species.

Morphology and Staining.—Gram-positive cocci resembling aerobic streptococci but frequently much smaller ($0.5\text{ }\mu$ or less) and exhibiting pleomorphism in artificial culture.

Cultural Characters.—After anaerobic incubation for forty-eight hours, colonies in blood agar are smooth, low-convex, approximately 1–2 mm. in diameter; no alteration occurs in the medium. Cultures, e.g. in meat-broth, usually give off an exceptionally foul odour.

Biochemical Reactions.—Attempts have been made to classify the anaerobic streptococci on the basis of such reactions; provided that a sulphur compound is present (e.g. 0.1 per cent. sodium thioglycollate) in the medium, *Pepto. putridus* strains ferment glucose, maltose and fructose with abundant gas production.

Occurrence.—It would appear that their normal habitat is the vagina; so far they have not been isolated from the upper respiratory tract, intestinal contents or the skin of healthy persons. *Pepto. putridus* is incriminated in puerperal sepsis, probably as an endogenous infection precipitated by trauma and the presence of necrotic material; it has also been isolated from brain abscess, infected wounds, e.g. post-operative synergistic bacterial gangrene and anaerobic streptococcal myositis.

Laboratory Diagnosis.—Strictly anaerobic methods are required if isolation is to be successful. Inoculation of blood-agar plates and incubation for forty-eight hours in a McIntosh and Fildes' jar produces colonies as described above; for details of the biochemical reactions of species other than *Pepto. putridus* the papers of Hare and his colleagues should be consulted.^{1, 2}

Chemotherapy.—Strains are sensitive to penicillin and it should be employed therapeutically.

DIPLOCOCCUS PNEUMONIAE

(*Pneumococcus*)

The causative organism of lobar pneumonia; also incriminated in catarrhal conditions of the upper respiratory tract, in conjunctivitis, otitis media, meningitis, peritonitis, arthritis, etc.

Morphology and Staining.—Characteristically appears as an oval or lanceolate Gram-positive coccus in pairs with the long axes in line with each other; approximately $1\text{ }\mu$ in its long diameter and capsulate. In culture the appearance is less typical, the cocci being more rounded and occurring in chains; capsulation is not so evident.

Cultural Characters.—Aerobe and facultative anaerobe;

¹ Hare, R., et al. (1952), *J. Hyg. (Lond.)*, 50, 295.

² Thomas, C. G. A., & Hare, R. (1954), *J. clin. Path.*, 7, 300.

temperature range 25°–40° C., optimum 37° C.; grows on ordinary media but best on blood or serum-enriched media. The addition of glucose (e.g. 0·1 per cent.) to culture media promotes growth and similarly cultivation in an atmosphere of 5 per cent. CO₂ is advantageous. It should be noted in the preparation of broth for the cultivation of pneumococci that they may be inhibited by an oxidised constituent of the peptone; this can be prevented by adding the peptone to the medium *before* heating so that it is later subjected to the reducing action of the meat infusion (p. 191); commercial peptones may also contain metallic impurities which are responsible for inhibitory effects on the growth of the pneumococcus.

Blood agar. Colonies are small (1 mm. in diameter), semi-transparent and are usually surrounded by a zone of *a*-haemolysis which may cause confusion with *Strept. viridans*; unlike the latter, the colonies are at first plateau-shaped and later develop elevated margins and concentric ridges—the so-called draughtsman colony. Green pigmentation is more obvious when the organism is growing on heated blood agar.

Viability.—Thermal death-point is about 52° C. for fifteen minutes. Ordinary laboratory cultures lose viability rapidly; cultivation in a semi-solid agar containing blood ensures longer survival and for maintenance of culture over long periods, rapid drying *in vacuo* eliminates the need for frequent subculture. Repeated *in vitro* cultivation leads to transformation from the smooth (S) to the rough (R) form, a change associated with loss of capsule formation, type-specificity and virulence.

Biochemical Reactions.—Ferments various carbohydrates and differs from the majority of *Strept. viridans* strains by frequently fermenting inulin; such tests may be performed in Hiss's serum-water (p. 208) or in the agar medium used for testing the biochemical reactions of gonococci (p. 210). There are other more specific tests for identification.

Bile Solubility.—Pneumococci are soluble in bile. The test consists of adding 1 part of a sterilised 10 per cent. solution of sodium taurocholate in normal saline to 10 parts of a broth culture. Alternatively, 0·1 ml. of a 10 per cent. solution of sodium desoxycholate may be added to 5 ml. of a broth culture which should not be more acid than pH 6·8; this method gives very satisfactory results, lysis occurring within fifteen minutes at 37° C.

Optochin Sensitivity.—Pneumococci are sensitive to optochin. Disks of filter paper, 8 mm. in diameter, sterilised by dry heat at 160° C. are impregnated with a 1 in 4000 aqueous solution of optochin (ethyl hydrocuprein hydrochloride), each disk containing

approximately 0·02 ml. The solution can be sterilised in the autoclave at 15 lb. pressure for thirty minutes without appreciable effect on its potency. Organisms are tested by making radial stroke cultures on a blood-agar plate, a disk being placed in the centre of the plate; a known sensitive strain is included in each set of tests. Pneumococci are inhibited in a zone of at least 5 mm. from the circumference of the disk, whereas strains of *Strept. viridans* grow up to the disk margin; occasionally a few colonies of pneumococci, resistant to optochin, will be noted in the zone of inhibition.

Antigenic Characters.—At least 77 specific serotypes of pneumococci have been recognised. Type-specificity is dependent on chemically specific polysaccharides contained in the capsule of the organism and type identification can be established by means of agglutination tests or by "capsule-swelling" reactions. For the latter technique, a loopful of broth culture or a saline suspension of growth from a blood-agar plate is mixed with a loopful of diagnostic antiserum and the mixture covered with a No. 1 cover-slip and examined with an oil-immersion lens, the substage condenser being suitably lowered and the diaphragm reduced in aperture. The enlarged delineation of the capsules, in the presence of type-specific antiserum, can be readily observed within one to two minutes; the sharpness of outline of the capsule is more significant than any apparent enlargement.

Typing sera usually contain methylene-blue so that the capsules remain unstained and present a ground-glass appearance; a set of sera may be purchased from The State Serum Institute, Copenhagen, and comprises 9 pooled sera (A-I) and 44 constituent specific sera; strains are first tested in the pooled sera and then in the type-specific sera comprising the pooled serum with which a reaction is obtained.

Virulence.—Pathologic material containing pneumococci (e.g. pneumonic sputum) or a young virulent culture, injected subcutaneously into rabbits or mice, produces a rapidly developing septicaemia and death in one to three days; at autopsy, typical capsulated diplococci are present in large numbers in the heart blood. The virulence for animals rapidly decreases if the organism is grown on media without blood or even on blood media if *in vitro* cultivation is prolonged; such avirulent strains no longer react with type-specific antiserum.

Occurrence.—In lobar pneumonia the pneumococcus is present, often in considerable numbers, in the consolidated areas and can easily be detected in the sputum. In a proportion of cases it can be demonstrated in the blood by blood culture, and also occurs

in the pathologic complications of pneumonia, e.g. empyema, peri- and endocarditis, meningitis, etc. Pneumococcal meningitis is often associated with middle ear infections or with traumatic or congenital defects in the skull, e.g. absence of cribriform plate; such cases tend to suffer recurrent attacks.

Epidemiology.—Lobar pneumonia is a communicable infection occurring particularly in the age range 10–50 years and caused predominantly by certain pneumococcus types (e.g. types 1, 2, 3, 5, 7, 14) which seem to be endowed with invasive properties; epidemics of lobar pneumonia have occurred in semi-closed communities (barracks, institutions, factories, etc.). The pneumococcus is essentially a human parasite and sources of infection are cases and carriers; carriers convalescing from lobar pneumonia may continue to harbour the organism for considerable periods, particularly if they are predisposed to chronic catarrhal pharyngitis or nasal sinusitis. Contact carriers, who have never suffered clinically apparent infection, may also become carriers of "epidemic" pneumococci.

Type-distribution studies have shown that, in Britain, types 1 and 2 are together responsible for more than 50 per cent. of cases of lobar pneumonia and type 3 strains are incriminated in less than 10 per cent. of cases.

Case-fatality rates in lobar pneumonia shows that type 3 is the most virulent and type 2 is more virulent than type 1. At the same time, types 1 and 2 are more invasive than other types, as shown by their prevalence in primary meningitis and peritonitis.

The modes of spread are similar to those of other organisms excreted from the respiratory tract, e.g. *Strept. pyogenes* (p. 483) and the incidence of lobar pneumonia is at its highest in the spring. Bronchopneumonia, in which pneumococci are commonly involved as secondary bacterial pathogens, occurs most often at the extremes of life or after primary virus respiratory infections (influenza, measles, etc.). The infecting pneumococci are those types found in the upper respiratory tract (e.g. types 6, 19, 23), and this is an endogenous, not a communicable, infection. Infections occur mostly in the winter months and are more common in economically poor communities. High case-fatality rates in the older age-groups occur in spite of therapeutic advances.

Laboratory Diagnosis.—A specimen of sputum is obtained and a mucopurulent portion used to inoculate a blood-agar plate; films stained by Gram's method are also prepared and examined to obtain an impression of the predominant bacterial flora.

Mouse Inoculation.—Intraperitoneal injection into mice of sputum or a young broth culture of a supralaryngeal swab is a

useful and reliable method for isolating pneumococci when they are scantily present.

On the blood-agar plate, characteristic colonies can be recognised among the other organisms that are frequently present; bile-solubility and/or optochin sensitivity tests may be performed as confirmatory procedures.

The need for serotyping of strains as a preliminary to giving specific therapeutic antiserum disappeared with the introduction of sulphonamide therapy, and type identification is now only performed in epidemiologic investigations.

Chemotherapy.—Most pneumococcal infections are amenable to chemotherapy with sulphonamides, penicillin, tetracyclines. In cases of pneumococcal meningitis, penicillin must be given intrathecally as well as systemically.

Prophylaxis.—The prophylaxis of lobar pneumonia has been attempted by the use of combined vaccines of the prevalent pneumococcus types in circumstances where there is a high incidence of infection, e.g. among native labourers in the South African mines, where the results were disappointing, and in Army camps, where more encouraging results have been obtained. In particular, a controlled trial during the Second World War of a combined antigen of purified polysaccharides prepared from four of the main epidemic types (types 1, 2, 5 and 7) indicated that a high degree of protection could be obtained against infection with these types after a single injection of 0.06 mg. of each of the polysaccharides.¹ But lobar pneumonia is a sporadic infection in civilian communities, where prophylactic vaccination would not be a practicable procedure.

Since a large proportion of pneumococcal infections supervene on antecedent virus respiratory infections, measures for the control of these virus infections, e.g. prophylactic vaccination against influenza, particularly in elderly persons with chronic chest and heart disease, may help to reduce the morbidity and mortality of secondary pneumonias. Improvements in social and environmental conditions and protection against sudden changes in climate will contribute to the control of bronchopneumonia in young children.

BACTERIOLOGICAL DIAGNOSIS OF PYOGENIC INFECTIONS

Collection of Specimens and Microscopic Examination.—Films are made on microscope slides from the pus or inflammatory exudate,

¹ Hedges, R. G., & McLeod, C. M. (1946), *Amer. J. Hyg.*, 44, 183.

dried and fixed by heat. The films should, if possible, be made directly from the lesion, a sterile wire loop or sterile capillary pipette being used to collect the exudate and transfer it to the slides. For transmission to the laboratory the material is placed (by means of a pipette) in a suitable sterile container. If only a minute amount of exudate can be obtained for transmission to the laboratory, a convenient method is to collect it in sterile capillary tubes, the ends of which are then sealed in a flame. The exudate runs into the tube by capillarity when one end is dipped into it. Swabs are very convenient for collecting specimens of exudate, but drying of the material may occur if there is much delay in transmission to the laboratory. In the case of ulcers, sinuses, etc., exudate may be taken with a sterile wire loop and films and cultures made at once; or the exudate is collected in capillary tubes or on swabs.

A simple and effective method of sampling the flora on a wound surface is based on the replica plating technique of Lederberg, and employs pads of sterile velvet or other suitable material to transfer the bacteria from the wound to the culture plate.¹ Circles 3 in. in diameter are cut from rigid aluminium sheet. On one face a 4-in. rod of aluminium is fixed to serve as a handle. On the other face is stuck a disk of furnishing velvet, using a suitable adhesive (*e.g.* "Evostick"; Evode Ltd., Stafford, England). The surface of the velvet is protected by covering with a Petri dish and the whole wrapped in paper and sterilised in the hot oven. The sterile pad is gently applied to the wound area and the charged pad is then printed at once on a suitable range of culture plates. It is important that the texture of the velvet remains soft and uncaked.

In the case of *pleural and peritoneal fluids*, the fluid should be withdrawn into a 1-oz. screw-capped bottle containing 4 drops of 20 per cent. sodium citrate solution in order to avoid coagulation, which renders cytological and bacteriological examination difficult. The material is centrifuged and films are made from the deposit.

Satisfactory specimens of *urine* may be obtained from the male by cleansing the urinary meatus, and, after a portion of the urine has been voided, collecting a sample directly into a sterile 8-oz. screw-capped bottle (mid-stream specimen). The collection of mid-stream urine from the female is more difficult, and much greater care is required to avoid contamination.² In all cases the collection of urine without catheterisation is preferable as it avoids

¹ Gorrill, R. H., & Penikett, E. J. K. (1957), *Lancet*, **ii**, 370.

² Murdoch, J. McC., et al. (1959), *Brit. med. J.*, **2**, 1055.

the danger of contamination of the bladder. When necessary, specimens of urine may be drawn, with aseptic precautions, by a catheter smeared with sterile lubricant—e.g. glycerol jelly. No antiseptics are used.

All specimens of urine must be submitted for examination *without delay*, since many bacteria grow readily in urine. The urine is centrifuged at 2000 r.p.m. for ten minutes, and the deposit examined wet for cells and casts, etc. Dried and fixed films of the urine should be gently washed in water to remove crystalline material, and stained by Gram.

Sputum should be expectorated directly into a suitable container as described on p. 433. Films are made as in the case of pus and stained by Gram's method. The distribution of micro-organisms in the sputum samples may be made more uniform, and the isolation of pathogens easier, by treating the sputum with equal volumes of sterile 1 per cent. buffered pancreatin solution and heating in the water-bath at 37° C. for one to one and a half hours.^{1, 2}

Cultivation.—Successive stroke inoculations are made on blood agar plates, one being incubated aerobically, the other anaerobically to recover microaerophilic and anaerobic organisms, e.g. streptococci. In the bacteriological examination of wounds the possibility of sporing anaerobic bacilli being present must be considered; the methods applicable for this purpose are detailed on p. 697 *et seq.* Special selective and differential media will be inoculated where indicated. *Esch. coli* and allied organisms can generally be recognised at once when cultures are made on MacConkey's agar. For the rapid identification of *Proteus*, Christensen's medium may be used (p. 224).

A proportion of the growth after incubation will be represented by separate colonies, and the colonial characters of the organisms recognised. Films are also made from the colonies and stained by Gram's method. In this way many organisms can usually be identified. If a mixed growth results, single colonies can be subcultured so that pure cultures are available for further examination.

If *septicaemia* or *pyaemia* is suspected, blood culture is carried out (p. 260). A quantitative estimate of bacteria per ml. of blood should be made by inoculating two 1·0 ml. quantities of blood into tubes of melted agar which are then poured into Petri dishes.

If inoculation of the blood into suitable culture media cannot be done immediately, the blood should be added to a sterile

¹ Rawlins, G. A. (1953), *J. med. Lab. Technol.*, 13, 133.

² Murdoch, J. McC., *et al.* (1959), *Brit. med. J.*, 2, 1277.

screw-cap containing 0·05 per cent. "Liquoid" (sodium poly-anethol sulphonate).

A difficulty frequently encountered in the bacteriological examination of pus and infected urine, is the overgrowth of pyogenic cocci by other organisms present, especially the swarming *Proteus*. The procedures given on p. 621 can prevent this.

CHAPTER XXI

THE NEISSERIAE

Definition.—The Neisseria are Gram-negative cocci, usually arranged in pairs with long axes parallel: strict parasites, often growing poorly on ordinary culture media. The two principal pathogenic members of the group are *Neisseria meningitidis* and *N. gonorrhoeae*; *N. meningitidis* is the causal organism of Epidemic Cerebrospinal Meningitis (or Cerebrospinal Fever), sometimes called Spotted Fever because of the frequent presence of a purpuric rash; it may also produce an acute or chronic septicaemia without meningitis. *N. gonorrhoeae* causes gonorrhœa, a venereal infection characterised by urethritis in the male and urethritis and cervicitis in the female; there may be local and metastatic complications.

NEISSERIA MENINGITIDIS (*MENINGOCOCCUS*)

Morphology and Staining.—Oval diplococci with opposed surfaces flattened or concave; sometimes in tetrads; cocci are about $0.8\text{--}1\mu$ in diameter; the long axes of the cocci in pairs are parallel, not in line as in the case of the pneumococcus; Gram-negative. Morphological capsules are not evident, but when the organisms react with specific antiserum, capsule-like structures become apparent, this effect corresponding to the "capsule-swelling" reaction of the pneumococcus (p. 496). In cerebro-spinal fluid the intracellular position in polymorph leucocytes is characteristic. In culture the usual shape and arrangement seen in the spinal fluid may be lost, and involution forms may be present.

Cultural Characters.—Aerobe; primary cultures are obtained most readily in an atmosphere containing 5 per cent. of carbon dioxide; temperature range is $25^{\circ}\text{--}42^{\circ}$ C., and the optimum is about 37° C.; although it may grow on good nutrient agar, growth is enhanced by the addition of blood or serum; optimum pH is 7.0–7.4. A specially suitable medium is a nutrient agar prepared from a digest basis, and containing 5 per cent. blood added to the melted agar at 90° C.

Colonies on serum-agar, after twenty-four hours, are small, greyish, transparent, circular, disks about 2 mm. in diameter,

i.e. larger than colonies of streptococci; later, the centre of the colony becomes more opaque and raised, while the periphery remains thin and transparent; the borders may become crenated. While this is the common type of colony, considerable variation in the appearances may be noted.

Colonies on blood agar are like those on serum agar but somewhat larger; they are smooth, grey and semi-transparent; no haemolysis occurs.

Viability.—When first cultured artificially, the meningococcus tends to die quickly in culture, *e.g.* within two or three days, probably due to alkali production. In culture it persists best at incubator temperature on moist egg medium. Alternatively, 1 per cent. agar in digest-broth *plus* 20 per cent. serum gives good results. It is killed by heat at 55° C. in five minutes or less. When desiccated under ordinary atmospheric conditions the meningococcus usually dies within two hours, but cultures can be preserved by rapid drying *in vacuo* (p. 268). The organism is sensitive to the sulphonamides and most antibiotics.

Biochemical Reactions.—Can be tested by growing on peptone-water agar slopes containing 5 per cent. serum, 1 per cent. of the particular sugar, and an indicator. The medium described on p. 210 is recommended for these tests.

Ferments glucose and maltose with acid production, but has no action on lactose, sucrose or inulin.

Cultures of the meningococcus give the oxidase reaction like those of the gonococcus (p. 510).

Antigenic Characters.—Gordon and Murray described four serological types (I-IV) of the meningococcus, but later, Griffith reduced these to two groups (I and II), his group I corresponding with the earlier types I and III, and group II with types II and IV. A more recent classification¹ recognises four main groups, now designated A, B, C and D, of which A corresponds to Griffith's group I, B to the old type II and D to the old type IV.² Group C is identified with a group C described by French workers.

The majority of cases of cerebrospinal meningitis during epidemics are due to group A, whereas many strains found in the nasopharynx of persons who have not been in contact with cases belong to the other groups. It has been concluded that the latter are of lower pathogenicity than group A.

Virulence; Animal Pathogenicity.—The meningococcus is

¹ Branham (1953), *Bact. Rev.*, 17, 175.

² Specific agglutinating antisera may be obtained from the Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W.9.

actively toxigenic, and potent products have been obtained from cultures. The toxin has been classified as an endotoxin though it is readily diffusible from the organisms, probably as a result of their rapid autolysis in culture.

In general, it has been found difficult to establish an active infection in laboratory animals by inoculation with cultures. However, intraperitoneal injection in mice of even small doses of the meningococcus *suspended in a solution of gastric mucin* brings about a rapidly fatal general infection.

Occurrence.—The natural habitat of the meningococcus is in the nasopharynx or post-nasal space, where it may be found in 5–10 per cent. of healthy persons. When outbreaks of cerebrospinal fever occur, the carrier rates may increase to 50–90 per cent. The route of spread of the meningococcus from the nasopharynx to the meninges is a controversial matter; the organism may either spread directly through the cribriform plate to the subarachnoid space by the perineural sheaths of the olfactory nerve, or it may be blood-borne. In favour of the latter route are the frequent positive blood-cultures in the early stages of infection, the purpuric rash in many cases, with the isolation of meningococci from the skin lesions, and the occurrence, particularly during epidemics, of meningococcal septicaemia with rash but no clinical meningitis. Rare types of meningococcal infection are primary conjunctivitis, endocarditis and pneumonia. Complications of the typical disease are labyrinthitis, arthritis and teno-synovitis.

Epidemiology.—Outbreaks of cerebrospinal fever have occurred particularly under conditions of overcrowding, e.g. among recruits in training camps and in ships and gaols. When cases occur under such circumstances, bacteriological examination usually reveals high nasopharyngeal carrier rates of the epidemic strain among healthy contacts. It has been stated that clinical cases are likely to occur when the carrier rate exceeds 20 per cent., but the case/cARRIER ratio may vary considerably. Meningeal infection is probably facilitated by fatigue and other factors which lower physical well-being and may be preceded or accompanied by a local nasopharyngitis. Apart from localised outbreaks, the highest attack rate (and also the highest case fatality) is in infancy. Cases occur most commonly in the spring in the United Kingdom and other countries with temperate climates. Large-scale epidemics spread over wide areas of East and West Africa during the dry season and end abruptly with the onset of the rains.

Laboratory Diagnosis.—In the early stages of cerebrospinal meningitis the organisms are present usually in considerable numbers in the cerebrospinal fluid and can be recognised by

microscopic examination. At a later stage they may be scanty and even apparently absent.

In a case of cerebrospinal meningitis the spinal fluid is under pressure, and turbid in appearance due to the large number of pus cells present. When there is no increased pressure, up to 10 ml. are withdrawn, and the volume of the sample should never exceed 25 ml.

In the laboratory the fluid is centrifuged, and films are made from the sediment and stained by (*a*) methylene blue, (*b*) Gram's method (with Sandiford's counterstain, p. 113). Cultures should be made on one of the blood or serum media referred to above. Films are made from the resulting growth and stained by Gram's method. The colony characters should be ascertained, and subcultures for further tests are obtained by picking off single colonies on to blood- or serum-agar slopes. The biochemical reactions should be tested and the serological group identified by agglutination tests with the appropriate antisera.

For clinical diagnosis the microscopic examination is often sufficient—*i.e.* if Gram-negative, intracellular diplococci with the characteristic shape of the meningococcus are observed. While the meningococcus is usually present in large number at an early stage of the untreated illness, it may become relatively scanty in the cerebrospinal fluid at a later stage and even undetectable by microscopic methods. It should be emphasised that the same change may occur within twenty-four hours of administering sulphonamide drugs. The meningococcus may be demonstrated by cultural methods when difficult to find by microscopic examination. A method which sometimes facilitates the cultivation of the organism is to add an equal volume of glucose-broth to the cerebrospinal fluid and incubate the mixture for eighteen hours; thereafter sub-inoculations are made on a solid medium as described above.

In cases of suspected meningococcal septicaemia, blood cultures should be carried out and subcultures made on blood agar every day for four to seven days.

Chemotherapy.—Meningococcal infections respond well to treatment with the sulphonamide compounds, which diffuse readily into the C.S.F.

Prophylaxis.—The early recognition of a high carrier rate, particularly of the more invasive group A, in a closed or semi-closed community, *e.g.* military recruits in barracks where apparently sporadic cases of meningitis are occurring, may help to prevent an outbreak, since the carriers can be quickly and effectively treated with small doses of sulphonamide.

Cultures should not be made within an hour after a meal or within twenty-four hours after the application of antiseptics to the throat. The specimen is best obtained by means of a swab with a longer wire-holder than the usual throat swab and with the terminal $\frac{3}{4}$ in., carrying the cotton-wool plectet, bent through an angle of about forty-five degrees. The swab is enclosed in a stoppered test-tube of sufficient width to admit the bent end.

The tongue is depressed, and the swab is passed behind the soft palate and introduced into the nasopharynx. Before withdrawal, the swab is also rubbed over the posterior wall of the nasopharynx. The swab must be introduced and removed from the mouth without touching the tongue.

The swab is rubbed *at once* over a small area at the edge of a serum- or blood-agar plate already prepared and warmed to 37° C., and then successive stroke inoculations are made on the remainder of the plate by means of a wire loop, the loop being charged several times from the area inoculated directly with the swab. The plate must be incubated without delay.

When it is impossible to make an immediate culture, a convenient method of maintaining the viability of the meningococcus for twenty-four hours or so is to place in the foot of the swab-tube a small amount of blood agar so that the swab, when returned to the tube, is kept in contact with the medium. In the laboratory the swab is used to inoculate a blood-agar plate, and the swab-tube is also incubated. Alternatively, the swab may be placed in Stuart's transport medium (see p. 242).

Suspected colonies are examined by means of Gram-stained films, and subcultures from single colonies are made on serum- or blood-agar slopes. The resulting pure cultures are then available for identification by biochemical and serological tests.

Other Causes of Meningeal Infection

In routine investigations of meningitis, other causal organisms must be considered—*e.g. Haemophilus influenzae*, pyogenic cocci, pneumococcus, tubercle bacillus, viruses, etc. If the C.S.F. is not distinctly turbid and no organisms are detectable in films stained by methylene blue or Gram's method, a Ziehl-Neelsen preparation should be examined for tubercle bacilli (p. 546). In tuberculosis the cellular exudate does not usually exceed 200–500 cells per cu. mm. and is mainly lymphocytic, as compared with the heavy polymorph exudate in meningococcal and other acute forms of bacterial meningitis. In meningococcal meningitis at a later stage of the illness there may be a fair number of lymphocytes

and mononuclear cells in the spinal fluid, and in tuberculous meningitis polymorph leucocytes may sometimes be fairly numerous. The acute meningitis caused by *H. influenzae* occurs mostly in children under three years of age. This organism often develops elongated filaments in such infections, and these are seen among the polymorph leucocytes of the exudate in the spinal fluid.

Among the viruses that may produce an inflammatory condition of the meninges are the entero-viruses (poliomyelitis, Coxsackie, ECHO), the virus of lymphocytic choriomeningitis (L.C.M.), of mumps and of louping-ill and the similar Russian Spring-Summer Encephalitis (see p. 831).

NEISSERIA GONORRHOEAE (GONOCOCCUS)

Morphology and Staining.—Oval diplococci with opposed surfaces flattened or concave. The diameter of the coccus is about 0·8–1 μ : Gram-negative. Morphologically the gonococcus is identical with the meningococcus. In inflammatory exudates the intracellular position of the organism is characteristic and some pus cells appear to be almost filled with ingested diplococci. In culture, involution forms are frequent.

Cultural Characters.—Aerobe; temperature range, 30°–39° C.; optimum about 37° C.; requires blood or serum for growth—*e.g.* agar containing 10 per cent. blood (heated at 55° C.), serum-agar prepared from fresh sterile serum (p. 210) or agar containing 10 per cent. hydrocele fluid. Various special media have been recommended, but the above-mentioned serve satisfactorily in the routine cultivation of the organism. The agar should be carefully standardised to pH 7·5.

Most strains grow better in an atmosphere containing carbon dioxide (*e.g.* 5 per cent.) than in ordinary air (p. 259). Incubation inside a closed jar also helps to maintain a moist surface on the culture medium, which helps the growth of gonococci. The CO₂ may also prevent the development of alkalinity.

Colonies on serum agar are semi-transparent disks about the size of a pin head, tending to remain discrete, circular in outline at first, but later showing a "scalloped" or crenated margin, a raised more opaque centre, and sometimes radial and concentric markings. Papillae may be noted after some days' growth.

In primary cultures, colonies may be slow in developing and growth may not appear for two or three days.

Viability.—The thermal death-point is about 55° C for 5 minutes. The gonococcus is a strict parasite and tends to die rapidly (*e.g.* in a

few hours) when discharged from the body, especially if subjected to cooling and drying. It has been found, however, that under certain conditions, e.g. in pus on linen or other fabric, the gonococcus may remain viable for periods up to three days. When first isolated, cultures have a feeble viability, and subcultures should be made every three or four days to maintain the strain. When accustomed to artificial growth, cultures survive longer if kept at 37° C. and in a moist condition. Cultures die at room temperature in two days.

Biochemical Reactions.—Can be tested for as in the case of the meningococcus, the medium described on p. 210 being used; ferments glucose but not maltose.

Antigenic Characters.—The gonococcus is not antigenically homogeneous; probably two main groups with intermediate types. There is an antigenic relationship with the meningococcus.

Pathogenicity.—The gonococcus is a strictly human parasite and all attempts to infect animals from mice to monkeys have failed. Its toxicity to animals is due to an endotoxin like that of the meningococcus.

Occurrence.—In the *male* the organism infects the mucosa of the urethra and produces a suppurative inflammation with purulent discharge. The cocci are present in large numbers in the discharge at an early stage, but later diminish, and are then associated with secondary infecting organisms—e.g. pyogenic cocci, coliform bacilli, diphtheroid bacilli. They may invade the prostate, seminal vesicles, epididymis, and peri-urethral tissue (producing a peri-urethral abscess).

In the adult *female* the urethra and cervix uteri are infected, but rarely the vaginal mucosa. The vestibular glands, the endometrium and the Fallopian tubes (salpingitis) may be invaded, and even the peritoneal cavity.

Blood invasion may result from primary gonorrhoeal infections, and arthritis and tenosynovitis may occur as complications. While the gonococcus has on occasion been cultivated from the joint fluid in arthritis, the possibility of gonorrhoeal arthritis being a manifestation of allergy must also be considered. Ulcerative endocarditis has been noted as a rare sequela. Purulent conjunctivitis may sometimes occur as a complication.

In female infants and children the gonococcus may produce a persistent vulvo-vaginitis with involvement sometimes of the rectum. Outbreaks of this infection used to occur in hospitals and institutions, but are now rare.

In newborn infants gonorrhoeal ophthalmia (acute purulent conjunctivitis) may result from direct infection at birth.

Epidemiology.—Gonorrhoea is a venereal infection spread by

sexual intercourse. Incidence, which increased sharply during the Second World War, fell steadily in the post-war years, but is increasing again. Incidence is about four times more frequent in males than in females, probably related to spread from promiscuous women.

Laboratory Diagnosis.—Films are made from the discharge.—In the *male*: from the urethral discharge; the meatus should be cleansed with sterile gauze soaked in saline solution, and specimens are taken either with a wire loop or directly on slides. In the *female*: from the urethra and cervix uteri, with a wire loop or swab and with the aid of a vaginal speculum.

The films are stained by (*a*) methylene blue, and (*b*) Gram's method (with neutral red or Sandiford's stain as the counter-stain), and in the acute stage, both in the male and female, the occurrence of the *characteristic Gram-negative intracellular organisms* is strongly suggestive of a gonorrhoeal infection, though it must be borne in mind that Gram-negative diplococci other than the gonococcus may occur on the mucous membranes of the genital passages, *e.g.* the commensal species to be described.

In *chronic infections*, particularly in the female, the cocci may be relatively scanty in films and difficult to identify accurately among the secondary infecting organisms. In the male the "morning drop" of secretion from the urethra should be examined, or films made from the centrifuged urinary deposit or the discharge after prostatic massage. In the female the secretion from the cervix uteri should be examined.

The diagnosis must be confirmed by cultivation, the organism being identified by cultural and biochemical characters, and differentiated from non-pathogenic diplococci by fermentation tests; but where there is a mixed infection isolation of the organism may be technically difficult. Inoculation with material to be cultivated should, if possible, *be made directly from the patient on a suitable medium*, and the culture should be incubated at once. If the material is kept at room temperature for some time before inoculation and incubation, or if it is allowed to dry, the organisms, being strict parasites, may die and fail to grow on the culture medium.

When it is impracticable to make direct cultures and necessary to use a swab for the transport of exudate to the laboratory, a swab on a wooden applicator may be broken into a tube of Stuart's transport medium (see p. 242).

It should be remembered that in cases already treated with penicillin it may not be possible to demonstrate the organism in discharges.

The Oxidase Reaction in the Detection of Colonies of the Gonococcus.—Cultures are made on plates of heated blood agar and after two days' incubation, freshly prepared 1 per cent. tetramethyl-*p*-phenylenediamine solution is poured on to the plate so as to cover the surface, and then decanted. The colonies of the gonococcus rapidly develop a purple colour (oxidase reaction). If subcultures are required from the colonies, these should be made immediately; after five minutes it may not be possible to subcultivate them. This method is specially useful in dealing with heavily contaminated material containing only scanty gonococci (*e.g.* cases of chronic cervicitis). Colonies of non-pathogenic neisserial also give a positive oxidase reaction, but the purple colour develops more slowly and is less intense.

Serum Diagnosis.—The complement-fixation test is applicable for diagnosis of chronic infections and complications and may be of value to the clinician, particularly in the differential diagnosis of such conditions as salpingitis and arthritis. However, positive reactions are much less common since the introduction of the sulphonamides and antibiotics. The technique of the test is described on p. 346.

Chemotherapy.—Although the sulphonamides were at first very effective chemotherapeutic agents, the development of sulphonamide-resistance, particularly in the Second World War, greatly reduced their value. Penicillin therapy has been applied with conspicuous success, although in recent years there has been evidence of the development of penicillin-resistant strains. Gonococcal infections are also amenable to treatment with certain other antibiotics, *e.g.* streptomycin and the tetracyclines, though this would be a second choice to penicillin.

Non-specific Urethritis

Much attention has been given in recent years to urethritis, usually of more chronic form, in which the gonococcus cannot be demonstrated, and other agents may be causally concerned. This condition has increased considerably in incidence since separate notification was begun in 1951, probably associated with a greater awareness and more accurate diagnosis; thus 16,066 new cases were reported in the United Kingdom in 1957. The infection differs epidemiologically in certain respects from gonorrhoea and a proportion of cases are probably non-venereal. It may form part of a more generalised disease with arthritis and conjunctivitis as major features (Reiter's syndrome).

Pleuro-pneumonia-like organisms (pp. 668-669) have been most

often incriminated and are present in a variable proportion of both male and female cases (and also in cases of prostatitis and cystitis). However, these organisms are also found in a smaller proportion of healthy persons, particularly females, and their aetiological relationship to non-specific urethritis is still not proven. Other possible causative agents are *Trichomonas* and *Haemophilus vaginalis*, both of which are associated with leucorrhoeal discharges in the female and, less often, with urethritis in the male. Attempts to find a virus aetiology for non-specific urethritis have so far been unsuccessful.

THE COMMENSAL GRAM-NEGATIVE DIPLOCOCCI

These organisms occur on various mucous surfaces of the body and are found with great regularity in the mucous secretions of the throat, nose and mouth; they may likewise occur on the genital mucosae. When inflammatory or other pathological conditions affect these mucous membranes, such commensals often flourish in large numbers and constitute a prominent feature of the local bacterial flora. They may possibly act as secondary infecting agents in such conditions.

Classification

It should be noted that there is some uncertainty regarding the biological classification of this group of organisms, and the taxonomic significance to be attached to colony characters, pigmentation and fermentation of different carbohydrates is doubtful. The group, however, can be broadly divided into two subgroups: (1) characterised by complete absence of fermentative properties, e.g. the classical *N. catarrhalis*, and (2) possessing such properties, e.g. *N. flava* and related types (see Table).

Fermentative Reactions of Neisseria Group

	<i>Glucose</i>	<i>Maltose</i>	<i>Lactose</i>	<i>Sucrose</i>
<i>Meningococcus</i>	.	.	+	—
<i>Gonococcus</i>	.	.	+	—
<i>N. catarrhalis</i>	.	.	—	—
<i>N. flava</i> and related types	+	+	—	+
<i>N. crassa</i>	.	.	+	+

(+ = acid; - = variation in reaction among different types.)

Neisseria catarrhalis

A frequent commensal in the throat and nose and often present in large numbers in catarrhal inflammations of the respiratory tract.

Morphology and Staining.—Practically identical with the meningococcus. In some strains the cocci are relatively large.

Cultural Characters.—Grows on ordinary media without serum and at room temperature; the colonies may be larger than those of the meningococcus, especially when fully grown, and are thicker and more opaque. The colony characters, however, may vary considerably, and both "smooth" and "rough" forms are observed. The organism exhibits no fermentative properties. Cultures when emulsified in saline tend to be auto-agglutinable.

N. catarrhalis is not agglutinated by meningococcus antisera.

Neisseria flava

The morphology of these organisms is like that of *N. catarrhalis* and they grow on ordinary media at room temperature. Cultures develop, after forty-eight hours, greenish-yellow or greenish-grey colours. Young colonies may simulate closely those of the meningococcus.

Biochemical reactions, which vary according to the type, are shown in the Table on page 511.

Neisseria flavescens.—This organism has been described as the causative organism in a group of cases of meningitis in America. It resembles the meningococcus in morphology, but on blood agar produces golden-yellow colonies. It does not ferment carbohydrates. It may be biologically related to *N. flava*.

Neisseria sicca.—Resembles *N. catarrhalis*, but its colonies are markedly dry, tough and adherent to the medium. It seems possible that this organism is not a separate species, but a "rough" variant of some other member of the group.

Neisseria mucosa.—Differs from the other members of the group in being definitely capsulate and producing mucoid colonies. This type also may represent a variant of one of the other members of the group. Strains corresponding to it have been reported in cases of meningitis.

Neisseria crassa.—Resembles *N. catarrhalis*, but shows marked variation in its staining by Gram's method, some individual cells staining Gram-positive, others Gram-negative. The colonies are small and rather like those of the streptococci. Growth occurs

at room temperature. It is open to question whether this organism should be assigned to the *Neisseria* group.

Organisms of Genus *Veillonella*

These are of some interest in view of their occurrence as commensals in natural cavities of man and animals, particularly the mouth and alimentary tract. They have not been definitely proved pathogenic though sometimes isolated from cases of appendicitis, pyorrhoea, pulmonary lesions, etc., and regarded as potentially pathogenic.

They are minute Gram-negative cocci about 0.3μ in diameter and occurring in masses. In cultural characters they are anaerobic and grow best at $37^\circ C$. The type species is *Veill. parvula*, whose distinctive characters are: the formation of hydrogen, carbon dioxide, hydrogen sulphide and indole from polypeptides, the fermentation of glucose and certain other sugars, haemolytic action, and the reduction of nitrate to nitrite.

CHAPTER XXII

CORYNEBACTERIA

Definition.—Gram-positive rods, arranged in pairs or palisades; often with club-shaped swellings at the poles; generally staining irregularly (metachromatic granules); non-motile, non-sporing, non-capsulated; aerobic, micro-aerophilic or facultative anaerobe; some species produce a powerful exotoxin.

Corynebacterium diphtheriae is the causative organism of diphtheria, which, in its characteristic form, consists of a localised inflammation in the throat with adherent exudate (false membrane) and a toxæmia due to the secretion and dissemination of a highly potent exotoxin. Certain species (*C. pyogenes*, *C. renale*, *C. ovis*, etc.) cause acute or chronic suppurative lesions in various domestic and laboratory animals. *Erysipelothrix* and *Listeria*, which are closely related members of the Corynebacteriaceae family, are primary pathogens in animals but sometimes cause infection in man.

CORYNEBACTERIUM DIPHTHERIAE

Morphology and Staining.—Slender rod-shaped organism, straight or slightly curved; the average size is $3\ \mu$ by $0.3\ \mu$, but longer and shorter forms may be noted; the ends are often expanded; it is non-motile and non-sporing. In culture, involution forms may be observed which are pear-shaped, club-shaped or even globular. The bacillus is Gram-positive, though more readily decolorised than many other Gram-positive organisms; stained with methylene blue it shows a "beaded" or "barred" appearance. "Barred" staining is characteristic of the *intermedius* type of diphtheria bacillus (p. 515). By Neisser's method (p. 120) volutin (metachromatic) granules are characteristic, staining blue-black in contrast with the light-brown coloration of the rest of the organism; the granules are mainly polar in situation. If over-decolorised in the Gram method the granules tend to retain the violet stain, while the rest of the organism is decolorised. These characteristic staining reactions depend on environment; thus, in culture, volutin granules are most pronounced when the bacillus is growing on a serum medium such as Löffler's, and by electron microscopy they can be demonstrated

as well-defined structures. Appearances also vary among strains, some of which exhibit very short forms with poorly developed granules (*e.g. gravis* type).

By phase contrast microscopy one or more septa can be observed in a single diphtheria bacillus, and the *intermedius* type appears to be multicellular. Branched forms have also been observed.

Cultural Characters.—Aerobe; temperature range, 20°–40° C.; optimum, 37° C.; grows on ordinary nutrient media, but best on serum media.

Colonies on Löffler's serum—at first small, circular, white, opaque disks with regular borders; later the centres become thicker and the borders crenated; they may reach 3–4 mm. in diameter after several days' growth; sometimes the growth on serum shows a distinct yellow tint.

Broth—some strains grow in small white masses, which sediment in the tube and also adhere to the side; a surface film of growth may also develop. Other strains produce a uniform growth in broth.

Does not liquefy gelatin.

On a medium containing potassium tellurite the diphtheria bacillus reduces the tellurite and yields greyish or black colonies. Potassium tellurite is also selective in certain concentrations for this organism and the allied diphtheroid bacilli (pp. 529–33).

On blood-tellurite media the colonies tend to show distinctive appearances. Three types of colony have been recognised as characteristic of different biological types, designated *C. diphtheriae gravis*, *mitis* and *intermedius*. The designations *gravis* and *mitis* have been applied in virtue of the association of these types with severe and mild forms respectively of the disease. The *gravis* type produces relatively large greyish-black, flat, lustreless colonies exhibiting often a "daisy-head" formation. Growth in broth is granular. The *mitis* type yields a convex, smooth, translucent colony, and growth in broth presents a uniform turbidity. The *intermedius* type is represented by relatively small, black, lustreless colonies with domed centre and flat, irregular margin, like a poached egg.

The colony characters on Hoyle's tellurite medium and blood agar (pp. 525–27) are described later in relation to the diagnosis of diphtheria.

Viability.—In culture, diphtheria bacilli may remain alive for two or more months at room temperature. In the moist condition they are comparatively easily killed by heat (in ten minutes at 60° C.), but when dry survive for much longer periods. Diphtheria bacilli may remain alive and virulent for a considerable period in

the dust of premises. The organism is sensitive to penicillin (pp. 400, 528) and to other antibiotics, e.g. erythromycin, acting on Gram-positive bacteria.

Biochemical Reactions.—Ferments with acid production, glucose, galactose, maltose and dextrin, but not lactose, sucrose or mannitol. (Some strains of proved virulent diphtheria bacilli have been stated to ferment sucrose.)

Gravis strains ferment starch and glycogen, and these reactions are characteristic features of this type. The *mitis* and *intermedius* types have no action on these carbohydrates.

These reactions can be elicited by using Hiss's serum medium (p. 208) made with 0·1 per cent. peptone-water. Some workers use phenol red as the indicator, the initial pH being 7·6. (Acid production also brings about clotting of the medium.)

Haemolysis.—Strains of the *mitis* type are generally haemolytic when growing in a medium containing ox or rabbit blood; the *intermedius* strains are invariably non-haemolytic; strains of the *gravis* type usually lyse rabbit but not ox blood.

Strains which do not fit into these three types have been isolated from both cases and carriers.

It should be noted that the stability of the supposed biological types of the diphtheria bacillus has been questioned; thus, it is stated that strains undergo variation in colony form and starch fermentation.

Antigenic Characters.—By agglutination reactions with antisera *gravis* strains have been classified into thirteen types, of which type I has been found to be by far the commonest in Great Britain. Among *intermedius* strains four types have been recognised, and *mitis* strains have been allocated to forty different types, one type being related apparently to a particular *gravis* type (Hewitt).

Diphtheria Toxin

The diphtheria bacillus produces a powerful exotoxin with specialised toxic properties. While the bacillus remains localised at the site of infection, the diffusible toxin is absorbed into the blood stream and leads to the various systemic disturbances of diphtheria (in particular myocardial damage) and to such sequelae as post-diphtheritic paralysis.

When diphtheria bacilli are grown in suitable fluid media, abundant toxin is produced. The strain used is, however, of considerable importance, for in artificial culture the organism may not adapt itself so readily to toxin production as it does in the human body. A single strain (Park-Williams No. 8) of the

intermedius type is almost universally used for toxin production on a large scale. The bacilli are removed from the culture by filtration or other means, and the bacteria-free liquid, which contains the exotoxin mixed with the culture medium and other products of bacterial growth, is referred to as *toxin*.

Certain cultural conditions are required for maximal production of diphtheria toxin. It was thought at one time that peptone-like substances produced by enzymic digestion of meat were essential for toxin production, but the work of Meuller on the nutritional requirements of the diphtheria bacillus has led to great advances in our knowledge. It is now possible to produce very potent toxin in a medium containing known amino acids, inorganic salts, maltose and, in addition, certain growth factors (pimelic acid, nicotinic acid and β -alanine). The toxin produced in this type of synthetic medium has been isolated in a purified state; it has the properties of a labile protein with a molecular weight of 15,000 to 72,000 and a lethal dose of 0.0001 mg. for a 250-gram guinea-pig.

The toxin is unstable and its potency diminishes on exposure to air and light. In sealed tubes and in the dark it may remain unaltered for several weeks. The reduction in toxicity is due to the spontaneous conversion of toxin into *toxoid*, which has no pathogenic effect on animals but still retains the power of combining with antitoxin and of stimulating immunity.

This process of conversion of toxin into toxoid can be carried out by adding 0.3 per cent. of formalin and incubating the toxin for two to three weeks at 37° C. The change from the toxic into the non-toxic state is assessed by injecting the material into guinea-pigs; when 5 ml. injected subcutaneously or intra-peritoneally produce no symptoms, the change is regarded as complete. Toxoid produced through the action of formalin in this way is a valuable immunising agent.

Animal Pathogenicity.—When a very small amount of diphtheria toxin, e.g. 0.00025 ml., of a culture-filtrate is injected into a guinea-pig, there develops at the site of inoculation a greyish necrotic focus surrounded by an area of congestion, while the subcutaneous tissue shows marked inflammatory oedema. The neighbouring lymph glands are swollen and congested, the suprarenals are enlarged, very congested and may show haemorrhages; the lungs are usually congested and there is effusion into the pleural cavities; the kidneys and liver show degenerative changes.

The same pathogenic effects are produced by the subcutaneous injection of a living culture of virulent diphtheria bacilli. The organisms remain more or less localised at the site of inoculation, while the toxin is absorbed into the circulation.

Tests for Toxigenicity.—When organisms morphologically resembling the diphtheria bacillus are found in the throat or nose unassociated with active disease (*i.e.* in carriers), it is important to determine whether they are virulent. The virulence test is usually done by the intradermal injection of a pure culture into guinea-pigs as follows.

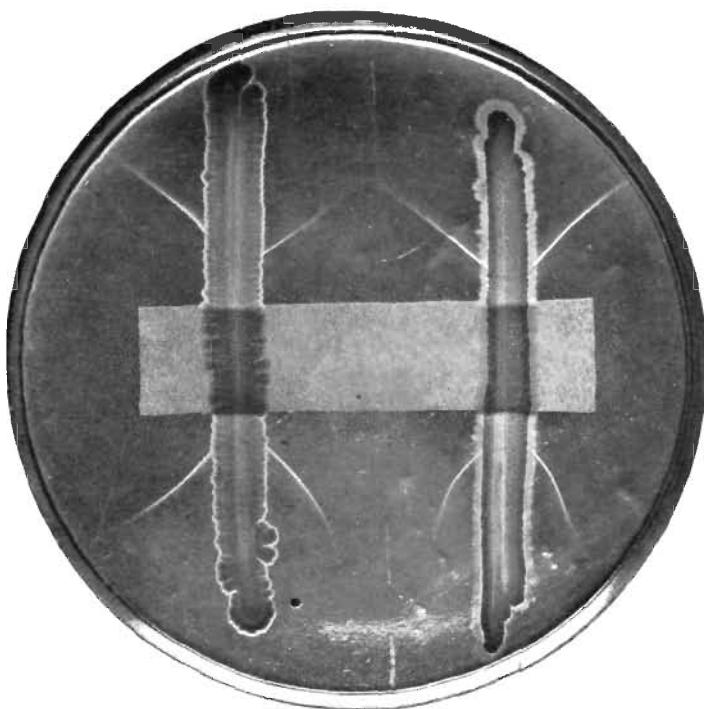
The primary culture from the nose or throat is plated out, and a single colony is subcultured to obtain a pure growth. Tellurite media, such as Hoyle's (p. 229), are recommended for the easy isolation of the diphtheria bacillus. The fermentative powers of the selected pure growth are tested, and if the organism ferments glucose, but not sucrose, a suspension from a culture on Löffler's serum or serum agar is made in *broth* of such a strength that the fluid is distinctly opalescent (about 5×10^8 bacilli per ml.). Two white guinea-pigs of about 400 grams weight are selected, and the hair is removed from the flanks as described on p. 418. The day before the test is carried out, one of the animals—the control guinea-pig—is injected intraperitoneally with 1000 units of diphtheria antitoxin (*vide infra*). For the actual test each guinea-pig is injected intradermally with 0.2 ml. of the suspension of organisms, a 1-ml. syringe and fine-bore needle (26 gauge, $\frac{3}{8}$ in. long) being used. Up to 8–10 different cultures may be tested on each guinea-pig, and the injections should be about one inch apart. A careful note of the position of the injection of each different culture should be made. The test guinea-pig is now injected with $\frac{1}{50}$ th unit of antitoxin per gram of body-weight. The guinea-pigs are examined twenty-four, forty-eight and seventy-two hours after inoculation.

Virulent diphtheria bacilli produce in the test animal a well-defined red area about 15 mm. in diameter. After the third or fourth day the colour fades, leaving a necrotic patch with a scab surrounded by growing hair. The control guinea-pig shows no such reaction, the puncture wound caused by the needle being generally the only evidence of injection. If the organism is non-virulent there is no reaction in either the test or control animal. A reaction in both animals shows that the organism is not the diphtheria bacillus, because the products of growth are not neutralised by diphtheria antitoxin, as indicated by the reaction of the control animal.

Rabbits are also suitable for virulence tests, and some workers have used these animals for the purpose.

The Gel-precipitin Test.—The following method by Elek¹ shows

¹ Elek, S. D. (1948), *Brit. med. J.*, **1**, 493; Ouchterlony, Ö. (1949), *Lancet*, **1**, 346.



Photograph showing the recognition of toxigenic strains of the diphtheria bacillus by Elek's method. In the centre is the horizontal strip of filter paper containing the antitoxin with the growths of the diphtheria bacillus at right angles to it. The fine white lines showing a positive reaction are well defined.

the production of toxin in a Petri dish culture and can be used as a reliable substitute for the animal test. This *in vitro* test depends on the formation of a precipitate when diphtheria toxin interacts with its homologous antitoxin in appropriate neutralising amounts like the Ramon reaction (*vide infra*—flocculation). Where toxin and antitoxin so interact in culture medium an opaque line of precipitate is observed.

Culture Medium.

A. Peptone (Difco proteose)	4.0 g.
Maltose	0.6 g.
Lactic acid (B.P.)	0.14 ml.
Distilled water	100.0 ml.

Adjust pH to 7.8

B. Agar	3.0 g.
Sodium chloride	1.0 g.
Distilled water	100.0 ml.

Dissolve by heat, filter, adjust pH to 7.8

Mix equal parts of A and B, distribute in 10 ml. amounts in screw-capped bottles, sterilise by steaming for thirty minutes on each of three successive days.

The Test.

- (1) Melt 10 ml. agar medium, cool to 55° C., add 2 ml. normal horse serum, Burroughs Wellcome No. 2, and pour into a Petri dish.
- (2) Introduce immediately (before agar has time to set) a strip of filter paper 60 mm. × 15 mm. which has previously been immersed in diphtheria antitoxin of a strength of 1000 units per ml. and the surplus antitoxin allowed to drain off.
- (3) Dry the surface of the medium for forty-five minutes in the incubator.
- (4) Inoculate the plate by stroking a heavy inoculum of the diphtheria bacillus to be tested across the plate at right angles to the paper strip. Two or three strains can be thus stroked, and a known virulent (toxigenic) strain is also inoculated at the same time. The line of inoculum should be as narrow as possible.
- (5) Incubate and examine after twenty-four and forty-eight hours.

Note.—Plates should be freshly prepared immediately before each test.

A positive reaction is denoted by fine white lines commencing from the stroke about $\frac{1}{2}$ in. from the filter-paper (see illustration). It appears at about twenty-four hours' incubation and in its early stages must be looked for with a hand lens. The plate is best examined against a dark background. After forty-eight hours the white line of precipitation is easily seen. The toxin diffuses sideways from the stroke, and the antitoxin diffuses from the filter paper and where these meet at

optimum neutralising concentration, a precipitate is formed. If the plate is then left at room temperature, secondary lines may be seen representing the interaction of other bacterial substances, diffusing out from the growth, with antibodies corresponding to them in the antitoxic serum. It should be realised that the diphtheria toxin used for the production of antitoxin is not a pure substance but may contain several antigens in addition to the actual toxin itself.

It should be noted also that not all makes of peptone are suitable and that some samples of horse serum prevent the development of the reaction.

Diphtheria Antitoxin

By immunising horses with toxoid and then with toxin, both in repeated doses, an antitoxin is produced from the tissues and is present often in large amount in the blood; the serum of such animals constitutes *diphtheria antitoxin*. For regulating the dosage of this antitoxin, an arbitrary *immunity unit* has been adopted and the number of such units in a given volume of serum denotes its antitoxic value. The unit was originally defined as that amount of antitoxin (or antitoxic serum) which just neutralises 100 M.L.D. of a certain toxin, the M.L.D. (minimum lethal dose) being the minimum amount which kills a guinea-pig of 250 grams weight in four days. It is not feasible, however, to preserve a standard toxin for testing antitoxin, but by means of a preserved standard antitoxin any toxin preparation can be standardised by neutralisation tests in guinea-pigs, and in turn the value of a new antitoxin can be estimated. The usual method for the purpose is to ascertain first the "L+" dose of the toxin; this is the quantity which when mixed with one unit of standard antitoxin is just sufficient to kill a 250-gram guinea-pig in four days. Varying dilutions of the new antitoxin are then mixed with the L+ dose and injected into guinea-pigs. Thus, the neutralising power of the new antitoxin can be compared quantitatively with the standard and the number of units in a given volume stated. Antitoxin may also be titrated by the intracutaneous injection of mixtures of toxin and antitoxin in the guinea-pig or rabbit, using an "Lr" dose of toxin. This is the amount of toxin which when injected into the skin of the experimental animal along with 1 unit of antitoxin causes a localised erythema 5 mm. in diameter within thirty-six hours. The amount of the unknown antitoxin producing the same result will contain 1 unit of antitoxin.

Diphtheria antitoxin may also be assayed *in vitro* by the Ramon flocculation method which is based on the rapid precipitation with optimal proportions of toxin and antitoxin. The "Lf" dose of toxin is first determined as the amount of toxin which flocculates

most quickly with 1 unit of antitoxin. The unknown antitoxin is then tested to find what dilution flocculates first with 1 Lf of toxin; this dilution will contain 1 unit of antitoxin.

The antitoxic property of serum is contained in the globulin fraction, and by precipitation in half-saturated ammonium sulphate the serum can be concentrated and much of the protein material which gives rise to serum sickness (p. 74) is removed. This was the method originally used, but *refined* antitoxins are now obtained by pepsin digestion and critical heat denaturation to precipitate non-antitoxic protein, the remaining material being then precipitated by ammonium sulphate, followed by dialysis and concentration by ultrafiltration. The volume to be injected is thus reduced, and the same number of units are contained in only a fraction of the amount of protein present in the concentrated sera previously used. As a result the incidence of serum sickness following injections of antitoxin has been reduced to 3-5 per cent.

The Regulations under the Therapeutic Substances Act define the strength, quality and testing of diphtheria antitoxin, and these regulations must be observed by manufacturers supplying serum for therapeutic use.

It has been observed that diphtheria due to the *gravis* type of bacillus (*vide supra*) may be of a hypertoxic type and refractory to antitoxin treatment. All strains of the diphtheria bacillus produce the same toxin, but it has been claimed by O'Meara that two distinct substances, designated A and B, enter into its constitution and that the toxins from different strains may vary in the relative amounts of these constituents. According to O'Meara the A substance is the toxin which is lethal to the guinea-pig; the B substance is not definitely toxic *per se* but acts by promoting the spread of A in the tissues, and if it is formed in sufficient amount renders the latter hypertoxic. O'Meara points out that the avidity of antitoxin depends on its power to neutralise the B substance and that for the production of an avid antitoxin, the toxin used must be rich in the B constituent.¹

Occurrence.—The local infection occurs typically in the faucial region. Infection often begins on one side of the fauces, usually on the tonsils, and gradually extends to involve the whole oropharynx. The bacilli are present in large numbers in the adherent exudate or false membrane and in the throat secretions. They do not invade the lymphatics to any extent and there is no blood infection. In nasal diphtheria, characterised by crust-formation, the organisms can be detected in the blood-stained nasal discharge.

¹ O'Meara, R. A. Q., et al., *Lancet*, 1947, 1, 212.

Infection of wounds, the conjunctiva, vulva and vagina may occasionally occur. A diphtheritic paronychia is sometimes met with.

The more severe forms of diphtheria are mostly caused by the *gravis* and *intermedius* types. The *mitis* type is usually associated with mild infections except when it involves the larynx and trachea (laryngeal diphtheria), when it may cause severe illness by obstruction and anoxia. The relative prevalence of these types varies in different areas and varies also at different times.

Epidemiology.—Before immunisation was introduced on a national scale in this country, the incidence of clinical diphtheria ranged from 50,000 to 60,000 cases annually, with 2500 to 3000 deaths. It is a disease of early childhood with peak incidence around four to six years of age, but when the large-scale immunisation of children has been well developed there is a shift of infection to young adults. Infection is rare in early infancy because of the passive immunity transferred via the placenta from the mother. Infection is spread by close contact, e.g. in schools, families and institutions, but the actual mode of transference has not been clearly defined. Besides direct droplet infection, spread by contaminated dust and fomites may play an important part. Nasal cases and carriers have long been recognised as particularly dangerous sources of infection and there is evidence that, like heavy nasal carriers of *Strept. pyogenes*, they may disperse very large numbers of diphtheria bacilli in their immediate environment. Persistent throat or combined nose and throat carriers follow clinical infection in 5–10 per cent. of cases and no doubt contribute to the spread of infection. In investigating outbreaks of infection, nose and throat swabs from all contacts should be examined and Schick tests (q.v.) done, so that (a) the carriers may be segregated and (b) the susceptibles protected by combined active-passive immunisation (see p. 58).

Schick Test

When a minute quantity of diphtheria toxin is injected intradermally, a local reaction follows in persons with less than a certain content of antitoxin in the blood. The average amount of antitoxin required to ensure neutralisation of the test dose of toxin is about 1/200 of a unit per ml. of blood, but there may, of course, be wide variations in antitoxin content among non-reactors. This reaction has been extensively applied with a view to gauging immunity or susceptibility to diphtheria, and likewise for ascertaining whether an individual requires artificial immunisation against

the disease. As an indicator of the amount of antitoxin in the blood and of actual resistance or susceptibility to diphtheria its value must be regarded as relative.

For the test a matured and fairly stabilised toxin preparation is chosen, and the selected dose is that amount which is just neutralised by approximately 1/1000 unit of antitoxin. The toxin preparation is diluted with a special buffer solution¹ so that 0.2 ml. contains the test dose. This is injected intradermally in the left forearm, and, as a control, an equal amount of a similar dilution of the same toxin previously heated at 70°–85° C. for thirty minutes is injected intradermally in the right forearm. A positive Schick reaction consists in an area of redness and swelling appearing after twenty-four to forty-eight hours, reaching its maximum about the fourth day, when it measures 1–5 cm. in diameter. It persists for seven to fifteen days, and on fading shows superficial scaling and a persistent brownish pigmentation. The absence of reaction on either arm (*i.e.* "negative Schick reaction") indicates that the toxin has been neutralised, sufficient antitoxin being present in the blood of the individual. A "pseudo-reaction" may occur, *i.e.* an area of redness appearing early (within six to twelve hours), which is less intense and usually disappears in one to three days. If this appears in a Schick-negative person, both forearms show similar reactions; in a positive reactor, the unheated toxin produces a reaction which is more pronounced and more persistent than that due to the heated material. A convenient time to examine results is on the seventh day, when true reactions are still visible and most pseudo-reactions have faded.

Pseudo-reactions are more common in older children and adults, because they have apparently been sensitised by exposure to diphtheria bacilli. Psuedo-reactors are usually immune to diphtheria. Children between six months and eight years of age are generally non-immune and rarely suffer from reactions after prophylactic immunisation. However, in older children a preliminary Schick test should be carried out to ascertain whether immunisation is required; pseudo-reactors should not receive any diphtheria prophylactic because of the risk of severe reactions.

Laboratory Diagnosis.—A specimen of the throat secretion should be obtained. No antiseptics (*e.g.* in form of gargles, etc.) must have been applied within twelve hours. A sterile throat

¹ A mixture of 57 grams crystal borax, 84 grams boric acid, 99 grams sodium chloride is made; 1.5 grams of this mixture are dissolved in 100 ml. of distilled water. A small amount of human serum (containing no detectable antitoxin) may also be added as a stabiliser.

swab should be rubbed over the affected area, or, where there is no definitely localised lesion, over the mucous membrane of the pharynx and tonsils.

In recent years various diagnostic methods for cultivating and identifying the diphtheria bacillus have been advocated. The following procedures may be recommended.

- A. Löffler's medium is inoculated as in the original method of diphtheria diagnosis and at the same time direct plating is made on Hoyle's medium.
- B. Direct plating is made on blood agar, and Monckton's enrichment method may be used as a supplementary procedure.

A. A tube of Löffler's medium is inoculated by smearing the infected swab over the whole surface of the medium, moistening the swab in the condensation water at the foot of the tube. The tube is incubated for eighteen to twenty-four hours at 37° C. If an earlier result is urgently required, the culture may be examined after six to twelve hours: if this should be negative, however, the examination must be repeated after eighteen to twenty-four hours.

The resulting growth is mixed by emulsifying it with a wire loop in the condensation fluid, and from this, films are made and stained by Neisser's method or Laybourn's modification (p. 119) of Albert's method.

Films may also be made directly from the swab and stained by the above methods, but only in a small proportion of cases can positive results be obtained in this way, and cultures should always be made as a routine procedure, irrespective of direct examination.

In the case of suspected throat diphtheria, the appearance in cultures of bacilli showing the characteristic morphology and staining reactions (especially the metachromatic granules by Neisser's or Albert's stain) may be regarded as significant in confirming the clinical diagnosis.

It should be noted that in some cases other organisms may overgrow the diphtheria bacillus in culture and lead to an apparently negative result. Moreover, the *gravis* type is often difficult to recognise in early growths, the bacilli being short and thick with absence of metachromatic granules. In mild cases and carriers the bacilli may be scanty and easily missed.

In the case of supposed nasal diphtheria, diphtheria carriers, diphtheria affecting mucous surfaces other than the throat, and wound-diphtheria, the microscopic examination of cultures is not conclusive. The suspected organism must be isolated in pure

culture and its virulence or toxigenicity determined as described on pp. 518-19.

The above method cannot be relied on in all cases, and it is advisable to cultivate the swab at the same time on a tellurite medium, incubating for twenty-four to forty-eight hours and making a diagnosis by recognition of the characteristic colonies, including the identification of the different colony-types, *gravis*, *mitis* and *intermedius*. (On tellurite media the typical metachromatic granules may not be demonstrable.) The two methods used together serve to check one another. It should be remembered that while tellurite inhibits many other organisms, diphtheroid organisms may grow on it as well as the diphtheria bacillus, and must be carefully differentiated from the latter. Hoyle's tellurite medium may be recommended. Growths are only just visible after twelve hours, but by using a plate-culture microscope colonies of *C. diphtheriae* can often be recognised by certain characters (*vide infra*); at this stage, however, the value of the plate is mainly in attracting attention to the presence of diphtheria bacilli missed on the Löffler's medium. After eighteen to twenty-four hours the growth is more abundant, the characters of the colonies are more distinct and by the combined use of the two methods a high degree of diagnostic accuracy is attained. When the bacilli are scanty or when nasal or aural swabs are examined, thirty-six to forty-eight hours may be required for the recognition of *C. diphtheriae* colonies, and if there is any doubt at twenty-four hours, further incubation should be allowed before reporting. The tellurite plate also facilitates the isolation of pure cultures, and when there is difficulty in identifying the diphtheria bacillus by colony characters, and where this organism occurs in a carrier, a pure culture must be obtained and tested for its biochemical reactions and virulence. If, however, the organisms present all the characters of the *gravis* or *intermedius* type, in bacteriological practice in Britain, virulence has been generally assumed without resorting to a toxigenicity test.

The Recognition of Colonies of *C. diphtheriae* on Hoyle's Medium¹

After Twelve Hours' Incubation.—To the naked eye, growths of the *gravis* and *mitis* types represent a grey "haze" hardly distinguishable from growths of diphtheroid bacilli, though the latter are somewhat blacker and more glistening. Magnified and by reflected light, the colonies are matt and not smooth like those of diphtheroid organisms.

¹ See Wright, H. A. (1944), *Edinb. med. J.*, 50, 737.

The *intermedius* type show very tiny colonies which are strikingly uniform in size and appearance.

After Twenty-four to Forty-eight Hours.—*Gravis type*—seen by daylight, growths are slate-grey with a bluish tinge; individual colonies have a paler border and if well separated attain a diameter of 3 mm. after thirty-six to forty-eight hours. Magnified and by reflected light from an electric bulb, the surface of the colonies has a ground-glass appearance which only indistinctly reflects the image of the bulb; touched with a wire and observed with the plate-culture microscope the colonies are seen to break up very readily; the shape may approximate to the daisy-head formation as originally described (p. 515), but often the colonies show merely a radial striation sloping from the raised centre to the slightly crenated periphery; sometimes the colonies are convex and circular in outline, with only slight striation and crenation.

Mitis Type—the colour, consistence and size of colonies are similar to those of the *gravis* type and they also show a ground-glass appearance, though more glistening; the colonies, however, are convex and have a perfectly circular outline.

Intermedius Type—the colonies are never larger than 2 mm. and growths are more delicate than those of the other types; magnified and by transmitted light, the colonies are very uniform in size and seem "pricked out" on the surface of the medium; they are blacker than the other types, but are of the same roughness and consistency; they are usually domed and circular with a tendency to crenation, but occasionally the margin is flattened and they show a "poached egg" appearance; after forty-eight hours they are frequently papillate.

It should be noted that these colonies are sometimes difficult to distinguish from those of certain streptococci growing from throat swabs, but the latter are black or brown and somewhat flatter. A microscopic examination should be made if there is any doubt.

Diphtheroid Bacilli.—Growths are generally more glistening than those of the diphtheria bacillus; magnified and by reflected light, colonies usually have a smooth surface on which the image of the electric bulb is sharply delineated; in colour, they range from black or dark-brown to pale grey or greyish-white; the characteristic slate-grey colour of the *gravis* type of diphtheria bacillus is seldom seen; colonies are sometimes tough or mucoid in consistence, or soft and butyrous.

In examining plates a uniform procedure should be used throughout, since the precise appearance and colour vary with the nature of the illuminant and the angle of the light. Colour is best seen with the naked eye in the confluent parts of the growth. It must also be remembered that variations in batches of medium may influence colony characters; and in diagnostic work it is advantageous to use, for comparison, plates on which known strains of *gravis*, *mitis* and *intermedius* types have been inoculated and grown at the same time as the cultures under examination.

B. Direct plating on blood agar: as tellurite has some degree

F

of inhibitory effect on the diphtheria bacillus, the swab may be plated on digest agar containing 10 per cent. horse blood, which also helps to clarify a differential diagnosis of sore throat due to *Strept. pyogenes*. The diphtheria bacillus grows well on this medium, and, after incubation overnight, colonies can often be recognised and differentiated from those of other organisms. If no colonies can be recognised, a film is made from the confluent part of the growth on the plate and stained by Albert's method. This gives the same information as the microscopic examination of a Löffler's serum culture. As a supplementary procedure some of the confluent growth may be inoculated into Monckton's enrichment medium (*vide infra*).

*Monckton's Enrichment Method*¹.—The swab or a Löffler's serum culture is inoculated into blood-copper-sulphate-tellurite broth (B.C.T. broth)—p. 230—and incubated for six hours. During this time the diphtheria and diphtheroid bacilli grow vigorously while other organisms are suppressed. The B.C.T. culture is then subcultured on an ordinary blood-agar plate which is incubated overnight. The diphtheria bacilli grow well overnight as there is no inhibitory substance in the medium; the preliminary enrichment ensures abundant colonies. The staining appearance by Neisser's or similar method is typical. The resultant growth is practically a pure culture, and enough can be obtained from the plate to make a broth suspension for the virulence test. By this method an identification of the diphtheria bacillus can be made within twenty-four hours of receiving the swab or incubated Löffler's serum culture, and the result of a virulence or toxigenicity test is obtainable in forty-eight hours.

When swabs arrive at the laboratory too late for the necessary incubation in B.C.T. broth, they should be inoculated on Löffler's serum and also on an ordinary blood-agar plate, the colonies developing on the latter being examined for the characters described above. The next day the Löffler's serum culture or blood-agar growth can, if necessary, be sub-inoculated into B.C.T. broth.

It must be emphasised here that the responsibility for the diagnosis of diphtheria rests entirely with the clinician. The bacteriologist can merely state, by certain routine methods, whether organisms morphologically resembling the diphtheria bacillus are present in cultures from the specimen submitted to him or whether the growths on a tellurite medium are typical of this organism. Failure to find such organisms does not necessarily exclude diphtheria, nor does their presence prove the disease to be diphtheria.² *If the clinician considers a case to be diphtheria it is*

¹ Monckton, J. C. (1947), *Bull. Inst. med. lab. Tech.*, 13, 2.

² *A System of Bacteriology* (1930), 5, 100. London: Medical Research Council.

his duty to administer antitoxin at once, and continue to do so even if a negative laboratory report is received. The mortality from the disease is directly correlated with the delay in administering antitoxin, and where there is reasonable suspicion that the case may be diphtheria, antitoxin must immediately be used without waiting for a bacteriological report. It is also emphasised that a reliable laboratory report, particularly in cases where there is doubt clinically, cannot be made under eighteen to twenty-four hours, and at that stage the bacteriologist can report on morphological or cultural appearances only. To prove conclusively the identity and virulence of the organism may necessitate tests extending over several days. In order that the bacteriological report should be as helpful as possible, the utmost care must be taken that a suitable specimen is submitted and precise details as to the nature and source of the material should be furnished.

Allowance being made for the possible limitations of the routine diagnostic methods described, the results of such examination have undoubtedly proved of the greatest value as an aid to, and confirmation of the clinical diagnosis.

Chemotherapy.—Although diphtheria bacilli are sensitive to penicillin and other antibiotics, these drugs have no direct antagonistic action on diphtheria toxin and cannot be substituted for antitoxin therapy. Penicillin and erythromycin have, however, been used with some success to eliminate diphtheria bacilli from the respiratory tracts of both cases and carriers.

Prophylaxis.—Individuals having little or no antitoxin in the blood stream, e.g. as indicated by a positive Schick reaction, may be actively immunised by the injection of one of the following preparations:—

(1) Formol-toxoid (F.T.): diphtheria toxin modified in toxicity by formalin (p. 517). The recommended dosage is three intramuscular or deep subcutaneous injections each of 1·0 ml. at four-weekly intervals. The sensitivity to toxoid may be tested by the intradermal injection of 0·2 ml. of a 1 in 100 dilution of toxoid. This test (sometimes designated the Moloney test) should be negative if the full dose is to be given.

(2) Toxoid-antitoxin floccules (T.A.F.): a suspension of the precipitate of floccules formed when toxoid and antitoxin are mixed in appropriate "neutralising" amounts. Its tendency to cause reactions is slight, and a good immunity follows the injection of three doses, each of 1 ml., given at intervals of four weeks. T.A.F. has been recommended for the immunisation of adolescents and adults.

(3) Alum-precipitated toxoid (A.P.T.): a suspension of the

washed precipitate produced by the addition of a small amount of aluminium hydroxide to toxoid. The precipitate is relatively insoluble and the toxoid is gradually liberated from the site of injection. Reactions are negligible in children under eight years, but in older children and adults they may be somewhat more severe than with T.A.F. Two doses, each of 0.5 ml. are given at an interval of four weeks. The interval may be lengthened, e.g. to three or even six months.

(4) Another prophylactic which has been advocated is a suspension of purified toxoid adsorbed on hydrated aluminium phosphate (P.T.A.P.). Recent evidence has shown that this preparation is a more potent and more reproducible antigen than A.P.T.¹

Whatever prophylactic is used, a subsequent Schick test should be carried out on a sample of the inoculated community to test the immunizing potency of the antigen.

When an outbreak of diphtheria has occurred in a school or institution, the susceptible contacts should, after preliminary Schick-testing, be given passive immunity by the injection of a small dose of antitoxin (500–1000 units) followed by active immunisation, which may be begun immediately or after two weeks.

DIPHTHEROID BACILLI

These are non-toxigenic corynebacteria with little or no pathogenicity.

Corynebacterium hofmannii

A commensal of the throat.

Morphology and Staining.—Compared with the diphtheria bacillus it is shorter (about 2 μ) and may present a somewhat oval shape; stained with Löffler's methylene blue, an unstained bar in the middle of the organism is a frequent character and renders it not unlike a diplococcus. It is strongly Gram-positive; usually no volutin granules are detected by Neisser's or Albert's stains.

Cultural Characters.—Grows aerobically on ordinary media; growths are more abundant than those of the diphtheria bacillus, and the colonies are larger and more opaque. For appearances on one of the tellurite media and on blood agar, see pp. 526-7.

Biochemical Reactions.—No fermentation of glucose or sucrose. It is non-pathogenic to laboratory animals.

¹ For further details of diphtheria prophylactics, see Parish, H. J. (1958), *Antisera, Toxoids, Vaccines and Tuberculins*. Edinburgh: Livingstone.

Corynebacterium xerosis

A commensal in the conjunctival sac. Closely resembles the diphtheria bacillus, and may show volutin granules.

Can be differentiated from the diphtheria bacillus by its production of acid in sucrose and by its non-pathogenicity to laboratory animals.

Corynebacterium acnes

An organism associated with acne, but its aetiological relationship to the disease is doubtful. It is Gram-positive, rod-shaped, and measures about $1.5\ \mu$ by $0.5\ \mu$. It is markedly pleomorphic and frequently shows a beaded appearance.

Other Diphtheroid Types

Certain of these present a close morphological similarity to the diphtheria bacillus, and may exhibit the characteristic volutin granules by Neisser's staining method (p. 120), though differing in fermentative reactions, e.g. fermenting sucrose. They are mostly non-pathogenic, and have been isolated from the secretions of the nose and nasopharynx, the external ear, conjunctival sac, the skin, lymph glands (apart from disease) and other tissues, pus, wounds, etc.

Barratt has described diphtheroid bacilli in the nasopharynx which tend to resemble *Corynebacterium ovis*, the Preisz-Nocard bacillus (*vide infra*); these organisms liquefy gelatin; they are virulent to guinea-pigs and rats, but diphtheria antitoxin has no protective action against them. Cook and Jebb have reported somewhat similar organisms which ferment starch and might seem to be intermediate between *C. diphtheriae gravis* and *C. ovis*.¹

The following corynebacteria are common pathogens among domestic animals.

Corynebacterium ovis

(*Pseudotuberculosis ovis*)

This, the *Preisz-Nocard bacillus*, is the causative organism of caseous lymphadenitis and pseudotuberculosis in sheep. A similar organism is associated also with ulcerative lymphangitis of horses (*vide infra*).

This organism is allied to *C. diphtheriae* in its biological characters.

¹ See Cook, G. T., & Jebb, W. H. H., *J. clin. Path.*, 1952, 5, 161.

Morphology and Staining.—Non-motile, slender rod-shaped organism $1\text{--}3 \mu$ in length by 0.4μ in breadth. When stained, it shows beading or a barred appearance like other diphtheroid bacilli, and is Gram-positive. By Neisser's method volutin granules can be demonstrated. Club-shaped forms may be noted.

Cultural Characters.—Growth occurs at 37° C . under both aerobic and anaerobic conditions on ordinary nutrient media.

Agar—growth is at first scanty; the colonies are small, thin, dry and greyish-white in colour, folded and granular and often show concentric rings.

Löffler's medium—colonies are similar to those on agar, but exhibit a yellowish colour; there is no liquefaction.

Broth—a granular growth occurs with sometimes a surface pellicle.

Gelatin—slow liquefaction occurs with most strains.

Glucose, maltose and dextrin are fermented, but usually not sucrose, lactose or mannitol. A haemolysin is produced.

Occurrence.—The associated disease in sheep is chronic and characterised by involvement of lymphatic glands, which are enlarged and caseous. Caseous nodules are seen also in the internal organs, e.g. lungs, spleen, liver and kidneys. The organism can be demonstrated in films or sections prepared from the various lesions.

Experimental Inoculation.—Laboratory animals, e.g. guinea-pig and rat, are susceptible to experimental infection with cultures. Intravenous injection in the guinea-pig produces a lethal effect within about ten days, and at autopsy caseous areas are noted in internal organs, e.g. lungs and liver. Intraperitoneal injection in a male animal leads to involvement of the tunica vaginalis as in the case of experimental glanders (p. 571). Subcutaneous injection is followed by lymphatic gland involvement, the glands showing the characteristic caseation. In rats, inoculation produces a fatal septicaemia. Sheep are also susceptible to experimental inoculation and die with an intense icterus associated with intravascular haemolysis.

This organism produces an exotoxin resembling, to some extent, that of the diphtheria bacillus, but not neutralisable by diphtheria antitoxin. Guinea-pigs are highly susceptible to this toxin and show at the site of subcutaneous inoculation an inflammatory lesion with oedema and haemorrhage, while the internal organs are congested and often contain small haemorrhages; there is, however, no change in the suprarenals and no pleural effusion (cf. diphtheria).

Diagnosis.—Films are prepared from the lesions and stained by

Gram's method, methylene blue and by Ziehl-Neelsen's method (to exclude acid-fast bacilli). Cultures are made, and pure growths from single colonies are investigated as regards cultural characters and experimental pathogenesis.

Similar organisms are found in equine ulcerative lymphangitis (pseudo-farcy), and in pseudo-tuberculosis (caseous lymphadenitis) of bovines (*Corynebacterium bovis*). These organisms form a group of closely related types. They have frequently been classified as one species—the "*Preisz-Nocard bacillus*". A similar organism (*C. murisepticum*) produces a septicaemic disease in mice.

Corynebacterium pyogenes

An organism associated with suppurative lesions in pigs, cattle and certain other animals. It may occur in mastitis of cattle and sheep.

Morphology and Staining.—Non-motile, rod-shaped organism not usually exceeding 2μ in length. Shows great pleomorphism. Gram-positive in young cultures. Stained with methylene blue, diphtheroid forms may be seen with deeply stained bands or granules, but volutin granules are not usually observed in preparations stained by Neisser's method.

Cultural Characters.—Aerobe and facultative anaerobe, but some strains grow better under anaerobic conditions. Optimum temperature about 37° C. Generally requires media containing blood or serum. The colonies on serum media are at first minute, but after several days' growth may attain a size of 2–3 mm. in diameter. They present no specially characteristic appearances. Inspissated serum, egg medium and gelatin are all liquefied. This organism is haemolytic when growing on blood agar, and a filterable haemolytic toxin demonstrable in suitable culture medium is lethal for mice and rabbits. In milk, acid and clot result in three days and after a time the clot is digested. Glucose, lactose and, in some cases, sucrose but not mannitol are fermented.

A variant type which lacks proteolytic action has been described.

Pathogenesis.—In the natural infection in swine, suppurative lesions may occur in various parts of the body, liver abscesses and arthritis being specially frequent. In cattle, the organism has been found associated with a variety of suppurative lesions, e.g. abscesses, pyaemia, pyelitis, mastitis, endometritis, etc.

Rabbits can be infected experimentally; intravenous injection of cultures produces a pyaemic condition with bone and joint lesions. Guinea-pigs are less susceptible.

Corynebacterium renale

This organism has been described in cystitis and in pyelonephritis of cattle. It resembles other organisms of the diphtheroid group in general characters. It digests milk casein, but has no action on gelatin or coagulated serum and no haemolysin is produced. Litmus milk is rendered alkaline after two or three days' incubation and a deposit is formed with a clear supernatant fluid having the colour of burgundy. Urea is actively converted to ammonia. Glucose is fermented; some strains also ferment laevulose and mannose. Injected intravenously in large doses into mice and rabbits, it may give rise to a fatal pyelonephritis within four weeks, the lesions produced being confined exclusively to the medulla.

Corynebacterium equi

This organism has been reported as the causative organism of pneumonia in foals. It has also been isolated from a number of other animal species as in pyometra in cattle and from suspected tuberculous lesions in the cervical lymph nodes of pigs.

It differs from other members of the diphtheroid group in its profuse viscid growth and the production of a red pigment; there is no haemolysis and no liquefaction of coagulated serum or gelatin. Carbohydrates are not fermented and urea is not hydrolysed. Some workers have found it to be weakly acid-fast and unusually resistant to oxalic acid.

Subcutaneous inoculation produces abscess formation in horses, pigs and goats, but there is no evidence of the formation of a toxin. Intraperitoneal inoculation into guinea-pigs gives rise to peritonitis.

ERYSIPLOTHRIX: LISTERIA

Erysiplothrix and *Listeria*, which are members of the family *Corynebacteriaceae*, have many similar biological characters and some workers would place them in the same species. They are common pathogens of both domestic and wild animals and birds and sometimes produce infection in man.

Erysiplothrix rhusiopathiae

(*Ery. insidiosa*)

The causative organism of Swine Erysipelas.

Morphology and Staining.—Slender, Gram-positive, non-motile rod-shaped organism $1\text{--}2\mu$ by $0\cdot2\text{--}0\cdot4\mu$, occurring singly and in chains. In culture media, longer and filamentous forms are

observed. True branching has been described, and on this account the organism was once classified as an *Actinomycete*.

Cultural Characters.—Growth occurs on ordinary media even at room temperature, though the optimum is about 37° C. The organism shows a tendency to be micro-aerophilic when first isolated, and in agar-shake cultures may grow best just below the surface, but is able to grow under both aerobic and anaerobic conditions. In gelatin-stab culture a line of growth occurs along the wire track with lateral spikes or disks radiating from the central growth. Surface colonies on plates are of two types: one exceedingly minute and dewdrop-like, with a smooth surface; it does not exceed 0.5 mm. in diameter when growing on agar. The other is larger and has a granular appearance. Various carbohydrates are fermented (without gas production), e.g. glucose, lactose; sucrose and mannitol are not fermented. Different groups of the organism have been recognised according to their antigenic characters.

Animal Pathogenicity.—Mice, rats, rabbits and pigeons are susceptible to inoculation. Mice and pigeons are specially susceptible, and usually die of an acute septicaemia within four or five days after experimental inoculation. Subcutaneous injection in rabbits produces a spreading inflammation and oedema with a fatal result. Experimental inoculation (with cultures) in swine reproduces the disease as it occurs naturally. The smooth-colony type of culture is the more pathogenic.

Occurrence.—In pigs the bacilli can be observed in the characteristic diamond-shaped skin lesion, and in internal organs, e.g. lungs, spleen and kidney. In some cases there is a septicaemic condition and the organism is detectable in blood films, particularly in leucocytes. In the chronic form of the disease, in which a "verrucose" endocarditis occurs, the bacilli may be confined to the cardiac lesions.

A similar organism, *Erysipelothrix muriseptica*, is responsible for epizootic septicaemia in mice. It is doubtful whether this organism constitutes a separate species.

Ery. rhusiopathiae may occur in apparently healthy pigs, and has been isolated from the tonsils, intestines and faeces. It has a wide distribution in other animals and in birds. It is also found, apparently as a commensal, on the skin and scales of many fish (particularly members of the perch family).

Laboratory Diagnosis.—For diagnostic purposes an attempt should be made to cultivate the organism from lesions and in acute cases from the blood; inoculation tests should also be carried out in mice or pigeons. An agglutination test is applicable.

Artificial immunisation against the disease is carried out by the injection of killed vaccine in which the organisms are adsorbed on aluminium hydroxide and suspended in an oily base. The organism is sensitive to penicillin, which has largely replaced the use of immune serum for therapeutic purposes.

Cases of human infection with this organism are known as "Erysipeloid" and have a distinctive clinical picture. There is very severe pain and swelling of a finger or a part of the hand with a dusky, greyish discolouration of the skin of the affected area. The condition is an occupational hazard for those who handle infected animals or fish; the majority of recorded cases have been in abattoir workers, butchers, fishmongers, laboratory workers and veterinary surgeons. According to Sneath *et al.*¹ (1951) it is seldom possible to recover *Erysipelothrix rhusiopathiae* from swabs, and the most satisfactory method is to obtain a biopsy from the actively growing edge of the lesion and to incubate this for forty-eight hours in 1 per cent. glucose broth, subculturing on to blood agar.

Listeria monocytogenes

This organism owes its specific name to the fact that infection by it in laboratory animals, *e.g.* rabbits and guinea-pigs, produces a monocytosis in the blood. Although it was originally isolated from these animals it is responsible for disease in a wide variety of hosts, *e.g.* foxes, dogs, gerbils, guinea-pigs and recently in chinchillas imported into this country. In farm animals the disease is mainly an encephalitis, although the organism has been found in foetuses from cases of abortion in cattle and sheep; in rodents and poultry necrotic hepatitis and myocarditis are frequently seen. It is a rare cause of meningo-encephalitis in man and of granulomatosis infantiseptica in the newborn.

Morphology.—It occurs as a Gram-positive straight or slightly curved non-sporing rod, 2–3 μ by 0·5 μ (average), often in pairs, end to end at an acute angle. Sometimes elongated filaments may be observed, particularly in solid medium at room temperature. It is feebly motile at 37° C., but in young broth cultures at 25° C. it is more active and exhibits up to four flagella. Young cultures of the organism are Gram-positive, but after forty-eight hours many are Gram-negative, while in older cultures they may be entirely Gram-negative.

Cultural Characters.—Cultures can be obtained at 37° C. under

¹ Sneath, P. H. A., Abbott, J. D., & Cunliffe, A. C. (1951), *Brit. med. J.*, ii, 1063.

aerobic conditions on ordinary media, but growth is better on media containing liver extract, blood, serum or glucose. The colonies are at first very small and droplet-like; after a few days' growth they may attain a diameter of 2 mm., being smooth and transparent, though later they may be more opaque. Surface colonies on blood agar are surrounded by a narrow zone of complete haemolysis. Gelatin and Löffler's serum are not liquefied. In stab culture, growth occurs evenly along the length of the stab. A scant colourless growth is obtained on MacConkey's medium, while on tellurite medium the colonies are small, black and glistening, surrounded by a characteristic zone of green coloration. In glucose, maltose and certain other common sugars acid is promptly produced without gas; lactose and sucrose are fermented slowly, but mannitol is not acted on.

Listeria is susceptible *in vitro* to penicillin, streptomycin, the tetracyclines, chloromycetin and erythromycin, but resistant to sulphonamides, bacitracin and polymyxin.

Animal Pathogenicity.—No demonstrable exotoxin or endotoxin is produced. Experimentally, the organism is pathogenic for rabbits, mice and guinea-pigs, but not for rats and pigeons. It gives rise to focal lesions on the chorio-allantoic membrane of chick embryos. It has been suggested that for the primary isolation of *Listeria* from the brains of sheep and cattle, refrigeration of the tissues is essential before inoculation of enriched medium.

Human Infections.—Cases of meningo-encephalitis in man have been described, characterised by a suppurative meningitis with mostly a mononuclear or polymorphonuclear exudate in the C.S.F. and a monocytosis. There is no confirmation of the claim that *Listeria* is causally related to infectious mononucleosis.

An intra-uterine infection, characterised by extensive focal necrosis especially of liver and spleen, known as granulomatosis infantiseptica has been described by Continental workers. It causes a high mortality in the affected foetus or newborn child.

CHAPTER XXIII

MYCOBACTERIA

Definition.—Slender rods occurring mostly in pairs or small clumps; tendency to clubbing and even true branching; difficult to stain because of waxy constituents, but once stained resist decolorisation with acid (acid-fast); non-motile, non-capsulated, non-sporing; very slow growth; aerobic; some species have not been cultivated on artificial media.

Pathogenic species affect man, mammals, birds and reptiles and are widely distributed throughout the world. The pathogenic members of *Mycobacterium tuberculosis* which merit particular attention here are the *human* and *bovine* strains; the *avian* strain very rarely affects man and more often pigs; the *murine* strain affects voles and has been used as a prophylactic vaccine in man. *Myco. leprae* is the cause of leprosy, which today occurs mostly in tropical and sub-tropical countries. *Myco. johnei* causes a chronic enteritis in cattle and sheep.

Mycobacteria characteristically produce chronic granulomatous lesions, which, in the case of tuberculosis, break down by caseation. The most common lesion is pulmonary tuberculosis. Leprosy occurs in either a tubercular or maculo-anaesthetic form and is a very chronic infection.

MYCOBACTERIUM TUBERCULOSIS

Human Type

Morphology.—Slender, straight or slightly curved rod-shaped organisms, $2\cdot5$ – $3\cdot5 \mu$ by $0\cdot3 \mu$, with rounded, pointed or sometimes expanded ends. In the tissues they may occur singly, or in pairs often forming an obtuse angle, or in small bundles of parallel bacilli. The organism is non-motile and non-sporing, though it possesses considerable powers of resistance to drying. In old cultures, individual cells may grow into long filaments and show branching.

Staining.—The tubercle bacillus is more difficult to stain than other bacteria. A strong dye with a mordant is required (e.g. carbol fuchsin), and either prolonged staining or the application of heat. It may stain uniformly or show marked beading. When

stained it resists decolorisation with 20–25 per cent. sulphuric or nitric acid, and also with alcohol, and is therefore described as "acid- and alcohol-fast" (pp. 116, 118). The tubercle bacillus is Gram-positive, but can be demonstrated only with difficulty by Gram's method.

It seems possible that the tubercle bacillus may occur in the tissues in a non-acid-fast form which is not demonstrable by the Ziehl-Neelsen method. It was originally claimed by Much that a granular phase of the organism could be recognised; and it has been stated that a filterable form of the organism can be demonstrated, but this lacks confirmation.

Cultural Characters.—Aerobe; temperature range, 30°–41° C.; optimum, 37°–38° C.

Does not grow on ordinary media. Primary growths may be obtained on blood or serum media, on pieces of animal tissue or on a medium containing egg-yolk (p. 211). In secondary culture, growths may result on ordinary media (agar, broth, potato) with 5–6 per cent. glycerol added; growth is slow—e.g. ten days may elapse after primary inoculation, or even sub-inoculation, before growth is apparent.

A most convenient medium for artificial culture in ordinary laboratory work is one of the *glycerol-egg media* (pp. 211–13); the growth is luxuriant and presents the following appearance: dry, irregular, tough and tenacious, wrinkled or mammillated, at first white, later buff-coloured.

If a fragment of inoculum is floated on the surface of *glycerol broth*, growth spreads *on the surface of the medium* as a white wrinkled pellicle.

Fairly rapid *submerged growth* can be obtained in a fluid medium designed by Dubos (p. 214) containing casein hydrolysate, bovine serum-albumin, asparagine and certain salts along with a surface-active water-soluble lipoid commercially known as "Tween 80", which is a polyoxyethylene sorbitan mono-oleate. This medium can be solidified and used for surface growths by incorporating agar.

Viability.—The thermal death-point is 60° C. for 15–20 mins. While many individual bacilli die when desiccated, a proportion survive for several weeks or months, if protected from daylight. The organism is relatively resistant to injurious chemical substances; it can survive in putrefying material, and in sputum may resist 5 per cent. phenol for several hours. It is highly susceptible to sunlight and ultra-violet radiation, and ordinary daylight, even through glass, has a lethal effect. *Myco. tuberculosis* is sensitive to streptomycin, viomycin and cycloserine among the antibiotics

and to para-amino-salicylic acid (PAS), iso-nicotinic acid hydrazide (INAH or isoniazid), thiosemicarbozone and pyrazinamide among chemotherapeutic agents.

Biochemical reactions are not ordinarily used in the identification or classification of tubercle bacilli. Antigenic analysis has shown that there are four main serological groups—mammalian (human, bovine and murine), avian, reptilian and saprophytic.

Animal Pathogenicity.—The guinea-pig is highly susceptible to experimental infection. If injected subcutaneously with the bacilli either in pathological material or in culture, after a few days a local swelling results consisting of tubercle nodules, which become confluent, undergo caseation and finally ulcerate. The neighbouring lymph glands become involved by spread of the bacilli along lymphatic channels and, later, lymph glands in other parts of the body are affected, showing the characteristic tuberculous lesions. The animal begins to lose weight and dies in six weeks to three months. At autopsy, a generalised tuberculosis is noted; the spleen is enlarged and contains greyish-white tuberculous nodules or larger necrotic lesions. The liver presents a similar condition. The lungs, however, may show relatively slight lesions and the kidneys may be practically free from tubercle nodules.

If the animal is killed four or five weeks after injection, secondary tuberculous nodules may be present only in the spleen and on the peritoneum. Smears must always be made from one or more of the lesions and stained by Ziehl-Neelsen's method to demonstrate acid-fast bacilli.

Guinea-pigs and rabbits can be infected also by inhalation and by feeding.

Mice are much less susceptible to experimental tuberculosis than are guinea-pigs but after intraperitoneal or intravenous (or intracerebral) inoculation they develop progressive or chronic lesions according to the dose and virulence of the strain.

The human type of tubercle bacillus, apart from its occurrence in human disease, has been found also in natural tuberculosis of monkeys, cattle, pigs and dogs.

Bovine Type

Morphology and Staining reactions are practically identical with those of the human type.

Cultural Characters.—As compared with the human type, growth is less luxuriant, and the bovine type is described as

"dysgonic" (the human type being referred to as "eugonic"). On egg medium it forms a thin, white, smooth, slightly moist, granular and easily broken-up growth. The difference between the human and bovine types is accentuated by using a glycerol-egg medium. Glycerol favours the growth of the human type, but has no such effect on the bovine variety.

Pathogenicity to Animals.—The bovine type is more virulent to cattle and other domestic animals than the human type. In the ox it produces a fatal tuberculosis, whereas the human type causes only a localised lesion which heals spontaneously.

The difference between the two types can be elicited by injecting a rabbit intravenously with an emulsion in saline of 0·01–0·1 mg. of dried bacilli (from a culture). The bovine type produces an acute generalised tuberculosis, and the animals usually die within three to six weeks; in the case of the human type the animals survive, or die only after several months, with slight lesions confined usually to the lungs and kidneys.

The differentiation may also be made by injecting 10 mg. of culture subcutaneously in the rabbit; the bovine type leads to a general tuberculosis, which is fatal usually within ten weeks, whereas the human type produces only a local lesion.

It is to be noted that strains which deviate in their characters from the standard human and bovine types may be met with. Thus, strains isolated from lupus are frequently of attenuated virulence for laboratory animals.

The bovine type of tubercle bacillus, in addition to its association with tuberculosis of cattle, is the commonest variety found in tuberculosis of most other domesticated animals (*e.g.* pigs, horses, cats.)

Occurrence.—The most common form of primary infection with the tubercle bacillus is a pulmonary lesion, known as the primary complex or Ghon focus. The organisms are inhaled in very small particles (not more than 5 μ in diameter) into the terminal bronchioles or alveoli, and the primary lesion may occur in any part of the lungs. From it, the organisms are carried by lymphatic drainage to the regional mediastinal glands in which there may be progressive enlargement and involvement, followed by caseation and later calcification. If the glandular lesion erupts into a blood vessel or bronchus, there results either miliary or bronchopneumonic tuberculosis, usually with lesions in other organs, *e.g.* meninges (tuberculous meningitis), spleen, liver, etc. Primary infection may also occur via the intestine with involvement of the mesenteric glands or via the tonsils with secondary cervical adenitis, usually from ingestion of infected milk. These forms of

tuberculosis have become much rarer now that most milk supplies are pasteurised and bovine tuberculosis is virtually eradicated. From studies during the Second World War about a quarter of the cases of cervical adenitis in children under ten years of age were at that time caused by the human type and about three-quarters of the cases of meningeal and bone and joint tuberculosis were also due to the human type. Primary infection of the skin (*lupus vulgaris*) is now a rare condition; infection via skin lesions sometimes occurs from handling infected materials (laboratory workers and veterinarians) while intracutaneous B.C.G. vaccination may produce a form of primary complex with local skin lesion and an associated adenitis.

While tuberculous meningitis occurs characteristically as a complication of the primary lung infection in very young children, it may also develop, *e.g.* in older children and young adults, as an apparently primary infection. In such cases, as in many instances of renal and bone and joint tuberculosis, there has presumably been an early infection, blood-borne from the primary lesion in the lung or elsewhere, which has lain latent until some factor has encouraged fresh activity. Sometimes, too, an active lesion in one area may help to reactivate a latent infection in another organ.

The secondary (or adult) form of pulmonary infection is the most common form of tuberculosis. It is characterised by one or more lung lesions which break down (caseation and cavitation) and, involving the bronchial tree, create a case of open tuberculosis. This clinical lesion occurs characteristically in young adults and is most often due to a fresh exogenous infection; in older people pulmonary tuberculosis is more likely to be a reactivation of an earlier healed primary or secondary lesion. Tuberculous ulceration in larynx and intestine are usually sequelae of pulmonary tuberculosis spread by infected sputum; similarly secondary infections of ureter, bladder, etc., follow renal tuberculosis.

Epidemiology.—Tuberculosis has a world-wide distribution and today probably takes precedence over malaria as *the* most important contributor to death and disability among the specific infections. Yet, the mortality from tuberculosis has been falling dramatically in the more developed countries due to effective chemotherapy, so that deaths in Britain have been reduced by over 80 per cent. during the decade 1948-58. On the other hand, morbidity rates are just beginning to show a downward trend or, where there has been intensive case finding with mass radiography may actually be increasing (as in Scotland during 1957-58; see chart, p. 542).

With the control of bovine tuberculosis which in earlier years

TUBERCULOSIS (NOTIFICATIONS AND DEATHS) IN SCOTLAND

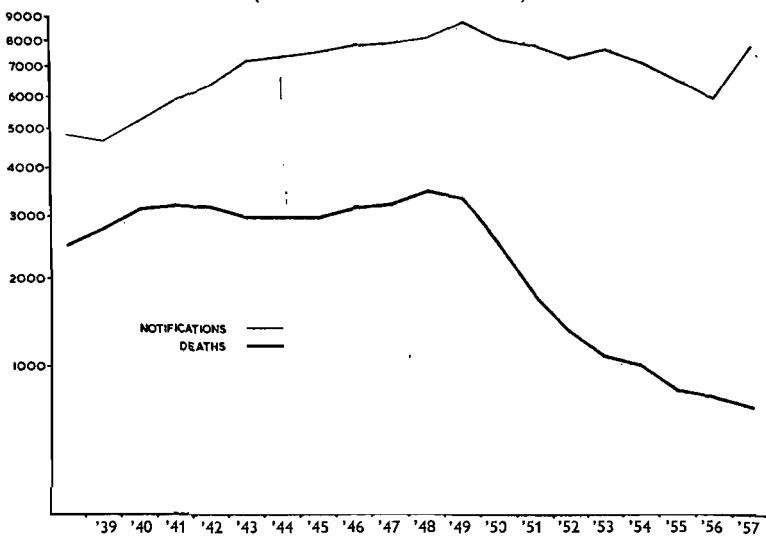


FIG. 24

accounted for 5–10 per cent. of all deaths and about a quarter of the cases of childhood tuberculosis, infection is nowadays predominantly due to the human type of *Myco. tuberculosis* and is spread mostly from open cases of pulmonary tuberculosis. The organism is expectorated in sputum and expelled in droplets during coughing and speaking. Since very small droplets that can be inhaled directly from the infective patient are less likely to carry tubercle bacilli than larger droplets or sputum, infection may occur more often indirectly from dried dust particles than directly from moist droplets. Tubercle bacilli survive slow drying for days or weeks if protected from the bactericidal daylight or sunlight so that the spread of infection from infected case to susceptible contacts by contaminated dust or fomites would be facilitated in overcrowded, badly lit rooms or factory buildings. Primary infection may occur at any age; if it occurs in early life (0–3 years) it is often associated with signs of disease, e.g. hilar tuberculosis or the more serious systemic infections. At school age (5–15 years) infection usually occurs in an inapparent form, but in adolescents and young adults is again more likely to result in clinical disease. The death-rates in five-yearly periods (infancy to 35 years of age) from all forms of tuberculosis in 1951 are shown in the following Table to indicate the relationship of age to severity of infection.

	Age in Years						
	0-4	5-9	10-14	15-19	20-24	25-29	30-34
Death-rate per 100,000	16.5	4.5	3.4	12.4	26.1	38.5	35.8

Infection occurs earlier and is more likely to result in clinical disease among susceptibles living in close contact with open cases, but many personal and environmental factors may contribute to overt tuberculosis, e.g. age, malnutrition, other respiratory disease, hormonal dysfunction, pregnancy, stress, genetic constitution, etc.

Workers exposed to the inhalation of stone-dust, etc., containing silica have a high incidence of tuberculosis. Nurses, medical students, doctors and workers in pathological laboratories are more exposed and tend to have a higher than average rate of infection.

The Tuberculin Test

Koch made the original observation that when a living, and later a killed, culture of tubercle bacilli was injected subcutaneously into a guinea-pig infected some weeks earlier with tubercle bacilli (resulting in a slowly progressive disease), there rapidly developed a local inflammatory lesion followed by necrosis and ulceration. This has been called the *Koch phenomenon*, and Koch went on to show that the same phenomenon could be elicited with an extract containing the specific protein of the tubercle bacilli, which he called *tuberculin*. The tuberculin reaction is due to the development of tissue hypersensitivity—or bacterial allergy—and is used in man and animals to find if they have or have had tuberculosis in an active or latent form.

Tuberculin was originally obtained from a six-week-old culture in glycerol-broth, evaporated to one-tenth of its volume, sterilised by heat and filtered (*Old Tuberculin—O.T.*). Various other methods have since been employed in its preparation. The specific tubculo-protein can now be separated from other constituents and products of culture in a synthetic medium and then purified. This *Purified Protein Derivative (P.P.D.)* is preferable to Old Tuberculin as it is constant in composition and potency, and there is an absence of non-specific substances. It is

issued in the dry state, from which it is easy to prepare dilutions by the addition of a borate buffer solvent.

It should be noted that tuberculins prepared from the human and bovine types of tubercle bacillus are indistinguishable by the usually accepted methods of standardisation as they contain the same specific substance.

Tuberculins are standardised under T.S.A. Regulations in such a way that a dilution of 1:10,000 of O.T. is equivalent to 1 tuberculin unit (T.U.), while 0.000028 mg. of P.P.D. equals 1 unit. A common practice in using tuberculin is to test first with 5 T.U. and if the individual gives a negative reaction to re-test with a dose of 100 T.U.

Tuberculin tests in man are carried out on the skin by different techniques; those most commonly used in Britain are the Mantoux, Heaf and Jelly tests. The Mantoux test consists in the intradermal injection of 0.2 ml. of the appropriate dilution of tuberculin; the test is positive when there is an area of induration measuring 5 mm. in diameter two to three days after the injection. With the Heaf test, a multiple puncture spring release gun is used to prick the tuberculin into the skin; a positive reaction may range from 4-6 discrete papules to solid induration. These two tests are usually done on the forearm. In the Jelly test, a tuft of erculin jelly is applied in the form of a "V" in the intrascapular area of the back and covered with plaster; with it, but not with the other two tests, a control test is used.

In a recent comparison¹ of these three and the Vod Pirquet scarification test, in which such factors as sensitivity, reproducibility, ease of performance and consistency were compared, only the Mantoux and Heaf tests were shown to be reliable and acceptable for large-scale epidemiological investigations: in general, the Heaf was preferred to the Mantoux test. The tuberculin test may be used: (a) epidemiologically, to determine the incidence of tuberculous infection in a community; In a large-scale Medical Research Council survey² in England and Wales in 1949-50 to cover the age-period 5-20 years, there was a steadily increasing proportion of positive reactors from around 15 per cent. in the 5-year-olds to 33 per cent. in the 14-year-olds and 70 per cent. in those aged 20 years. Among young children, the incidence was highest in rural areas (especially in counties with much bovine tuberculosis) and was higher in industrial urban

¹ A Report to the Research Committee of the British Tuberculosis Association. (1958) *Tubercle (Lond.)*, 39, 76; (1959) *ibid.*, 40, 317.

² *Lancet* (1952), i, 775.

than in non-industrial urban areas. There is evidence of a steady reduction in the incidence of positive reactors in more recent years; (b) diagnostically in young children with suspected clinical infection. A positive reaction in a young child may also be used for case-finding among the family contacts; (c) in immunisation campaigns in order to separate the positive and negative reactors and to assess the response to vaccination by sample testing afterwards.

The Tuberculin Test in Cattle.—The tuberculin reactions have been utilised also in the recognition of tuberculosis in cattle, and are of great importance in testing milk cows. Thus, in England *tuberculin-tested milk*, and in Scotland *certified milk* and *tuberculin-tested milk*, must be obtained from tuberculin-tested animals which yield a negative reaction. Until recently the test in cattle has usually been carried out in this country by the *double intradermal method*, but today the *single intradermal comparative test* is preferred. P.P.D. tuberculins are used, doses of 0·1 ml. being injected intradermally in the neck. Two sites are chosen, the upper one for the avian tuberculin and the lower site (5 in. below) for the mammalian tuberculin: the skin thickness in the area is measured. The test is read at the seventy-second hour, the skin thickness being again measured and the reaction examined for evidence of oedema. Any swelling showing oedema or an increase of 4 mm. or more in skin thickness should be regarded as positive. The interpretation of this test depends on the presence or absence in the herd of non-specific infections such as Johne's disease or so-called skin tuberculosis that may sensitise the animals to tuberculin. In cases where the comparison between the reactions to the two tuberculins proves inconclusive, the animals concerned should be isolated and re-tested not earlier than thirty days following completion of the herd test.

Great progress has been made in the eradication of bovine tuberculosis in Great Britain and it is anticipated that the last eradication area will become an *attested area* towards the end of 1960, which means the virtual elimination of the disease.

Laboratory Diagnosis

Tubercle bacilli are most numerous in acute lesions showing rapid caseation, e.g. acute phthisis. In acute miliary tuberculosis they appear to be relatively scanty. In chronic closed lesions few tubercle bacilli are observed, and they may not be detectable microscopically though demonstrable by animal inoculation, e.g. in the pus from a tuberculous abscess.

media for the primary cultivation of tubercle bacilli have been described; it is advisable to use a medium which experience has proved to give constantly good results with a minimum of contaminations.

(b) Nassau's modification of Jungmann and Gruschka's method
—Prepare the following solutions:

Solution A.

Ferrous sulphate	:	20 gm.
Sulphuric acid 20 per cent. (vol. per cent.) . . .	:	100 ml.

Solution B.

Hydrogen peroxide	3 per cent. w/v (10 vols.)
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Solution A can be made up in bulk and keeps indefinitely. Solution B must be made up fresh on each occasion from a standard pharmacopoeal 6 per cent. w/v solution kept in the dark. For use place 2 ml. of sputum in a universal container. Add 1·2 ml. solution A and 1·2 ml. solution B. Shake the container for thirty seconds and allow to stand on the bench for twenty minutes, shaking at intervals. Centrifuge at 3000 r.p.m. for thirty minutes and discard the supernatant fluid. Fill the container to the shoulder with 5 per cent. sterile sodium citrate, shake vigorously, and again centrifuge. Decant the supernatant fluid and inoculate the deposit on two slopes of Löwenstein-Jensen medium.

Note.—In applying the method of direct cultivation the possibility must be borne in mind of *saprophytic* or *atypical* acid-fast bacilli (*vide infra*) occurring in cultures from the various materials examined. Since these organisms sometimes yield growths not unlike that of the tubercle bacillus, careful scrutiny of the culture should be made, and if there is any doubt further tests including animal pathogenicity tests should be carried out.

Micro-Culture.—Methods have been devised for cultivating tubercle bacilli directly from sputum by smearing the specimen thickly on glass slides, e.g., microscopic slides divided longitudinally in two, treating them with some agent to destroy organisms other than tubercle bacilli, e.g. 6 per cent. sulphuric acid for twenty minutes, and then washing thoroughly with sterile distilled water, and finally placing the slides in a suitable fluid medium, e.g. citrated human blood lysed in an equal volume of 1 per cent. saponin. After incubation for about a week the slides are stained by the Ziehl-Neelsen method and young colonies of the tubercle bacillus can be seen by microscopic examination first with the low-

power and then the oil-immersion lens. This method has also been applied in testing resistance to chemotherapeutic drugs.¹

Guinea-pig Inoculation.—In laboratory diagnosis, where tubercle bacilli cannot be detected in specimens by microscopic examination, guinea-pig inoculation in addition to direct cultivation may be used, as one or other (or both) of these procedures often yields positive results when microscopic examination is negative. With most materials, culture gives at least as high a proportion of positive results as guinea-pig inoculation and has the advantages that a positive finding can be reported earlier and the culture is immediately available for sensitivity tests. In suspected renal tuberculosis, on the other hand, guinea-pig inoculation of microscopically negative urinary deposits seems to be more often positive than culture.

The usual method of carrying out the guinea-pig inoculation test is to inject material subcutaneously in the flank or intramuscularly in the thigh of two guinea-pigs, one of which is killed at four weeks and the other at eight weeks. The result of the inoculation test can sometimes be expedited by intradermal inoculation, 0·4 ml. of the material being injected into the shaved abdominal skin. If the specimen is tuberculous, a nodule appears in seven to twenty-one days; this is incised, and films of the lesion are examined for tubercle bacilli.

Laryngeal Swab; Gastric Lavage

Where sputum is absent or, if present, is being swallowed, material for culture may be obtained either by the laryngeal swab or by stomach wash-out.

The laryngeal swab consists of a piece of nickel or "Nichrome" wire 9 in. long and $\frac{1}{16}$ in. in diameter, bent at an angle of 120° an inch and a half from the end. Cotton-wool is wrapped round this end of the wire, which has been flattened and spirally twisted to hold the cotton-wool firmly. Each swab is placed in a boiling-tube (7 x 1 in.) the open end of which is plugged with cotton-wool. The tubes with swabs in position are sterilised in the autoclave. Immediately before use the swab is moistened with sterile distilled water.

The swab may be passed into the larynx either with the guidance of a laryngeal mirror, or blindly. For both methods the patient is seated and supplied with a piece of gauze with which to hold his tongue fully protruded. The operator, if using a mirror, sits

¹ Pryce, D. M. (1941), *J. Path. Bact.*, 53, 327; Oeding, P. (1951), *Acta tuberc. scand.*, 25, 208.

opposite the patient and with the aid of the mirror guides the tip of the swab over the epiglottis into the larynx. If working blindly, the operator stands at the side of the patient and passes the swab through the mouth backwards and downwards strictly in the mid-line; the manœuvre is facilitated by asking the patient to take panting breaths. Reflex cough usually develops as the swab enters the larynx, and absence of cough suggests that the swab has not entered the larynx. Two consecutive swabs should be taken from each patient.

In the laboratory sufficient 10 per cent. H_2SO_4 (v/v) is added to the boiling-tube to cover the cotton-wool on the swab. After five minutes in acid the swab is removed, excess of fluid is expressed on the side of the tube, and the swab is transferred for a further five minutes to a second boiling-tube containing a similar amount of 2 per cent. NaOH. The swab is again drained of excess fluid and is rubbed over the surface of a slope of Löwenstein-Jensen medium. The inoculated medium is incubated at 37° C. for six to eight weeks and examined at weekly intervals.

An alternative procedure is to immerse the swab in a saturated solution of trisodium phosphate and incubate at 37° C. overnight. The swab is then removed and the fluid centrifuged at 3000 r.p.m. for twenty minutes. The supernatant is decanted and the deposit is inoculated on to two Löwenstein-Jensen slopes.

It is inadvisable to leave a long delay between the collection of the swabs and inoculation on to culture medium.

Fasting stomach contents may be aspirated with a Ryle tube in the morning after a period of coughing and swallowing. The material should be despatched to the laboratory as quickly as possible, or if there is likely to be a long delay, the contents should be neutralised with caustic soda. The deposit is homogenised by either the modified Petroff or the modified Jungmann method (*vide supra*) and inoculated on two slopes of Löwenstein-Jensen medium. Patients prefer the laryngeal swab to gastric lavage, which, however, may give at least as high a proportion of positive results. Neither of these methods will give as good results as scanty expectoration.¹

Serological tests for specific antibodies following tuberculous infection have not proved of much diagnostic value. The complement-fixation test becomes positive in a high proportion of established cases but is usually negative in early or suspected cases. In 1948, Middlebrook and Dubos² described a haemagglutination test which depends on the agglutinating effect of patients' serum

¹ Forbes, G. B., et al. (1948), *Lancet*, ii, 141.

² Middlebrook, G., & Dubos, R. J. (1948), *J. exp. Med.*, 88, 521.

on sheep or group O human red blood cells sensitised with an extract of tubercle bacilli.

Haemagglutination Test.—Sheep red cells are *sensitised* with an antigenic extract of the tubercle bacillus or with commercial "Old Tuberculin"; the cells are then mixed with varying dilutions of patient's serum, from which any heterophile antibody has been removed by absorption (p. 61); if specific antibody is present, the cells are agglutinated and the degree of the reaction is indicated by the highest dilution of serum producing agglutination. This test has received a good deal of study and clinical trial, but its practical value in the diagnosis of clinical tuberculosis is doubtful because of its lack of both specificity and sensitivity.¹

Chemotherapy.—Although the tubercle bacillus is sensitive to an increasing range of antimicrobial drugs, clinical experience has shown that the most effective and least toxic chemotherapeutic substances in tuberculosis are streptomycin, para-amino-salicylic acid (PAS) and isonicotinic acid hydrozide (Isoniazid). Because resistant variants of the tubercle bacillus may emerge within two to three months after commencing treatment with any *one* of these drugs, it is essential that (*a*) a combination of two—or three—antimicrobial drugs be used from the onset of treatment in cases of tuberculosis in order to minimise this risk and (*b*) every effort must be made to culture and test the sensitivity of infecting strains, since a strain that is already resistant to one of a pair of drugs being used will soon develop resistance to the other.

Prophylaxis.—Killed vaccines of the tubercle bacillus give little or no protection against tuberculosis. In 1921, two French workers, Calmette and Guérin, introduced a living vaccine prepared from a bovine strain that had been cultured for many years on a bile-potato medium. This Bacille-Calmette-Guérin (B.C.G.) vaccine was at first given orally, but following Scandinavian practice is now given mostly by intracutaneous injection. Its value in the prophylaxis of tuberculosis has been much disputed, but in recent years controlled trials in different countries and with different communities and age-groups have demonstrated conclusively that B.C.G. vaccination gives protection of the order of 80 per cent. against clinical infection. Thus, in large-scale trials among school-leavers (14 years of age) in industrial areas in England, the incidence of clinical disease was reduced by approximately 85 per cent. in the vaccinated groups during a follow-up period of six to seven years (see pp. 939-40).² In a study among

¹ Hilson, G. R. F., & Elek, S. D. (1951), *J. clin. Path.*, **4**, 158.

² Medical Research Council Report (1959), *Brit. med. J.*, ii, 379.

North American Indians (0-20 years old) a similar degree of protection persisted for some ten years after vaccination.¹

Fresh liquid vaccine deteriorates on storage and it is recommended that it be used within fourteen days of preparation. It loses viability quickly in warm atmospheres ($30^{\circ}\text{ C}.$) or if exposed to light; this reduces its usefulness in under-developed tropical and sub-tropical countries.¹ Another disadvantage is that tests for safety and potency cannot be completed before the liquid vaccine is released for use. To overcome these difficulties, freeze-dried vaccines have been developed and clinical trials with a British freeze-dried vaccine have shown that it is comparable in potency with the Danish liquid vaccine.

Laboratory estimates of potency depend on the direct relationship that has been demonstrated between protection and the degree of post-vaccination tuberculin sensitivity in the guinea-pig. The degree of sensitivity required is defined by T.S.A. Regulations. For the freeze-dried vaccine, the tuberculin sensitivity produced in children is tested for each batch at the B.C.G. Control Centre in the Oxford Region. The dose of liquid B.C.G. vaccine is a single dose, given intracutaneously, of 0.1 ml. containing 0.05 to 0.1 mg. moist weight of the constituent bacilli (= 1-2 million viable organisms). A small inflammatory lesion develops at the site of inoculation and goes on to superficial ulceration after some weeks. Adenitis of the regional lymph nodes may follow in infants (rarely in older children). Occasionally an erysipeloid lesion (lupus) may occur, particularly if vaccination has been done by multiple puncture. Oral vaccination with repeated large doses is practised in some countries, but there have been no properly controlled trials to substantiate claims for the protective value of the oral vaccine.

Mycobacteria of Ulcerative Lesions in the Human Subject.—Ulcerative lesions of the skin have been reported in Australia and Sweden from which mycobacteria, clearly distinguishable from the tubercle bacillus, have been isolated. The Australian strains, *Myco. ulcerans*, were found to be pathogenic to mice and rats but not guinea-pigs. In culture the optimum temperature was $33^{\circ}\text{ C}.$ ² The infections described in Sweden were derived from swimming pools, and the strains of mycobacteria isolated, *Myco. balnei*, were highly pathogenic to mice; in guinea-pigs they were only slightly pathogenic; in rabbits inoculation of the skin produced ulcers similar to the

¹ Aronson, J. D., et al. (1958), *Arch. intern. Med.*, **101**, 881.

² MacCallum, P., et al. (1948), *J. Path. Bact.*, **60**, 93.

human lesions. The Swedish strains grew at 31° C. but not 37° C.¹

Mycobacterium avium (*Avian tubercle bacillus*).—The causative organism of tuberculosis in birds. Its morphology and staining reactions are the same as those of the other types of tubercle bacilli. Its optimum temperature is 40°–43° C., and on glycerol-agar the growth is more rapid in development, moister, more homogeneous and more luxuriant than that of the mammalian types, the culture presenting a somewhat creamy appearance. Individual colonies are large, raised, hemispherical, with a smooth shiny surface and a yellow or brownish-yellow colour.

It is highly virulent to fowls, which are resistant to the mammalian tubercle bacilli. For testing purposes, 0·001 mg. of culture is injected intravenously or the bacilli are administered by feeding. The guinea-pig, which is highly susceptible to the human and bovine types, is resistant to the avian bacillus. The rabbit shows a moderate degree of susceptibility.

This type of tubercle bacillus also occurs in pigs, and has been reported in other domesticated mammals, including cattle. Human tuberculosis due to the avian type has been recorded, but is an extreme rarity.

Mycobacterium tuberculosis var. **muris**.—This organism (*Vole tubercle bacillus*) has been found in a tubercle-like disease of voles, which is fairly widespread. Morphologically the bacilli are longer and thinner than the typical tubercle bacillus and often somewhat curved. Growth on egg medium is very slow and is not enhanced by the presence of glycerol. Antigenically the organism is similar to the human and bovine types. Inoculated experimentally in voles it produces typical tuberculosis, but in guinea-pigs, rabbits and calves is of low virulence, moderate doses producing only a local lesion. The vole tubercle bacillus is practically non-pathogenic to man. Inoculation of the organism in guinea-pigs and calves brings about immunity to virulent tubercle bacilli; and it is now being used as a vaccine as an alternative to B.C.G.

Mycobacterium piscium.—Acid-fast bacilli resembling the tubercle bacillus have been isolated from fish and other cold-blooded animals, e.g. frogs, turtles, etc., and have been regarded as aetiologically associated with a tubercle-like disease in such animals. These organisms grow best at 25° C. and flourish even at 15° C. In cultural characters they correspond to the avian type (*vide supra*). They are not pathogenic

¹ Linell, F., & Norden, A. (1954), *Acta tuberc. scand. Suppl.*, 33.

to mammals or birds, but produce lesions on experimental inoculation in frogs, fish, etc.

Atypical Mycobacteria

Acid-fast bacilli which cannot be identified as either human or bovine tubercle bacilli and which may be associated with human disease have attracted considerable attention in recent years and have been given the unsatisfactory name *atypical mycobacteria*. (The alternative term "anonymous" has also been proposed.) These mycobacteria have been divided into four main groups: group I are photochromogens, which develop lemon or orange-yellow pigment only when exposed to light; group II are scotochromogens, which develop yellow or orange-red pigment in the dark; group III are not deeply pigmented and have resemblances to the avian bacillus; group IV grow rapidly at both 37° C. and at room temperature in contrast to groups I-III; they are not usually pigmented. Atypical mycobacteria associated with disease processes are relatively rare in this country (probably less than 1 per cent. of cultures isolated from sputum) and belong mostly to the photochromogenic group I. Their pathogenicity may be increased in association with pneumoconiosis. Identification will depend on (a) careful scrutiny of primary cultures, noting particularly morphology (beading, pleomorphism), growth characteristics and pigmentation; (b) the effect of exposure to light on pigment production; (c) drug sensitivity: atypical strains are usually resistant to thiosemicarbazone; (d) the catalase test in association with isoniazid sensitivity; (e) the Niacin test; and (f) virulence in the mouse (positive) and the guinea-pig (negative or localised abscess).¹

Saprophytic and Commensal Mycobacteria.—Non-pathogenic acid-fast bacilli may be found in milk, butter, manure, water, grass (e.g. *Mycobacterium phlei*) and the smegma of man and animals. They are similar in morphology to the tubercle bacillus, but their growth on culture medium is rapid; they develop on ordinary media and at room temperature (though the optimum may be 37° C.), producing an abundant dry or slightly moist growth which is irregular, coarsely granular and sometimes wrinkled; most strains are definitely pigmented—yellow, pink or brown.

Acid-fast bacilli have been frequently demonstrated in the deposits from the interior of laboratory taps. This possibility

¹ Gilani, S., & Selkon, J. B. (1958), *Tubercle*, 39, 396.

must be borne in mind in using tap water for preparing films and staining solutions. It has also been pointed out that such organisms may be present on bark and rubber corks and rubber washers such as are used with specimen containers.

Mycobacterium smegmatis (*Smegma bacillus*).—This is a commensal organism found in smegma and sometimes on the skin. It conforms in biological characters to the saprophytic types described above. As it may occur in specimens of urine, it has to be differentiated carefully from the tubercle bacillus. It is generally shorter and thicker than the latter, and shows greater variation in size and shape. The smegma bacillus is acid-fast, but in urinary deposits is often decolorised by alcohol (p. 118), which has no effect on the tubercle bacillus. It is also less resistant to antiformin than the tubercle bacillus.

Mycobacterium leprae

The causative organism of Leprosy.

Morphology and Staining.—A straight or slightly curved slender bacillus, about the same size as the tubercle bacillus, with pointed, rounded or club-shaped ends; so far as is known it is non-motile and non-sporing. Like the tubercle bacillus it requires, as a rule, a strong stain, and is acid-fast, though not to the same degree; it may stain uniformly, but usually shows marked beading, which may be coarser than that of the tubercle bacillus; it is Gram-positive, and can be stained fairly readily by the ordinary Gram's method (*cf.* tubercle bacillus).

Cultivation.—A great many attempts have been made by various workers to cultivate this organism; the majority have been unsuccessful, and though successful results have been claimed and cultures of acid-fast bacilli have apparently been isolated from leprous lesions, it is doubtful whether these strains represent the true leprosy bacillus. Some success in culturing the organism of rat leprosy on tissue cultures has recently been claimed.

Occurrence and Distribution.—Leprosy is an infective granuloma, developing as (1) the "nodular" type, in which granulomatous nodules ("lepromata") form in the skin, mucous membranes and various organs (*e.g.* lungs, liver, spleen, testes), or (2) the "maculo-anaesthetic" type, where the granulation tissue infiltrates certain nerves and leads to motor and sensory paralysis, with characteristic trophic changes (*e.g.* anaesthetic skin areas—"maculae"). Both types of the disease may occur in the same patient.

The organisms are found in the granulomatous lesions, being particularly numerous in the nodular form. They are distributed intracellularly for the most part, parallel bacilli occurring in bundles which may completely fill up cells. They may be found also in the tissue spaces, in the walls of small vessels, in skin glands, lymph glands and in the secretions of the nose, throat and mouth, due to the fact that the mucosal lesions ulcerate readily and discharge bacilli into the mucous secretions. The organisms do not occur in the maculae which are essentially trophic and not primarily leprosy lesions. In leprosy the bacilli have actually been observed in organs without associated lesions.

The bacilli are present in the nerve granulomata, but are less numerous than in the nodular lesions.

Laboratory Diagnosis.—Films are made from any ulcerated nodule on the skin, or a non-ulcerated nodule can be punctured with a needle and squeezed till lymph exudes, from which films are made. Films can be prepared also from a scraping of an excised piece of tissue, or sections may be prepared as for histological examination. A convenient method is to remove, with curved scissors, a piece of skin (about 2 mm. deep) overlying a nodule, and prepare films from the deep surface.

Films should be made in all cases from the nasal mucosa or secretion, as diagnostic information may be obtained in this way even when nodules are not present in the skin. This also applies to the maculo-anaesthetic cases.

The films or sections are stained by the Ziehl-Neelsen method, substituting 5 per cent. sulphuric acid for 20 per cent. Stained leprosy bacilli may resist decolorisation with 20 per cent. sulphuric acid, though in general they are less strongly acid-fast than the tubercle bacillus.) The presence of the characteristic acid-fast bacilli, especially when they occur in large numbers and are situated inside cells, is generally diagnostic.

When the lungs are affected the bacilli may be demonstrated in the sputum, but require to be differentiated from the tubercle bacillus by animal inoculation; the leprosy bacilli do not produce any pathogenic effects in laboratory animals.

Mycobacterium lepraeumurium.—The organism of "rat leprosy" which presents some pathological similarity to human leprosy, is an acid-fast bacillus related to, but not identical with, the leprosy bacillus. This disease of rats is transmissible experimentally to animals of the same species, but not readily to other species, though transmission to the hamster has been recorded. It should be noted that human leprosy cannot be transmitted to rats.

Mycobacterium johnei*(Bacillus of Johne's Disease)*

The causative organism of a chronic enteritis of cattle, and a similar disease of sheep; sometimes called *Myco. paratuberculosis*.

Morphology and Staining.—A Gram-positive, acid-fast and alcohol-fast bacillus like the tubercle bacillus, but more readily stained by the Ziehl-Neelsen method. It is often comparatively short (1 to 2 μ), but cannot be distinguished microscopically from the tubercle bacillus. It stains uniformly, though the longer forms may stain irregularly.

Cultural Characters.—Has proved difficult to cultivate artificially. Growths can be obtained on glycerol-egg medium containing 1 per cent. killed *Myco. tuberculosis* or other acid-fast bacilli, e.g. *Myco. phlei* (a grass bacillus), or extracts of these organisms.¹ The organism requires a growth factor which is synthesised by other members of the acid-fast group; this factor is also present in certain vegetable tissues and in some fungi. It is very stable and can be partially replaced by vitamin K. Primary growths develop very slowly and four weeks may elapse before they are definitely visible. The optimum temperature is about 38° C. After continued cultivation in this way, subcultures may be obtained on egg media without the addition of another acid-fast organism or its products. Cultures resemble those of the tubercle bacillus.

Experimental Inoculation.—The disease is transmissible experimentally to calves and young goats, the incubation period being several months. Lambs can also be successfully infected. Laboratory animals are mostly refractory although in recent years mice and young rabbits have been artificially infected with the production of lesions, containing the bacilli, in the intestines, mesenteric lymph nodes, liver and spleen.

Occurrence.—The lesions are of a granulomatous nature and lead to corrugated thickening of the mucosa of the intestine; the small bowel is primarily affected. The bacilli are present in large numbers, usually packed inside the cells of the lesions (as in leprosy).

The disease in sheep may be caused by either of two types; the classical *Myco. johnei* as isolated from cattle, or a variant which has proved more difficult to grow artificially and produces an

¹ Taylor, A. Wilson (1950), *J. Path. Bact.*, **62**, 647.

orange-coloured pigment.¹ The disease has also been reported in goats, horses, deer, gnu, antelope, camels, and a llama.

Diagnosis.—At autopsy, the characteristic acid-fast bacilli may be demonstrated in the mucous membrane of the bowel by the appropriate staining methods and cultivated from this tissue after treatment with antiformin or oxalic acid.

During life, the organism may be observed in the faeces; microscopically, groups of small acid-alcohol-fast bacilli may generally be regarded as diagnostic. Tuberculosis may be excluded by the inoculation of material containing the acid-fast bacilli into laboratory animals.

An allergic skin reaction evoked by "Johnin", a preparation (from cultures) analogous to tuberculin, has been utilised in diagnosis, but its diagnostic specificity is doubtful. Tuberculin prepared from the avian type of tubercle bacillus yields a similar reaction.

The Johne bacillus, in common with other pathogenic mycobacteria, is capable of stimulating the production of complement-fixing antibodies which can be demonstrated in the blood serum of cattle. The value of the complement fixation test for the diagnosis of Johne's disease in tuberculosis-free herds has been under investigation for a number of years and today a negative complement-fixation reaction is one of the requirements for the importation of cattle to a number of countries.

¹ Taylor, A. Wilson (1951), *J. Path. Bact.*, **63**, 333.

CHAPTER XXIV

THE ANTHRAX BACILLUS

MALLEOMYCES

LARGE straight Gram-positive rods occurring in chains which grow aerobically and form heat-resistant spores belong to the genus *Bacillus*. The Gram-positive property of strains is variable. The spores are ubiquitous and are extremely common in dust so that a large proportion of bacteria contaminating cultures belong to this group. These organisms exist as saprophytes in soil, water, air and on vegetation—e.g. *Bacillus mycoides* and *Bacillus subtilis*. *Bacillus anthracis*, the causative organism of anthrax in man and animals is the only pathogen of the group, though very occasionally species such as *B. subtilis* have been isolated from the tissues in terminal disease.

Anthrax is primarily an infectious disease of domestic herbivores; in them it occurs in various forms from a fulminating septicaemia to a subacute or chronic fever with localising pustular lesions. Man contracts the disease sporadically by coming into contact with infected animals or contaminated animal products. Anthrax is uncommon in the United Kingdom and North America but is relatively common in many other parts of the world.

BACILLUS ANTHRACIS

Morphology.—A non-motile, straight, rod-shaped, sporing bacterium, rectangular in shape and of relatively large size— $4-8\ \mu$ by $1-1.5\ \mu$. The bacilli tend to be arranged in chains end to end (streptobacilli), but may occur singly and in pairs. In blood and tissue they exhibit a distinct capsule when suitably stained. Unlike the capsules of many other bacteria (which are of polysaccharide nature), the capsule of the anthrax bacillus contains a polypeptide of D-glutamic acid. The spore, when fully developed, can be seen as a refractile oval structure, central in position and of the same cross-diameter as that of the bacillus. Sporulation occurs readily when the organism is discharged from the body of an infected animal, and spores are a morphological feature of the bacilli when growing in artificial culture, but *sporulation does not occur in the tissues*. After the spore is fully formed, the residual protoplasm of the bacillus disintegrates and

the spore becomes a free structure. The spore represents a highly resistant phase of the organism, and can survive under conditions which would be unfavourable to the vegetative form. When replaced in favourable conditions, the envelope of the spore ruptures at one pole and the vegetative phase is reproduced.

Staining.—The organism in the tissues is strongly Gram-positive. In films prepared from cultures the staining reaction of individual bacilli is variable; older cells and the vegetative remnants of sporulating cells may be Gram-negative. The spore is unstained by the ordinary methods, but can be stained differentially by special methods (p. 121).

Methylene-blue Reaction of McFadyean.—This staining reaction has been utilised in veterinary work for the recognition of anthrax bacilli in blood films. The films are made in the usual way on slides, dried and passed rapidly three times through the flame; they are then stained with polychrome methylene blue for a few seconds (p. 110), washed and dried. Between the bacteria an amorphous purplish material is noted, representing the disintegrated capsules of the organisms; this appearance is characteristic of the anthrax bacillus.

Cultural Characters.—Aerobe and facultative anaerobe; temperature range, 12°–45° C.; optimum, 35° C.; grows on all ordinary media; aerobic conditions are necessary for sporulation, for which the optimum temperature is 25°–30° C. Germination of spores takes place under both aerobic and anaerobic conditions. Rapid germination occurs in the presence of certain amino acids such as adenosine, *l*-alanine and *l*-tyrosine.

Colonies on agar—white, granular, circular disks (about 3 mm. in diameter after twenty-four hours' growth) which, under the low power of the microscope, show a wavy margin, often likened to locks of hair, and presenting the "medusa-head" appearance. The colony is one continuous convoluted thread of bacilli in chain formation.

Agar stroke—thick, white, opaque, somewhat dry, friable growth with irregular edges, showing the same microscopic characters as the colonies. To the naked eye this growth presents a ground-glass appearance.

Gelatin stab—a line of growth along the wire puncture from which fine lateral spikes radiate, longest towards the top. This is the so-called "inverted fir-tree growth"; liquefaction occurs later, starting at the top of the growth.

Coagulated serum is partially liquefied.

Broth—growth develops as white flakes which sediment, and sometimes shows pellicle formation.

Growing on blood agar the anthrax bacillus is only slightly haemolytic as compared with the "anthracoid" bacilli (p. 569), which are markedly lytic.

Variation.—Capsule formation is subject to variation, and when the capsule is absent or imperfectly developed the colonies tend to be moist and slimy and may be devoid of the characteristic wreathed margins. This is well seen in cultures which have been attenuated in virulence by growth at temperatures above the optimum, e.g. 42°–43° C., as in Pasteur's method of attenuating the organism for prophylactic vaccination.

The typical colony, as described above, is of the "rough" form; the variant is small, "smooth" and without the characteristic wreathed appearance, while the bacilli in this type of colony are arranged in bundles, not in a convoluted chain. Virulence is associated with the "rough" form, the "smooth" variant being relatively avirulent.

Viability.—The vegetative cells are as susceptible as other non-sporing bacteria. The thermal death-point is about 60° C. for half an hour. The spore is highly resistant to chemical and physical changes in the environment, though there is a marked strain variation in this respect. The spores of many strains will resist dry heat at 140° C. for one to three hours and 100° C. moist heat (steam or boiling) for five to ten minutes. Five per cent. phenol requires several weeks to kill the spores, but 1: 1,000 mercuric chloride destroys them in thirty minutes and 4 per cent. potassium permanganate in fifteen minutes. For the disinfection of wool a 2 per cent. formaldehyde solution can be used and allowed to act for twenty minutes at 102°–105° F. (39°–40.5° C.). Animal hair and bristles can also be disinfected of anthrax spores by six hours' treatment with 0.25 per cent. formaldehyde solution at 60° C., and such treatment does not affect the colour and texture of the material.

It should be noted that the usual heat fixation and staining of microscopic preparations from cultures of the anthrax bacillus may not affect the viability of the spores, and laboratory infection from handling such material has been recorded. The fixation of films by 1: 1000 mercuric chloride for five minutes has been claimed to kill the spores and does not interfere with the staining reactions.

The germination and vegetative growth of spores of most strains is inhibited by penicillin (0.1 µg./ml. or less), streptomycin (0.5–2 µg./ml.), the tetracyclines (0.1–0.5 µg./ml.), erythromycin (1 µg./ml.), chloramphenicol (2.5–10 µg./ml.) and sulphonamides, in *in vitro* tests.

Biochemical Reactions.—Glucose, sucrose, maltose, trehalose and dextrin are fermented with acid but no gas production. Nitrates are reduced to nitrites.

Antigenic Structure.—Three distinct antigenic components have been recognised, a somatic protein, somatic polysaccharides and a capsular polypeptide.

The protein somatic antigen (protective antigen) stimulates immunity in most animals and it can be shown to be present in the oedema fluid of anthrax lesions. This antigen cannot be recognised by the usual antigen-antibody tests, but has been shown by *in vivo* neutralisation of the toxic power of the antigen. However, on agar-diffusion plates a line of precipitation occurs which correlates with the immunising activity of preparations containing the antigen.

The capsular polypeptide is composed mainly of D-glutamic acid, and this substance is found only in virulent strains. Antisera prepared by inoculating animals with encapsulated organisms react with the isolated polypeptide which by itself is not an antigen (hapten). Capsular antibody is not protective. Polypeptides of related chemical and immunological nature are found in other species of *Bacillus*—e.g. *B. subtilis*. The capsular polypeptide of this organism contains L-glutamic acid as well as the D-form.

The somatic polysaccharide is combined with a peptide moiety containing α and ξ -diaminopimelic acid to form a complex included in the cell wall of the organism. By itself the isolated polysaccharide acts as a hapten and will react with antisera produced against the whole bacillus—e.g. in precipitin reactions.

Additional antigens are undoubtedly present, but as yet are uncharacterised.

Animal Pathogenicity.—All mammals are susceptible, though to a varying degree. Some cold-blooded animals can also be infected. Guinea-pigs and mice are highly susceptible to experimental inoculation. If a guinea-pig is injected subcutaneously with pathological material containing the bacilli, or cultures, the animal dies, usually within two days, showing a marked inflammatory lesion at the site of inoculation and extensive gelatinous oedema in the subcutaneous tissues. Large numbers of the bacilli are present in the local lesion. The animal exhibits a profound septicaemia and the anthrax bacilli are present in large numbers in the heart blood and in the capillaries of the internal organs. They are specially numerous in the spleen, which is enlarged and soft, and in the kidneys. With virulent strains the LD₅₀ by the subcutaneous route is of the order of five bacteria or less.

Experimental production of anthrax by inhalation of con-

taminated aerosols has also been studied. Spores deposited on the alveolar walls are taken up by phagocytes and carried to the tracheo-bronchial glands. Infection spreads via the lymphatics to the general circulation. The LD₅₀ is about 20,000 organisms if the particle size of the aerosol is less than 5 μ , since the smaller particles are more likely to penetrate in the air-stream to the alveolar walls, but is much higher if the particles are larger.

Infection of some species of animal may be produced by the oral route with a relatively large number of spores (e.g. 10⁸⁻⁹), but guinea-pigs are resistant to infection by this route.

Bacillus anthracis appears to be pathogenic because of at least three substances,¹ all of which have been shown to have aggressive activity and are lethal. First is the polypeptide composed of D-glutamic acid which occurs in the capsule; second is an impure lipo-protein fraction from the body of the bacillus, and third an extracellular specific lethal factor which can produce secondary shock, and whose effect may be neutralised by specific antiserum. These substances inhibit the bactericidal and phagocytic defence mechanisms of the host and allow the organism to multiply unhindered. Strains are only fully pathogenic when they produce all of these substances, and the absence of one may make the strain relatively non-virulent.

Death of infected animals is due to the production of the lethal factor, part of which appears to be associated with the somatic protein antigen (protective antigen), which produces classical secondary shock with fall in blood pressure, haemoconcentration, haemorrhage and oedema, disturbances in electrolyte balance and carbohydrate metabolism. This factor can be detected in young cultures but appears to be destroyed after five hours' incubation.

Occurrence.—The anthrax bacillus produces an epizootic disease in herbivorous animals, particularly among sheep and cattle, but no species is completely immune. The condition is usually septicaemic in nature, and *post-mortem*, the bacilli are found in large numbers in the heart blood and internal organs, especially the spleen, which is enlarged and soft (splenic fever). Subacute and chronic disease also occurs in animals as do localising pustules which are analogous to the malignant pustule in man. In animals the portal of entry is the mouth and intestinal tract, the spores being ingested with coarse vegetation which probably predisposes to trauma of the mucosa.

The spores germinate at the site of entry and the vegetative cells produce "toxins" leading to the formation of gelatinous oedema

¹ Smith, H., & Keppie, J. (1955), *Mechanisms of Microbial Pathogenicity*, p. 126. Camb. University Press.

and haemorrhage. In the susceptible animal the bacilli resist phagocytosis and reach the lymphatics and thence the blood stream. Before death the bacilli multiply freely in the blood and tissues. In the resistant animal there is a more profuse leucocyte response with phagocytosis and decapsulation of the organism.

In man infection is acquired from animal sources, usually through damaged skin or mucous membranes, or more rarely by inhalation of spores into the lungs. Infection thus occurs most commonly through the skin in persons such as farmers and veterinarians handling infected animals, or among dock workers, factory workers and farmers from handling carcasses and hides, animal hair and bristles, shaving-brushes, feeding-stuffs, bone-meal, etc. The resulting lesion is usually described as a *malignant pustule*. This starts as a papule and becomes a blister within twelve to forty-eight hours and then a pustule with an increasing area of inflammation depending upon the resistance of the host. Coagulation necrosis of the centre results in the formation of a dark-coloured *eschar* which is later surrounded by a ring of vesicles containing serous or sero-sanguineous fluid, and outside this is an area of oedema and induration which may become very extensive.

Infection may result from inhalation of spores carried in dust or filaments of wool from infected animals, as in the wool factories—“wool-sorters’ disease”. The organisms settle in the lower part of the trachea or in a large bronchus, and an intense inflammatory lesion results, with haemorrhage, oedema, spread to the thoracic glands, involvement of the lungs, and effusion into the pericardial and pleural cavities; the organisms are present in considerable numbers in the lesions; a septicaemic condition or a haemorrhagic meningitis may supervene.

In general, the infectivity of the anthrax bacillus for man is not of a very high order. When a case of anthrax occurs in an industrial establishment, spores of the bacillus are often widely distributed and in large numbers in the environment.

Infection may occur by the intestinal route, but this is relatively uncommon except in primitive societies using infected animals for food in which outbreaks with a high mortality may occur.

Epidemiology.—Anthrax is primarily a disease of animals, and man is only secondarily infected.

In the terminal stages of the disease in animals the bacilli are present in very large numbers in faeces, urine and saliva, and these may contaminate ground and pasture and be ingested directly by other animals, though direct spread of this type is rare. Pasturage may also be contaminated from the carcasses of dead animals. The vegetative cells rapidly sporulate and remain viable

for many years, constituting enzootic foci of infection. The spores are ingested by cattle and sheep, pass the stomach and invade the small intestine mucosa of the new hosts which serve to perpetuate the disease. In the United Kingdom the disease is sporadic amongst cattle and is commonest in the winter months when it can usually be traced to imported feeding-stuffs that have been contaminated with anthrax spores, especially bone-meal imported from areas where animal anthrax is common, e.g. the Far East.¹

In countries where the disease is relatively rare in animals, industrial anthrax from contamination with imported materials is the commonest form of infection in man.

Labarotory Diagnosis.—Malignant Pustule—Films are made from the exudate and stained by Gram's method; the finding of bacilli morphologically like *B. anthracis* is suggestive but not conclusive. If there are unbroken vesicles round the lesion, fluid from these should be examined.

Successive-stroke inoculations should be made on an agar plate. The resulting colonies are recognised by examining them with the low power of the microscope, and films are made and stained by Gram's method. Spores are noted in cultures.

In all cases the identity of the suspected organism must be confirmed by inoculation of a guinea-pig or mouse with exudate from the lesion, or with the isolated culture. A small dose of culture is sufficient to produce a lethal effect. The occurrence of the bacilli in the heart blood and in the spleen in considerable numbers, and the other *post-mortem* appearances described above, are diagnostic.

In carrying out the *post-mortem* examination, the precautions detailed on p. 425 should be observed.

If exudate used for inoculation contains other organisms, it is advisable to inoculate it on a scarified area of skin in preference to subcutaneous injection.

Diagnosis of Anthrax in Domestic Animals (post-mortem).—The usual form of *post-mortem* examination must not be made, in order to prevent any distribution of sporing bacilli from the carcase. In the body no sporulation occurs, but spores are readily formed when the bacilli are exposed to air. Films of blood taken from a superficial vein in the ear are prepared, and stained by Gram's method and by McFadyean's methylene-blue method (*vide supra*). The finding of characteristic bacilli in the blood giving the methylene-blue reaction is diagnostic. In pigs and horses the bacilli may not be detectable in the blood. If necessary, the

¹ Jamieson, W. M., & Green, D. M. (1955), *Lancet*, i, 560.

organism can be cultivated and identified by the procedure described above, a specimen of blood from the ear being used for the investigation.

Isolation of Bacillus Anthracis from Heavily Contaminated Material.—It should be noted that after eighteen hours in the dead animal anthrax bacilli may alter and appear like putrefactive organisms. If the material is heavily contaminated, shake a portion with water and allow it to stand for three to four hours with occasional shaking. Squeeze or tease the material and heat the supernatant fluid to 70° C. for ten minutes. Add different volumes (0·2 ml.-2·0 ml.) of this fluid to melted agar, pour plates and incubate at 37° C., from evening to early morning. It is essential to examine the plates early. Examine for deep colonies which have a typical filamentous appearance. A rich culture medium is essential and plates should not be too crowded with colonies. Confirmation is obtained by inoculation subcutaneously in the mouse or guinea-pig.

For direct isolation by animal inoculation centrifuge 50 ml. (or more) of the heat-treated fluid described above at high speed (3000 r.p.m.) for fifteen minutes. Discard the supernatant and inoculate the residue intramuscularly into a guinea-pig which has been passively immunised twenty-four hours previously with *Cl. welchii* antitoxin 1000 units, *Cl. septicum* antitoxin 500 units, *Cl. oedematiens* antitoxin 1000 units and tetanus antitoxin 500 units; or a polyvalent gas-gangrene serum with added tetanus antitoxin. Death due to anthrax occurs in two to three days. Death from gas-gangrene (usually due to *Cl. septicum* or *Cl. bifermentans*) occurs earlier. In the latter cases aerobic cultures from the local lesion and spleen should be made as they may yield *B. anthracis*. The minimum infecting dose is 30-50 spores.

Precipitin Test.—This test was first used by Ascoli in the recognition of anthrax infection in organs and tissues from suspected carcases, and may be applicable even in the case of putrefied material. It depends on the occurrence of a specific precipitin (p. 64) in the serum of an artificially immunised animal. Immune sera, however, vary in their precipitin content, and for the test a serum with known precipitating properties must be selected. About 2 grams of the tissue are boiled for five minutes with 5 ml. of normal saline, to which acetic acid has been added in the proportion of 1: 1000. The fluid is cooled and then filtered through paper. 0·5 ml. of the serum is placed in a narrow tube and the filtrate is carefully run on to the top. The development within fifteen minutes of a white ring of precipitate at the junction of the two fluids denotes a positive result.

Examination of Shaving Brushes, Wool, etc.—The bristles or wool are cut up with scissors into small fragments and shaken thoroughly with several volumes of sterile salt solution or soaked in a 3 per cent. solution of caustic potash, so as to obtain "washings" from the material. The fluid is decanted and centrifuged. The sediment is suspended in about 1 ml. of saline and heated at 70° C. for ten minutes to eliminate non-sporing organisms present. (It is inadvisable to heat at higher temperatures or for longer periods as some of the spores of the anthrax bacillus may not survive under these conditions.) Cultures on agar plates are then made from the suspended sediment, and guinea-pigs are injected subcutaneously with the material, and later, with cultures of any suspected organism isolated, if the direct inoculation test is negative. It is to be noted that in such examinations, organisms very similar in their morphological and cultural characters to the anthrax bacillus may be encountered, e.g. "*B. anthracoides*" (*vide infra*). Such organisms, if injected in large doses, may also produce fatal effects in mice and guinea-pigs. Their differentiation from *B. anthracis* is referred to later.

Chemotherapy.—Most antibiotics to which the anthrax bacillus is sensitive in *in vitro* tests have been used successfully in the treatment of anthrax in man. Chemotherapeutic agents have no effect upon the "toxins" already produced, therefore it is important to institute therapy as soon as possible.

The serum of artificially immunised animals e.g. Sclavo's serum has been used in the treatment of human anthrax combined with antibacterial drugs. Doses of 50–100 ml. given intravenously, and repeated daily if necessary in severe cases, have been recommended.

In animals, treatment is not often possible as most cases are not diagnosed till moribund or dead. Penicillin in large doses and chlortetracycline should be given combined with immune serum.

*Prophylaxis and Control.*¹—In outbreaks of animal anthrax, affected animals must be promptly diagnosed and isolated. Carcasses must be disposed of by deep burial in quicklime or cremation to limit sporulation of the organism from the tissues and spread to pasturage and other animals. The limitation of the import of animal hides and hair to a single port (Liverpool), where facilities for mechanical handling and disinfection are available ("Duckering"), has done much to reduce the danger of anthrax in the United Kingdom (see Anthrax Order, 1938).

The eradication of anthrax in animals can be assisted by active

¹ Spears, H. N., & Davidson, J. C. (1959), *Vet. Rec.*, 71, 637.

immunisation procedures. There are numerous vaccines in use. Pasteur's attenuated type has been extensively used. This vaccine is a culture of the bacillus whose virulence has been reduced by continuous growth at 42°–43° C., but a great drawback is that it is difficult to regulate the degree of attenuation. This vaccine is being replaced by the avirulent vaccine ("spore vaccine" of Sterne¹), prepared from a non-capsulate strain of *B. anthracis* which is therefore avirulent but produces "toxin" sufficient to give a strong immunity when inoculated as living spores. This vaccine has many advantages particularly as it is harmless; it may be used in all species of domesticated animals and only one dose is required to immunise.

More recently a "sterile anthrax antigen" vaccine has been introduced,² consisting of a fraction isolated from *B. anthracis* with powerful immunising properties but which is sterile and non-toxic. It is of additional interest in that it has been used successfully to immunise humans.

THE AEROBIC GRAM-POSITIVE SPORING BACILLI BIOLOGICALLY ALLIED TO BACILLUS ANTHRACIS

(These organisms are saprophytes, and represent a large number of different species. They are found in soil, water, dust and air. Being ubiquitous, they are frequent contaminants of culture medium in the laboratory, and bacteriological workers should be acquainted with their general biological characters.)

Classical types representative of this group are *B. subtilis* (the "Hay bacillus"), *B. mycoides*, *B. mesentericus* and *B. megatherium*. The type-species is *B. subtilis*, and for convenience these organisms are sometimes spoken of as the "*B. subtilis* group". For the detailed differential features of the various species, reference can be made to *Bergey's Manual of Determinative Bacteriology* (6th edition). The general characters of the commoner types met with in laboratory work may be summarised as follows, and for general purposes it is unnecessary to identify a particular species.

Morphology and Staining.—Certain large-cell types tend to resemble the anthrax bacillus, e.g. *B. mesentericus*. Others, e.g. *B. cereus*, *B. mycoides* and *B. megatherium*, are shorter with rounded ends, and several motile species with peritrichous flagella are met with (e.g. *B. subtilis*). The spore is central or excentric

¹ Sterne, M. (1939), *Onderstepoort J. vet. Sci.*, **13**, 313.

² Wright, G. G., Hedberg, M. A., & Slein, J. B. (1951), *J. Immunol.*, **72**, 263; Belton, F., Strange, R. E. (1954), *Brit. J. exp. Path.*, **35**, 144.

(e.g. *B. subtilis*, *B. mycoides*), subterminal or terminal. It may be relatively small, not exceeding $0.8\ \mu$ (e.g. *B. mesentericus*), or large, up to $1.8\ \mu$ (e.g. *B. megatherium*).

Cultural Characters.—The optimum temperature is usually low, e.g. about 20°C ., but certain types grow best between 30° and 37°C . and some are thermophilic, with their optimum temperature at 55°C .; they are characteristic aerobes, but, usually, also facultative anaerobes; abundant growth occurs on all the ordinary culture media. The appearance of the growth varies considerably among different types. *B. subtilis* produces a white, glistening, adherent, somewhat membranous growth, which tends to spread, and somewhat similar growths are seen among other species. Certain types produce colonies and growths practically similar to *B. anthracis*, with the same "medusa-head" appearance, e.g. "*B. anthracoides*". The colonies of *B. mycoides* are at first similar to those of the anthrax bacillus, but are easily differentiated by their feathery appearance, due to long projecting and branching threads radiating out from the central growth. The growths may be dry, gummy or moist, and white, greyish-white, yellowish or brown. Certain species producing a black pigment have been described. On potato, characteristic cultural appearances may be noted, e.g. *B. mesentericus* develops a thick wrinkled or folded layer of growth which assumes a brownish colour. Pellicle formation in broth is a frequent character. Generally, gelatin is liquefied and proteolytic action is well developed. Some types ferment carbohydrates. Starch may be hydrolysed.

It has been shown by McGaughey and Chu¹ that of the group of aerobic sporing bacilli only *B. mycoides*, *B. cereus*, and to a lesser extent *B. anthracis*, are capable of splitting the lecithin of egg-yolk incorporated in a culture medium. This reaction, which is due to an enzyme, phospholipinase, defines them quite sharply from *B. subtilis* and other members of the group.

These organisms are usually non-pathogenic on experimental inoculation into laboratory animals.

Bacillus anthracoides.—This designation has been applied to a type of organism of the above group, which in morphological and cultural characters closely resembles *B. anthracis* and may exhibit the "medusa-head" colonies characteristic of the latter. Under certain conditions this organism might at first be confused with the anthrax bacillus. It can be differentiated, however, by its motility. Colonies on blood agar are usually haemolytic (*cf.* anthrax bacillus). It should be noted that if a large dose of culture

¹ McGaughey, C. A., & Chu, H. P. (1948), *J. gen. Microbiol.*, **2**, 334.

of this type of organism is injected into a guinea-pig or mouse, a local inflammatory lesion with inflammatory oedema may be produced, and septicaemia with a lethal effect. The organism can be detected in the heart blood and internal organs, though *in small number* (*cf.* anthrax). In blood or tissues it does not exhibit the McFadyean methylene-blue reaction (p. 560).

B. cereus is a saprophytic widely spread organism commonly found in heat-treated milk; as it has the property of reducing methylene blue, it may be necessary to identify it, and this is easily done on the egg-yolk agar plate. The power to split lecithin is very much greater in *B. cereus* than in *B. anthracis* in which it is only slight, and an egg-yolk-agar¹ plate will distinguish easily *B. cereus* from the other saprophytic aerobic sporing bacilli and from *B. anthracis* itself.

MALLEOMYCES MALLEI (*Actinobacillus mallei*)

The causative organism of Glanders. This was at one time a very common disease of horses in this country, but has now been completely eradicated. The disease still occurs in Eastern Europe and various parts of Asia.

Morphology.—A straight or slightly curved bacillus with rounded ends, 2–3 μ by 0·4 μ . Pleomorphism is common and involution occurs in old cultures. The bacillus is non-sporing and non-motile.

Staining.—Gram-negative. In the tissues the appearance is granular or beaded.

Cultural Characters.—An aerobe which does not grow below 20° C. Grows on ordinary media, but better on media containing glycerol, serum or blood. Colonies are about 1 mm. diameter after two to three days' growth. They are white in colour, semi-transparent, later becoming more opaque and yellow-brown in colour. Some colonies may be slimy in appearance, other variants are dry and wrinkled.

Growth does not occur readily in primary culture and the organism dies out quickly, but after several subcultures it becomes adapted to a saprophytic existence and may live for two months.

Occurrence and Epidemiology.—Glanders is an infective granuloma with a marked tendency to suppuration. It is essentially a disease of horses, asses and mules, and is only occasionally trans-

¹ E.g. Add 15 ml. egg yolk saline suspension (p. 232) to 500 ml. molten nutrient agar at 50°–55° C. before pouring the plates.

mitted to man, usually by direct infection from an animal source. Under natural conditions the usual mode of transmission appears to be ingestion but infection by inhalation can also occur.

In acute and subacute glanders in animals ulcerating nodules occur in the nasal mucosa and later in the lungs and internal organs. The bacilli are present in considerable numbers in all lesions, situated for the most part extracellularly. Latent infections are not infrequent in animals and have also been observed in the human subject.

In human glanders, the infection usually originates in the skin (*e.g.* wound, abrasion, etc.), more rarely in the mucosa of the mouth or nose. The bacilli are found in the local inflammatory lesion and spread by the lymphatics, producing an acute lymphangitis. Ultimately a pyaemic condition results with secondary foci in which the bacilli are numerous.

Experimental Inoculation.—Guinea-pigs are markedly susceptible, and after subcutaneous injection die in a week or two with generalised lesions, as in acute animal glanders. If a male guinea-pig is inoculated intraperitoneally, the tunica vaginalis is rapidly invaded, and, externally, swelling of the testis is noted (Straus reaction).

Diagnosis of Glanders.—Films are prepared from the pus, discharge from sores, etc., or from nodules in internal organs, found at *post-mortem*; these are stained with methylene blue and by Gram's method. The appearance of beaded Gram-negative organisms corresponding to the glanders bacillus is suggestive.

Cultures are also made on glycerol agar or blood agar, and if a mixed growth results, pure cultures are obtained from single colonies.

In all cases the nature of the infection must be confirmed by animal inoculation. A male guinea-pig is injected intraperitoneally with the pathological material or the isolated culture; in two to three days an enlargement of the testis results, and the animal subsequently dies, showing the lesions of acute glanders. If the inoculum contains a large number of other organisms, it may be introduced by subcutaneous injection. The organism can then be recovered from the enlarged regional glands and tested further by intraperitoneal injection of pure cultures.

Agglutination and complement-fixation tests can also be used in the diagnosis of glanders in horses.

Mallein is a preparation from the glanders bacillus analogous to tuberculin; when inoculated into the skin or subcutaneously a positive delayed reaction has proved to be of value in the diagnosis of infection, particularly in horses.

MALLEOMYCES PSEUDOMALLEI

(*Bacillus whitmori*; *Pseudomonas pseudomallei*)

This is the causative organism of Melioidosis—a glanders-like disease occurring in Burma, Thailand, Indo-China, Malaya, Ceylon and parts of the East Indies. A few cases have also been described in the United States. The disease occurs as an epizootic among rodents; rats seem to be the most important source of the infection in man, and the contamination of food with their excreta may be an important method of transmission. Experimentally the disease can be transmitted by the rat-flea.

The organism is similar to the glanders bacillus but is motile and grows well in gelatin at 20° C., liquefying the medium. The flagella are usually polar. Growth on agar may be mucoid or dry and corrugated, and on potato a brown coloured growth similar to that of *M. mallei* is produced. Glucose, lactose, dulcitol, sucrose and mannitol are fermented with acid, but no gas is produced. Susceptible animals such as the guinea-pig and white rat may be infected experimentally, and a Straus reaction occurs in the male animal similar to that produced by *M. mallei*. *Malleomyces pseudomallei* is serologically distinct from *M. mallei*. The organism is resistant to high concentration of penicillin, streptomycin, chlortetracycline and chloramphenicol.

Ordinarily the disease develops in man as an acute pulmonary infection followed by blood spread to the viscera, the development of miliary abscesses and death. There are relatively few cases of chronic melioidosis described, and most of these have survived what was probably an acute phase of the disease. Other cases are pyaemic with cutaneous eruptions which may last for two to three months before death.

CHAPTER XXV

SALMONELLAE

THE family Enterobacteriaceae is composed of numerous inter-related bacteria all of which are Gram-negative rods, either motile with peritrichous flagella or non-motile. They are non-sporing and grow on ordinary media; all ferment glucose rapidly with or without gas production and reduce nitrates to nitrites. Many species are intestinal pathogens or commensals, whilst a few are saprophytic and found in soil and water.

A classification of the Enterobacteriaceae is essential for diagnostic and epidemiologic purposes, and several groups or genera are recognised within the family. Each genus is made up of biochemically similar strains which are antigenically related; it must be noted, however, that the biochemical interrelationships of the family do not allow all strains to be assigned to one or another genus, and intermediate strains are frequently encountered. With this proviso, and recognising that there are intergeneric relationships among typical strains of the several genera, the family can be divided into the following groups: *Salmonella*, *Arizona*, *Shigella*, *Escherichia*, *Citrobacter*, *Klebsiella*, *Cloaca*, *Hafnia*, *Proteus* and *Providencia*. In the present chapter, only *Salmonella* and *Arizona* species will be considered.

THE GENUS SALMONELLA

More than 400 types are included in this genus; *Salmonella typhi* or the typhoid bacillus was the first member to be described and is the causative organism of typhoid fever. The improvements in environmental sanitation and a better knowledge of the epidemiology of the disease led to dramatic reductions in morbidity; this has been followed more recently by a marked reduction in case fatality rates effected by chloramphenicol therapy.

Paratyphoid fever is clinically similar to typhoid fever and in Britain is caused almost exclusively by *Salmonella paratyphi B* (the paratyphoid B bacillus); the other aetiological agents of paratyphoid fever are *Salmonella paratyphi A* and *Salmonella paratyphi C*, but these are rarely encountered in this country. The term "enteric fever" includes typhoid and the paratyphoid fevers; in addition to their clinical similarity, the gross pathology is the same irrespective of the infecting organism and the distinction depends on bacteriological investigation.

SALMONELLA TYPHI(S. *typhosa*)

Morphology and Staining.—A Gram-negative, non-sporing bacillus, about $2\text{--}4\ \mu$ by $0.5\ \mu$, actively motile with numerous long peritrichous flagella; does not possess a capsule. Most strains are fimbriate.

Cultural Characters.—Aerobe and facultative anaerobe; temperature range, $15^\circ\text{--}41^\circ\text{ C.}$, optimum, 37° C. Grows well on ordinary media.

Colonies on Agar—moderately large, thick, greyish-white, moist, circular disks, dome-shaped and smooth; the opacity and size vary with different strains. Stock laboratory cultures may show a mixture of these smooth colonies with rough colonies which in the extreme are irregular, dull, effuse and dry.

Colonies on MacConkey's Medium.—Similar to above and “pale” or colourless, since *S. typhi* does not ferment lactose.

Desoxycholate-citrate Medium.—Colonies also “pale” or colourless.

Viability.—The thermal death-point is about 56° C. The majority of individual bacilli die within a few hours when subjected to drying. In water the bacilli gradually die, but may survive for some time; thus, in sewage-polluted sea- and fresh-water, viable bacilli have been found after four weeks. In soil, survival may occasionally occur for six weeks or longer; in culture, the organism survives for long periods, e.g. several months. Although *S. typhi* is sensitive to several antibiotics *in vitro*, chloramphenicol is the only satisfactory drug in therapy.

Biochemical Reactions.—Glucose and mannitol are fermented without gas production; lactose and sucrose are not fermented, nor is indole produced. By extending biochemical testing to other substrates (particularly organic acids) it is possible to differentiate *S. typhi* from the paratyphoid bacilli (p. 577); biochemical subtypes of *S. typhi* can be recognised using arabinose and xylose as substrates and such subtypes are epidemiologically significant, e.g. strains usually ferment xylose promptly, but some split xylose late or not at all; similarly arabinose is rarely fermented and then only lately.

Antigenic Characters.—The serum of animals immunised with *S. typhi* contains agglutinating antibodies which are specific for the characteristic antigens, somatic (O) and flagellar (H), of the organism, and agglutination reactions are therefore employed in the identification of this species; these are more conveniently considered in relation to other *Salmonella* types (p. 589).

Vi Antigen and Agglutinin.—Freshly isolated strains of *S. typhi* possess a somatic antigen designated "Vi", occurring as a surface antigen; such strains are more virulent for mice than strains lacking the Vi antigen. The Vi antigen renders the organism relatively inagglutinable by an O antiserum, and serological identification with the latter must be made with a fresh saline suspension of *S. typhi* which has been boiled for one hour and washed by centrifugation. In addition, an unheated suspension should be tested with Vi agglutinating antiserum, which is obtained by immunising animals with an alcohol-acetone treated saline suspension of *S. typhi* known to contain the Vi antigen; after harvesting the serum, H and O agglutinins are absorbed from it with a strain possessing H and O antigens but devoid of the Vi component. Alternatively, an unabsorbed serum made from a Vi containing Ballerup culture may be used.¹

Conversely, for the detection of Vi agglutinin in serum (*e.g.* in a carrier of *S. typhi*), a Vi-containing suspension of *S. typhi* must be employed.² The diagnostic application of the Vi Widal reaction is dealt with on p. 584.

Bacteriophage Typing of S. typhi.—Craigie and Yen³ elaborated a method of differentiating Vi strains into types by means of an anti-Vi phage which, on serial cultivation with one of these types, acquires an increased activity to strains of this type. Thus, type-specific phages can be obtained which, in certain dilutions, act selectively. On this basis, 33 phage types have been recognised and in addition several provisional types have been identified. In this way, freshly isolated strains possessing Vi antigen can be classified and the method has proved valuable in epidemiologic studies of typhoid fever, *e.g.* in correlating cases and in tracing the source of an outbreak and the mode of spread. The technique of phage-typing defies summary treatment and reference should be made to published work.^{4, 5}

Vi-phage typing is carried out by the Director of the International Reference Laboratory for Enteric Phage Typing, Central Public Health Laboratory, Colindale, London, to whom cultures should be sent.

Virulence and Animal Pathogenicity.—In common with other

¹ Edwards, P. R., & Bruner, D. W. (1942), *Kentucky Agr. exp. Sta. Circ.*, **54**.

² Such a suspension and other diagnostic suspensions and antisera are obtainable from The Standards Laboratory for Serological Reagents, Central Public Health Laboratory, Colindale, London, N.W.9.

³ Craigie, J., & Yen, C. H. (1938), *Canad. pub. Hlth J.*, 448 and 484.

⁴ Craigie, J., & Felix, A. (1947), *Lancet*, i, 823.

⁵ Anderson, E. S., & Williams, R. E. O. (1956), *J. clin. Path.*, **9**, 94.

members of the genus, *S. typhi* is primarily an intestinal parasite. It shows a strong host specificity for man; *S. typhi* does not appear to infect animals under natural conditions, and ordinary laboratory animals, e.g. rabbit, rat, mouse and guinea-pig, when dosed orally with *S. typhi* do not suffer any harmful result unless massive doses are employed, and even then the illness is quite unlike that of typhoid fever. When adequate doses are administered to mice by the intraperitoneal or intravenous route, death results, but this is probably toxæmic; with non-lethal doses the disease in mice shows little or no tendency to spread by contact from mouse to mouse in distinction to the epizootic spread following on the introduction of *Salmonella typhimurium*.

Occurrence.—Infection is by ingestion; from the small intestine the organisms pass via the lymphatics to the mesenteric glands, whence after a period of multiplication they invade the blood stream via the thoracic duct; the liver, gall-bladder, spleen, kidney and bone-marrow become infected during this bacteraemic phase in the first seven to ten days of the disease. From the gall-bladder a further invasion of the intestine results, and lymphoid tissue—Peyer's patches and lymphoid follicles—are particularly involved in an acute inflammatory reaction, and infiltration with mononuclear cells, followed by necrosis, sloughing and the formation of characteristic typhoid ulcers. Haemorrhage of varying degree may occur and, less frequently, perforation through a necrotic Peyer's patch will complicate the illness.

S. typhi is present in large numbers in the inflamed tissue in the ulcers and is found in the intestinal contents and the dejecta; it may localise in the kidney and appear in the urine, sometimes producing a marked bacilluria. The bacillus is found in other lesions occurring as complications or sequelæ of typhoid fever—e.g. acute suppurative periosteitis and osteitis, abscess of the kidney, acute cholecystitis, bronchopneumonia, empyema, ulcerative endocarditis and even in the suppurative lesions, it may be present in pure culture.

In 2–5 per cent. of convalescents, the typhoid bacillus persists in the body, sometimes for an indefinite period. In such carriers, the bacilli are present in the gall-bladder or in the urinary tract and are excreted in the faeces or urine.

Epidemiology.—The sources of infection are patients suffering from the disease, including the mild and ambulatory forms and carriers. Typhoid fever is predominantly a water-borne infection and so, in communities where adequate treatment of water supplies is undertaken and where a water-carriage system of sewage disposal is operating, the recognised case of typhoid fever

is unlikely to act as a focus of epidemic spread since he should be admitted to hospital and nursed with aseptic precautions.

Carriers of *S. typhi* are a more likely source of infection in a community with well developed environmental services. Bacilli present on the hands of the carrier can be transferred to many vehicles; a carrier engaged in dairy work may contaminate milk, which can serve as culture medium and a likely mode of spread¹ (there is no evidence that *S. typhi* causes bovine infection). Similarly, carriers engaged in food-handling either in preparation or distribution of foodstuffs, have been incriminated as sources in epidemic outbreaks. Shell-fish harvested from sewage-polluted sea-water, and vegetables, salads and water-cress contaminated with human excreta have all been noted as vehicles of infection.

In materially less favoured countries the absence of community services results in low sanitary standards and greater opportunities for epidemic spread from cases and carriers; in these circumstances also, flies may transmit the bacilli from excreta to foodstuffs.

SALMONELLA PARATYPHI A (*S. paratyphi*)

SALMONELLA PARATYPHI B (*S. schottmüller*)

SALMONELLA PARATYPHI C (*S. hirschfeldii*)

These are the causal organisms of paratyphoid fever which is essentially similar to typhoid fever but clinically is milder and of shorter duration. In their morphology and general cultural characters the paratyphoid bacilli are identical with *S. typhi*, but they can be differentiated from the latter by their ability to produce gas in fermentation reactions, and the following table exemplifies this feature and also the differentiation of the paratyphoid bacilli one from the other.

	Glucose	Mannitol	Xylose	d-tartrate	Mucate
<i>S. typhi</i>	—	—	V	—	V
<i>S. paratyphi A</i>	+	+	—	—	—
„ B	+	+	+	—	+
„ C	+	+	+	+	—

Keys: —=acid, no gas; + = acid and gas; V = variable reaction; —=no reaction.

¹ Bradley, W. H. (1943), *Brit. med. J.*, 1, 438.

However, as with all members of the genus *Salmonella*, the ultimate identification of type depends on serological examination.

Antigenic Characters.—These bacilli possess different antigens and agglutinating antisera for known strains are used for the identification of the respective types. This is dealt with on p. 589.

Bacteriophage Typing of S. paratyphi B.—Phage typing of this organism is used for epidemiologic purposes.^{1, 2} More than 30 distinct types, subtypes and variations can be recognised. Cultures for phage typing should be sent to the Reference Laboratory, Colindale (p. 575).

Occurrence.—The occurrence and distribution of the paratyphoid bacilli in the body of an infected person or carrier are the same as in the case of *S. typhi*.

Epidemiology.—The sources of paratyphoid infections are human cases and carriers, but there is evidence that, in comparison with *S. typhi*, many more paratyphoid bacilli require to be ingested before infection results. Hence water-borne spread of paratyphoid fever is rare and most episodes result from the ingestion of food stuffs with peak incidence in the summer months.³

LABORATORY DIAGNOSIS OF ENTERIC FEVERS⁴

The bacteriologic diagnosis depends on (1) the isolation from the body, and the identification, of the causative organism, or (2) the demonstration of its presence in the body by the Widal agglutination reaction which is based on the occurrence of specific agglutinins to the organism in the serum of the infected person.

Blood Culture.—In the early stages of the illness, blood culture is the most conclusive diagnostic method, and should be employed in all cases met with during the first seven to ten days, and in relapses (where a bacteriological diagnosis has not previously been established). The use of blood culture bottles containing bile-salts (0.5 per cent. sodium taurocholate) is recommended; the technique of inoculation and laboratory procedure is referred to on p. 260. Another method⁵ of isolating the bacilli from the blood stream has been advocated; blood obtained by venepuncture is distributed in 5 ml. quantities to Universal containers and allowed to clot when the separated serum is aseptically removed.

¹ Felix, A., & Callow, B. R. (1943), *Brit. med. J.*, 2, 127.

² Anderson, E. S., & Williams, R. E. O. (1956), *J. clin. Path.*, 9, 94.

³ Savage, W. G. (1942), *J. Hyg. (Lond.)*, 42, 393; *Brit. med. J.*, 1956, 2, 317.

⁴ Felix, A., Laboratory Control of the Enteric Fevers. *Brit. med. Bull.* (1951), 7, 153.

⁵ Watson, K. C. (1955), *J. Lab. clin. Med.*, 46, 128.

To each bottle is added 15 ml. of 0·5 per cent. bile-salt broth containing 100 units per ml. of streptokinase; the latter causes rapid lysis of the clot with release of any organisms present. Incubation and further examination is performed as for blood culture.

The probability of demonstrating the causative organism in the blood lessens as the disease progresses. If the result is positive, the strain is isolated in pure culture, and identified by morphological, cultural and biochemical characters and by testing it with agglutinating antisera (O and H) as on p. 589.

Faeces.—*S. typhi* and the paratyphoid bacilli can be isolated from the faeces throughout the illness, but are most frequent during the second and third weeks. Repeated examination of the faeces may be required before isolation is successful.

If there is likely to be a delay of some hours before specimens of faeces for culture reach the laboratory, 2 volumes of 30 per cent. neutral glycerol in buffered 0·6 per cent. sodium chloride solution (p. 433) should be added to 1 volume of the faeces and thoroughly mixed with it. This prevents other intestinal organisms from overgrowing the enteric fever bacilli. The glycerol solution is apt to become acid on storage and is then unsuitable for use; as a safeguard the solution may be tinted with phenol red, and if there is a change to yellow the fluid should be discarded.

Direct Plating of Faeces.—A medium is required which will differentiate colonies of typhoid-paratyphoid bacilli from those of the normal bowel flora; in the past, MacConkey's bile-salt neutral red lactose agar (p. 216) has been extensively used for this purpose, but more recently has been superseded by other selective media which are inhibitory to the normal flora, e.g. Desoxycholate-citrate agar (D.C.A.), which, like MacConkey's medium, also contains lactose and neutral red (p. 218). On both of these media *Salmonella* colonies are "pale" as compared with the pink colonies of most of the commensal bowel organisms.

It is essential that the surface of such media should be sufficiently dry before inoculation, otherwise a confluent growth may result instead of separate colonies. When the D.C.A. medium is used the inoculation is made as follows :

Several loopfuls of the specimen (liquid faeces or a dense emulsion in saline of solid or semi-solid faeces) are smeared over area A of the plate. The loop is sterilised in a flame, re-charged by rubbing it over area A, and then used to inoculate the remainder of the plate by successive parallel strokes B, C and D, drawn in the directions indicated in the diagram. The wire should be held so that the whole loop is in contact with the surface of the medium. In this way the resulting colonies are evenly distributed

over the plate; by this method a heavy inoculation can be made, with the resulting colonies well separated except, of course, in area A.

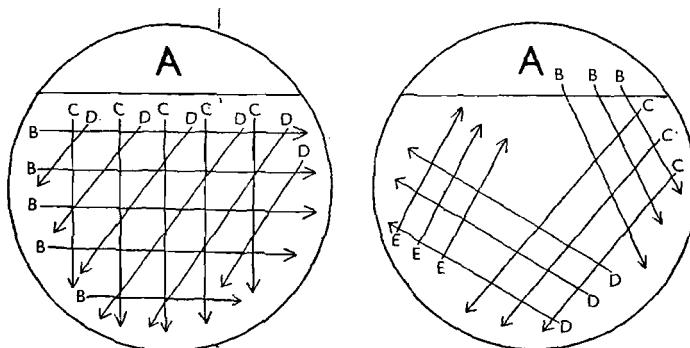


FIG. 25

D.C.A. Plate

MacConkey Plate

MacConkey's medium may still be used for the direct cultivation of faeces in other infections, e.g. due to gastro-enteritis producing strains of *Escherichia coli* (p. 612). Not only must the surface of MacConkey's medium be free of all traces of condensation water, but a lighter inoculation is required; the technique generally employed for plate-inoculation is adequate, since at each stage in the process the inoculum density is reduced by sterilising the loop and re-charging it from the preceding inoculum line; alternatively, the inoculum may, with advantage, be distributed over two plates of MacConkey's medium.

After eighteen to twenty-four hours' incubation the colonies are usually sufficiently large for subinoculating those that are considered likely to be enteric fever bacilli. The plates may also be incubated for longer periods if no suspicious colonies are noted after twenty-four hours. The colonies of *S. typhi* and the paratyphoid bacilli present a pale or colourless appearance, but other intestinal organisms may produce not dissimilar colonies; several colonies are therefore subcultured on agar slopes, using a straight wire for transfer from the diagnostic plate. The pure cultures isolated are tested and identified as indicated under blood culture (*vide supra*).

Wilson and Blair's bismuth-sulphite medium may also be used with advantage for direct plating and should always be employed in the attempted isolation of *S. typhi*, since it is the most reliable medium so far devised for this purpose. It is recommended that the inoculum be distributed over two plates of this medium;

furthermore, it is advisable to incubate for forty-eight hours before discarding, since, although *S. typhi* will usually appear within twenty-four hours, *S. paratyphi B* frequently requires the longer incubation period before colonies are recognisable.

Enrichment Methods.—These have proved invaluable for isolating enteric fever bacilli when present in small numbers in faeces, as in convalescent cases, or in the detection of carriers. Fluid media are used, incorporating substances which inhibit the commensal flora, while allowing the enteric fever bacilli to flourish. Tetrathionate broth and selenite F broth are widely used; these are inoculated with 2-3 loopfuls of faecal suspension at the same time as direct plating and are subcultured to D.C.A. medium after incubation for twelve to eighteen hours. In this way an enriched culture of enteric fever bacilli is obtained and sometimes an almost pure growth.

In the examination of faeces from cases of enteric fever the best results are obtained by employing two or three different methods simultaneously. This gives a higher percentage of positive results than when one method only is used. Thus it is recommended that routine inoculation of D.C.A. and Wilson and Blair plates and of both enrichment media should be undertaken in the attempted isolation of enteric fever bacilli.

Urine.—The bacilli may also be isolated from urine. The specimen is centrifuged, several loopfuls of the deposit being used to inoculate the recommended media. In enteric fever there appear to be transient bacilluric periods, and daily examinations for a week of the morning urine are of particular value where the isolation of the causative organism is aimed at and where other methods have been unsuccessful. In carrier detection and in determining the site of carriage, it should be remembered that even small quantities of the urine from a urinary carrier mixing with his stool may lead to the patient being regarded as both a urinary and faecal carrier; care in collecting specimens will eliminate such errors.

Bile.—By means of a duodenal tube, bile may be aspirated and cultured in an attempt to isolate enteric fever bacilli. This technique is of value in the later stages of the illness and in carrier detection.

Widal Reaction.—The reaction becomes definitely manifest usually about the 7th to 10th day. Occasionally it is earlier in development (*e.g.* 5th day), but may be delayed. A negative result at an early stage of the illness therefore may be inconclusive. The technique is described in Chapter XIII; it is customary to test the patient's serum against standard H and O suspensions (in parallel)

of each enteric fever organism likely to be encountered—*e.g.* in Britain, *S. typhi* and *S. paratyphi B*. As a rule, both O and H agglutinins are developed, but in some cases only one of these agglutinins is detected, particularly in the early stages of the illness.

The Widal reaction, tested quantitatively, is also progressive up to a certain point, *i.e.* the titre of the reaction rises from the time of the first appearance of agglutinins in the serum and reaches its maximum about the end of the third week. A “rising titre”, on repeated testing, is therefore highly significant.

For determining the type of infection, H agglutination is more to be relied on than the O reaction, since the enteric organisms have some O antigenic components in common (table, p. 590); thus in a typhoid infection, O agglutination may sometimes be pronounced with the test suspension of *S. paratyphi B* as well as that of *S. typhi* (O antigen 12), while in a paratyphoid B infection, marked O agglutination may also occur with both test suspensions. However, the presence of O agglutinins usually reflects recent infection and both H and O agglutinins should be tested for routinely.

In interpreting the result of a Widal test, certain additional facts must be kept in mind:

(1) Normal serum may agglutinate the test suspensions in low dilutions, and no diagnostic significance can be attached to such reactions. In Britain, the usual limits of such normal agglutination of Standard Suspensions¹ are *S. typhi* and *S. paratyphi B*, H-1 in 30, O-1 in 50 and *S. paratyphi A*, O and H, 1 in 10. Normal agglutination of these organisms varies in degree in different communities and different countries. If agglutination occurs only in low dilutions within the possible range of normal agglutination, the test should be repeated as later results may show higher titres and are therefore more conclusive.

In this regard, it should be noted that, where clot culture (p. 578) is practised for isolation of enteric bacilli early in the disease, the serum withdrawn from the specimen can be used for Widal testing and the results used as a base-line against which tests with subsequent specimens can be assessed.

(2) Non-specific antigens, such as fimbrial antigens, may be present in test suspensions and react with an agglutinin in human sera. This may cause a fallacy in the Widal test unless the sus-

¹ Standard Suspensions and Diagnostic Antisera are obtainable from The Standards Laboratory for Serological Reagents, Central Public Health Laboratory, Colindale, London. The provision of such reagents ensures that results are comparable among laboratories using them.

pensions employed are known to be free from such antigens (p. 607).

(3) Persons inoculated with typhoid-paratyphoid vaccine (T.A.B.) also show specific agglutinins in their sera, and this may complicate the interpretation of the Widal reaction in such persons. In previously vaccinated cases a definitely rising titre for any one of the organisms has been regarded as significant from the diagnostic viewpoint; however, non-specific factors, such as a non-enteric febrile condition, may cause an increase of agglutinins already present as a result of vaccination, and enteric infection by one organism may lead to an increased agglutination titre for the others.

It has been claimed that in such persons, several months after vaccination, the agglutinins are mainly of the H type, whereas in infected subjects both O and H agglutinins can be demonstrated. Thus, in the application of the Widal reaction in vaccinated persons, the results may be of doubtful significance, but if over six months have passed since the date of vaccination and if the O agglutination titre is higher than 1 in 100 and rises on repeated testing, such a result may be considered significant.

The bacteriologist should be informed of the clinical history and any T.A.B. vaccinations; with this liaison, the physician can in turn receive an evaluation of the Widal result rather than a formal statement of reaction titres.

Diagnosis of Enteric Carriers

The proof that a person is a carrier depends on the isolation of *S. typhi* or one of the paratyphoid bacilli from the faeces or urine, and in view of the intermittency of excretion 6 consecutive examinations of such specimens should be made before the result is declared negative. The methods employed are as described above; since the bacilli are likely to be most numerous in the bile and in the contents of the small intestine, examination of aspirated bile is valuable or alternatively the subject should be given 3 grains of calomel followed by a saline purgative, and after catharsis the second, or preferably the third stool is examined.

In a considerable proportion of carriers, the Widal reaction is positive, and the test is of some value as a preliminary one provided that the subject's experience of T.A.B. vaccination is known. A negative Widal reaction does not, of course, exclude the carrier state; if the subject's serum is tested with a Vi-containing strain of *S. typhi*, the presence of Vi agglutinin can be detected and such a test has considerable value in recognising carriers of *S. typhi*.

A concentrated Vi suspension is obtainable from The Standards Laboratory; as this suspension loses sensitiveness on keeping, it should not be used after two months. The test mixtures are prepared and incubated in $3 \times \frac{1}{2}$ in. tubes. A series of doubling dilutions of the serum is prepared, ranging from 1 in 10 to 1 in 640, with the usual control tube containing saline only. Each dilution should be in a 1 ml. volume. 0.05 ml. of the test suspension is then added to each tube including the saline control and the tubes are incubated at 37° C. for two hours and then allowed to stand overnight at room temperature. To observe the result, the tubes are held over a mirror and the image of the sediment examined; in the control tube the sedimented organisms should form a small, circular, well-defined, compact deposit; in the test proper, if marked agglutination has occurred, the deposit (consisting of agglutinated bacilli) is spread over the foot of the tube. Intermediate degrees are also observed. "Standard agglutination" is denoted by absence of the central deposit with bacterial clumps occupying about half the area of the foot of the tube. Sera containing lysed blood may give false positive reactions in low dilutions.

In suspected typhoid carriers a titre of 1 in 10 is regarded as significant; however, the introduction of alcoholised T.A.B. vaccine (p. 585) containing Vi antigen, has complicated the interpretation of Vi agglutination reactions, since individuals to whom alcoholised vaccine has been given may possess Vi agglutinins.

Chemotherapy.—In spite of contrary evidence from animal experiment, none of the sulphonamides is of value in treating cases of enteric fever nor in the sterilisation of chronic carriers. Chloramphenicol, in adequate doses, leads to rapid clinical cure, but has also resulted in an increase in the relapse-rate on discontinuing therapy, and second attacks of enteric fever have been reported.

Prophylaxis.—*S. typhi* and the paratyphoid bacilli are never parasitic on hosts other than man; therefore preventive measures against the spread of enteric fever are more readily undertaken and attended with greater success than in diseases in which host specificity is less strict.

General preventive measures include the following:

(1) The institution and maintenance of safe water supplies and water-borne sewage disposal systems.

(2) The supervision of personnel engaged in water-works, dairy-farms and in the food industry; and their instruction in the elementary epidemiology of enteric infections.

(3) Protection of foodstuffs from flies and the storage of food in refrigerators.

(4) Bacteriologic control of imported foods, e.g. eggs.

Specific preventive measures:

(1) Adequate nursing of cases and the maintenance of an Enteric Register for each community, in which is listed all known chronic carriers; the phage-type of *S. typhi* or *S. paratyphi B* which carriers are excreting should be noted in the Register, since this information may be invaluable in the investigation of future outbreaks.

(2) The control of known carriers in relation to their employment; carriers may be "cleared" by antibiotic therapy, e.g. massive dosage of penicillin, and/or surgical measures, e.g. cholecystectomy.

(3) The administration of T.A.B. vaccine to individuals at special risk, e.g. (a) troops and other persons travelling into or residing in areas where the standard of sanitation is low or where enteric fevers are common; (b) laboratory workers handling specimens or live suspensions; (c) individuals residing with known chronic carriers.

T.A.B. vaccine is prepared from selected smooth cultures of *S. typhi*, *S. paratyphi A* and *B* according to the method described on p. 181. The vaccine is usually sterilised at 60° C. (thirty minutes) and 0·5 per cent. phenol added as a preservative; it is standardised to contain 1,000 million *S. typhi* and 750 million each of *S. paratyphi A* and *B* per ml. The first immunising dose of 0·5 ml. is followed not less than ten days later by a second dose of 1 ml., both administered subcutaneously; for those continually at risk, a booster dose (0·5 ml.) or 0·2 ml. intracutaneously should be given biennially. *S. paratyphi C* may be incorporated in the vaccine (750 million bacilli per ml.) which is then designated "T.A.B.C."

The prophylactic value of T.A.B. vaccination was regarded as well established although valid field trials had not been undertaken. It has been assumed from experimental studies on mice that the full immunising potency of typhoid vaccine depends on the use of virulent strains containing adequate O and Vi antigen, but sterilisation by heat and preservation with phenol tend to destroy the Vi antigen. To preserve this antigen Felix and his team advocated a less severe treatment for the vaccine cultures by killing with 75 per cent. alcohol and preservation with 25 per cent. alcohol; this alcoholised vaccine stimulates the formation of Vi antibody in a substantial proportion of vaccinated subjects, whereas such

antibody was rarely found after administration of the heat-phenol treated vaccine.¹

The comparative efficacy of the two types of vaccine has recently been the subject of strictly controlled field trials in Yugoslavia under the auspices of the World Health Organisation. The results indicate that phenolised typhoid vaccine is superior to the alcoholised vaccine, since it gave a 70 per cent. protection rate; the alcoholised preparation appeared to be little better than a control vaccine prepared against *Shigella flexneri*, which theoretically should have no protective value in typhoid fever (see pp. 940-942).

ORGANISMS OF BACTERIAL ENTERITIS OR FOOD POISONING

In addition to the enteric fever organisms, more than 400 *Salmonella* serotypes have been recognised and these are associated with cases of food poisoning in which the illness takes the form of an acute gastro-enteritis. These serotypes are identical with the paratyphoid bacilli not only morphologically and in cultural characteristics but also in their general biochemical activities; extended biochemical tests, with a wide range of substrates, are of some value in differentiation, but only antigenic analysis with type-specific sera allows each member of the genus to be identified with certainty.

Virulence.—Different types vary to some extent in their invasiveness. It should be noted that unlike the typhoid-paratyphoid bacilli, these *Salmonella* serotypes are frequently encountered enzootically in nature and also readily infect laboratory animals when intentionally administered.

Occurrence.—In cases of food poisoning, these organisms are found in the intestinal contents during the disease, and in some cases bacteraemia and septicaemia may occur. Cholecystitis may also result and meningitis has been recorded not infrequently as a result of infection; persons with sickle-cell anaemia appear to be more liable to *Salmonella* infections of bone than individuals free of this condition.²

Epidemiology.—Unlike typhoid fever, in which human cases and carriers are the sole source of infection, *Salmonella* food poisoning usually originates from animal sources. Domestic animals such as cows, sheep and pigs may be infected clinically or subclinically; in apparently healthy cattle a carrier rate of 1 per cent. has been

¹ Felix, A., Rainsford, S. G., & Stokes, E. J. (1941), *Brit. med. J.*, i, 435.

² Hendrickse, R. G., & Collard, P. (1960), *Lancet*, i, 80.

found. Investigations in the U.S.A.¹ and in Britain² have shown an increase in the carrier state as animals are moved from farm, through market to abattoir, and it is thought that the carrier state proceeds to frank infection and septicaemia during transit when the animal is deprived of food and water; similar findings have been made in sheep.³ The source of epizootic salmonella infection is variable, but feeding-stuffs have been incriminated⁴ and the maintenance of such infections enzootically is readily explained by the finding that *S. typhimurium* remains viable in water, pasture and faeces commonly for four weeks and sometimes as long as twenty-eight weeks.⁵

After meat has been marketed, whether or not it is already infected with salmonellae, it can be contaminated at all stages of preparation before being consumed; in this regard it is reported that the frequency of salmonella isolations from sausages offered for retail is ten times greater than from the gut of the healthy animals from which the sausage meat was obtained.⁶ The handling of contaminated meat along with clean produce may result in the latter becoming contaminated; if, on purchase, meat and meat products are free of salmonellae, they may still become contaminated in the final stages of preparation at home, in canteens, school-meal kitchens and the like, if any individual involved in cooking or serving the meal is excreting salmonellae either as a carrier or as an ambulant case of infection. Cooking is not always a safe means of destroying salmonellae in foodstuffs.⁷ The food rarely shows any obvious sign of bacterial contamination.

In addition to meat, cow's milk and milk products may be a source of infection; furthermore, epizootic salmonella infections in rats, mice and other rodents allow such animals to infect foodstuffs with their excreta. The use of *Salmonella enteritidis* as a rodent poison for eradication purposes implies that this organism may sometimes be transmitted to man in this way.

Birds, particularly hens, ducks and turkeys, suffer from Salmonella infection and their carcasses may be a source of infection to man; likewise duck and hen eggs may be the source, the egg being infected either during formation in the oviduct, as in the

¹ Galton, M. M., et al. (1954), *J. infect. Dis.*, **95**, 236.

² Hobbs, B. C., & Wilson, J. G. (1959), *Monthly Bull. Minist. Hlth Lab. Serv.*, **18**, 198.

³ Salisbury, R. M. (1958), *N.Z. vet. J.*, **6**, 76.

⁴ Report (1959), *Monthly Bull. Minist. Hlth Lab. Serv.*, **18**, 26.

⁵ Josland, S. W. (1951), *Aust. vet. J.* **27**, 264.

⁶ Report (1959), *Monthly Bull. Minist. Hlth Lab. Serv.*, **18**, 26.

⁷ Miller, A. A., & Ramsden, F. (1955), *J. appl. Bact.*, **18**, 565.

case of the duck, or by the organisms passing through the shell from cloacal discharges lying in the nesting-box. Contamination through the shell is most likely to occur immediately after laying whilst it is still warm and moist externally; during the cooling period, bacteria are readily sucked through the shell.¹

Unless infected eggs are used for preparation of custards or other communally consumed products, infection is restricted to the individual consumer and such single case infections will rarely attract attention. The pooling of eggs, either as raw whole egg for the baking industry or as spray-dried powder for home use as well as for manufacturing concerns, allows contamination of otherwise clean material with a minority of infected eggs. Circumstantial evidence of the importance of such bulk material in promoting epidemic salmonella infections has occurred simultaneously with the appearance of serotypes which were rarely or never encountered prior to importation of such material; such a circumstance followed the introduction of dried egg powder from the U.S.A. during the early years of the last war.

The majority of epidemics investigated bacteriologically are caused by one of the following: *S. typhimurium*, *S. enteritidis*, *S. thompson*, *S. newport* or *S. dublin*.

S. typhimurium alone accounts for more than 70 per cent. of these outbreaks, and phage-typing of strains has been useful in detecting the source of infection. The close biochemical similarity to *S. paratyphi B* emphasises the need for accurate serological identification in the genus.

S. enteritidis is the next most frequently encountered in food poisoning outbreaks. This serological entity can be subdivided into four biochemical types, and these types are epidemiologically significant. Similarly, three biochemical types can be recognised of the serotype *S. dublin*.

Diagnosis of Salmonella Food Poisoning

Faeces and other specimens are dealt with as in the diagnosis of enteric fevers. Blood culture rarely yields positive results and should be carried out only in cases where recovery is delayed.

In convalescence, the serum of patients agglutinates the homologous organism, but the agglutination test is not applicable during the acute stage as the agglutinins take some days to make their appearance in the blood. The test may be applied retrospectively in convalescent cases in which either the diagnosis has been delayed or from which no *Salmonella* has been isolated during the acute

¹ Haines, R. B., & Moran, T. (1940), *J. Hyg. (Lond.)*, **40**, 453.

phase; thus, gaps in the chain of epidemiologic findings may be filled by such evidence.

The bacteriological examination of suspected food-stuffs, if available, should be carried out as on page 391. It must be emphasised that in the investigation of food poisoning outbreaks, the bacteriologist is one of a team including clinician, epidemiologist and others; only by close co-operation will such investigations be carried to a successful conclusion.

Prophylaxis.—The various procedures are tabulated below, and for a detailed account of these reference should be made to a textbook of hygiene.

(1) Supervision of the health of animals on farms with the segregation and disposal of infected members of a herd.

(2) Adequate meat inspection at abattoirs.

(3) Maintenance of all premises in a clean state, combined with rodent-proofing and anti-fly measures.

(4) Adequate cooking and subsequent storage of food which is not consumed immediately after preparation.

(5) Supervision of the health of food-handlers and their education in methods of preventing contamination of foodstuffs.

Identification of *Salmonella* Serotypes

When a lactose non-fermenting bacillus presenting the general characters of the genus has been isolated, its precise identification may involve a considerable amount of detailed testing, and such work has tended to become highly specialised and beyond the scope of the smaller laboratories. The following description outlines the procedures adopted and their underlying principles.

The presence or absence of motility of the strain under investigation is noted by inoculating a tube of semi-solid agar (p. 197) and a set of sugars is inoculated. Christensen's urea medium is also inoculated to exclude organisms of the genus *Proteus*. As a preliminary to testing the strain in an extended range of biochemical tests it is recommended that composite sugar media be employed (p. 222). Other biochemical reactions which may be tested are the fermentation of xylose, arabinose, trehalose, inositol and rhamnose and the utilisation of *d*-tartrate, *l*-tartrate, *i*-tartrate, citrate and mucate.

Determination of O Group.—As will be seen from the table (p. 590), the identification of the O antigen provides a means of placing any member of the genus in one of a number of groups designated A-I. Inspection of the O-antigenic formulae exemplified in this table will show that all members of a particular group possess an O-antigen factor which is common throughout

the group and not shared with other groups, e.g. Group A—O antigen 2, Group B—O antigen 4, etc. This is designated the determining antigen, and, correspondingly, diagnostic sera which have been absorbed to remove all antibodies except that for the group-specific antigen are designated as "Factor 2" serum, "Factor 4" serum, etc.

Some Representatives of the genus Salmonella
(Kauffmann-White Classification)

Group	Type	Somatic antigens	Flagellar antigens	
			Phase 1	Phase 2
A	<i>S. paratyphi A</i>	1, 2, 12	a	—
B	<i>S. paratyphi B</i>	1, 4, 5, 12	b	1, 2
	<i>S. typhimurium</i>	1, 4, 5, 12	i	1, 2
	<i>S. stanley</i>	4, 5, 12	d	1, 2
	<i>S. heidelberg</i>	4, 5, 12	r	1, 2
	<i>S. abortus-equi</i>	4, 12	—	e, n, x
	<i>S. abortus-ovis</i>	4, 12	c	1, 6
C ₁	<i>S. paratyphi C</i>	6, 7, Vi	c	1, 5
	<i>S. cholerae-suis</i>	6, 7	c	1, 5
	<i>S. thompson</i>	6, 7	k	1, 5
	<i>S. bareilly</i>	6, 7	y	1, 5
C ₂	<i>S. newport</i>	6, 8	e, h	1, 2
	<i>S. bovis-morbificans</i>	6, 8	r	1, 5
D	<i>S. typhi</i>	9, 12, Vi	d	—
	<i>S. dublin</i>	1, 9, 12	g, p	—
	<i>S. enteritidis</i>	1, 9, 12	g, m	—
	<i>S. gallinarum</i> ¹	1, 9, 12	(non-flagellate)	
E ₁	<i>S. anatum</i>	3, 10	e, h	1, 6
	<i>S. meleagridis</i>	3, 10	e, h	1, w
	<i>S. london</i>	3, 10	l, v	1, 6
E ₂	<i>S. senftenberg</i>	1, 3, 19	g, s, t	—
F	<i>S. aberdeen</i>	11	i	1, 2
G	<i>S. poona</i>	13, 22	z	1, 6

¹ *S. pullorum* is serologically identical.

Confirmation that the organism is a member of the genus is obtained by slide agglutination tests with polyvalent O serum, and Vi serum. If agglutination occurs only in Vi serum, the culture should be heated in saline at 100° C. for one hour and centrifuged, a fresh saline suspension of the heated deposit should be retested with polyvalent O serum. In either event, the culture suspension should now be tested with the absorbed "Factor" sera in one of which agglutination will be noted and further testing restricted to identifying the specific type within this particular O group. Cultures which are initially agglutinated by the Vi serum should be in group C if they are *S. paratyphi* C and in group D if they are *S. typhi*. On occasion, a rough culture of *S. typhi* is isolated which does not agglutinate with group D serum after heating and it is essential to determine biochemically whether a culture, which in the live state agglutinates in Vi serum but after heating is inagglutinable in group C, group D and Vi serum, is in fact a rough culture of *S. typhi*.

Determination of Type.—Before proceeding to type the organism by identifying its H-antigen structure, it is necessary to determine whether the culture (if diphasic) is in the specific or non-specific phase (p. 66). An H-agglutinating serum prepared against the non-specific, mono-phasic variety of *S. cholerae-suis* is a convenient reagent for this purpose. If a formolised suspension of the unknown organism is agglutinated in large floccules to any extent by this serum, it is in the non-specific phase, and an effort must be made to secure a specific-phase subculture of it before further identification is attempted.

The Craigie tube is a convenient method for obtaining the specific form of a *Salmonella* organism: 5 ml. amounts of nutrient agar (0·2 per cent. agar) are placed in stoppered $6 \times \frac{1}{2}$ in. test-tubes with a small inner tube open at both ends and with the upper end projecting well above the agar; the medium is sterilised, cooled to 50° C. and then 0·5 ml. and 1 ml. of a 1 in 5 dilution of the non-specific phase serum (filtered to ensure sterility) are added, giving a final concentration of 1 in 50 and 1 in 25 respectively. The medium is allowed to solidify in the upright

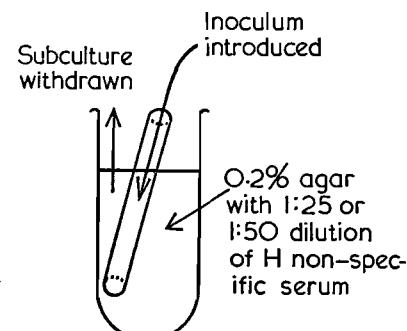


FIG. 26
Craigie Tube.

position. The agar *inside* the inner tube is inoculated by the stab-method with non-specific, group-phase culture. The specific forms can then be separated by incubating and subculturing from the agar *outside* the inner tube. The best results are obtained by employing the shortest period of incubation, e.g. from early forenoon to evening or from late evening to early morning.¹ When the specific phase has been obtained, formalised suspensions are used in agglutination reactions with pure specific-phase H sera prepared against the *Salmonella* types in the group to which the already determined O antigen belongs. In many cases the evidence thus obtained, in association with the biochemical reactions, will serve to identify the unknown strain if it is one of the usual types of *Salmonellae*.

The set of Standard agglutinating sera recommended is as follows:

O antisera:

S. typhi Vi.

Polyvalent O serum containing factors 1-13, 19 and 22.

Factor sera—

2*	for <i>Salmonella</i> O group A
4	" " "
7	" " "
8	" " "
9	" " "
3, 10	" " "
19*	" " "
11*	" " "
13,22	" " "
	A B C ₁ C ₂ D E ₁ & ₂ E ₄ F G

H antisera:

Polyvalent *Salmonella* serum, composite H specific and non-specific.
Salmonella H phase-2 polyvalent serum (H factors 1-7).

Specific H sera:

	Antigens		Antigens
<i>S. paratyphi A</i>	a	Factor serum 1, v, w
<i>S. paratyphi B</i>	b	<i>S. bovis-morbificans*</i>	. . . r
<i>S. paratyphi C*</i>	c	<i>S. bareilly*</i>	. . . y
<i>S. typhi</i>	d	<i>S. poona*</i>	. . . z
<i>S. newport</i>	e, h	<i>S. glostrup*</i>	. . . <i>z</i> ₁₀
Factor serum	g, m, p	<i>S. cerro*</i>	. . . <i>z</i> ₄ , <i>z</i> ₂₃
<i>S. enteritidis</i>	g, m		
<i>S. typhimurium</i>	i		
<i>S. thompson</i>	k	<i>S. tennessee*</i>	. . . <i>z</i> ₂₉

*. These sera are not issued routinely by the Standards Laboratory, but only on special request.

¹ Tulloch, W. J. (1939), *J. Hyg. (Lond.)*, 39, 324.

THE ARIZONA GROUP

This group is composed of lactose-fermenting organisms which are closely linked, biochemically and serologically, to the genus *Salmonella*. The first member of the group was isolated from reptiles and described in 1939.¹

These organisms, most frequently associated with reptiles, have also been isolated from fowls, mammals and man, but have so far attracted little attention in Britain although it is recognised that they can produce severe and fatal human infections.

In their morphology and staining reactions, and in their general cultural characteristics, members of the group are indistinguishable from *Salmonellae*. With the exception of one serotype, these organisms ferment lactose with gas production; so prompt is this activity that many isolates produce pink colonies on the primary D.C.A. plate after overnight incubation; with others, lactose-fermentation may be delayed for three to five days, and occasional strains show no activity until after two to three weeks' growth. Their constant ability to liquefy gelatin is also in contrast to *salmonellae*, in which gelatinase production occurs only rarely; conversely, their failure to ferment dulcrite further distinguishes Arizona strains from the vast majority of *Salmonellae*. Their biochemical similarity implies that Arizona strains may be mistakenly identified as *salmonellae*, the more so since antigenic overlapping between the two is extensive as regards both H and O antigens; diagnostic *Salmonella* sera can be used as substitutes for many of the Arizona antisera.²

At least 32 sero-groups of Arizona are recognised on the basis of different O antigens; most of these groups contain 2 or more serotypes identifiable by flagellar antigens which, as in the genus *Salmonella*, may occur in two phases. 180 serotypes are at present recognised and these are epidemiologically significant.³

The error in identifying a culture as a *Salmonella* rather than as an Arizona strain is of no great importance since, in man, the organisms produce similar clinical syndromes and can be regarded as very similar from the epidemiologic point of view and for prophylactic measures.

¹ Caldwell, M. E., & Ryerson, D. L. (1939), *J. infect. Dis.*, **65**, 242.

² Edwards, P. R., & Ewing, W. H. (1957), *Identification of Enterobacteriaceae*. Minneapolis. Burgess Publ. Co.

³ Edwards, P. R., West, M. G., & Bruner, D. W. (1947), *J. infect. Dis.*, **81**, 19.

A more significant error is the rejection of an organism as "non-pathogenic" on the basis of prompt lactose-fermentation on primary diagnostic media.¹ At present, our knowledge of the distribution of Arizona serotypes in man is elementary but of their pathogenicity there can be no doubt.

¹ Edwards, P. R., Fife, M. A., & Ramsey, C. H. (1959), *Bact. Rev.*, **23**, 155.

CHAPTER XXVI

SHIGELLAE

Antigenic Relationships in Enterobacteriaceae

THE members of this genus are the causative organisms of acute bacillary dysentery, a disease which is widespread throughout the world and which has become increasingly common in Britain in the last thirty years. The severity of the illness is, to some extent, related to the particular group or species incriminated; the *Shigella dysenteriae* group is most commonly associated with the classical syndrome of sudden onset of abdominal pain, tenesmus, pyrexia and prostration, with the passage of frequent stools which rapidly lose their faecal nature and become composed of blood and mucus. The illness associated with *Shigella sonnei* infection may be confined to the passage of a few loose motions and is frequently so mild that the individual continues at work or school. The stool is seldom much altered except in consistency, some blood and mucus being frequently present with it, but rarely as the sole constituents.

Two other groups—*Shigella flexneri* and *Shigella boydii*—are encountered as causative agents in bacillary dysentery. The illness associated with members of these groups tends to be more severe than with *Sh. sonnei*.

Morphology and Staining.—Identical with *S. typhi* except that they do not possess flagella and are non-motile. Fimbriae occur only in *Sh. flexneri* types.

Cultural Characters.—Resemble the genus *Salmonella* with the exception that *Sh. sonnei* is a late fermenter of lactose—thus, colonies of the latter on MacConkey or D.C.A. media become pink when incubation is prolonged beyond eighteen to twenty-four hours.

Viability.—The thermal death-point is about 55° C. for 1 hr.; within the genus, *Sh. sonnei* is more resistant to adverse environmental factors than are the other members.

Biochemical Reactions.—The 4 groups within the genus *Shigella* display the activities shown in the table.

Group	Glucose	Lactose	Mannitol	Indole	No. of serotypes
A. <i>Sh. dysenteriae</i>	+	-	-	v	8
B. <i>Sh. flexneri</i>	+	-	+	v	6
C. <i>Sh. boydii</i>	+	-	+	v	11
D. <i>Sh. sonnei</i>	+	(+)	+	-	1

Key: + = acid, no gas; (+) = delayed reaction; v = variable in different types.

The members of group A differ from those in other groups by constantly failing to ferment mannitol; groups B and C comprise species which are biochemically similar to each other whereas group D strains are late fermenters of lactose and never produce indole. With the exception of certain strains of *Sh. flexneri* type 6, dysentery bacilli are anaerogenic.

The practical classification of Shigellae into mannitol-fermenting and mannitol non-fermenting strains is retained, notwithstanding the fact that various exceptions have been recognised; indeed, the relative rarity of *Sh. flexneri* strains which do not ferment mannitol gives the latter feature value as an epidemiologic marker. This circumstance is best exemplified by the biochemical subdivision of the serologically homogeneous *Sh. flexneri* type 6 as in the table.

Biochemical Variety	Glucose	Mannitol	Dulcitol
No. 88	+	+	-
No. 88	+	+	(+)
Manchester	+	+	(+)
Newcastle	+	-	(+)

Key: + = acid and gas; - = acid only; (+) = delayed reaction.

Within the Flexner group, mannitol non-fermenting strains are also found in serotypes 1, 2, 3 and 4, but only in the latter type are they as common as such variants in type 6; mannitol non-fermenting strains of *Sh. flexneri* type 4 were previously designated *Shigella rabaulensis* and *Shigella rio*.

The inclusion of dulcitol as a substrate in biochemical typing of type 6 strains allows strains designated No. 88 to be divided into two bio-types; one-third of such strains do not ferment dulcitol,

whereas the others produce acid in this substrate after two to four days' incubation.

Antigenic Characters.—Group A strains can be recognised as one of at least 8 specific serotypes by agglutination tests with type-specific antisera. These types do not show significant intra- or inter-group sharing of antigens; unabsorbed sera can thus be used in type identification.

Group B strains: 6 serotypes are recognised—these are significantly interrelated by possession of a common group antigen (group antigen 1). Each serotype, when freshly isolated, also possesses a type-specific component (type antigens I, II, III, etc.) as originally shown by Boyd.¹ These findings were confirmed by Wheeler,² who also divided types 1, 2 and 4 each into two subtypes on the basis of minor group antigens, e.g. *Sh. flexneri* 1a possesses the type-specific antigen I as does type 1b, strains designated subtype 1a do not possess the minor group antigen 6, whereas subtype 1b strains constantly contain this minor antigenic factor. Group B strains tend to lose their type-specific component although retaining their group antigens; thus, two variants, X and Y, of *Sh. flexneri* are also recognised. Diagnostic sera for determining the type of a strain must be absorbed to remove group agglutinins.

Group C strains: The 11 recognised serotypes comprising this group are biochemically indistinguishable from group B strains, but are serologically distinct from the latter, since they do not possess the *Sh. flexneri* group antigen 1; however, some serotypes in group C show significant serological relationships with other group C strains as well as with certain types of *Sh. flexneri*, and it is essential that (with the exception of *Sh. boydii* types 2, 3, 7 and 8) diagnostic antisera should be absorbed to remove agglutinins which react non-specifically.

Group D strains: Smooth cultures of *Sh. sonnei* are serologically distinct from other shigellae; the study of smooth-rough (S-R) variation of *Sh. sonnei* has revealed that each form is antigenically distinct and that the recognised relationship with *Sh. boydii* type 6 strains is solely a function of the R form. *Sh. sonnei* strains cannot be subdivided into types by serological methods; attempts have been made to type or characterise strains by other methods (p. 600).

Animal Pathogenicity.—Cultures of dysentery bacilli are

¹ Boyd, J. S. K. (1938), *J. Hyg. (Lond.)*, **38**, 477, which contains references to his earlier studies.

² Wheeler, K. M. (1944), *J. Immunol.*, **48**, 87.

generally non-pathogenic when introduced orally in laboratory animals. Intravenous injection produces a haemorrhagic enteritis and, if the animal survives, muscular paralysis may result. These effects are particularly marked in the case of *Sh. dysenteriae* type 1 (*Shigella shigae*), which forms a potent, diffusible toxin. Apart from man, only monkeys and chimpanzees suffer naturally from bacillary dysentery.¹

Occurrence.—Infection occurs by ingestion; it is probable that the infecting dose is of a much lower order than for salmonellae (except *S. typhi*). After reaching the large intestine the shigellae multiply locally with resultant inflammation of the mucosa which may, in severe cases, progress to ulcer formation and sloughing of large areas of mucous membrane. The cellular response is characteristically by polymorphonuclear leucocytes and these are readily noted on microscopic examination of the dejecta. Dysentery bacilli rarely invade other tissues and bacteraemia has only occasionally been reported.

On clinical recovery, the patients may continue to excrete the organisms in their stools for a few weeks and some patients may become persistent carriers. It is worth noting that persons carrying *Sh. dysenteriae* serotypes excrete these organisms persistently compared with carriers of *Sh. flexneri* and *Sh. sonnei* in whom intermittent excretion is more common, *i.e.* the organisms may be isolated from the faeces for two or three days consecutively and then disappear only to reappear after some days or weeks. When comparing earlier studies² on carrier rates in bacillary dysentery with more recent reports,³ it should be remembered that the introduction of more selective media, *e.g.* D.C.A., ensures the isolation of shigellae when these are present in such small numbers that otherwise they would not be detected.

Epidemiology.—Sources of infection are human cases and carriers; the mild nature of Sonne infections, often amounting to little more than a social inconvenience, allows infected patients to remain ambulant with a greatly increased opportunity for disseminating the organisms compared with the person who is confined to bed. The role of the healthy convalescent and symptomless carrier as a source of infection has been the subject of much debate; provided that such persons are passing formed

¹ Rewell, R. E. (1949), *Lancet*, i, 220.

² Fletcher, W., & Mackinnon, D. L. (1919), *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 29.

³ Watt, J., Hardy, A. V., & DeCapito, T. M. (1942), *Publ. Hlth Rep. (Wash.)*, 60, 261.

stools, they are much less important in the spread of the disease than cases with fluid or loose stools.

In Britain, bacillary dysentery is endemic with epidemic waves occurring in the early part of the year and, to a lesser extent, in late autumn. This contrasts with experience in tropical and subtropical countries where the disease is most prevalent during the warmer months. The lack of modern sanitation has been invoked to explain the frequency of epidemics in certain countries, and certainly the construction of elementary toilet facilities, e.g. bore-hole latrines, has reduced the incidence in such circumstances; by contrast, in Britain, we have seen the almost unrelenting increase in bacillary dysentery in the last thirty years during which time environmental hygiene has been well above the standard obtained in materially less-favoured communities. Similarly, the spread of organisms by flies from faeces to food, although a potential danger in less satisfactory circumstances, must rarely be important in our community, where the peak incidence of the disease occurs during the months of the year when house flies are uncommon. Dysentery bacilli, being excreted in the faeces, may be transmitted to food through the soiled hands of a case or carrier, or they may pass from such sources to other individuals via inanimate objects such as door-handles, toilet chains and seats, pencils and crockery. A comparative study¹ of the dissemination of *Sh. sonnei* by cases revealed that contamination was heaviest in the immediate vicinity of the toilet pedestal, that the organisms passed through toilet paper on to fingers and that they could be recovered from fingers at least three hours after these had been contaminated.

In summary, there is sufficient evidence to confirm that, in Britain at least, bacillary dysentery is a hand-to-mouth infection depending for its spread on poor personal hygiene rather than on low standards of community services, although sanitary arrangements in many schools leave much to be desired. Whilst bacillary dysentery is uncommon in the first year of life, the majority of cases occur in the under-5 age-group; in recent years, the increasing incidence in the 5-14 years group has drawn attention to the role of primary schools in promoting the spread of dysentery.² Males are more frequently infected than females, except in the 15-45 years group, when the intimacy of contact between mother and sick child ensures that women are more exposed to infection, and suffer more commonly, than men.

¹ Hutchinson, R. I. (1956), *Monthly Bull. Minist. Hlth Lab. Serv.*, 15, 110.

² Taylor, I. (1957), *Proc. roy. Soc. Med.*, 50, 31.

Serological identification of specific types within groups A-C has been valuable in tracing the source of epidemics due to these types. The predominance of *Sh. sonnei* as the principal infecting agent in Britain in the last twenty to thirty years has stimulated the search for some means of characterising this serologically homogeneous species for epidemiologic purposes. The subdivision of *Sh. sonnei* strains on their ability to ferment xylose has been employed in tracing infection,^{1, 2} but is of limited value since only two types can thus be identified. Phage typing of *Sh. sonnei* has similarly been undertaken,^{3, 2} but instability of type and the predominance of one phage-type did not encourage its use.

The ability of Sonne strains to produce various specific colicines⁴ has been employed recently for epidemiologic purposes and encouraging results have been obtained.⁵ Our experience of colicine typing is entirely in agreement with the published account.

The method recommended is based on that of Abbott and Shannon.

Media:

1. For colicine production. Tryptone Soya (T.S.) Agar (Oxoid) is reconstituted according to the manufacturer's instructions and horse blood is added to give a final concentration of 2·5 per cent. The medium is dispensed in Petri dishes.
2. For cultivation of Indicator Strains. Infusion broth (p. 190) is used for this purpose.

Technique:

The strain to be tested is inoculated by a single diametrical streak on T.S. agar and the plate incubated at 35° C. for twenty-four hours. The growth is then removed with the edge of a glass slide and 2-3 ml. of chloroform are poured on to the base of the plate and the medium-containing portion replaced. After ten to fifteen minutes' exposure to the chloroform vapour, the residual liquid chloroform is decanted and the plate exposed to air for a further ten to fifteen minutes; thereafter the indicator

¹ Cruickshank, R., & Swyer, R. (1940), *Lancet*, ii, 803.

² Tee, G. H. (1955), *J. Hyg. (Lond.)*, 53, 54.

³ Mayr-Hartig, A. (1952), *J. gen. Microbiol.*, 7, 382.

⁴ Colicines (bacteriocines) are naturally occurring antibiotic substances elaborated by many members of the family *Enterobacteriaceae*. The colicine activity of a strain is often directed against members of another genus, although usually such activity is most marked against strains of the same genus as that of the producing strain. See: Frederiq, P. (1949), *Rev. belge Path.*, 19, Suppl. 4.

⁵ Abbott, J. D., & Shannon, R. (1958), *J. clin. Path.*, 11, 71.

(passive) strains are seeded on to the plate at right angles to the original growth line. Incubation at 37° C. for eight to twelve hours reveals various patterns of inhibition of the indicator strains by the colicines in the medium. At least 16 colicine-types of *Sh. sonnei* can thus be identified, and these appear to be stable. Indicator strains may be obtained from Dr. J. D. Abbott, Public Health Laboratory, 42 Hamilton Square, Birkenhead, or from Dr. R. R. Gillies, Bacteriology Department, University of Edinburgh.

Laboratory Diagnosis.—The collection of faecal specimens and their transmission to the laboratory demands the same care as for specimens from cases of enteric fever (p. 579). Specimens obtained by rectal swabbing are less reliable than faeces unless they have been taken expertly and cultured without delay. The use of moist, serum-coated rectal swabs¹ enhances their value, but, in general, the use of rectal swabs should be restricted to the investigation of outbreaks in institutions where laboratory facilities are immediately available. A further disadvantage of swab specimens is that they prohibit macro- and microscopic examination of the stool and require the skill of a qualified person for satisfactory results to ensue; the onus of collecting faeces can be placed on the patient or his relatives.²

Microscopic Examination.—A wet film preparation of a saline suspension of faeces or a mucoid portion of the specimen will usually reveal an abundant and characteristic cellular exudate. The cells present are mostly polymorph leucocytes with a varying number of red blood cells, and in the early stages numerous epithelial cells. In addition to these, macrophages are frequently a characteristic feature of the exudate; the leucocytes, as a rule, show marked degeneration.

Cultivation.—If the faeces are formed it is essential that they be emulsified in saline prior to plating out on D.C.A. and inoculating a selenite enrichment broth;³ the latter is subcultured on to a fresh plate of D.C.A. medium after incubation for eighteen to twenty-four hours. If mucus is present in the specimen, it should be employed as an inoculum. Colonies of dysentery bacilli are pale or colourless after primary incubation on D.C.A., and at least three such colonies should be submitted to further investigation. Tubes of the following media are inoculated: (1) peptone water;

¹ Rubbo, S. D., & Benjamin, M. (1951), *Brit. med. J.*, i, 983.

² Thomas, M. E. M. (1954), *Brit. med. J.*, ii, 394.

³ Armstrong, E. C. (1954), *Monthly Bull. Minist. Hlth Lab. Serv.*, 13, 70.

(2) glucose peptone water; (3) lactose peptone water; (4) sucrose peptone water; (5) mannitol peptone water with phenol red or Andrade's indicator and Durham tube (p. 202), and these are incubated for twelve to twenty-four hours. The peptone water is examined after six to seven hours for motility of the organisms. Alternatively, tests may be made using composite media (p. 222); this latter technique obviates the need for making a hanging-drop preparation to study motility and otherwise is as satisfactory as the first method.

Cultures with the biochemical characteristics of one or other of the *Shigella* groups must have their identity confirmed by serological investigation. If the strain is mannitol non-fermenting, it should be tested for agglutination with the individual type-specific sera comprising group A. If the strain ferments mannitol, then it may be a member of groups B-D and should be tested with polyvalent sera for *flexneri* and *boydii* serotypes and with a Sonne antiserum; in the case of positive reactions with one or other of the polyvalent sera, the strain is further tested with the type-specific sera within the particular group. Positive results obtained by slide agglutination must be confirmed by the tube-dilution method to ensure that agglutination occurs at or beyond the stated diagnostic titre of the particular antiserum employed.

Slide agglutination tests with colonies picked directly from diagnostic plates may be used for making early presumptive verbal reports before undertaking confirmatory biochemical and serological investigations.

Agglutination Tests with Patient's Serum.—Such tests do not have the same value as the Widal reaction in the enteric fevers, since, even in cases from which the causative organism has been isolated, the patient's serum frequently shows no increased level of specific antibody compared with sera from healthy individuals; furthermore, the presence of certain natural antibodies in human sera (p. 607) may give non-specific agglutination of the test suspensions.

The following sera are available from the Standards Laboratory for Serological Reagents:

- Group A. Individual specific sera for types 1-7.*
- Group B. Polyvalent serum.
 - Individual sera for types 2, 3, 6 and X variant.
 - Individual sera for types 1, 4, 5 and Y variant.*
- Group C. Polyvalent Boyd I (types 1-6).*
 - Polyvalent Boyd II (types 7-11).*
 - Individual sera for types 1-11.*

- Group D. S-R Sonne serum.
Phase I (S) serum.*
Phase II (R) serum.*

* Available only on special request.

Chemotherapy.—The value of sulphonamides in treating Sonne dysentery has diminished since, in Britain, the majority of strains are now resistant to these agents. Streptomycin in adequate dosage and for a sufficient time gives satisfactory clinical and bacteriological cure, but inadequate dosage or too brief a treatment period allows *Sh. sonnei* to develop resistance *in vivo*. The tetracyclines or chloramphenicol should be reserved for such cases; area laboratories can usefully check the sensitivity of Sonne strains and advise practitioners regarding the emergence of strains resistant to one or other therapeutic agent.

Prophylaxis.—The epidemiology of bacillary dysentery in temperate climates implies that improvement in personal hygiene, particularly hand-washing after defaecation and before handling foodstuffs, would achieve much in the prevention of spread. Children must be trained in such elementary procedures, and adequate facilities must be provided in schools and other communal centres. The adaptation of existing hand-operated toilet-flushing systems to foot-operated mechanisms and the installation of similar mechanisms for manipulating toilet seats would eliminate the handling of structures which act as vehicles of infection. The replacement of roller-towels with disposable paper-towels or continuous-feed roller systems will encourage hand-washing, but these and wash-basins must be in the same annexe as the toilet otherwise the handling of intervening doors will defeat their purpose.

Sulphonamides have been administered prophylactically in closed community outbreaks, but with little advantage; in many reports of such "trials" no mention is made of whether the epidemic strain was tested for sensitivity to the drug. Since the majority of Sonne strains are resistant to sulphonamides, it is not surprising that their employment in prophylaxis has rarely been successful.

In areas where toilet facilities are primitive or non-existent, either the provision of bored-hole latrines or chemical toilets will reduce the likelihood of insect-borne modes of spread and mechanical and chemical fly-control techniques may be applied to domestic and other premises.

Specific vaccines have no prophylactic value.

Antigenic Relationships in Enterobacteriaceae

The serological identification of enterobacteria depends mainly upon specific agglutination reactions involving group or type-specific somatic (O) and flagellar (H) antigens, e.g. in *Salmonellae* (p. 589).

However, certain other kinds of surface antigen may be present; some are associated with morphological features of the bacteria, e.g. capsular or envelope K antigens of Kauffmann¹ (L, A, B and Vi types) and fimbrial antigens,² whilst others, such as the α antigen of Stamp and Stone³ and the β antigen of Mushin,⁴ are not characterised morphologically. Such antigens may cause difficulties in serological identification of the bacteria either by covering and masking the O antigen so that the bacilli are inagglutinable by O antibodies or by producing non-specific cross reactions because of their presence in otherwise unrelated organisms.

Fimbrial antigens.—These are borne on fimbriae (p. 16) and have been found in *Esch. coli*, *Sh. flexneri*, *Cl. cloacae*, *Klebsiella* species, *Salmonellae*, *Proteus* and *Ps. pyocyanea*⁵⁻⁸. In many species fimbriation is a variable state; serial aerobic cultivation in fluid media selects a fimbriate mutant while the non-fimbriate mutant is selected by serial cultivation on nutrient agar plates. Fimbriae can be demonstrated only by the electron-microscope but their presence is associated with haemagglutinating activity and their absence with lack of such activity, so that the demonstration of direct bacterial haemagglutination (often specifically inhibited by 2 per cent. D-mannose) affords evidence of fimbriation.

Many *Esch. coli* strains remain partially fimbriate on agar and their fimbrial antigens may give rise to non-specific reactions; K antigens occurring in such strains have some general resemblances to fimbrial antigens in thermolability, anatomical location and liability to mask the O antigen and confer relative somatic inagglutinability (see p. 610).

The main differences between the three types of K antigen are:
L antigens do not combine with L antibodies after heating at

¹ Kauffmann, F. (1954), *Enterobacteriaceae*. Copenhagen, Munksgaard.

² Gillies, R. R., & Duguid, J. P. (1958), *J. Hyg. (Lond.)*, **56**, 303.

³ Stamp, Lord and Stone, D. M. (1944), *J. Hyg. (Lond.)*, **43**, 266.

⁴ Mushin, R. (1949), *J. Hyg. (Lond.)*, **47**, 227.

⁵ Duguid, J. P., et al. (1955), *J. Path. Bact.*, **70**, 335.

⁶ Duguid, J. P., & Gillies, R. R. (1957), *J. Path. Bact.*, **74**, 3-7.

⁷ Constable, F. L. (1956), *J. Path. Bact.*, **72**, 133.

⁸ Duguid, J. P. (1959), *J. gen. Microbiol.*, **21**, 271.

100° C. for one hour, whereas A and B antigens retain agglutinin-binding capacity after such treatment.

Strains possessing L or B antigens become O agglutinable after heating at 100° C. for one hour, but those with A antigens remain inagglutinable in somatic antisera after heating at 100° C. for two and a half hours.

The antigenicity of the L type is destroyed by heating at 100° C. for one hour and that of the A antigens is only reduced.

There are particular differences between L, A and B antigens on the one hand, and fimbrial antigens on the other; these are shown in the table.

Activity and Stability of Superficial Antigens of Enterobacteria (which may mask the O antigen)

Treatment of bacilli	Antigens				
	L	A	B	H	F
Living or 0·5 per cent. formalin	1. + + + + +				
	2. + + + + +				
60° C./1 hr.	1. ⊥ + + + +				
	2. ⊥ + + + +				
100° C./1 hr.	1. - + - - -				
	2. - + + - -				
50 per cent. alcohol, 20 hr./37° C.	1. - + + - ⊥				
	2. ⊥ .. + ⊥ ..				

Key: L.A.B. = K antigens; H = flagellar antigens; F = fimbrial antigens.

1. Agglutinability } of washed bacilli. + = unaffected;
 2. Agglutinin-binding } ⊥ = reduced;
 ⊥ = almost eliminated;
 ⊥ = abolished;
 .. = not tested.

The rapid, loosely floccular nature of fimbrial agglutination renders it liable to confusion with flagellar agglutination in species such as *Esch. coli*; it may be distinguished from the latter by treating the bacilli either with 0·005 N HCl¹ or with 50 per cent.

¹ Duncan, J. T. (1935), *Brit. J. exp. Path.*, **16**, 405.

ethyl alcohol, since fimbriae resist such reagents, whereas flagella are destroyed by them.

Fimbrial antigens differ from α antigen which does not react with natural agglutinins in normal human sera, and from β antigen which has not been detected in fimbriate genera, such as *Salmonella*, yet occurs in some non-fimbriate species, e.g. *Sh. boydii* type 6; furthermore, α and β antigens are not subject to predictable variation in appearance and disappearance under different cultural conditions.

The X antigens^{1 2, 3} of *Salmonellae* are very similar to fimbrial antigens in thermostability, type of agglutination, variability in differing cultural conditions and in occurring as precipitinogens in the supernatants of cultures heated at 100° C.

Difficulties in Diagnostic Serology caused by Fimbrial Antigens.—In homologous antiserum containing O and fimbrial agglutinins, the agglutination of fimbriate bacilli is mainly due to fimbrial antibodies; somatic agglutination reactions are obscured, since fimbrial agglutination occurs more rapidly and is loosely floccular. Among the *Shigellae*, fimbriae frequently occur but only in *Sh. flexneri* serotypes 1a, 2a, 2b, 3, 4a, 4b, 5, X and Y; the fimbrial antigens in these types are identical, irrespective of O serotype.

It is important, therefore, when undertaking identification of the O serotype of a *Sh. flexneri* isolate, to avoid fimbrial agglutination. This can be ensured by using baeillary suspensions that are devoid of fimbriae, either by serial aerobic cultivation on well dried agar plates to select non-fimbriate phase bacilli or by defimbriating the suspension by heating at 100° C. followed by centrifugation to harvest the defimbriated cells.

In normal diagnostic laboratory practice, it is unlikely that such difficulties will be encountered, since the procedures of isolation on solid media and subcultivation on agar prior to serological testing usually ensure that *Sh. flexneri* is in the non-fimbriate phase.

An additional safeguard would be the use of diagnostic typing sera free from immune and natural fimbrial antibodies; such sera could be prepared by absorption with a fimbriate culture of *Sh. flexneri* of heterologous O serotype. However, the use of such sera alone would not completely answer the problem, since in non-fimbrial sera fimbriation of test suspensions masks somatic agglutination to a greater or less degree.

Suspensions used for the preparation of diagnostic *Sh. flexneri*

¹ Topley, W. W. C., & Ayrton, J. (1924), *J. Hyg. (Lond.)*, 23, 198.

² Happold, F. C. (1929), *Brit. J. exp. Path.*, 10, 263.

³ Cruickshank, J. C. (1939), *J. Hyg. (Lond.)*, 39, 224.

antisera in rabbits must be non-fimbriate; even then, since the majority of rabbits possess *Esch. coli* fimbrial antibodies which may cross-react with those of *Sh. flexneri*, agglutinating sera may contain fimbrial antibodies. Tests on pre-immunisation bleedings will allow selection of animals whose sera do not contain fimbrial antibodies; alternatively, the fimbrial antibodies may be absorbed with a fimbriate strain of heterologous O serotype. Sera containing fimbrial antibodies for *Sh. flexneri* do not react with fimbriate strains of *Salmonella*, *Proteus* or *Cloaca*.

Another circumstance in which fimbriae and fimbrial antibodies can cause confusion is in Widal testing of human sera. Fimbrial antibodies have been noted in the sera of healthy persons and such sera have been found to react non-specifically in tests with diagnostic suspensions of *Salmonellae* if the latter are fimbriate; such anomalous serological findings are unrelated to the patient's condition. The use of standard agglutinable suspensions which are non-fimbriate or at most not more than poorly fimbriate, will prevent the occurrence of such non-specific reactions; *Salmonellae* subcultured from agar to broth and incubated for six hours show maximum flagellation and minimal fimbriation. The source of fimbrial antibodies in healthy persons may be from the commensal *Esch. coli* in the gut or as a result of past infections with fimbriate *Sh. flexneri* or *Salmonellae*. The presence of fimbriae in some T.A.B. suspensions may explain the presence of antibodies in individuals receiving injections of T.A.B. vaccines.

In addition to direct examination of suspensions by electron-microscopy, two other methods of controlling production are available; namely, testing for agglutinability with pure fimbrial antiserum and, also, a simple haemagglutination test for the presence of fimbriae.

CHAPTER XXVII

ESCHERICHIA COLI; OTHER ENTEROBACTERIACEAE

THE members of the Enterobacteriaceae to be considered in this chapter have been the subject of much dispute in so far as their classification is concerned. That used here is given in the table.

Group or Genus	Features						
	Gas from Glucose	Motility	Indole	Gelatin liq.	V.-P.	M.-R.	Citrate
Escherichia	+	+	+	-	-	+	-
Alkalescens- Dispar	-	-	+	-	-	+	-
Citrobacter	+	+	-	-	-	+	+
Klebsiella	+	-	-	-	+	-	+
Cloaca	+	+	-	+	+	-	+
Hafnia*	+	+	-	-	+	-	+

* Results when tests are performed at 20° C. (see p. 618).

ESCHERICHIA COLI

Morphology and Staining.—Identical with *Salmonella* species; most strains are flagellate and fimbriate and a few capsulate.

Cultural Characters.—Generally similar to *Salmonella* species although on nutrient agar, colonies are relatively larger and more opaque than the latter.

MacConkey's medium.—Colonies are rose-pink on account of lactose fermentation. Strains of *Esch. coli* grow poorly, if at all, on D.C.A. medium on which they also produce pink, but smaller and opaque colonies.

Biochemical Reactions.—These have been referred to in the table, and it will be noted that members of the genus *Escherichia* bear a close resemblance to those in the *Alkalescens-Dispar* (A-D) group (p. 614) except that the latter are never motile and in addition never produce gas in fermentation reactions; the two groups are also closely related serologically.

Indole Production.—This can be tested for by growing the

organism in peptone water, and, after two days, withdrawing 2 or 3 ml. into a test-tube. An equal volume of Ehrlich's rosindole reagent is then added:

Para-dimethyl-amido-benzaldehyde	4 g.
Absolute alcohol	380 ml.
Pure hydrochloric acid	80 ml.

A rose-pink colour develops in the presence of indole and can be separated out with amyl alcohol. The addition of a saturated solution of potassium persulphate hastens the reaction. If the indole reaction is negative after two days' growth, the test should also be repeated after seven days, as some strains are slow in their production of indole.

An alternative procedure is to add 1 ml. of ether to the culture, which is then vigorously shaken. The ether extracts the indole, and after it has been separated, the culture having been allowed to stand for a few minutes, about 0·5 ml. of Ehrlich's reagent is added.

Kovacs' reagent can also be employed for detecting indole production; the test may be performed as above or as a paper indicator test (p. 223).

Citrate Utilisation Reaction.—*Esch. coli* is unable to utilise citrate as a source of carbon¹ and cannot grow in a synthetic medium containing this salt; with the exception of A-D strains, the other members of Enterobacteriaceae described in this chapter can utilise citrate as a sole source of carbon and produce a visible turbidity in the medium. The medium recommended by Koser² consists of 1·5 g. sodium ammonium hydrogen phosphate (microcosmic salt), 1 g. potassium dihydrogen phosphate, 0·2 g. magnesium sulphate and 2 g. sodium citrate in 1 litre of distilled water; an alternative formula is given on p. 218. One loopful of a saline suspension of the culture is used as an inoculum.

Two other tests of value in differentiating *Esch. coli* from other closely related organisms are the Voges-Proskauer and the Methylated reactions.

Voges-Proskauer Reaction.—To 0·5 ml. of a two days' culture in glucose-phosphate medium (p. 218) is added 1 ml. of a 10 per cent. solution of caustic potash and the culture held at room temperature for some hours. If the reaction is positive, a salmon-pink colour develops due to a reaction between diacetyl (formed by oxidation of acetyl-methyl-carbinol) and a guanidine residue in the peptone.

¹ Brown, H. C. (1921), *Lancet*, i, 22.

² Koser, S. A. (1923), *J. Bact.*, 8, 493; *ibid.*, 9, 59.

Barritt's modification is highly sensitive and gives the colour reaction more rapidly than the original method:—To 5 ml. of a two days' culture in glucose-phosphate medium, add 3 ml. of a 5 per cent. solution of α -naphthol in absolute alcohol and 1 ml. 40 per cent. potassium hydroxide solution. A positive reaction is denoted by a bright pink colour appearing at the surface of the culture in five to fifteen minutes.

Methyl-red Reaction.—This reaction indicates the hydrogen-ion concentration produced by growth in a standard glucose medium. The organism is grown for three days in glucose-phosphate medium. To 5 ml. of culture is added 1 drop of methyl-red solution (0·1 g. methyl-red dissolved in 300 ml. alcohol and made up to 500 ml. with distilled water); a red colour, denoting a pH of 4·5 or less, is described as positive whilst yellow, signifying a low hydrogen-ion concentration, is described as negative.

It has been suggested that the optimum temperature for growth of cultures to be tested for V.-P. and M.-R. reactions is about 30° C., incubating for five days.

Antigenic Characters.—In addition to O and H antigens, the majority of commonly encountered *Esch. coli* strains possess K antigens. The term K antigen describes collectively a group of antigens designated L, A or B on the basis of physical characteristics (p. 604); these are somatic antigens which occur as envelopes or capsules and prevent O agglutination of living strains by their homologous O antisera. The serological classification of *Esch. coli* has only been established since the discovery of the K antigens and their recognition as the cause of irregularities and discrepancies in earlier attempts at serotyping members of this genus. The pathogenicity of many strains of *Esch. coli* is related to the possession of K antigens.

Occurrence.—*Esch. coli* strains predominate among the aerobic commensal organisms present in the healthy gut. Serological studies have shown that the types present are not only numerous at any one examination but that over a period of time the coli types fluctuate: some types persist over relatively long periods of time, whereas others are quite transient.^{1, 2, 3}

These organisms are also incriminated as pathogens. They are found most frequently in pyogenic infections of the urinary tract (pyelitis, cystitis, etc.) either in pure culture or mixed with faecal streptococci; they also occur in appendix abscess, peritonitis, cholecystitis, septic wounds and bed-sores.

¹ Kauffmann, F., & Perch, B. (1943), *Acta path. microbiol. scand.*, **20**, 201.

² Wallick, H., & Stuart, C. A. (1943), *J. Bact.*, **45**, 121.

³ Sears, H. J., Brownlee, I., & Uchiyama, J. K. (1950), *J. Bact.*, **59**, 293.

Serotype distribution studies,^{1, 2, 3} reveal that strains isolated from pathological material usually belong to a relatively few O groups and the majority are O-inagglutinable due almost invariably to the possession of L antigens; by contrast, strains isolated from healthy faeces are distributed over many more O groups and only a minority are O-inagglutinable, and this is due to the presence of L antigen in only 50 per cent. of such strains.

In contrast to the finding of numerous O groups of *Esch. coli* present in a specimen of healthy faeces, testing of numerous colonies from the urine in a case of cystitis usually reveals that they are all of the same O group; such serological homogeneity, although to a lesser degree, is noted also in peritonitis and cases of appendix abscess. The infections mentioned above are of an endogenous nature and the aetiological role of *Esch. coli* in such had long been accepted before adequate techniques of serotyping had been introduced.

The suspicion that *Esch. coli* might also be capable of causing gastro-enteritis in man has only been confirmed as a result of recent serologic evidence. The pathogenic role of certain coli types in human cases of gastro-enteritis was first emphasised by Bray⁴; this strain is now known as *Esch. coli* O 111 and in common with other enteropathogenic types (e.g. O 26, O 55, O 119 and O 128) it possesses a B type of K antigen. The antigenic analysis of these types is discussed on p. 612.

In the veterinary field also, *Esch. coli* is a recognised pathogen; careful and protracted studies in Sweden⁵ revealed that in calves dying from "coli-septicaemia", compared with healthy calves, the differences in type distribution and the relative frequencies of O-inagglutinable strains was analogous to those in similar studies in man (*vide supra*).

Esch. coli is associated with the condition of white scours in calves; enteritis in piglets, mastitis in cows, uterine infections in bitches and occasional abortion in ewes can also be attributed to these organisms. Hjarre's disease—a granulomatous condition resembling tuberculosis and occurring in poultry may be caused by certain types of *Esch. coli*.

Epidemiology.—The essentially endogenous nature of many infections caused by *Esch. coli* has already been mentioned; how-

¹ Kauffmann, F. (1944), *Acta path. microbiol. scand.*, **21**, 20.

² Ewertsen, H. W., & Knipschildt, H. E. (1946), *ibid.*, **23**, 170.

³ Vahlne, G. (1945), *Serological Typing of the Colon Bacteria*. Lund, Gleerupska Univ., Bokhandeln.

⁴ Bray, J. (1945), *J. Path. Bact.*, **57**, 239.

⁵ Wramby, G. (1948), *Investigations into the Antigenic Structure of Bact. coli Isolated from Calves*. Uppsala, Appelbergs Boktryckeriaktiebolag.

ever, exogenous infection of the urinary tract may occur, the organisms being introduced during diagnostic or therapeutic catheterisation. In such instances the instruments may have been inadequately sterilised or insufficient care taken in the pre-operative cleansing of the patient or the operator.

In gastro-enteritis, the majority of reported epidemics have occurred among infants under 18 months of age; that the pathogenicity of such *Esch. coli* strains is not necessarily so restricted is shown by reports of enteritis in adult hospital patients¹ and of experimentally induced infections in adult volunteers,^{2, 3} whose sera showed the presence of specific antibodies after recovery.

Artificially-fed babies are much more likely to be infected than those fed naturally; the sources of infection in babies are both cases and carriers of the infection and spread may occur by contaminated fomites. Contamination of the milk-bottle or introduction of the organisms into the milk-feed during its preparation will ensure ingestion and if the feed has been prepared some hours before, the original inoculum will have the opportunity to multiply to a greater or less extent depending on the conditions of storage.

Laboratory Diagnosis.—In film preparations from pus, urinary sediment, etc., stained by Gram's method, *Esch. coli* can be recognised as Gram-negative bacilli but indistinguishable from any other member of the Enterobacteriaceae. Specimens are plated on to blood-agar and MacConkey's medium; on the latter, *Esch. coli* yields pink, lactose-fermenting colonies, and if confirmation of its identity is required then the reactions listed in the table (p. 608) must be tested.

Identification of gastro-enteritis-producing strains.—Specimens of faeces submitted from cases of diarrhoea will also be examined for Salmonellae and Shigellae; since *Esch. coli* usually do not grow on the highly selective D.C.A. medium it is essential that the less inhibitory differential MacConkey's medium should also be inoculated. A blood-agar plate should be inoculated lightly with faeces since, on occasion, growth appears on this medium when the enteropathogenic *Esch. coli* strain cannot be identified on the parallel MacConkey plate; further, it is easier to avoid contaminants when subculturing from the blood-agar medium.

After incubation for eighteen to twenty-four hours, the blood-agar and MacConkey media are examined for colonies of *Esch. coli*; since colonies of enteropathogenic strains do not differ in appearance either among themselves or from other *coli* strains it is

¹ McNaught, W., & Stevenson, J. S. (1953), *Brit. med. J.*, ii, 182.

² Ferguson, W. W., & June, R. C. (1952), *Amer. J. Hyg.*, 55, 155.

³ June, R. C., Ferguson, W. W., & Worfel, M. T. (1953), *ibid.*, 57, 222.

essential that at least 10 colonies be subjected to serological testing. Slide tests are performed with a fragment of each colony against a polyclonal antiserum. If agglutination is noted, then further tests are made with the individual type-specific sera; when living, freshly isolated strains are tested in OB antisera, agglutination in the specific serum occurs rapidly.

Sera available from the Standards Laboratory are as follows:

Polyvalent *Esch. coli* serum containing factors O 26, O 55, O 111, O 119, O 127, O 128, and their related B agglutinins.
Type specific sera: O 26: B 6; O 55: B 5; O 111: B 4;
O 119: B 14; O 127: B 8; O 128: B 12.

A modified MacConkey agar medium,¹ in which sorbitol is substituted for lactose, has been used for the differentiation of serotypes O 55: B 5, and O 111: B 4, since it was considered that fresh isolates of these serotypes did not ferment sorbitol and therefore gave pale colonies on this medium whereas many other *Esch. coli* types fermented sorbitol and yielded red colonies; this distinction is by no means complete, and the only circumstance in which such a medium should be employed (in addition to the usual media) is in the search for further cases and carriers in an outbreak where the epidemic strain has been characterised fully and is a sorbitol non-fermenting strain.

Chemotherapy.—Since there is much variation in the sensitivity of *Esch. coli* strains to the commonly employed agents, chemotherapy should whenever possible be guided by an *in vitro* assessment of the sensitivity of the particular strain. It should be noted that the presence of pathological changes e.g. renal stone, bladder diverticulum, hypertrophied prostate, may prevent the eradication of *Esch. coli* from the urinary tract even although the infection is treated with a chemotherapeutic agent to which the strain is highly sensitive.

Prophylaxis.—The prevention of infection by *Esch. coli* in wounds, etc., and in exogenously acquired urinary tract infections depends upon the provision of properly sterilised dressings and instruments, thorough debridement of wounds and preparation of patients and attendants before operation or wound-dressing.

The measures taken to prevent the spread of gastro-enteritis are those applicable in other types of bowel infection. The preparation of feeds and cleansing and sterilisation of feeding-bottles demands constant care and attention;² the mother must

¹ Rappaport, F., & Henig, E. (1952), *J. clin. Path.*, **5**, 361.

² Report. *Monthly Bull. Minist. Hlth Lab. Serv.* (1953), **12**, 214.

be instructed in personal cleanliness and urged to wash her hands before preparing a feed. Whenever possible, each feed should be prepared immediately before it is required, and if storage of prepared feeds is unavoidable the container should be carefully wrapped to prevent accidental contamination and unnecessary handling. Some form of terminal heat-treatment of feeds for babies in hospital such as is used in U.S.A. is to be recommended.¹

THE ALKALESCENS-DISPAR GROUP

In Chapter XXV it was stated that although a classification of Enterobacteriaceae was essential for practical purposes of diagnosis and epidemiological studies, the biochemical and serological interrelationships of members of the family do not allow all cultures to be assigned to one or other genus. The close relationship of many Arizona strains to Salmonellae (p. 593) is paralleled in the case of the Alkalescens-Dispar (A-D) group whose members bear close resemblances, both biochemical and serological, to Escherichiae; apart from their lack of flagella and motility and their failure to produce gas in fermentation reactions, strains of the A-D group behave as Escherichia strains.

It has been shown also that the O antigens of the A-D group are either identical with or bear strong relationships to Escherichia O antigens^{2, 3}; similar findings have been made with regard to the K antigens in the two groups.

Because of this very close similarity it has been recommended that any future cultures with the characters of the A-D group should be classified as anaerogenic *Esch. coli*; thus the A-D group will not be extended beyond its present limits.

The first description of these organisms was in 1918⁴ and their relationship to cases of bacillary dysentery was mooted; both Alkalescens (lactose non-fermenting or late fermenting) and Dispar (lactose-fermenting) strains occur in normal faeces and their presence in cases of enteritis (in which recognised bacterial pathogens cannot be found) is regarded as incidental. Conversely, their role as incitants of urinary tract infections has been suggested.

¹ Wright, Joyce (1951), *Brit. med. J.*, **2**, 138.

² Frantzen, E. (1951), *Biochemical and Serological Studies of the Alkalescens-Dispar Group*, Copenhagen, Einer Munksgaard.

³ Ewing, W. H., Taylor, M. H., & Hucks, M. C. (1950), *Publ. Hlth Rep. (Wash.)*, **65**, 1474.

⁴ Andrewes, F. W. (1918), *Lancet*, **1**, 560.

Citrobacter freundii (Escherichia freundii)
(including the Bethesda-Ballerup group)

Members of this group can utilise citrate and produce H₂S; a further feature distinguishing them from *Esch. coli* is their ability to grow in Moeller's KCN medium.¹ Although certain members have been suspected of causing enteric infections, their aetiological relationship remains to be established. At present the importance of *Cit. freundii* species lies in the fact that many indole negative strains attack lactose slowly, if at all, so that their colonies resemble those of Salmonellae and Shigellae. Their motility, however, rapidly resolves any confusion with Shigella strains, but many bear a close resemblance to Salmonellae on biochemical testing; these are the members which comprise the Bethesda-Ballerup group and they have been studied more intensively than other *Cit. freundii* species which attack lactose promptly.

False-positive reports of Salmonella isolations and the delay in issuing negative reports attending the establishment of identity of lactose non-fermenting *Cit. freundii* strains can be eliminated by using the KCN test; Salmonellae fail to grow in this medium whilst *Cit. freundii* cultures flourish.

Cyanide broth (Modified Moeller Medium)

Bacto Proteose Peptone No. 3	3 g.
NaCl	5 g.
KH ₂ PO ₄	0.225 g.
Na ₂ HPO ₄ (2H ₂ O)	5.64 g.
Distilled water	1 l.

Adjust pH to 7.6

The medium is autoclaved in flasks. To the cold medium is added 15 ml. 0.5 per cent. KCN solution (0.5 g. KCN dissolved in 100 ml. sterile distilled water). One ml. of this medium is then placed in sterile tubes (1 × 10 cm.) which are stoppered quickly with corks sterilised by heating in paraffin. In such tubes the medium can be stored for two weeks at 4° C. The tubes are inoculated with one loopful of a twenty-four-hour broth culture grown at 37° C. Control tubes of medium without KCN are inoculated in parallel and the pairs of tubes incubated at 37° C. The tubes are inspected daily for two to four days.

Certain extra-generic relationships are recognised with O antigens of Salmonella and Arizona serotypes,² but these need

¹ Moeller, V. (1954), *Acta path. microbiol. scand.*, **34**, 115.

² West, M. G., & Edwards, P. R. (1954), *U.S. Publ. Hlth Service Monographs*, 22.

not occasion any great difficulty in identification; reference to the publication of West and Edwards¹ should be made regarding the serological characteristics of these organisms. The fact that the Vi antigen of *S. typhi* is identical with that of a Ballerup strain² has already been noted (p. 575).

THE GENUS KLEBSIELLA

Members of this genus conform to the definition of the family Enterobacteriaceae and are, without exception, non-motile. They differ from the groups and genera already described in this chapter by generally giving a positive Voges-Proskauer reaction and a negative Methyl-red reaction. The genus includes strains of parasitic and saprophytic origin, some of which are given species designation while others are characterised solely by their antigenic formulae.

Microscopically these organisms can be shown to possess large capsules and produce an abundance of extracellular slime which result in their colonies being mucoid or viscid; by means of capsular antisera the genus can be divided into at least 72 serotypes—tests may be performed as agglutination reactions or more usually by the “capsule-swelling” reaction (p. 496). Most strains are fimbriate.

Two species (*Klebsiella pneumoniae* and *Klebsiella rhinoscleromatis*) can be defined within the genus by biochemical means and a third species (*Klebsiella ozaenae*), intermediate in biochemical behaviour, may be recognised.

Klebsiella pneumoniae (Friedlander's Bacillus; Kl. aerogenes)

First isolated from fatal cases of lobar pneumonia in which disease it is only rarely incriminated; occurs as a commensal in the upper respiratory tract and intestine and may be associated with catarrhal conditions in the respiratory tract and with para-nasal sinusitis, conjunctivitis, etc.

Morphology and Staining.—Gram-negative bacillus varying greatly in size—1–4 µ by 0·5–1 µ. Capsulate both in the tissues and in artificial culture. Non-motile and non-sporing.

Cultural Characters.—Colonies are large, raised and viscid; on MacConkey's medium colonies are pink due to fermentation of lactose.

¹ West, M. G., & Edwards, R. (1954), *U.S. Publ. Hlth Service Monographs*, 22.

² Kauffmann, F., & Moeller, E. (1940), *J. Hyg. (Lond.)*, 40, 246.

Biochemical Reactions.—In addition to the characters listed on p. 608, *Kl. pneumoniae* usually produces gas in glucose, lactose and sucrose. Characteristically, strains hydrolyse urea but not with the rapidity displayed by *Proteus* strains; their lack of motility and inability to deaminate phenylalanine serve to differentiate them from *Proteus*.

Antigenic Characteristics.—All smooth forms of *Kl. pneumoniae* (and other members of the genus) possess O antigens; in addition, capsulate, non-mucoid smooth forms possess a K antigen, non-capsulate, mucoid forms possess an M antigen, and mucoid and capsulate forms possess both an M and K antigen in addition to O antigen. Similarly, rough forms may possess only R antigen, or in addition one or other or both M and K antigens. It has been shown that in any one strain, the M and K antigens are identical^{1, 2}; the frequent presence of these antigens and their masking of O and R somatic antigens dictates the use of capsular antisera in typing procedures. Some 72 or more capsular types of Klebsiellae are recognised; *Kl. pneumoniae* strains belong to types 1 and 2.

Strains previously designated *Aerobacter aerogenes* (*Bacillus lactic aerogenes*) are biochemically indistinguishable from *Kl. pneumoniae*; their serological similarity extends to identity of O antigens although they possess different K antigens. To avoid confusion in reporting such strains when isolated from water supplies or other saprophytic sources they should be reported as *Kl. aerogenes*.³

Klebsiella rhinoscleromatis

Cultures of this species, belonging to capsular serotypes 3, 4 and 5, contrast biochemically with *Kl. pneumoniae* in being anaerogenic, failing to ferment lactose, giving a negative V-P and positive M-R reaction and not utilizing citrate; *Kl. rhinoscleromatis* is associated with a chronic granuloma, rhinoscleroma, which is prevalent in south-eastern Europe; the bacilli are situated intracellularly in the lesions which occur in the mucous membrane of the nose, throat and mouth.

Klebsiella ozaenae

Such strains are not confined to cases of ozaena so that its causal relationship to the condition is in doubt. Biochemically, *Kl.*

¹ Edwards, P. R., & Fife, M. A. (1952), *J. infect. Dis.*, **91**, 92.

² Wilkinson, J. F., Duguid, J. P., & Edmunds, P. N. (1954), *J. gen. Microbiol.*, **11**, 59.

³ Cowan, S. T. (1956), *J. gen. Microbiol.*, **15**, 345.

ozaenae strains are intermediate between the Klebsiella species described above; they belong to capsule types 4, 5 and 6.

Species	Glucose	Lactose	V-P	M-R	Citrate
<i>Kl. pneumoniae</i>	+	+	+	-	+
<i>Kl. rhinoscleromatis</i>	-	-	-	+	-
<i>Kl. ozaenae</i>	- or +	- or +	-	+	+

The Genus Cloaca

Consequent to the incorporation of *Aero. aerogenes* in the genus Klebsiella it was necessary to define the position of strains previously designated *Aerobacter cloacae*. This has been accomplished by resurrecting the genus Cloaca, of which *Cloaca cloacae* is the type species.

Members of this genus are microscopically indistinguishable from other Enterobacteriaceae; they are seldom capsulate and frequently motile, flagellate and fimbriate, and their ability to liquefy gelatin is a further characteristic assisting the differentiation from Klebsiella species.

More definitive studies, both biochemical and serological, are required to clarify interrelationships with other genera of Enterobacteriaceae. Members of the genus *Cloaca* are widely distributed in nature and are commonly encountered in water and grasses; they are occasionally isolated in small numbers from healthy faeces but are not regarded as pathogenic for man or animals.

The Genus Hafnia

Members of this genus are probably non-pathogenic for man; their interest to medical bacteriologists lies in their ubiquity and similarity to other lactose non-fermenting Enterobacteriaceae. The temperature at which biochemical tests are performed with *Hafnia* strains has a marked effect on the results; tests of motility and for V-P, M-R reactions as well as various fermentation reactions are variable when performed at 37° C. whereas if these are carried out at 20-22° C. the results for any one test are uniform.

In addition to the reactions noted in the table (p. 608), it should be noted that *Hafnia* cultures invariably effect acid fermentation of glucose and mannitol with accompanying gas production, give a positive KCN result but do not hydrolyze urea.

CHAPTER XXVIII

PROTEUS; PYOCYANEA; LACTOBACILLI

MEMBERS of the genus *Proteus* are widely distributed throughout man's environment and can be detected in sewage, soil and on garden vegetables. They are found in the faeces of higher animals and man, and may be present in large numbers following bowel infections; their relationship to various disease processes is dealt with later (p. 621).

Morphology and Staining.—Gram-negative bacilli; 1·5–3 μ by 0·5 μ (average) but displaying pleomorphism; motile with numerous lateral flagella; non-sporing and non-capsulate. Most strains are fimbriate.

Cultural Characters.—Aerobe and facultative anaerobe; grow well at 37° C. on ordinary media. A strong seminal odour is often noticeable. Characteristically, discrete colonies are seen only in the earliest stages of incubation, thereafter a thin film of growth extends round the colony and rapidly spreads all over the available surface of the medium; during this spreading or swarming phase, the bacilli are characteristically long and slender and these are replaced by short forms when the surface of the medium has been covered.

Swarming tends to occur in successive waves so that ultimately a plate inoculated at one central point shows a series of concentric rings of thick growth between which there are smooth translucent areas of spreading growth. Swarming is associated with active motility although its cause is not clearly established¹; non-motile variants do not spread.

Biochemical Reactions.—All members of the genus ferment glucose but have no action on lactose; four biochemical types can be recognised as indicated in the table.

Substrate	<i>Pr. vulgaris</i>	<i>Pr. mirabilis</i>	<i>Pr. morganii</i>	<i>Pr. rettgeri</i>
Mannitol	—	—	—	1 or +
Maltose	+	—	—	—
Gelatin	+	+	—	—
Indole	+	—	+	+
Citrate	—	—	—	+

Key: Fermentation reactions: + = acid and gas; 1 = acid only; — = negative.
Other reactions: + = liquefaction of gelatin, production of indole or growth in citrate medium.

¹ Lominski, I., & Lendrum, A. C. (1947), *J. Path. Bact.*, 59, 688.

It should be noted that, with the exception of *Pr. rettgeri*, all species usually produce gas; strains of *Pr. rettgeri* are frequently anaerogenic.

The ability of *Proteus* strains to decompose urea rapidly has been used as a test to differentiate them from other enterobacteriaceae; urease activity is also displayed by most *Klebsiella* strains and by some strains of *Escherichia* and *Citrobacter* species. Whilst the absence of urease activity in *Salmonellae* and *Shigellae* allows their distinction from *Proteus* for diagnostic purposes, a more useful and more specific test is the ability of *Proteus* to transform phenylalanine to phenyl-pyruvic acid; no other members of the family *Enterobacteriaceae* possess such activity.¹

The phenylpyruvic acid (P.P.A.) reaction. This is performed as follows:

To 0.5 ml. of a dense suspension (10^{11} organisms per ml.) of organisms add 0.5 ml. of 0.2 per cent. DL-phenylalanine in saline; add 1 drop of 0.01 per cent. phenol red and make alkaline with 0.1 M Na_2CO_3 . Shake vigorously and incubate the tube in an almost horizontal position; after four hours add 10 per cent. H_2SO_4 until the colour changes from yellow to pink. Add sufficient $(\text{NH}_4)_2\text{SO}_4$ to saturate the solution and then 5 drops of half-saturated $\text{FeNH}_4(\text{SO}_4)_2$. Shake thoroughly and read the results after one minute. Positive reactions are designated + to +++, depending on the intensity of green colouration developing in the fluid.

Antigenic Characters.—A diagnostic antigenic schema for *Pr. vulgaris* and *Pr. mirabilis* has been established² and extended³ to comprise 47 O groups, which can be subdivided on the basis of H antigens to allow 119 serotypes to be recognised.

Much attention has been given to the agglutination of certain *Proteus* types by the blood serum of cases of typhus fever. This reaction is employed as a diagnostic test (Weil-Felix reaction); the *Proteus* strain X 19 which is employed in the reaction is biochemically *Pr. vulgaris* and the reaction is dependent on the specific O antigen present in this strain, which has no aetiological relationship to the disease. The Weil-Felix reaction is explained on the basis of a common antigen shared by *Proteus* X 19 and the rickettsiae of typhus fever (p. 735).

Another *Proteus* type designated XK (biochemically *Pr. mirabilis*) is agglutinated by the serum of patients suffering from

¹ Henriksen, S. D. (1950), *J. Bact.*, **60**, 225.

² Kauffmann, F., & Perch, B. (1947), *Acta path. microbiol. scand.*, **24**, 135.

³ Perch, B. (1948), *Acta path. microbiol. scand.*, **25**, 703.

the disease "Scrub typhus", occurring in the Far East. A diagnostic agglutination test similar to the Weil-Felix reaction is based on this antigenic relationship.

Occurrence.—The majority of strains isolated from pathological specimens and human faeces are biochemically typical of *Pr. mirabilis*. The specific relationship of *Pr. morganii* to summer diarrhoea of infants is still in doubt, but it is recognised that, along with other *Proteus* species, this organism is often a concomitant of *Shigellae*, making its appearance in the stools as the case improves and the dysentery bacilli become scanty. Secondary infection of wounds, bed-sores, etc., is probably endogenous in origin; although such a source may account for cases of cystitis in which *Proteus* is incriminated, there are many instances in which exogenous infection takes place following diagnostic or therapeutic instrumentation.

Laboratory Diagnosis.—The spreading growth of *Proteus* strains on media such as blood-agar is sufficient for identification in routine diagnostic work; the disadvantage of this characteristic is that other organisms when present may be completely overgrown by the spreading growth and their subcultivation for purposes of identification and antibiotic sensitivity testing delayed. Swarming may be inhibited by several methods; discrete colonies of *Proteus* can be obtained on a blood-agar medium by increasing the agar concentration to 4 per cent.; similarly, inhibition of swarming may be obtained by incorporating chloral hydrate (1 in 500), sodium azide (1 in 5000) or one of the sulphonamide drugs.¹ Desoxycholate-citrate agar inhibits swarming and, provided that the other organisms present are capable of growing on this medium, its use in allowing separation from *Proteus* species is valuable.

If necessary, the biochemical type of a *Proteus* strain can be determined as indicated in the table (p. 619).

Chemotherapy.—*Proteus* infections, e.g. of the urinary tract, may respond to treatment with chloramphenicol, streptomycin and the tetracyclines; chemotherapy should whenever possible be guided by *in vitro* sensitivity tests, since strains vary markedly. As in the case of *Esch. coli* infections (p. 613), structural or pathological abnormalities impede the eradication of organisms and in many instances, although the original infecting organism is eradicated, there is often replacement with some other species.²

¹ Holman, R. A. (1957), *J. Path. Bact.*, **73**, 91.

² Gould, J. C., Macleod, J. G., & O'Flynn, J. D. (1952), *Brit. J. Urol.*, **24**, 138.

Providencia (*Proteus inconstans*)

These Gram-negative, motile bacilli are closely allied to the genus *Proteus*; they alone among the Enterobacteriaceae share with *Proteus* species the ability to deaminate phenylalanine although only rarely do *Providencia* strains decompose urea, and they never exhibit spreading on ordinary agar. They occur in normal faeces and in urinary tract infections. *Providencia* cultures may be allocated to one of two biochemical groups depending on their ability to produce gas from glucose and their action on substrates such as adonitol and inositol.

For details of biochemical and serological characteristics of this group the publication of Ewing *et al.*¹ should be consulted.

**PSEUDOMONAS PYOCYANEA
(*Ps. aeruginosa*)**

This species does not belong to the family *Enterobacteriaceae* but is conveniently considered along with these, since, in occurrence and other respects, it is not dissimilar.

Morphology, and Staining.—Identical with *Proteus* species except that the flagella are terminal in situation and few (one to three) in number.

Cultural Characters.—Essentially aerobic—a few strains give very slight growth anaerobically. Temperature range, 5°–43° C.; optimum 37° C. Grows on ordinary media, producing a peculiar musty odour.

Agar.—Stroke inoculation produces an abundant, moist, greenish-blue fluorescent growth; the pigments (pyocyanin and fluorescin), on which the colour depends, also diffuse through the medium and are most abundantly produced at room temperature.

MacConkey Medium.—Similar to growth on agar; colonies are pale since lactose is not fermented.

Biochemical Reactions.—With the exception of glucose, none of the usual sugars is fermented. Gelatin is usually liquefied and growth occurs in inorganic media with citrate as a sole source of carbon. Indole and H₂S are not produced and the Voges-Proskauer and Methyl-red reactions are negative.

Antigenic Characters.—Difficulty in differentiating between flagellar and somatic antigens has delayed the introduction of serotyping as a method of dividing the species for epidemiologic

¹ Ewing, W. H., Tanner, K. E., & Dennard, D. A. (1954), *J. infect. Dis.*, 94,

purposes; phage typing may be of value in recognising "types" of *Ps. pyocyanea*.¹

Occurrence.—*Ps. pyocyanea* is the only member of the *Pseudomonas* group which is pathogenic to man; it is commonly found in suppurating wounds, infected burns and otitis media, usually in association with pyogenic cocci. It is frequently incriminated in urinary tract infections, either alone or with a member of the family *Enterobacteriaceae* or Gram positive cocci. It is occasionally found as a commensal in the intestine of man and animals and also in sewage; from the latter source and from water and soil, a similar but purely saprophytic organism, *Pseudomonas fluorescens*, may be isolated.

Chemotherapy.—Many strains are susceptible to polymyxin and some to streptomycin and chloramphenicol.

Ps. pyocyanea was one of the first organisms found to produce antibiotic substances. Although animal experiment showed that "pyocyanase" was too toxic for systemic administration, such products were applied locally to surface infections for some years. It has been shown² that preparations of pyocyanase inhibit the growth of *Strept. pyogenes* at a dilution of 1 in 24,000, and although this knowledge has not been applied therapeutically it explains the inhibition of such species in mixed growths with *Ps. pyocyanea*.

THE LACTOBACILLI

These organisms constitute a group of acid-resistant (aciduric), Gram-positive, non-sporing bacilli, which occur in the intestine of mammalian animals and are particularly prevalent during the stage of suckling. Thus, in breast-fed infants such organisms may constitute the predominant flora of the intestine, and two main types, *Lacto. acidophilus* and *Lacto. bifidus*, have been recognised and specially studied.

Organisms of this group are also found in cow's milk, in the human mouth, stomach and vagina, in soil, and in silage and bran.

***Lactobacillus acidophilus* (*Bacillus acidophilus*)**

So called because it is able to flourish in a highly acid medium (pH 4·0). It occurs in the faeces, saliva and milk. In

¹ Gould, J. C., & McLeod, J. W. (1960), *J. Path. Bact.*, **79**, 295.

² Rake, G., Jones, H., & McKee, C. M. (1943), *Proc. Soc. exp. Biol. (N.Y.)*, **52**, 136.

morphology it is a relatively large, non-sporing, non-motile, Gram-positive bacillus. The individual organisms vary in length, and may appear even in short coccal forms. Some are about $1\text{ }\mu$ broad, but slender forms may be noted, and there is a tendency to chain formation. The organism thus shows considerable pleomorphism. It may be cultivated under aerobic conditions on whey-agar at 37° C. (p. 235), but when first isolated it tends to be micro-aerophilic and grows best at a reduced oxygen tension. The colonies are small, and vary in appearance as seen under the low power of the microscope; two main types are described: (1) "feathery", in appearance not unlike a *Cl. tetani* colony (*q.v.*), and (2) rounded with projecting outgrowths ("crab-colony"). A convenient method of obtaining cultures from faeces is to inoculate broth, to which is added 0.5 per cent. of glacial acetic acid; after incubation, subcultures can be made on agar plates under aerobic conditions.

This organism produces acid fermentation of glucose and lactose without gas formation. It also ferments maltose, whereas *Lactobacillus bulgaricus*, a related organism originally isolated from Yoghurt (a fermented milk), has usually no action on maltose. The latter organism cannot grow in the intestine of man. It is a thermophile, the optimum temperature being $45^\circ\text{--}62^\circ\text{ C.}$

Closely related organisms are *Bacillus acidophilus odontolyticus* described in association with dental caries, the so-called Boas-Oppler bacillus found in the stomach contents in conditions in which the hydrochloric acid is absent or deficient, and *Döderlein's bacillus*, which is found normally in the vagina.

Lactobacilli Counts.—The detection and estimation of lactobacilli is of increasing importance in dentistry. The salivary flow is activated by the individual chewing a small piece of paraffin wax for a period of three minutes; saliva is collected in a sterile container as it is produced and the volume made up to 10 ml. with saline.

A lactobacillus count is the number of lactobacilli present per millilitre of the standard sample. The sample is serially diluted in broth or peptone water and distributed in 0.1 ml. quantities over a series of tomato-peptone-agar plates (p. 236). The colonies are counted after three or four days' incubation at 37° C. and the count adjusted to represent the number of bacilli in a 10 ml. sample of saliva.

***Lactobacillus bifidus* (*Bacillus bifidus*)**

Derives its name from the apparently bifid by the original observers. This org. | Key: lg gas | urea. |

numbers in the faeces of breast-fed infants. Its average dimensions are $4\ \mu$ by 0.5 - $0.7\ \mu$, but it displays considerable pleomorphism. The ends are often expanded. Three bacilli together may be arranged like a Y. Though usually Gram-positive, there is a certain amount of variation in its reaction to Gram's staining method.

In primary culture it is a strict anaerobe. Cultures have been obtained at $37^\circ\text{ C}.$ in tubes of neutral lactose-broth containing a piece of sterile rabbit kidney (p. 238), with a layer of sterile vaseline superimposed on the medium. After several days' growth, subcultures are made on glucose-agar plates which are incubated anaerobically. Pure cultures on glucose agar can be obtained from single colonies. The organisms may ultimately become microaerophilic. Glucose, sucrose, maltose and various other sugars are fermented with acid production, but no gas.

CHAPTER XXIX

CHOLERA VIBRIO AND ALLIED ORGANISMS

VIBRIO CHOLERAE (V. COMMA)

Vibrio cholerae, or the comma bacillus, is the causative organism of Asiatic Cholera, a disease now more restricted in incidence and distribution than formerly; characterised by an acute gastro-enteritis of sudden onset and often running a rapid and fatal course. The patient becomes acutely dehydrated, cachectic and prostrated within a few hours. In the absence of fluid replacement by intravenous administration, death may occur in spite of antibiotic therapy.

Morphology and Staining.—Curved or “comma-shaped” rod (vibrio) with rounded or slightly pointed ends, about $1\frac{1}{2}$ – 3μ by 0.5μ . It is actively motile, and the movement is of a “darting” or “scintillating” type. A single long terminal flagellum is a characteristic feature of the organism. The vibrios occur singly, in pairs, or in chains end to end with the curves alternating, *i.e.* presenting a somewhat spiral arrangement. “S” forms and spirals representing elongated undivided single cells may be noted. Involution occurs readily, especially in culture, and globular, club-shaped or irregular forms may be observed. No spores are produced. *V. cholerae* is Gram-negative.

When the organism has been growing in artificial culture for a time, the morphology becomes less typical and the curvature of the vibrios is less pronounced.

Cultural Characters.—Aerobe; slight growth also occurs under anaerobic conditions. Temperature range, 16° – 40° C.; optimum, 37° C. Grows on ordinary media. A slight trace of acid is inhibitory, but abundant growth occurs on alkaline media, *e.g.* Dieudonne’s medium (*q.v.*): The optimum reaction is about pH 8.2.

Colonies on agar—white circular disks about the size of the colonies of coliform bacilli, semi-transparent, with well-defined circular margins; older growths develop a brownish-yellow colour.

Other types of colony may be noted as variants from the standard form, *e.g.* an opaque yellowish-white colony, a “ring” colony with an opaque centre and transparent border, and “rugose” colony; in the last mentioned, the corrugated growth

is due to a gelatinous intercellular substance or a definite capsule.

Gelatin stab—at first there is a white line of growth along the track of the inoculating wire; then liquefaction occurs at the top and spreads downwards in funnel-shaped form.

Coagulated serum is liquefied.

Viability.—*V. cholerae* is killed at 56° C. within thirty minutes. It dies within two or three hours when subjected to drying. In stagnant water with an alkaline reaction the organism may survive for a considerable period, e.g. eighteen days or longer. The cholera vibrio is readily distributed by water supplies. It is sensitive to sulphonamides, streptomycin, chloramphenicol and the tetracyclines.

Biochemical Reactions.—The fermentative reactions are as follows:—

Glucose	Lactose	Dulcitol	Sucrose	Mannitol	Maltose	Mannose	Arabinose
—	—	—	—	—	—	—	—
(some strains — after several days' growth)							

(—=acid; no gas)

They can be tested for as in the case of the *Salmonella* organisms.

Cholera-red reaction—this depends on the production of indole and nitrites in peptone water. It can be elicited by adding a few drops of sulphuric acid to a four-days' peptone water culture. A reddish-pink colour develops, due to the formation of nitroso-indole.

Haemolysis—the classical type of *V. cholerae* is *non-haemolytic*, but haemolytic vibrios showing a serological relationship to *V. cholerae* may be met with in cholera-like cases and carriers. This is exemplified by the so-called "El Tor vibrio," which also differs from the classical cholera organism in yielding a positive Voges-Proskauer reaction.

It is advisable to test for haemolysis by adding varying amounts (0·1–1·0 ml.) of a two days' broth culture to 1 ml. of a 5 per cent. saline suspension of sheep's red blood corpuscles in tubes which are incubated for two hours and allowed to stand overnight in the refrigerator (Greig test).

Haemolytic vibrios, such as the El Tor variety, manifest their haemolytic action when growing on blood agar, but certain vibrios, including strains of *V. cholerae* which do not form haemolysin, also produce clearing or apparent laking when growing on this medium, due probably to a chemical alteration of the haemoglobin. Such vibrios clear heated blood media, e.g. "chocolate agar", in the same way as ordinary blood-agar.

Antigenic Characters.—For all practical purposes the cholera

vibrio may be regarded as a homogeneous species, and unknown strains can be identified by testing their agglutination reaction with an antiserum for a known *V. cholerae*.

The organism possesses both H and O antigens. The H antigen may be shared with certain other vibrios, e.g. paracholera vibrios (*vide infra*), though these organisms are distinct as regards their O antigens. This serological difference is best demonstrated with bacterial suspensions made up in plain saline solution (without formalin). Most of the El Tor strains (*vide supra*) possess the same H and O antigens as the classical cholera vibrio.

Within the serological subgroup represented by the classical *V. cholerae* and with a common O antigen, serological types can be recognised, distinguished by agglutinin-absorption tests with O antisera; this difference depends on a subsidiary O antigenic component characteristic of the type. Two such types have been recognised and designated according to the names of standard strains "Inaba" and "Ogawa". It is possible that a third type, "Hikojima", also exists, possessing the characteristic components of both the above-mentioned types.¹

Transformation of a *V. cholerae* strain to the "rough" form is associated with loss of the specific O antigen.

Occurrence.—Typical cholera is an acute disease of sudden onset, characterised by intense diarrhoea and tenesmus, vomiting, "rice-water" stools, muscular cramps and extreme collapse.

The vibrios multiply freely in the lumen of the small intestine and are present in large numbers in the intestinal contents and dejecta. The stools contain many white flakes consisting of mucus and epithelial cells. Numbers of vibrios are demonstrable in these flakes. The organism does not penetrate deeply in the bowel wall and practically never invades the blood stream. The gall-bladder may be infected.

Epidemiology.—Bengal, with the deltas of the Ganges and Brahmaputra rivers, is nowadays the principal area where cholera exists endemically throughout the year. There may also be an endemic focus in Burma (Irrawaddy delta). Pandemic spread occurred four times in the nineteenth century and on each occasion was traced back to Bengal. Besides local spread in India and East Pakistan, recent outbreaks have occurred in Egypt (1947) and in Thailand (1957-58). The maintenance of endemic foci probably depends on case-to-case infection in an unsanitary environment. The infection flares up and becomes epidemic in the early spring, reaching a peak in Bengal in April-May just before the monsoon.¹

¹ Gardner, A. D., & Venkatraman, K. V. (1935), *Lancet*, i, 265.

season. Spread of infection is facilitated by the large water-storage tanks which are the centre of community business and leisure. The alkaline reaction of these tanks favours survival of *V. cholerae* and is probably determined by the activity of algae present in the water.¹ Contamination of water supplies by cases is the greatest single source of epidemic spread, but case-to-case infection by contamination of fomites, food, etc., presumably occurs. Chronic carriers are very rare, if they exist at all; transient carriage during convalescence may continue for one to three weeks.

Laboratory Diagnosis.—The bacteriological diagnosis depends on the isolation and identification of the vibrio.

The organism may be detected microscopically in the intestinal dejecta, but this is not sufficient for accurate laboratory diagnosis, and inexperienced workers may easily be misled by slightly curved bacilli simulating vibrios.

Where there may be delay in the transmission of stools to a laboratory for examination, a preserving fluid has been found valuable in maintaining the viability of the vibrio and preventing overgrowth by other organisms.

Preserving Fluid.—Prepare the stock solution as follows:—Dissolve 12·405 grams boric acid and 14·912 grams potassium chloride in 800 ml. hot distilled water; after cooling make up the volume to a litre with distilled water. To 250 ml. of this stock add 133·5 ml. 0.2 M sodium hydroxide, make up the volume with distilled water to a litre, and add 20 grams dried sea-salt (*vide infra*). Filter the solution through paper, distribute in 10 ml. amounts in screw-capped bottles and autoclave. The final pH should be 9·2. Emulsify 1 to 3 grams of the stool in 10 ml. of the preserving fluid. The following mixture may be substituted for sea-salt: NaCl 27 grams, KCl 1 gram, MgCl₂,6H₂O 3 grams, MgSO₄,7H₂O 1·75 grams.

(a) A plate of Dieudonné's medium (p. 225) is inoculated directly from the stool, and incubated for eighteen to twenty-four hours. This medium is highly selective for vibrios, inhibiting the growth of most other intestinal bacteria. A practically pure culture of an intestinal vibrio can sometimes be obtained directly in this way from a stool containing large numbers of coliform bacilli. Aronson's medium (p. 226) and desoxycholate-citrate agar (p. 218) may also be used as alternatives to Dieudonné's medium and give successful results.

(b) At the same time a tube of peptone water is inoculated with a mucus flake from the stool, or, in the case of a fluid faecal stool, with a large loopful of the specimen. The peptone water used is

¹ Cockburn, T. A. (1960), *U.S. Publ. Hlth Rep.*, 75, 26.

a 1 per cent. peptone with 0·5 per cent. sodium chloride, standardised to pH 8·2. The tube is incubated for six to eight hours. Within this time vibrios, if present, grow freely and at the surface of the medium, and even outgrow other intestinal bacteria.

The peptone water culture is examined after six to eight hours by means of a stained film, made from a drop of the surface layer of the culture: a large loopful is placed on a slide and, without spreading, slowly dried at room temperature; the film is then fixed by heat, and washed in a stream of water to remove the dried peptone particles which stain deeply and obscure the organisms; the preparation is stained with dilute carbol fuchsin for one minute and examined microscopically. At the same time a hanging-drop preparation may be examined; at the edge of the drop, vibrios are easily detected by their characteristic morphology and "scintillating" or darting motility. If vibrios are present, a sub-inoculation is made on a Dieudonné plate. If no vibrios are detected, a sub-inoculation is made into a second peptone water tube; this tube is incubated for six to eight hours, and film from it is then examined as in the case of the primary culture: if vibrios are present, a Dieudonné plate is inoculated from the peptone culture. If no vibrios are detectable in the second peptone water culture the result may be regarded as negative.

Pure cultures on agar slopes are obtained from isolated colonies on the Dieudonné plates; the morphological, cultural and biochemical characters of the strains are then determined; the final identification of the organism depends on its agglutination by a specific anti-cholera serum obtained by immunising an animal with a known *V. cholerae* (p. 325). In view of the fact that the H antigen of *V. cholerae* may be common to other vibrios, the test may be made more specific by using an O-agglutinating serum. If the antiserum available gives both H and O reactions, the bacterial suspension should be heated at 100° C. for twenty minutes to inactivate the H antigen.

A rapid presumptive diagnosis can be made by slide-agglutination of colonies picked directly from the culture medium. At the same time it is essential to obtain cultures from single colonies and confirm the identity of the organism by detailed tests. It must be remembered that the El Tor vibrio is serologically similar to *V. cholerae* and that the Greig haemolytic test (*vide supra*) is required for the differentiation of the two organisms.

Isolation of Vibrio cholerae from Water.—100 ml. of a sterile alkaline (pH 9·0) 10 per cent. solution of peptone containing 5 per cent. sodium chloride are added to 900 ml. of the water specimen, which is then distributed in sterile stoppered flasks or

bottles. These are incubated, and sub-inoculations are made (from the surface growths) on Dieudonné's medium after twenty-four and forty-eight hours, as in the method described above for the isolation of *V. cholerae*. A larger quantity of water may be tested by filtering it through a Seitz disk or membrane filter and by using the disk as the inoculum for a peptone water culture.

Chemotherapy.—The need for fluid replacement and restoration of electrolyte balance is the most urgent therapeutic measure. *In vitro* sensitivity of *V. cholerae* to chloramphenicol and experimental infections in mice suggested that this antibiotic would be efficacious in therapy; in practice, however, patients do not respond more favourably than those treated only by intravenous fluids. Nevertheless, there is rapid clearance of vibrios from the stools, and this is important in reducing the danger to the community. Similar findings have resulted from the use of the tetracyclines.

Prophylaxis.—Cholera vaccine has been used in the prophylaxis of the disease. It is prepared from a twenty-four-hour culture on nutrient agar and the bacterial suspension is killed by heat at 55° C. (one hour) and then standardised, usually to 8000 million organisms/ml. Two doses each of 1·0 ml. are given at an interval of seven days; protection is believed to last for four to six months. The establishment and maintenance of purified water supplies which are free from the risk of contamination by cases or carriers eliminates the greatest single mode of spread of this disease. As a temporary expedient, wells and storage tanks may be treated with hypochlorite or other chlorine derivative. In the absence of a safe supply, all water for personal and culinary use must be boiled. Mineral waters and ice supplies must be carefully supervised. Medical and nursing attendants of cases must be scrupulous in their personal hygiene to avoid acquisition of this highly infectious disease. The rapid elimination of vibrios from the stools of cases subsequent to antibiotic therapy reduces the duration of infectivity and, whilst the therapeutic advantage is minimal (*vide supra*), such treatment may help to diminish community spread.

The Paracholera Vibrios

These are associated with choleraic conditions, usually of lesser severity than true cholera, and occurring as sporadic cases or in limited outbreaks.

Their morphological, cultural and biochemical characters generally correspond to those of *V. cholerae*, but the reactions with mannose and arabinose may differ from those of the cholera vibrio

(*q.v.*). Most of the types described are haemolytic, like the El Tor vibrio (*vide supra*).

These organisms may possess an H antigen in common with *V. cholerae* but their O constituents are distinct. The different serological races have distinctive O antigens.

The bacteriological diagnosis in paracholera is carried out in the same way as in true cholera.

Other Vibrios

Certain species of vibrio have been described in diseases of animals, e.g. *V. fetus*, *V. jejuni*, *V. coli* and *V. metchnikovi*.

V. fetus occurs in abortion of sheep and cattle, and can be isolated from the placenta and also from the foetus. The organism varies in length, the short forms being comma-shaped, the longer individuals exhibiting two to four coils. It is relatively slender and shows a flagellum at one or both ends. It stains Gram-negatively. This organism is micro-aerophilic and has been cultivated on agar slopes in sealed tubes containing in the condensation water a few drops of sterile defibrinated horse blood. Growth is not abundant and is most marked in the condensation water. When first cultivated growth may only develop between the agar and the wall of the tube; after repeated subculturing a surface growth is obtained. Laboratory animals are not usually susceptible to experimental inoculation, although pregnant guinea-pigs can be infected by a variety of routes.

Under natural conditions cattle are infected during insemination; on the other hand, sheep are most probably infected from contaminated food and water supplies. Diagnosis depends on the isolation of the organism from the stomach contents of the aborted foetus or from uterine exudates. Non-pathogenic strains resembling *V. fetus* have been isolated from bovine genitalia, e.g. *Vibrio bubulus*; such strains are not to be confused with *V. fetus*, since unlike the latter they do not form catalase, they produce H₂S and are strict anaerobes.

Chemotherapy.—Streptomycin, chloramphenicol and the tetracyclines are of value and may also be used in the treatment and storage of bull semen.

V. jejuni has been described in an infectious diarrhoea ("winter dysentery") of cows in America. It is a Gram-negative vibrio with a flagellum at one or both poles and, although similar to *V. fetus* in other respects, it is serologically distinct.

V. coli, an organism closely resembling *V. fetus*, is causally related to a form of dysentery in young pigs.

V. metchnikovi was first isolated from a septicaemic disease of fowls. It resembles closely *V. cholerae* in general biological characters, but differs serologically, and in its high virulence for guinea-pigs, pigeons and fowls: a minute amount of culture introduced intramuscularly or into a cutaneous wound produces in these animals a rapidly fatal septicaemia. *V. cholerae* does not show such degree of pathogenicity. Similar organisms have been isolated from choleraic cases and from water.

Various other vibrios and spirilla have also been described. These are mostly water forms. In certain parts of India, vibrios are regularly present in unprotected wells and rivers. These may present some similarity to the cholera vibrio but are serologically distinct and they occur in areas where cholera is not endemic. Many of them correspond to the vibrios described in paracholera though such water vibrios are apparently non-pathogenic. Certain water vibrios exhibit in culture marked phosphorescence, e.g. *V. phosphorescens*. Vibrios have been isolated from a variety of other sources, e.g. from sputum (*V. sputigenus*), from cheese (*V. tyrogenus*), from intestinal contents in "Cholera nostras" (*V. proteus*—Finkler and Prior's spirillum), and from infections in fish.

CHAPTER XXX

PASTEURELLA

THE Pasteurella family constitutes a group of closely related, biochemically inactive, Gram-negative rods showing bipolar staining; the first member to be studied was the bacillus of fowl cholera, which Pasteur used in his early studies on immunity. Besides plague in man, other members of the group are associated with acute and chronic systemic infections in a wide variety of animals and birds.

PASTEURELLA PESTIS

The organism of Oriental Plague, which has been one of the major pestilences of the world, with rats as the main reservoir and the flea as the intermediary between rodent and man. Typically, the severe forms of illness (*Pestis major*) occur either as *bubonic* plague or as *pneumonic* plague. In these cases there is a sudden onset with high fever, great prostration and varying degrees of delirium, with usually an associated septicaemia. There is also an ambulatory form (*Pestis minor*) in which the patient is only mildly pyrexial, with some lymphadenitis and a vesicle or pustule at the site of the flea bite.

Morphology and Staining.—In its most characteristic form this organism is a short, oval bacillus with rounded ends—*i.e.* coccobacillary—about $1\cdot5 \mu$ by $0\cdot7 \mu$, occurring singly and in pairs. In the tissues a typical capsule may be observed; in cultures grown at $37^\circ C$. capsular material can be demonstrated by means of India ink preparations, but is not well-defined.

It is Gram-negative, and when stained with a weak stain (*e.g.* methylene blue) shows characteristic bipolar staining which is an important feature in identification.

In culture the plague bacillus is less typical. Longer forms are frequent, and polar staining is less obvious. Pleomorphism is marked especially in old cultures, and involution or degeneration forms are particularly noticeable. These are markedly enlarged, stain faintly and include globular, pear-shaped, elongated or irregular forms. In fact, the microscopic picture of an old culture often suggests that of a yeast or mould. Involution in culture can be hastened by the presence of 3 per cent. sodium chloride,

and this has sometimes been utilised in identifying the organism.

In fluid culture the bacilli tend to be arranged in chains.

The organism is non-motile and non-sporing.

Cultural Characters.—Grows aerobically and anaerobically on ordinary culture medium. The optimum temperature of the plague bacillus, unlike most other pathogens, is below 37° C., and primary cultures grow best at 27° C. The minimum temperature is about 14° C.

The plague bacillus is somewhat sensitive to free oxygen and growth may not develop under aerobic conditions if the inoculum is small; this inhibition can be avoided by the addition of blood or sodium sulphite to the medium or by the exclusion of air.

Colonies on agar—at first very small, transparent, white, circular disks (1 mm. or less), later becoming larger (3–4 mm.) and opaque; they are not specially characteristic.

In older cultures some of the colonies may have outgrown the others and become more opaque. This appearance is not unlike that of a mixed growth.

Gelatin—no liquefaction occurs.

Broth—growth consists of a granular deposit at the foot and on the side of the tube, not unlike that of a streptococcus. If cultured in a flask of broth with drops of sterile oil on the surface, and provided the flask is not subjected to shaking or movement, a characteristic growth develops, consisting of “stalactites” hanging down into the fluid from the oil drops.

Viability.—The thermal death-point is about 55° C. for fifteen minutes. The organism dies quickly when subjected to drying. Laboratory cultures remain viable for long periods (*e.g.* months) if kept moist and at low temperatures.

Biochemical Reactions.—

Glucose	Lactose	Dulcitol	Sucrose	Mannitol	Milk	Indole
+	—	—	—	+	—	—

(+ = acid; no gas)

Growth occurs on a bile-salt medium—*e.g.* MacConkey's (*cf.* other members of the *Pasteurella* group).

It should be noted that the risk of laboratory infection from handling pathological material and cultures is considerable, and all manipulations should be carried out with the utmost care.

Antigenic Characters.—It has been shown that *P. pestis* contains two types of antigen, one somatic and heat-stable, the other heat-labile at 100° C. and associated with the capsule which is formed in cultures growing at 37° C. The capsular antigen may be of

importance in relation to the immunising properties of *P. pestis* vaccines; thus, a vaccine prepared from cultures grown at 37° C., in which capsular material is well developed, has greater immunising properties in mice than from cultures grown at lower temperatures, e.g. 25°–30° C., as in the preparation of the Haffkine plague vaccine which has been extensively used in India. On the other hand, the somatic or "residue" antigen is important in the protection of guinea-pigs. A non-toxic complex of antigenic fractions can be prepared which immunises both mice and guinea-pigs¹ and may prove useful as a protective vaccine against plague. Strains of *P. pestis* are serologically homogeneous.

Animal Pathogenicity.—The bacillus is pathogenic to monkeys, rats, guinea-pigs and other rodents, and plague is essentially an epizootic disease among wild rats and certain other rodent animals. A guinea-pig or white rat injected subcutaneously with a recently isolated culture dies in a few days, and at autopsy a marked local inflammatory condition is noted, with necrosis and oedema; the related lymph glands are also involved; the spleen is enlarged and congested and often shows small greyish-white areas in its substance; there is also septicaemia. The characteristic bacilli can be seen in large numbers in films from the local lesion, lymph glands, spleen pulp and heart blood. A similar condition is found in rats dying of epizootic plague (*vide infra*).

Rats and guinea-pigs can be successfully inoculated by applying infected material to a shaved area of skin or to a mucous membrane, e.g. of the nose.

Marked local and general toxic effects can be produced in animals by injection of dead cultures, but culture-filtrates are practically non-toxic. The "toxin" can be released by lysis of the bacterial cells and from it a toxoid can be prepared, but its relationship to the pathogenicity of the plague bacillus is doubtful.

Occurrence.—In *bubonic plague* the bacilli are initially present in large numbers in the affected lymph glands. When the bubo undergoes necrosis as the condition advances, they become less numerous, and may even disappear. Septicaemia may result, and then the bacilli can be detected in the blood during life by blood culture. *Post mortem* they are found in the spleen.

In *pneumonic plague* the bacilli are present in large numbers in the sputum and in the broncho-pneumonic areas in the lung.

Epidemiology.—Plague is epizootic in rats and certain other rodents. The infection is spread by rat fleas (e.g. *Xenopsylla cheopis*). The occurrence of bubonic plague in man is due to—

¹ Keppie, J., Cocking, E. C., & Smith, H. (1958), *Lancet*, i, 246.

transmission of the infection from rats by the same agency. The mechanism of transmission is briefly as follows: the flea sucks blood (containing plague bacilli) from an infected animal; the bacilli multiply in the stomach and proventriculus, which may become blocked with bacillary masses; when the insect again bites and sucks blood, regurgitation takes place from the blocked proventriculus into the bite wound, and so inoculation results.

The time during which the bacilli survive in a flea and the insect remains infective depends on temperature and humidity. A temperature of about 50° F. (10° C.) and a high degree of humidity have been found to be the most suitable conditions. A temperature over 80° F. (27° C.) is unfavourable.

Pneumonic plague is communicated from person to person by infected droplets from the respiratory passages. This form of the disease may be initiated from cases of bubonic plague in which the organisms localise in the lung and produce a pneumonic lesion.

Laboratory Diagnosis

Bubonic Plague.—The bubo is punctured with a hypodermic syringe and exudate withdrawn. From this material films are made and stained with methylene blue and by Gram's method. The appearance of the characteristic bacilli showing bipolar staining is highly suggestive.

Cultures are also made on blood-agar, and single colonies are subcultured. The resulting growths are then available for further investigation.

Some of the exudate should also, if possible, be injected subcutaneously into a guinea-pig or white rat. If plague bacilli are present, the inoculated animal will die, showing at autopsy the appearances described above.

The cultures obtained may be tested as regards biochemical reactions, involution on 3 per cent. salt-agar, chain formation in broth, and stalactite growth. The cultures can also be used for further animal inoculation experiments.

Pneumonic Plague.—The bacilli can be detected microscopically in the sputum, and for identification should be isolated in pure culture as in dealing with material from bubonic plague.

In carrying out animal inoculation with sputum, other virulent organisms may be present (*e.g.* pneumococcus); instead of injecting subcutaneously, successful inoculation with the plague bacillus can be effected by applying the material to the nasal mucosa, or to a shaved area of skin.

In septicaemic plague, the bacillus can be demonstrated and isolated by blood culture (p. 260).

Diagnosis of Plague Infection in Wild Rats

At autopsy the following appearances are noted:—enlargement of lymphatic glands, with periglandular inflammation and oedema, most frequently in the cervical glands owing to the fact that the neck is the common harbourage of fleas; serous effusion in the pleural cavity; enlargement of the spleen, which may show small white areas in the pulp; congestion and a mottled appearance of the liver; congestion and haemorrhage under the skin and in the internal organs.

Films are prepared from the heart blood, the glands and spleen, and stained by Gram's method and with methylene blue. Cultures should also be made, and the isolation of the organism attempted by the usual methods. Guinea-pigs should be inoculated subcutaneously with an emulsion of the splenic tissue. In rats found dead of plague it may be difficult to demonstrate the bacilli microscopically or to isolate them in culture. Carcasses in a state of decomposition may be heavily contaminated with other organisms which render the microscopic examination confusing and isolation difficult. Inoculation of a white rat or guinea-pig, by smearing the nasal mucous membrane or a shaved area of skin with material from the lesions, should be carried out.

Chemotherapy.—Although comparative trials have not been undertaken, there is no doubt of the efficacy of antibiotic therapy. Irrespective of the agent employed, therapy must be instituted early in the disease and continued for at least ten days if relapses are to be avoided. Tetracyclines have given spectacular results even in pneumonic plague, which, if untreated, is invariably fatal. Chloramphenicol, streptomycin and sulphonamides have also been found effective.

Prophylaxis.—Plague vaccine has been widely used for prophylactic purposes, particularly the preparation known as Haffkine's vaccine. It is a four weeks' culture of *P. pestis* grown at 27° C. in a goat's-flesh-digest broth. The culture is killed by heat at 55° C. (fifteen minutes) and 0.5 per cent. phenol is added as a preservative. Two doses of 1.0 and 2.0 ml. are injected subcutaneously at an interval of seven to ten days.

Living non-virulent cultures have also been employed as vaccines and seem to give a good degree of protection.

Rodent control on an organised and permanent basis combined with flea destruction will do much to eliminate epizootic conditions.

The need for personal protection of medical and nursing personnel, particularly those caring for cases of pneumonic plague, is vital and entails the wearing of protective clothing, masks, etc. Such persons may be given immediate temporary protection with anti-plague serum.

OTHER ORGANISMS OF PASTEURELLA GROUP

The plague bacillus is only one species in a biological group, *Pasteurella*, which includes three species of veterinary importance, namely *Pasteurella septica*, *Pasteurella haemolytica* and *Pasteurella pseudotuberculosis*. These organisms and *P. pestis* all show a similarity in morphology, staining reactions, cultural and biochemical characters, but differ in certain features and in their parasitism and virulence to different animal species.

Strains of *P. septica* (*P. multocida*) have been generally named according to the animal in which they occur (*P. bovisepctica*, *P. oviseptica*, *P. avicida*, etc.), but they are possibly members of the same species differing in their parasitic adaptations to particular hosts.

P. septica causes haemorrhagic septicaemia, transit fever and mastitis in cattle; pneumonia with septicaemia in pigs; septicaemia and snuffles in rabbits; "fowl cholera" and septicaemia in poultry. Characteristic bipolar staining is exhibited by the organisms which are present in the blood and tissues. They are readily differentiated from other members of the genus (see table, p. 640).

Strains from haemorrhagic septicaemia in cattle are usually highly pathogenic for mice and rabbits but non-pathogenic for ducks and chickens. Fowl cholera strains are equally pathogenic for mice, rabbits, ducks and fowls.

Strains from different countries can be divided into four types by cross-protection tests, e.g. type I strains occur only in cases of haemorrhagic septicaemia in cattle and buffaloes in the tropics, while type II comprises strains from diseases of animals in this country.

P. haemolytica strains have been isolated from pneumonic lungs of cattle and sheep and are responsible for septicaemia in lambs; they are non-pathogenic for rodents (cf. *P. septica*). *P. pseudotuberculosis* causes pseudo-tuberculosis in guinea-pigs, rats and certain other rodents and has been isolated from birds. It might be confused with *P. pestis* if isolated from wild rats, but can be distinguished by its motility when growing at 22° C.

Different serological types of *P. pseudotuberculosis* have been

recognised by agglutination reactions. The organism possesses three antigenic constituents: flagellar, somatic and type-specific, and somatic and common to the different types. This last is the same as the somatic antigen of *P. pestis*.

The following table shows how *P. pestis*, *P. septica*, *P. haemolytica* and *P. pseudotuberculosis* may be differentiated:

	Motility at 18°-22° C.	Maltose	Indole	Growth on Bile-salt Medium
<i>P. pestis</i>	-	+	-	+
<i>P. septica</i>	-	-	+	-
<i>P. haemolytica</i>	-	+	-	-
<i>P. pseudo-</i> <i>tuberculosis</i>	+	+	-	+

+=acid; no gas.

In freshly isolated culture *P. pestis* can be differentiated from *P. pseudotuberculosis* and other *Pasteurella* organisms by adding very small inocula (from dilutions of the culture) to rabbit blood agar and incubating at 37° C.: *P. pseudotuberculosis* grows well in twenty-four hours while *P. pestis* develops slowly at this temperature, small colonies appearing only after forty-eight hours. The optimum temperature for *P. pestis* when freshly isolated is 27° C.

Rare cases have been recorded of human infection by *P. septica* and *P. pseudotuberculosis*; the former in particular, has been found in septic wounds following cat and dog bites.¹

PASTEURELLA TULARENSIS

This organism is classified by some workers with the *Brucella* family because of its growth requirements, its biochemical behaviour and its serological relationship with that group. It is also, like the *Brucella*, highly pathogenic for laboratory workers handling cultures of the organism. However, in its ecology as an animal and human parasite and its possible transmission by insects, it comes closer to the *Pasteurella* group and is included in this family in Bergey's classification.

The organism is a small Gram-negative cocco-bacillus not usually exceeding 0.7 μ in length and 0.2 μ in width, with a

¹ Allott, E. N. et al. (1944), *J. Path. Bact.*, **56**, 411.

tendency to pleomorphism in artificial culture. It stains best with dilute carbol-fuchsin and shows bipolar staining. It is present in large numbers as a capsulated organism in the spleen and liver of infected animals. Its occurrence in large numbers inside cells in these organs has suggested that it may multiply as an intracellular parasite. *P. tularensis* cannot be cultivated on ordinary media. Cultures can be obtained, however, on a medium consisting of pure egg yolk, on blood-agar or serum-agar containing a piece of sterile rabbit spleen, and on horse-serum-agar containing 0·1 per cent. cystine and 1 per cent. glucose.

In the Western States of America it produces a plague-like disease (tularaemia) in wild rodents (*e.g.* rabbits, hares, ground-squirrels, etc.). The lesions are not unlike those found in plague-infected animals, and this infection has to be considered, therefore, in the diagnosis of plague in animals. The disease has also been observed in Japan, Russia, Norway and certain other parts of Europe. Various rodent and other wild animals may be infected.

This infection is also transmissible to man—*e.g.* from handling infected animals (*e.g.* rabbits and hares) and from laboratory cultures. A prolonged febrile illness results, sometimes with glandular lesions and ulcers of the skin. The serum of infected persons agglutinates the organism. It may be noted that the serum of cases with *Brucella* infections may contain agglutinins for *P. tularensis*. For diagnostic purposes guinea-pigs or mice may be inoculated with exudate from the glands or ulcers.

Infection is also spread by ticks and other biting arthropods, and *P. tularensis* has been cultivated from ticks. The disease is sometimes apparently water-borne; thus, water-rats may be infected and contaminate water by their excreta.

The tetracyclines can be used in the treatment of the infection.

CHAPTER XXXI

BRUCELLA: HAEMOPHILUS: BORDETELLA BRUCELLA GROUP

THE generic name *Brucella* is applied to a group of bacteria which include the organism of classical undulant fever ("Malta fever"), *Brucella melitensis*, and that of bovine contagious abortion, *Brucella abortus*. These organisms are generally classified as separate species but are similar in many of their features, and additional *Brucella* types occur which seem to be intermediate biologically between them, e.g. *Brucella suis*, originally described as the *porcine* type of *Br. abortus*.

Certain animals (e.g. goats, sheep, cattle and pigs) are the natural hosts of these organisms, and in cattle particularly, abortion is a common result of infection, though other infected animals, e.g. goats, may often show no recognisable illness. The main *Brucella* types differ in their habituation to certain animal species: *Br. melitensis* occurs usually in goats and sheep, *Br. abortus* in cattle, and *Br. suis* in pigs. Each of these may produce undulant fever in man: *Br. melitensis* is more infective and causes a more severe illness than *Br. abortus*, while *Br. suis* approaches *Br. melitensis* in its virulence to the human subject.

Brucella melitensis

The causative organism of undulant fever of the Mediterranean littoral and islands, France, India, China, South Africa and certain areas of North and South America.

Morphology and Staining.—A Gram-negative cocco-bacillus, usually appearing as round or oval forms about $0.4\ \mu$ in diameter. Definite bacillary forms ($1-2\ \mu$ in length) may be observed. The organism occurs singly, in pairs, or even short chains. It is non-motile and non-sporing.

Cultural Characters.—Aerobic; optimum temperature, 37° C. It can be cultivated on ordinary nutrient media, but a better growth is obtained on liver-infusion agar (pH 6.6-6.8). Colonies on agar in primary growth may not appear for two or three days; they are small transparent discs without special characters, about 1 mm. in diameter but increasing in size to 2-3 mm.

Gelatin stab—a delicate line of growth along the track of the inoculating wire, with little or no surface growth. No liquefaction occurs. Potato medium—after several days a characteristic chocolate-brown growth is produced.

Viability.—The thermal death-point is 60° C. for ten minutes. It may persist in dust or soil for two to three months. It is sensitive to sulphonamides, streptomycin, the tetracyclines and chloramphenicol.

Biochemical Reactions.—*Br. melitensis* exhibits no fermentative properties demonstrable by the ordinary methods.

Antigenic Analysis is considered later, along with other members of the *Brucella* group.

Animal Pathogenicity.—Laboratory animals are relatively resistant to experimental inoculation, but if a large dose of culture is injected intramuscularly in the guinea-pig, infection may be produced; this is not progressive and does not lead to a fatal result as a rule. If the animal is killed after about two months, necrotic areas are found in the liver and spleen, in which the living organisms are present.

Br. abortus: Br. suis

Brucella abortus is the organism of bovine contagious abortion; it has been observed occasionally in other animals, e.g. sheep, horses. *Brucella suis* produces a similar infection in pigs. In morphology and general biological characters these organisms closely resemble *Br. melitensis* and may cause undulant fever in the human subject.

When cultivation is attempted directly from the animal body, *Br. abortus* does not grow under ordinary aerobic conditions and requires an atmosphere containing 5–10 per cent. of carbon dioxide. This can be obtained by placing the inoculated tubes or plates in an air-tight jar containing 5–10 per cent. carbon dioxide (p. 259). After continued cultivation, however, the organism may be grown in the ordinary atmosphere. On the other hand, *Br. suis*, like *Br. melitensis*, can be grown under the usual aerobic conditions. Bang originally cultivated *Br. abortus* by preparing shake cultures in tubes of serum agar, the colonies developing best in a zone just below the surface of the medium. This is due to the fact that in this zone the partial pressure of carbon dioxide is at an optimum for the growth of the organism. A convenient method of producing sufficient carbon dioxide (2–3 per cent.) for the growth of this organism in a tube is to seal the mouth of the tube with paraffin-wax after flaming the cotton-wool stopper.

As with *Br. melitensis* the most suitable medium for these organisms is a liver-infusion agar (p. 231); the addition of gentian-violet in a concentration of 1:250,000 facilitates isolation from material likely to contain other bacteria.

On potato medium *Br. abortus* and *suis* produce a brown staining like that of *Br. melitensis*. They exhibit no obvious fermentative reactions.

Differential Tests for Brucella

Both *Br. abortus* and American strains of *Br. suis* form sulphuretted hydrogen (the latter more markedly): this can be detected by placing a piece of moistened lead acetate paper at the mouth of the culture tube and replacing it, if necessary, daily for four days. Danish strains of *Br. suis* and *Br. melitensis* do not produce H₂S.

Br. melitensis, *abortus* and *suis* have been differentiated by means of media containing 1:25,000 basic fuchsin and 1:30,000 thionin respectively. *Br. melitensis* is not inhibited to any extent by these dyes, *Br. abortus* is typically inhibited by thionin, not by fuchsin, whereas *Br. suis* is inhibited by fuchsin but not by thionin (see Table p. 645). Methyl violet, 1:50,000, and pyronin, 1:100,000, give results similar to those with basic fuchsin.

These dye-sensitivity tests can be carried out in the following way¹:

Sterilised strips of filter paper (6×0·5 cm.) are impregnated with the dye solutions, dried and stored for future use; the following concentrations have been found satisfactory: thionin 1:600, basic fuchsin 1:200. The strips are placed in parallel on the surface of a plate of liver-infusion agar and then covered by pouring the same medium (melted) over them to form an additional layer. Stroke inoculations from cultures of the strains to be tested are made at right angles to the strips. After incubation in 5–10 per cent. carbon dioxide for two to three days the results can be determined as follows: if the organism resists the dye it grows across the strip; if sensitive, growth is inhibited for some distance (up to 10 mm.) from the strip.

Antigenic Characters.—*Br. melitensis*, *abortus* and *suis* show a very close biological relationship. Direct agglutination tests with antisera fail to distinguish between them. Agglutinin-absorption tests, however, elicit a difference between *Br. melitensis* on the one hand and *Br. abortus* and *Br. suis* on the other; but the two latter cannot be distinguished serologically. This difference in

¹ Cruickshank, J. C. (1948), *J. Path. Bact.*, **60**, 328.

antigenic constitution is quantitative rather than qualitative. Thus, the three species possess two similar antigenic constituents though in different proportions, one constituent being dominant in *Br. melitensis*, while the other predominates in *Br. abortus* and *Br. suis*.

	CO ₂ requirement	H ₂ S production	Growth in presence of	
			Basic fuchsin 1:25,000	Thionin 1:30,000
<i>Br. melitensis</i> . .	—	— (or slight)	+	+
<i>Br. abortus</i> . .	+	+	+	—
<i>Br. suis</i> (American strains)	—	++	—	+
Danish strains of <i>Br. suis</i> are similar to the American strains but do not produce H ₂ S.				

For the practical identification of the two serological types of *Brucella*, agglutinating sera absorbed with the heterologous organisms respectively are used, the absorbing dose being adjusted according to the titre of the serum so that the minor agglutinin is removed without substantially altering the major agglutinin. These absorbed sera are monospecific and agglutinate only strains in which the particular antigen is dominant.¹

Animal Pathogenicity.—Experimental inoculation of cultures into guinea-pigs produces a non-lethal infection with tubercle-like lesions (e.g. in lymph glands, spleen, liver, etc.), and as *Br. abortus* may occur in cow's milk, this has to be remembered in relation to the animal inoculation test for the tubercle bacillus (p. 379).

Br. suis is more virulent for guinea-pigs than *Br. abortus*.

Br. abortus is less virulent to monkeys than *Br. melitensis*, which produces, under experimental conditions in these animals, a condition analogous to undulant fever. *Br. suis* resembles *Br. melitensis* in its virulence for monkeys.

¹ See Hamilton, A. V., & Hardy, A. V. (1950), *Amer. J. publ. Hlth*, **40**, 321.

Human Infection with *Brucella*

In the Mediterranean littoral and islands, human infection with *Br. melitensis* results usually from the ingestion of goat's milk and the organisms can be demonstrated in the milk of a considerable proportion of goats in the endemic areas. Sheep may also be infected naturally with *Br. melitensis*. In France, both sheep and goats constitute reservoirs of the infection though cows also may carry the organism. In that country, cases of undulant fever occur mostly in the rural population and among persons who come into contact with infected animals or their carcasses. Thus, the human infection may result either from the ingestion of milk or by contact with animals. *Br. melitensis* infection of cattle has been recently reported in England, but there has been no evidence of any overt cases of the human infection.

It is now well established that *Br. abortus* may, under certain conditions, produce undulant fever (*abortus* fever) in man, infection being derived from unpasteurised cow's milk, or through contact with cattle. The organism dies out quickly in butter and cheese. The disease therefore occurs mostly in farming communities and among veterinarians and butchers. The incidence is highest in adult males; it is rare in children. *Br. abortus* may occur in a considerable percentage of samples of unsterilised market milk, but the incidence of *abortus* fever in man is exceedingly low. Latent infections, however, may occur. It would appear, therefore, that the infectivity of *Br. abortus* must be comparatively low for the human subject. *Br. suis*, with the exception of Danish strains, possesses a higher virulence. Contact with pigs or pig-meat infected by *Br. suis* may lead to human infections by this organism.

Infection among laboratory workers handling cultures of *Br. melitensis* and *Br. abortus* is not uncommon; it may occur by inhalation or via the conjunctiva or abraded skin.

Laboratory Diagnosis.—*Blood culture* should be carried out repeatedly in all cases during the febrile phase and it is essential that at least 10 ml. of blood should be withdrawn for this purpose, as the organisms may be relatively scanty.

In suspected *Br. abortus* infections, the blood-culture must be incubated in an atmosphere of 10 per cent. carbon dioxide for three weeks or longer. Even so, not more than 20–30 per cent. of cultures will be positive. Castaneda's method for blood-culture may be recommended.¹

Castaneda's Method of Blood-culture in Brucella Infection.—3 per cent. melted agar is allowed to set on one of the narrow sides

¹ Proc. Soc. exp. Biol. (1947), 64, 115.

of a 120 ml. flat rectangular bottle with a perforated screw cap (p. 262); 20 ml. broth are then added. 5 ml. of blood are mixed with the broth and the mixture is allowed to flow over the agar. Carbon dioxide is introduced by a needle through the perforation in the cap to yield a 10 per cent. concentration. The bottle is incubated in the upright position, and the agar surface is examined daily for colonies; if no colonies are seen in forty-eight hours the blood-broth is allowed to flow gently over the agar by suitably tilting the bottle, which is again incubated in the upright position. If *Brucella* is present in the blood, colonies can usually be observed within a week.

In *Br. melitensis* infections the organism may be isolated from the urine.

A positive *agglutination reaction* may be elicited after seven to ten days from the onset of the illness. It has to be noted that apparently normal serum may agglutinate *Brucella* suspensions in low dilutions. In cases of undulant fever, however, the serum often agglutinates both *Br. abortus* and *Br. melitensis* in high dilutions, e.g. 1 in 1000. In a suspected case, if the reaction occurs only with low dilutions, e.g. less than 1: 80, the result cannot be regarded as conclusive. When the test is repeated, a rising titre may be observed and a more conclusive result obtained. Very rarely a positive blood-culture has been obtained in clinical cases with negative agglutination reaction.

The sera of some individuals contain a substance which prevents or "blocks" agglutination of *Brucella* organisms by specific agglutinins. False negative reactions may occur when such sera are tested for diagnostic purposes. Zone phenomena in agglutination tests may also be due to this "blocking" substance. The substance is heat-labile and can be completely or partially inactivated by heating the serum at 56° C. for fifteen to thirty minutes. Other methods of obviating this "blocking" effect in diagnostic tests have also been described, e.g. by using an anti-human globulin serum, as in the Coombs test for rhesus antibody.¹

It may be noted that agglutinins for *Brucella* organisms may be present in the serum of cases of tularaemia.

Brucellin Test.—The intradermal injection of a killed suspension of *Brucella* or of a purified extract may elicit a delayed tuberculin-like reaction (minimum diameter 5 mm. induration) which may mean past or present infection. However, non-specific positive reactions may occur so that this test should not *per se* be accepted as diagnostic.

¹ Wilson, M. M., & Merrifield, I. V. O. (1951), *Lancet*, ii, 913.

Chemotherapy.—*Brucella* infections respond best to a combination of streptomycin and tetracycline, continued for a period of two to three weeks. Successful results by combined therapy with streptomycin and sulphonamides have also been claimed.

Diagnosis in Animals.—The agglutination test with the serum of supposedly infected animals and standardised *Brucella* suspensions¹ has been used in diagnosis. Results in which agglutination occurs in dilutions of 1 in 20 or over are generally regarded as positive.

A convenient method of diagnosis of *Br. abortus* infection in cows (applicable also to other *Brucella* infections in other lactating animals) is the *whey-agglutination* test. Milk from each of the four quarters of the udder is mixed and clotted with rennin. The separated whey is then tested for agglutination of *Br. abortus* in the same way as serum. A titre of 1 in 80 or over is usually diagnostic of udder infection.

The *Brucella ring test* is a very sensitive means of detecting agglutinins in milk samples. The technique is as follows:—

(1) Mix the milk thoroughly and pour into a $3 \times \frac{3}{8}$ in. test-tube sufficient to give a column of milk about 1 in. high. (2) Add 1 drop of stained antigen (*vide infra*) and mix thoroughly by shaking. Avoid frothing which interferes with the reading of the test. (3) Incubate in a 37° C. water bath for about forty to fifty minutes, *i.e.* sufficient time for the cream to rise.

The stained antigen² is prepared as follows:—Make a concentrated suspension of *Br. abortus* by washing off mass cultures of a smooth aerobic strain with 0·5 per cent. phenol-saline; heat at 60° C. for thirty minutes in a water-bath; wash the cells and pack by centrifuging; stain with haematoxylin diluted 1 in 5 (Ehrlich's or Delafield's) for five minutes; 10 ml. of packed cells require 1200 ml. of diluted stain; finally suspend the washed stained cells as a 4 per cent. suspension in equal parts of glycerol and phenol-saline.

In milk containing *Brucella* agglutinins the bacteria are agglutinated and rise with the cream, forming a blue cream line leaving the skim-milk white. In samples in which there are no agglutinins there is a white cream line and the rest of the milk remains blue. The test depends on the presence of cream and may not work with fat-deficient milk from individual cows. This difficulty may be overcome by adding negatively reacting cream.

In animals that have aborted, the organism can be demonstrated

¹ Obtainable from the Standards Laboratory, Central Public Health Laboratory, Colindale, London, N.W.9.

² See Hamilton, A. V., & Hardy, A. V. (1950), *Amer. J. publ. Hlth*, **40**, 321.

microscopically in the uterine discharge¹ and also in the stomach contents of the foetus, and can be cultivated by the methods referred to above. Inoculation of a guinea-pig may be resorted to for demonstrating and isolating the organism; the inoculated animal is killed after four weeks, and cultures are made from the spleen. The inoculation test is also utilised for demonstrating the organism in milk.

Immunisation.—Certain avirulent strains of *Br. abortus*, e.g. S19, used in the living state as vaccines have been shown to produce an effective immunity against contagious abortion and are applied practically in controlling the disease in herds.

Other Types of Brucella

A type of *Br. abortus* has been reported in undulant fever in Rhodesia; this organism, however, resembles *Br. melitensis* in its virulence for man, and its ability to grow in the ordinary atmosphere. The infection is derived from cattle.

In the south-east of France, *Brucella* strains have been isolated with the biological characters of *Br. abortus* but the antigenic structure of *Br. melitensis*. In pathogenicity they resemble *Br. abortus*.

Br. ovis, a new species, has recently been reported as being responsible for epididymitis in rams in Australia and New Zealand. The causal organism has also been isolated from the placentae of infected ewes and from tissues of aborted lambs. It needs carbon dioxide for primary isolation; it does not produce hydrogen sulphide and it has a dye sensitivity not unlike that of *Br. suis* but is not inhibited by basic fuchsin in the usual concentrations.

HAEMOPHILUS

Haemophilus influenzae (Pfeiffer's influenza bacillus) was originally described as the causal organism of epidemic influenza and has been designated *haemophilic* in virtue of its inability to grow on culture medium without the addition of whole blood or certain growth-promoting substances present in blood. These growth factors, however, are not restricted to blood, but are present also in certain vegetable tissues. The Koch-Weeks bacillus (of con-

¹ A useful differential stain for the demonstration of *Br. abortus* in infected material is as follows: Dilute carbol-fuchsin (1:10) is allowed to act, without heating, for fifteen minutes. The slide is then decolorised with $\frac{1}{2}$ per cent. acetic acid solution for fifteen seconds, washed thoroughly and counterstained with Löffler's alkaline methylene blue for one minute. This staining method may also be used for demonstrating the elementary bodies of *enzootic abortion* of ewes in smears from the diseased cotyledons and chorion.

junctivitis) shows the same growth requirements as *Haemophilus influenzae*, and may appropriately be grouped with it. The bacillus of whooping-cough has also been placed in the *haemophilic* group, but its growth requirements are different from those of the influenza bacillus and it has now been given the family name of *Bordetella*. Similarly, the bacillus of Morax (of conjunctivitis) has been included by some systematists in this group, but this is not strictly justifiable. If the term *haemophilic* is used in a broad sense to designate organisms which require blood for their growth it would embrace a number of heterogeneous species, and it would be preferable to restrict the generic term *Haemophilus* to those organisms which are dependent on the growth factors required by *Haemophilus influenzae*.

Haemophilus influenzae

This organism is commonly found in the throats of healthy people and is associated with both acute and chronic infections of the respiratory tract. In its *smooth* phase, it may cause pyogenic meningitis and acute laryngeo-tracheitis.

Morphology.—A very small slender bacillus, usually about $1.5\ \mu$ by $0.3\ \mu$, occurring singly or in pairs; non-motile; non-sporing; capsulated types occur. Oval cocco-bacillary forms are noted, and in culture there is marked pleomorphism; some strains, e.g. those present in meningitis, show elongated, curved, thread-like forms.

Staining.—Gram-negative; carbol fuchsin in a 1 in 20 dilution should be used as the counter-stain and applied for five minutes.

Cultural Characters.—Aerobe. Optimum temperature, about 37° C . Does not grow on ordinary media, but can be cultivated in the presence of blood—e.g. on blood-agar or preferably heated-blood-agar.

It has been shown that two growth-promoting constituents present in blood are necessary for the cultivation of *H. influenzae*. One of these, termed the X-factor, is thermostable and resists autoclaving at 120° C . This factor is haematin and is supposed to act in virtue of its being required for the synthesis of catalase, which is necessary for the aerobic growth of the organism. It has been found that the X factor can be dispensed with under anaerobic conditions. The other factor, designated V, is more easily destroyed by heat. It has been identified as coenzyme I or II which is essential as a hydrogen acceptor in the oxidation-reduction processes of the organism.

It is noteworthy that *H. influenzae* grows better in symbiosis

with staphylococci. This is due to the fact that these organisms synthesise the V factor. Thus, in a mixed culture, colonies of the influenza bacillus are bigger in the neighbourhood of staphylococcal colonies: this appearance has been described as *satellitism*.

Blood-agar: very small, transparent, droplet-like colonies which tend to remain discrete. Optimum growth occurs in media in which the contents of the red blood cells are liberated either by heat—Levinthal's medium¹—or by peptic digestion—Fildes' medium (p. 232). *Smooth* strains have rather larger mucoid colonies with a characteristic iridescence which is best seen on translucent media (Levinthal or Fildes).

Glucose and various other carbohydrates are fermented. Indole is produced by some strains.

H. haemolyticus.—Certain strains have been found to differ from the typical form in producing a well-marked zone of haemolysis on blood agar. These may be coarser in microscopic appearance and tend to develop elongated threads. Most of these haemolytic strains require only the V factor for growth.

Some non-haemolytic strains also are independent of the X factor and require only the V substance; all strains which require only this factor have been designated *H. para-influenzae*. Strains of this type have been described in some cases of subacute bacterial endocarditis.

H. canis (H. haemoglobinophilus), an organism originally isolated from a purulent condition of the preputial sac in a dog, resembles *H. influenzae* in general characters, but requires only the X factor for its growth.

H. suis has been found in an influenza-like disease of pigs associated with a filterable virus (p. 798). It differs from *H. influenzae* in the lack of fermentative action.

Antigenic Characters.—Recently isolated *smooth* or capsulated forms of *H. influenzae* have been classified into a number of serological types possessing specific soluble substance (polysugar phosphates) as capsular constituents. In culture the organism readily undergoes transformation to the rough form which lacks such specific substance, and, under these conditions, strains assume considerable serological diversity. Pittman has examined serologically smooth strains of *H. influenzae*, and has subdivided them into six types, namely, *a*, *b*, *c*, *d*, *e* and *f*. Organisms isolated from meningitis or acute laryngo-tracheitis belong predominantly to type *b*. Straker in 60 cases of meningitis found that 58 were

¹ Levinthal, W. (1918), *Z. Hyg. InfektKr.*, 86, 1. (Five per cent. defibrinated rabbit or human blood is added to melted agar and boiled; the deposit is allowed to settle and the clear supernatant is used.)

due to type *b* organisms and the other 2 to types *a* and *f* respectively. Change of type can be effected by the action of DNA of one type on strains of another type; this phenomenon is similar to that first described for pneumococcus types (p. 41).

Animal Pathogenicity.—*H. influenzae* is not naturally pathogenic for laboratory animals. Mice may be infected by intraperitoneal injection of strains suspended in mucin. Capsulated, but not non-capsulated strains, resist the bactericidal action of normal rabbit blood.

Occurrence.—Rough strains of *H. influenzae* are found in a varying proportion (30–80 per cent.) of normal throats. They are opportunists, or potential pathogens, like the pneumococci and staphylococci found in the upper respiratory tract, in that they are associated with infection (sinusitis, bronchitis, bronchopneumonia) when the respiratory tract is less resistant to bacterial invasion, e.g. following a primary virus infection or at the extremes of life. The name "influenza bacillus" was given to this organism when it was found in many of the influenzal pneumonias in the pandemic of 1889–90. It was again associated with severe influenzal infections in the 1918–19 pandemic, but other pathogens (pneumococci, streptococci and staphylococci) were also commonly found; by that time it was becoming accepted that these bacteria were secondary invaders, although it was not until 1933 that the virus aetiology of influenza was established.

Besides its importance as a secondary pathogen in acute respiratory infections, *H. influenzae* is the organism most commonly found in cases of chronic bronchitis and bronchiectasis. Its causal relationship to these chronic infections is not clear, but it may be noted that clinical improvement following long-term chemotherapy in these conditions is usually accompanied by a reduction in the proportions of *H. influenzae* in the sputum. Antibodies to *H. influenzae* are commonly found in the blood of cases of chronic bronchitis.

As already mentioned, the smooth capsulated *H. influenzae*, particularly type *b*, may be the primary pathogen in two clinical syndromes—pyogenic meningitis and acute laryngo-tracheitis. These infections occur most commonly in young children (meningitis, 0–3 years; laryngo-tracheitis, 2–6 years) and are usually accompanied by bloodstream invasion. There is no association with epidemics of influenza.

Laboratory Diagnosis.—Cultivation from sputum is best carried out on heated (chocolate) blood-agar or on Levinthal's or Fildes' medium. Rough strains are resistant to penicillin, which may be incorporated in the medium to inhibit Gram-positive bacteria.

In smears from the deposit of cerebro-spinal fluid from a case of meningitis, small clumps of fine, pleomorphic, Gram-negative bacilli can be seen; blood-culture is usually positive. The iridescent colonies of these capsulated strains are best demonstrated on translucent media. The type may be identified by slide-agglutination with the specific antisera.

Chemotherapy.—Although smooth strains of *H. influenzae* are sensitive to penicillin as well as to streptomycin and the tetracyclines, the drugs of choice for the treatment of *H. influenzae* meningitis are chloramphenicol and a sulphonamide in combination, since both drugs, given orally, diffuse readily into the cerebro-spinal fluid. Good results have been reported from the use of small maintenance doses of the tetracyclines or penicillin V in the control of acute episodes in patients with chronic bronchitis.

Haemophilus aegyptius

(*Koch-Weeks Bacillus*)

Associated with an acute and often infectious form of conjunctivitis.

Morphology and Staining.—Short, slender, Gram-negative rods about 1–1.5 μ in length and similar to *H. influenzae*; intracellular position in polymorph leucocytes of the inflammatory exudate is characteristic.

Cultural Characters.—Does not grow on ordinary media but like *H. influenzae* (q.v.) can be cultivated on media containing blood; in its growth requirements and general cultural characters it is very similar to the influenza bacillus.

Moraxella lacunata

(*Diplobacillus of Morax*)

Associated with sub-acute or chronic conjunctivitis.

Morphology and Staining.—Gram-negative, rod-shaped organism measuring about 2 μ by 1 μ , in pairs end to end; non-motile.

Cultural Characters.—Aerobe; requires blood or serum for growth; optimum temperature is about 37° C., and no growth occurs at room temperature; on coagulated serum, growth produces liquefaction, and colonies develop "pits" or "lacunae" on the surface of the medium.

Moraxella liquefaciens is morphologically similar to the bacillus of Morax, but grows well on ordinary nutrient media at 20°–30° C. It liquefies coagulated serum, but, unlike the other, grows in

gelatin at 22° C. and liquefies it. It is associated with conjunctivitis in which there may be primary involvement of the cornea.

Other organisms found in conjunctivitis are: the gonococcus, pneumococcus, meningococcus, staphylococci, streptococci, coliform bacilli and pneumobacillus. *Staphylococcus albus* and diphtheroid bacilli, e.g. *C. xerosis* (*q.v.*), are frequent normal inhabitants of the conjunctival sac.

Haemophilus ducreyi

(*Bacillus of Ducrey*)

Associated with Chancroid or Soft Sore.

Morphology and Staining.—A Gram-negative, rod-shaped organism 1.5 μ by 0.4 μ; occurs in pairs and chains; present in the exudate from the sore, in the tissue lesion and in the secondary buboes; it is non-motile and non-sporing.

Cultural Characters.—This organism has proved difficult to cultivate artificially, and appears to be a strict parasite. It requires the X growth factor of blood, but not the V factor. It has been found that primary cultures can readily be obtained from the sore by inoculating directly tubes containing coagulated rabbit blood. These are prepared by distributing fresh rabbit blood, withdrawn from an ear vein or by cardiac puncture, in amounts of 1–2 ml. in small test-tubes. The tubes are sloped and, when the blood has clotted, are heated at 55° C. for five minutes. The inoculum is introduced into the serum which has separated from the clot. After growth in these, the organism can be isolated on blood agar plates. This method has been used for diagnostic purposes.

Ducrey's bacillus has also been cultivated directly by inoculating the surface of agar containing 20 to 30 per cent. of defibrinated rabbit blood sloped in wide tubes with a large surface exposed to air, the cultures being incubated at 35° C.

Pure cultures can be obtained by puncturing a bubo with a syringe, drawing up some of the pus, and with this, inoculating tubes of coagulated blood or blood agar.

Strains are all agglutinated by a specific antiserum.

An allergic skin reaction, produced by the intracutaneous injection of killed culture, has been utilised for diagnosis and has been regarded as specific.

BORDETELLA

The principal pathogen in this genus, the whooping-cough bacillus, has until recently been included in the genus *Haemophilus*,

because it required blood for its primary isolation. However, this organism is closely related antigenically to two other bacterial species, *parapertussis* and *bronchiseptica*, which are less fastidious in their growth requirements and should not be called *haemophilic*. A separate name for this group seemed to be justified and *Bordetella* has been adopted, in recognition of Bordet, who first described the whooping-cough bacillus.

Bordetella pertussis

This is the causative organism of whooping-cough, one of the commonest childhood specific fevers, affecting the respiratory tract and characterised by spasmodic coughing with a forced inspiration, or whoop, at the end of the paroxysm.

Morphology and Staining.—A small, oval, Gram-negative cocco-bacillus, slightly larger than *H. influenzae* and more uniform in size and shape; definite bacillary forms occur and become more numerous in *rough* cultures; non-motile; non-sporing; capsules are demonstrable in young cultures. In culture films, the organisms tend to form loose clumps with clearer spaces between, giving a "thumb-print" distribution.

Cultural Characters: Aerobe. It is usually first cultivated on media containing a large proportion of fresh blood; in subculture, however, growth may be obtained on media containing serum but without blood corpuscles or haemoglobin, and it is independent of the X and V factors required by the influenza bacillus. Catalase and a substance, e.g. albumin or charcoal, which will absorb toxic products (possibly unsaturated fatty acids) are essential for growth. For primary culture the special medium of Bordet and Gengou or a modification of this (p. 231) should be employed. On this medium, growth occurs slowly (two to three days) as small raised discrete colonies which are highly refractile to light, resembling a bisected pearl or a mercury drop. The colonies are cohesive and may be picked off entire. Stroke subcultures have been likened to "streaks of aluminium paint".

Semi-synthetic liquid media for large-scale growth for vaccines have been described.¹

Viability.—*Bord. pertussis* differs from the influenza bacillus in its continued viability at low temperatures (0°–10° C.). It is killed by heat at 55° C. for half an hour. It is sensitive to the tetracyclines and chloramphenicol and relatively resistant to penicillin.

¹ Cohen, S. M., & Wheeler, M. W. (1946), *Amer. J. publ. Hlth*, **36**, 371; Verwey, W. F., et al. (1949), *J. Bact.*, **58**, 127.

The organism has no fermentative properties.

Antigenic Characters.—Recently isolated strains appear to be identical in antigenic characters and react similarly with agglutinating and complement-fixing antisera. After artificial cultivation, however, antigenic variation occurs, associated with changes in colony characters analogous to the S→R transformation of other bacterial species.

A number of distinguishable antigenic fractions have been isolated from *Bord. pertussis*, e.g. agglutinogen, haemagglutinin, protective antigen and toxin. This last component is only liberated after bacterial disruption, e.g. by freezing and thawing or ultrasonic disintegration.

Animal Pathogenicity.—Intranasal inoculation of *Bord. pertussis* cultures in mice anaesthetised with ether produces an interstitial pneumonia which may be fatal. Mice are also highly susceptible to intracerebral injection of virulent strains. A condition similar to clinical whooping-cough has been produced in monkeys by introduction of cultures into the respiratory tract.

Occurrence.—Whooping-cough is predominantly an infection of the respiratory mucosa, running a protracted course of four to eight weeks, with convulsions, bronchopneumonia, lung-collapse and debility as possible complications. The organisms can be demonstrated on the epithelial surfaces in the trachea, with ulcers and presumably this surface infection is responsible for fits of paroxysmal coughing and the bronchial spasm that are outstanding features of the disease. The organisms are expelled in droplets during coughing or in scanty viscid sputum, and are easily demonstrable in the first two weeks of infection. Leucocytosis (total white blood cells: 15,000–30,000 and a relative leucocytosis of 70–80 per cent.) is a characteristic feature of the disease.

Epidemiology.—Whooping-cough affects some 70 per cent of children and is more common and more severe in females than males. It is an infection of early childhood and up to 10 per cent of children in urban areas may acquire the disease before their first birthday. There seems to be little or no passive protection by antibodies from the mother. The early infections are the most severe, so that about three-quarters of the deaths from whooping-cough occur in the first year of life. Infection is most commonly spread by direct droplet spray and requires close contact; the attack rate among susceptible children exposed at home is 80–90 per cent. Regularly recurring cycles of epidemic prevalence occur at two-yearly or longer intervals.

Laboratory Diagnosis.—For detecting and isolating the organism the "cough-plate" method may be used: a plate of the Bordet-

Gengou medium is held about 4 in. in front of the mouth of the patient while coughing; the plate is thus inoculated directly with the expelled droplets. After three days' incubation the "pearly" colonies of *Bord. pertussis* can frequently be recognised without difficulty, and identified by microscopic, cultural and serological methods.

While the cough-plate method has proved of practical value in the recognition of early cases of pertussis, it is more applicable in hospital cases than in general medical practice. A specimen for bacteriological diagnosis can be obtained more conveniently by using a pernasal swab. For this purpose, a fine pledget of cotton wool is mounted on a long flexible wire and the swab is passed into the post-nasal space from the anterior nares. Alternatively, an ordinary throat swab bent at the end and introduced behind the soft palate so as to collect secretion from the nasopharynx may be used. A plate of the Bordet-Gengou medium is inoculated by smearing a section of the plate directly with the swab and then distributing the inoculum from this area on the remainder of the surface with an inoculating wire. To prevent overgrowth of the plate by other organisms and to render colonies of *Bord. pertussis* more easily detectable, penicillin solution is added to the plate and spread evenly over the surface (6 units per 12 ml. of medium in a 4-in. plate). Pernasal swabs give rather better results than swabs passed through the mouth.

A total and differential white cell count can give useful confirmatory evidence of infection.

Both complement-fixation and agglutination tests have been applied. Such reactions are more likely to be positive at a late stage of the disease when the organisms are less easily isolated, and may be useful in confirming the diagnosis in atypical cases.

Chemotherapy.—The tetracyclines and chloramphenicol have been used in the treatment of pertussis and, though *in vitro* the specific organism is sensitive to these antibiotics, clinical response (reduction in number and severity of paroxysms) is largely limited to cases treated in the early stages of infection.

Prophylaxis.—Although whooping-cough vaccines have been used for many years, it is only recently that carefully controlled trials¹ have shown that certain pertussis vaccines can give a high degree of protection against whooping-cough. Vaccines, made from strains of *Bord. pertussis* in the smooth phase, grown on defined media and killed by merthiolate or formalin-merthiolate, are given in three doses (each of 20,000 M. organisms) at monthly

¹ *Brit. med. J.* (1959), i, 994.

intervals. Because of the severity of whooping-cough in infancy, it is recommended that vaccination be carried out between two and six months of age. With a good vaccine, the attack rate in home exposures may be less than 10 per cent. compared with 80-90 per cent. in the unvaccinated. Protection may be expected to last for at least two to three years. When whooping-cough occurs in a vaccinated child, the attack is usually milder and of shorter duration than in unprotected children (see pp. 937-939).

Bordetella parapertussis

This organism is related antigenically to *Bord. pertussis* but produces a milder form of whooping-cough which is apparently not common in this country. It differs from *Bord. pertussis* in its more rapid growth so that the "pearly" colonies are well-developed after two days' incubation on Bordet-Gengou medium. The underlying medium becomes greeny-black due to the production of a brown pigment. It actively produces catalase and on subculture grows readily on ordinary culture media. *Bord. parapertussis* can be specifically identified by agglutination with an absorbed antiserum.

Bordetella bronchiseptica

This organism was originally described in canine distemper and has been considered to have relationships to the *Brucella* group. It is, however, related in its antigenic characters and its toxin to *Bord. pertussis*, but differs from this organism in its motility and in possessing peritrichous flagella. It can grow on ordinary media without blood. In canine distemper it represents a secondary infection, but is frequently responsible for a bronchopneumonic condition in rodents; it may be found in snuffles of rabbits.

CHAPTER XXXII

ACTINOMYCETES AND ACTINOMYCOSIS

**NOCARDIA; ACTINOBACILLUS; STREPTOBACILLUS
MONILIFORMIS; PLEURO-PNEUMONIA ORGANISMS;
FUSOBACTERIUM; FUSIFORMIS NECROPHORUS;
BACTEROIDES.**

ACTINOMYCETES stand midway between true bacteria and the more complex fungi. They are characterised by a delicate branching mycelium which may fragment into bacillary and coccoid forms and are non-spore forming. Those varieties which are anaerobic or micro-aerophilic, non-acid-fast and obligate parasites are placed in the genus *Actinomyces*. Other varieties which are aerobic, partially or non-acid-fast, and saprophytic but facultative parasites are placed in the genus *Nocardia*. The differentiation of species is not yet on a very satisfactory basis and there are many problems in the taxonomy of these organisms.

The genus *Streptomyces* comprises over one hundred named species of aerobic organisms with branching mycelium. Fragmentation of the mycelium into bacillary or coccoid elements does not occur. Reproduction is by conidia in chains from branching aerial hyphae. The organisms are saprophytes and are usually found in soil; they have little or no pathogenicity for man or laboratory animals. The main importance of *Streptomyces* lies in the fact that some species produce antibiotics of great therapeutic value (e.g. *Streptomyces griseus* produces streptomycin).

ACTINOMYCES

The causative organism of actinomycosis in animals and man.

Morphology.—It tends to grow in the tissues in colonial form as a mycelium or felted mass of branching filaments which are comparatively slender ($0.8\text{ }\mu$ - $1\text{ }\mu$ thick). In the centre of this mycelial colony the filaments interlace irregularly, but at the periphery there is a tendency to radial arrangement. The organism shows true dichotomous branching. The mycelium is embedded in a groundwork or matrix. In old growths the fila-

ments become matted together into a structureless mass. They also show fragmentation into bacillary and coccoid forms. The formation of conidia has not been demonstrated.

In culture the typical mycelium may not be so obvious as in the tissues, and the growth may be composed largely of shorter bacillary forms resembling a diphtheroid bacillus; among these, however, are seen some longer branching filaments which reveal the true character of the organism.

Ørskov's method is a very suitable technique for studying the morphology of the organism in culture.

Growing in the tissues (especially in animals), the actinomycetes colony develops pyriform or club-shaped structures at the periphery, originally supposed to result from the swelling of the sheath at the extremities of peripheral radial filaments. These "clubs" lie radially with their wide end outwards, and as seen in tissue sections form a complete ring round the colony. In animal lesions the clubs may constitute the main morphological feature of the older colonies, owing to the degeneration of the filaments which become fused into structureless material in the centre of the colony. In human lesions club formation is much less frequent than in animals. It is probable that the clubs are the result of deposition of lipoid material from the tissues round the extremities of projecting filaments, rather than intrinsic structures developed from the organism itself.

Staining.—The filaments are Gram-positive. The clubs usually stain Gram-negatively, but are acid-fast and can be stained differentially by the Ziehl-Neelsen method, using 1 per cent. instead of 20 per cent. sulphuric acid for decolorisation.

Cultural Characters.—Two main cultural types have been recognised:—

(1) *Actinomyces israelii* is derived from human sources such as the mouth, tonsillar crypts and carious teeth. It is micro-aerophilic or anaerobic and the optimum temperature for growth is 37° C.; growth does not occur at temperatures much below the optimum. Increased carbon dioxide concentration favours growth. Blood agar, glucose agar, or 5–10 per cent. serum agar is a suitable medium for routine cultivation; and growth can be obtained conveniently by the ordinary anaerobic methods.

Colonies on agar are raised, nodular, cream-coloured and opaque; they show a rosette form or an irregular outline and are firmly adherent to the medium.

(2) *Actinomyces bovis* is found in the mouths of cattle and in lesions in the bones and internal organs of pigs as well as bovines. This species is more tolerant of oxygen than *Actinomyces israelii*.

and its colonies are softer, smoother in outline, and do not adhere to the medium.

A shake culture of either type in a tube of nutrient agar presents a characteristic distribution of the colonies, which are most numerous in a zone about 10–20 mm. below the surface, *i.e.* where there is only a trace of free oxygen present and an optimal concentration of carbon dioxide.

It should be noted that the cultural appearances of these organisms are subject to variation, and varying conditions of cultivation may be responsible for variability in cultural characters. Dissociation may also occur in culture and give rise to variants which differ from the parent strain.

Viability.—*Actinomyces bovis* resists drying at room temperature for eighteen to twenty-two days; Cultures at 37° C. live for one to four days. It is killed by moist heat at 60° C. in fifteen minutes. The organism is susceptible *in vitro* to sulphonamides, streptomycin, penicillin and tetracyclines; the last two are the most active. Strains vary in their sensitivity and prolonged treatment with one or more of these drugs may be needed to eradicate the infection.

As compared with the aerobic actinomycetes, these types are less active chemically: they are non-proteolytic, non-haemolytic and do not produce pigment. Saccharolytic action, however, can be demonstrated, various sugars being fermented (without gas).

Serological grouping.—Strains of bovine origin (*Actino. bovis*) form apparently a group separate from the human strains.

For further information regarding these organisms reference should be made to Erikson.¹

Animal pathogenicity.—By experimental inoculation of cattle and laboratory animals, *e.g.* rabbits and guinea-pigs, circumscribed nodular or tumour-like granulomatous lesions have been produced in which colonies of the organism are demonstrable. Laboratory animals, however, are not readily infected with this organism by experimental inoculation. Successful results are most likely to be obtained by intraperitoneal injection of rabbits.

Aerobic types.—From time to time aerobic strains of mycelial organisms may be recovered from typical cases of actinomycosis. The classical strain *Actinomyces graminis* of Bostroem is a typical example, but it has been suggested that its occurrence in lesions represents a secondary contamination; similar species, belonging to the genus *Nocardia*, are common saprophytes in soil, grain and grasses, and may be found in the mouth and alimentary tract of animals and also man.

Aerobic organisms described in typical cases of actinomycosis have

¹ Erikson, D. (1935), *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 203; (1940) *ibid.* No. 204.

been somewhat heterogeneous in biological characters and probably represent different species.¹ Only one of these will be described here, viz. *Actinomyces graminis*.

Temperature range, 20°–40° C.; optimum, 37° C. Grows on ordinary media, but the presence of serum or glycerol encourages growth.

Agar—grows slowly; colonies begin to appear after four or five days; when well developed, they stand out on the surface of the medium as discrete, rounded, yellow, transparent knobs, often likened to "amber drops"; they are firmly adherent to the medium; older colonies become umbilicated and assume a dry "powdered" appearance due to the development of aerial mycelium.

Gelatin—slow liquefaction occurs.

No pathogenic effects have been demonstrated by experimental inoculation.

Occurrence.—Actinomycosis is an infective granuloma, occurring mainly in cattle, sheep and pigs, and occasionally in man. In human cases the lesions usually show a suppurative tendency, and the pus contains colonies of the organism in the form of small round granules about the size of a pin-head, which are occasionally of a bright yellow colour (like grains of sulphur). These granules can be recognised by the naked eye if the pus is examined in the form of a thin layer on a slide. The commonest avenue of infection in man is through the mucosa of the mouth or throat. Not infrequently the infection starts in connection with a carious tooth or in the tonsil. The initial infectivity of the organism is probably weak, and invasion of the tissues may occur only in the presence of some additional factor. Primary foci have been noted in animals, and occasionally in man, around fragments of grain embedded in the mucous membrane of the mouth. It was at one time thought that grain was a primary source of the infection, but it is likely in these cases that the grain fragment merely facilitates the establishment of infection in the tissue. The prevalent type of *Actinomyces* (*vide supra*) is, in fact, a strict parasite and incapable of a saprophytic existence on grain. Where the avenue of infection is by the mouth or throat, the primary lesions involve the soft tissues of the mouth and neck, the periosteum of the jaw, and even the vertebrae. In some cases the avenue of infection may be through the mucosa of the bowel, e.g. caecum, or the primary lesions may be in the lung. There is a considerable amount of evidence that the actinomycetes sometimes occurs as a commensal in the mouth, throat, e.g. crypts of tonsils, and the alimentary tract, and that actinomycotic infection is endogenous.

¹ Erikson, D. (1935), *Spec. Rep. Ser. med. Res. Coun. (Lond.)*, No. 203; (1940) *ibid.*, No. 240.

Metastatic lesions are also liable to occur, e.g. in the liver, brain, kidney or lung, by blood-stream spread.

The organism is found in tissue lesions, as in pus, in the form of compact colonies or granules which are visible even to the naked eye, and these present the microscopic appearances described above, varying according to the age of the individual colonies.

Actinobacillus actinomycetemcomitans.—Besides the mycelial organism, a small Gram-negative cocco-bacillus (morphologically resembling the *Brucella* group) is sometimes present in large numbers in the *Actinomyces* colony. This organism can be cultivated independently. Growth occurs under aerobic conditions, and consists of small colonies somewhat like those of a streptococcus and entirely different from *Actinomyces*. Various carbohydrates are fermented with slow acid production. This organism has been regarded as a distinct species; it may, however, be identical with *Actinobacillus lignieresii* (*vide infra*). Experimental inoculation does not produce any specific lesions.

Laboratory Diagnosis.—If the pus from an actinomycotic lesion is spread out in a thin layer in a Petri dish or on a microscope slide, the characteristic colonies or granules can be recognised with the naked eye. For microscopic examination the granules in a drop of pus are crushed between two slides. In this way films can be prepared and then stained by Gram's method.

The granules can easily be separated by shaking up the pus with water in a test-tube, allowing them to sediment and collecting them in a capillary pipette. They are then deposited on a slide and films made by crushing. Preparations obtained in this way are more satisfactory than those made directly from pus in which the granules may be relatively scanty.

Microscopic demonstration of Gram-positive branching filaments arranged in the form of mycelium is generally sufficient for clinical diagnosis.

In tissue lesions the colonies can be recognised by preparing histological sections and staining by Gram's method, and, in the case of animal lesions, by the modified Ziehl-Neelsen method described above.

To cultivate the organism it is essential that actual granules should be used for inoculating the medium. For this purpose the pus is mixed with sterile water, the ~~grains~~ are allowed to sediment or deposited by centrifuging and ~~then~~ ^{that the granules} are removed with a pipette; this is repeated two or three times ~~particularly necessary~~ when there is mixed infection. Two blood-^{or plates} ~~cultured~~ are ^{are} inoculated with the separated granules. One is ^{anaerobically}, the other ^{aerobically}.

OTHER PATHOGENIC ACTINOMYCETES

Apart from the typical actinomycosis, granulomatous and suppurative conditions occur in animals and man, due to infection by mycelial organisms which differ biologically from the actinomycetes described above. The following organisms may be taken as examples. These are assigned to the genus *Nocardia* of the *Actinomycetaceae*. This genus has close similarities to *Actinomyces*, but the filaments readily divide into bacillary and coccoid form and the morphology of the organism often appears to be that of an ordinary bacterium; conidia are not formed. The filaments may show acid-fastness and growths tend to resemble those of the Mycobacteria; many species are chromogenic.

NOCARDIA ASTEROIDES

(*Actinomyces asteroides*; *Eppinger's Streptothrix*)

Originally isolated from a brain abscess. The filaments are relatively broad (1μ in diameter) and very readily break up in culture into bacillary forms. They stain Gram-positively and are slightly acid-fast. This organism can be cultivated aerobically on ordinary medium as a friable, white, dry, wrinkled or nodular growth, which later becomes pigmented (yellow or pink).

NOCARDIA FARCTINICA

(*Actinomyces farcinicus* or *nocardii*)

The organism of bovine "farcy", in which superficial lymph glands become swollen and ulcerate through the skin.

The organism shows mycelium formation, but in culture readily fragments into shorter bacillary and oval forms. A feature of the organism is the beaded, irregular appearance of the filaments when stained by Gram's stain. It is Gram-positive; some strains are strongly acid- and alcohol-fast whilst others are completely decolorised. It grows aerobically at 37° C . on ordinary media, producing raised irregular greyish-white colonies after two to three weeks. On Löwenst^{er}z^e medium, however, small yellow colonies appear after 10 days' incubation.

Guinea-pigs are inoculated in the mouth, throat and skin. In cattle, subcutaneous injection leads to a local lesion which breaks through the skin and produces a chronic *Spec. Rep. S.* lesion. Rabbits are not susceptible to inoculation.

THE ORGANISMS OF MYCETOMA OR MADURA FOOT

Mycetoma is an infective granuloma localised usually to the tissues of the foot and exhibiting no metastases. The condition occurs only in certain tropical and subtropical countries, e.g. India, some parts of Africa, etc.

In the tissue lesion and pus, granules or colonies are noted as in actinomycosis. These granules vary in colour; in some cases they are white or yellow ("pale variety"), in others black ("melanoid variety").

The pale granules usually represent colonies of an actinomycete, *Nocardia madurae*.

Morphologically this organism resembles the classical actinomycetes, but clubs are less frequently noted.

It is a strict aerobe. The optimum temperature is about 37° C. On nutrient agar the growth consists of circular raised colonies like those of other actinomycetes, at first yellowish, later pinkish.

Other species of *Actinomyces* have also been reported in cases of mycetoma.

Certain cases of mycetoma, particularly those with black granules, are caused by true fungi such as *Madurella* (see p. 895).

LEPTOTHRIX

(*Leptotrichia*)

This term has been used for organisms resembling the Actinomycetes but lacking branching of the filaments.

An organism of this type is a common inhabitant of the mouth cavity, and may be detected in films made from the secretion between the teeth or deposits of tartar. It has been designated *Leptotrichia*. Pathogenic properties have been claimed for it, but its invasive power is probably slight.

Lept. buccalis appears to be an established species with many of the characters of lactobacilli, with which organisms it is now classified.¹

ACTINOBACILLUS LIGNIERESI

This organism is the cause of slowly developing granulomata especially in the soft tissues of the lower jaw and neck in cattle. One characteristic form of the disease is the so-called "woody tongue". To such cases the term "actinobacillosis" has been

¹ Hamilton, R. D., & Zahler, S. A. (1957), *J. Bact.*, **73**, 386.

frequently applied. In contrast to *Actinomyces* this organism does not usually invade bones and shows a marked tendency to spread by lymphatics. In such cases typical mycelial organisms cannot be detected in the tissues and the granules may be composed almost entirely of club formations. No mycelial organism can be cultivated, but on ordinary media under aerobic conditions a small Gram-negative bacillus develops. This organism is about $1\cdot5\ \mu$ in length and is non-motile. The designation *Actinobacillus* has therefore been applied to it. In shake cultures in glucose agar, elongated filamentous but unbranched forms are noted. The colonies are small, circular and translucent. Glucose, maltose, sucrose and certain other sugars are fermented. Inoculation of cultures into cattle and guinea-pigs reproduces lesions characteristic of actinomycosis with typical colonies or granules in the tissue.

Actinobacillosis has also been described in sheep, and strains of *Actinobacillus* isolated from these animals appear to be similar to those of bovine origin.

A. lignieresi is sensitive to treatment with iodine, sulphonamides and penicillin.

STREPTOBACILLUS MONILIFORMIS

This organism occurs as a normal inhabitant in the nasopharynx of wild and laboratory rats and is the cause of a spontaneous disease of mice characterised by multiple arthritis often involving the joints of the feet and leading to swellings of the feet and legs. It is also the cause of a proportion of cases of "rat-bite fever" in man. Although the organism is usually introduced through a bite, this history cannot always be obtained; in some cases the infection seems to be acquired by the ingestion of contaminated food. A group of cases in America, characterised by fever, multiple arthritis and an erythematous eruption (Haverhill Fever), was shown to be associated with the organism which was swallowed in contaminated milk. Certain writers have named it *Actinomyces muris*, though its relationship to the Actinomycetes is doubtful.

Morphology and Staining.—The organism is a Gram-negative, pleomorphic bacterium, occurring as short rod-shaped forms ($1\cdot3\ \mu$ by $0\cdot3\text{--}0\cdot4\ \mu$) or as elongated filaments which are either undivided or consist of chained bacilli. They may show characteristic fusiform, oval or spherical enlargements sometimes projecting laterally from the filaments. Branching of filaments has been described by some writers, but it is doubtful if true branching occurs.

Cultural Characteristics.—Growth can be obtained in the

presence of blood, serum or ascitic fluid, and a high proportion of blood or serum is required in the medium. Löffler's serum medium serves well for cultivation. The colonies are small (1 mm.). Viability in culture is feeble and cultures die in two to four days.

On solid medium after two to three days' incubation raised granular colonies 1–5 mm. in diameter develop. Adjacent to these, and best seen with the plate microscope a variable number of minute colonies 0·1–0·2 mm. in diameter may be seen; they grow into the depths of the medium and can only be transferred by excising a small portion of the agar. These small colonies breed true on subculture and constitute the "L forms" or "L phase" of the organism's growth; they consist mainly of very small coccoid or coccobacillary elements but larger and bizarre forms may be present.

"L" organisms are extremely resistant to penicillin, while the streptobacilli are very sensitive to this antibiotic. However, both forms have identical fermentative properties and one antigen is common to them. L forms lack an antigen present in the streptobacillus and they have little or no virulence for laboratory animals. It is now generally accepted that L forms are variants of *Streptobacillus moniliformis* in which there is a defective mechanism of cell wall formation. It should be noted that L phase variation occurs spontaneously to a greater or lesser extent with all strains of *Streptobacillus moniliformis*. In other bacteria where L phase dissociation is recognised, abnormal cultural conditions are required to induce the production of L-type colonies.

In morphology and mode of reproduction L phase organisms have many similarities to the pleuropneumonia organisms.

Mice are susceptible to experimental inoculation and develop either a rapidly fatal general infection without focal lesions or a more slowly progressive disease with swelling of the feet and multiple inflammatory lesions of joints.

Laboratory Diagnosis.—In the human infection the organism has been isolated by blood culture, and from joint fluid in cases with arthritis. In fluid culture colonies of the organism take the form of "fluff balls" situated on the surface of the sedimented blood cells.

PLEUROPNEUMONIA ORGANISMS

(*Mycoplasma mycoides*)

These organisms are the cause of bovine pleuropneumonia. Originally they were classified with the filterable viruses, in view of their ability to pass the coarser filters; they are, however,

within the range of microscopic visibility and can be cultivated readily on artificial medium.

Morphology, which can be studied in impression preparations from cultures, depends on the stage of growth, and diverse forms have been observed. Successive phases have been described: a granular phase in which the organism appears coccoid or coccobacillary, not exceeding $0.4\text{ }\mu$ in diameter; a filamentous phase produced by the enlargement of the granules, their peripheral budding and the separation of the buds which remain attached to the parent structure by a delicate filament; a mycelial phase developed by the formation of new filaments which produce a branching meshwork; later the filaments seem to divide or their protoplasm becomes condensed into chains of coccus-like forms; finally, the chains disintegrate into granular forms like those of the first phase. Ring forms, vibrionic forms and large oval bodies have also been observed. According to Klieneberger and Smiles¹ the elementary granules enlarge to spheroids which become filamentous, and segment; deeply stained nucleus-like bodies appear in these segments and subdivide, and this is followed by the subdivision of the whole protoplasm into merozoite-like derivatives, which become free bodies from which the cytoplasm is lost and the elementary granule reformed. The filterability of the organism is probably due to the ability of the small granular bodies to pass through a filter.

The organism is best stained by Giemsa's stain, heat being applied. It is Gram-negative.

Morphological forms similar to those of the pleuropneumonia organisms have been found in cultures of *Streptobacillus moniliformis* (see p. 667). Similar forms have also been observed in cultures of other bacteria.

Cultural Characters.—Cultures can readily be obtained aerobically at 37° C. in serum broth or on serum agar (10–20 per cent. of horse or ox serum) at pH 7.6–7.8. Growth is visible in two to five days; in broth as a general cloudiness; on agar as very minute 'droplet-like' colonies which develop a raised centre; these later become larger (1 mm. diameter), white, umbilicate and somewhat tenacious. Cultivation leads to attenuation of virulence. The growth of contaminating Gram-positive and Gram-negative bacteria may be inhibited by the inclusion in the culture medium of thallium acetate at a concentration of 1 in 1000 without appreciable interference with the development of the pleuropneumonia organisms. The organisms grow readily in the developing chick embryo, usually without

¹ *J. Hyg. (Lond.)*, 42, 110.

producing lesions or killing the embryo. They grow luxuriantly in tissue cultures and are an important factor as contaminants in virological and cytological work. The organism is killed within one hour by heating at 58° C. and is usually susceptible to a concentration of neomycin of 50 units per ml.

Animal Pathogenicity.—Subcutaneous inoculation of cattle with exudate from the disease, or with a virulent culture, leads to a local inflammatory lesion; in some cases an inflammatory condition in the interstitial tissue of the lungs and a pleuritic exudate ensues with a fatal result. The disease has also been reproduced by intravenous inoculation. Laboratory animals resist experimental infection.

Cattle can be immunised against the disease by the use of a living vaccine of attenuated organisms cultivated in the chick embryo. The serum of immune animals contains protective antibodies.

An organism similar to that of pleuropneumonia may occur in a pulmonary infection of rats, arthritis in these animals, and in "rolling" disease of mice.

Organisms of this type have also been isolated from the human genital tract of male and female subjects (urethra, vagina, cervix uteri), usually in cases with non-specific inflammatory conditions, e.g. urethritis or *Trichomonas* infection, but occur also in the normal healthy urethra.

Saprophytic filter-passing organisms isolated from sewage are similar to the pleuropneumonia organism but show no antigenic relationship to it.

The Organism of Agalactia.—This infectious disease affects sheep and goats, and is transmitted by contagion. It is characterised by inflammatory lesions of the mammary glands, eyes and joints. The causative organism resembles that of bovine pleuropneumonia and can pass the coarser earthenware filters. It may be cultivated on serum agar. The disease has been produced in goats by experimental inoculation of cultures. Recovery from the infection produces a lasting immunity. For further reading on the pleuropneumonia organisms see a review by Edwards, 1954.¹

FUSOBACTERIUM FUSIFORME

(*Fusobacterium plauti-vincenti*)

This organism is referred to on p. 726 as a concomitant of a spirochaete in Vincent's angina, and is found in various necrotic

¹ Edwards, D. G. ff. (1954), *J. gen. Microbiol.*, **10**, 27.

inflammatory conditions along with this spirochaete, e.g. ulcerative gingivitis and stomatitis, etc., and occasionally in diphtheritic lesions of the throat.

Morphology.—It is a large, non-motile, fusiform bacillus, 5–14 μ by 1 μ .

Staining.—Gram-negative. The centre of the bacillus often stains less deeply than the poles, and a beaded or granular appearance may be noted.

Cultural Characters.—Strict anaerobe. It has been cultivated on a medium containing 1 part of blood to 3 of agar. The colonies are small white disks resembling a growth of streptococci.

Isolation has proved most difficult owing to the admixture with large numbers of other organisms present in inflammatory exudates.

FUSIFORMIS NECROPHORUS

(*Sphaerophorus necrophorus*)

The taxonomy of this organism is still doubtful and is not clearly distinguished from the *Bacteroides*. Some writers have classified it with the organisms described above as *Fusobacterium*, others with *Bacteroides* (*vide infra*).

It is responsible for diphtheritic and necrotic lesions ("necrobacillosis") in various animals, e.g. gangrenous dermatitis of equines, calf diphtheria, foot rot of sheep, necrotic stomatitis of pigs, lung abscesses in various domesticated animals, labial necrosis of rabbits.

The organism may appear in the form of elongated slender filaments varying in length and attaining sometimes to 50 or even 100 μ . Branching has occasionally been described. The filaments are Gram-negative and show a characteristic beaded appearance when stained by the ordinary stains. In addition to the filamentous form, the organism may be seen as small Gram-negative bacilli. Growth is obtained at an optimum temperature of 34°–36° C. on serum agar under strictly anaerobic conditions. The colonies are small, white, opaque disks with projecting wavy filaments. Cultures yield a characteristic "cheese-like" odour, especially in a milk medium. Indole is formed. Gelatin is not liquefied. A thermostable necrotising endotoxin is produced.

Rabbits and mice are highly susceptible to inoculation. Subcutaneous injection in rabbits produces an initial focus of necrosis at the site of inoculation and the animal dies in one to two weeks.

For diagnostic purposes stained films made from the edges of the necrosed tissue are examined,

Direct cultivation is difficult owing to the large numbers of other organisms present in the lesions. Pure cultures can be obtained readily by inoculating rabbits or mice from the necrotic tissue and isolating the organism on serum-agar from the inoculated animal at autopsy.

Human infections¹ with this type of organism are probably commoner than was at one time realised. Localised lesions in the skin and subcutaneous tissues are found particularly in workers who are obliged to handle infected animals; veterinary surgeons, meat inspectors, laboratory technicians and butchers are liable to this infection, especially when there are small abrasions of the skin on their hands. Ulceration of throat, often after tonsillectomy, and purulent gingivitis after dental extractions, are also manifestations of the infection. A larger and more important group of cases occur following abortion and less frequently after normal childbirth; puerperal fever due to suppuration in the genital tract results. After surgical operation on the abdomen these organisms may cause peritonitis and are sometimes associated with sloughing of edges of the incision. Occasionally appendicitis and urinary infection are associated with the organism. Another important group of cases suffer from empyema with or without lung abscess formation. In severe infections with *Fusiformis necrophorus* and also with members of the *Bacteroides* group (*vide infra*) septicaemia or pyaemia may follow a suppurative thrombophlebitis. Bacterial endocarditis has been reported and widespread abscess formation, osteomyelitis, purulent meningitis and suppurative arthritis may occur. The organism is sensitive to penicillin, moderately sensitive to chloramphenicol and relatively resistant to streptomycin.

Fusiformis nodosus.—Foot rot of sheep is a specific disease in which a fusiform (*F. nodosus*) and a spirochaete (*Spirochaeta penortha*) play a causal role. It is a large, Gram-negative rod, both ends of which are enlarged. Films stained with methylene blue show occasional red-staining granules either at the ends or along the rod. An obligate anaerobe, it prefers the addition of horse serum or yeast extract to the medium for surface growth; colonies are semi-opaque, smooth, convex and colourless with an entire edge. Growth in fluid medium is poor; there is no "cheese-like" odour or gas formation.

BACTEROIDES

Organisms of this genus (Gram-negative, non-sporing, motile or non-motile anaerobic bacilli) may occur in the intestine of mammals

¹ Alston, J. M. (1955), *Brit. med. J.*, ii, 1524.

and have sometimes been found associated in the human subject with appendicitis, urinary and puerperal infections, etc. *Bacteroides fragilis* (*Bacillus fragilis*) is a typical species. It is a small Gram-negative, non-motile, rod-shaped organism sometimes showing bipolar staining and is an obligatory anaerobe. Growth can be obtained on ordinary media at 37° C. but is scanty and the colonies are small. Acid (without gas) is produced from glucose, sucrose, maltose and certain other sugars. Gelatin is not liquefied. This species has been found in appendicitis and in suppuration of the urinary system, but its pathogenic rôle has not been clearly determined. When injected subcutaneously in guinea-pigs it may produce an abscess, and in rabbits extensive sloughing. Kangaroos and wallabies are frequently affected with a necrotic condition ("jaw disease"; nocardiosis) in which *F. necrophorus* and a member of the genus *Bacteroides* appear to be important pathogens. The latter organism forms Gram-negative filaments with rounded ends and is not pathogenic to rabbits or mice. Like *Bacteroides fragilis* it is resistant to penicillin but susceptible (*in vitro*) to streptomycin and tetracyclines.

It should be noted that *Bacteroides fragilis* has also been classified by some writers along with the organisms described above as *Fusobacterium*. The taxonomy and relationships of these organisms require further study.

Donovania granulomatis

(*Calymmatobacterium granulomatis*)

This organism, whose biological relationships are still doubtful, is responsible for a chronic granulomatous disease ("granuloma venereum") observed in tropical and subtropical countries. The initial lesion is on the genitalia. In the mononuclear cells of the lesions the organism is seen as a small Gram-negative pleomorphic bacillus (1 to 2 μ in length). It may show polar staining and appears to be capsulate. Extracellular forms are also observed. The organism has proved difficult to cultivate on the usual bacteriological media, but cultures have been readily obtained in the yolk sac of the chick embryo. Recently it has been grown in egg-yolk *in vitro*. Laboratory animals are not susceptible to inoculation, but the disease has been reproduced in man by inoculation with yolk sac cultures. The organism is not filterable. Sterilised cultures yield an allergic skin reaction in infected persons, and also give a complement-fixation reaction with patient's serum. A capsular material has also been found to fix complement with sera from patients with the disease. The organism

has morphological resemblances to *Klebsiella* and cross-reacts serologically with *Klebsiella rhinoscleromatis* (p. 617).

(It should be noted that this infection is quite different from lymphogranuloma inguinale—p. 766—and should not be confused with the latter disease.)

CHAPTER XXXIII

CLOSTRIDIA

THE genus *Clostridium* comprises the Gram-positive spore-bearing anaerobic bacilli. Most species of this genus are saprophytes which normally grow in soil, water and decomposing plant and animal matter, playing an important part in the process of putrefaction. Some are commensal inhabitants of the animal or human intestine, and a few species produce disease. The latter include: *Clostridium tetani*, the cause of tetanus; *Cl. welchii*, *Cl. septicum* and *Cl. oedematiens*, the causes of gas-gangrene and other infections; and *Cl. botulinum*, the cause of botulism. With only a few exceptions, the bacteria producing powerful exotoxins belong to this genus.

The bacilli are typically large, straight or slightly curved rods, 3–8 μ by 0·6–1 μ , with slightly rounded ends. Pleomorphism is common and a pure culture may contain many forms, including filaments, citron, spindle-shaped and club forms. Some members of this group tend to lose their Gram-positive reaction early in culture, especially in broth culture, and may then appear Gram-negative. All produce spores but vary widely in their readiness to do so. Information regarding the shape of the spore and its position in the bacillus is of use in classification. *Cl. welchii* and the type species, *Cl. butyricum* are the only capsulate members.

Almost all members of the genus are motile, but *Cl. welchii* is an important exception. Some motile species do not show active motility under the relatively aerobic conditions of the usual wet-film preparations. These may be examined in tissue fluid preparations following animal inoculation. A semi-solid nutrient agar medium for the demonstration of motility (p. 197) is recommended. Stab cultures in this medium freshly prepared with 1 per cent. glucose added to enhance anaerobiosis should be examined frequently before excessive gas production invalidates the test. It should be noted that a non-motile species (e.g. *Cl. welchii*) may show lateral spikes of growth along "faults" extending from the stab line, but the appearance is not likely to be confused with the diffuse growth of a truly motile species.

The clostridia are biochemically active, frequently possessing both saccharolytic (carbohydrate-decomposing) and proteolytic (protein-decomposing) properties. In general, one or other of

these activities is predominant in any one species, and two groups are thus recognised:

The *Saccharolytic Organisms* are characterised by their rapid and vigorous growth in carbohydrate media with the production of acid and abundant gas. Saccharolytic species ferment glucose and may, in addition, be usefully examined for fermentation of lactose, maltose, sucrose and salicin. The indicator dyes in such tests become reduced and may be irreversibly decolourised so that it is advisable to add a little fresh indicator to the tests when recording the results. When grown in cooked-meat broth, saccharolytic clostridia rapidly produce acid and gas but do not digest the meat; the cultures may have a slightly sour smell and the meat is often reddened. Gas production is not necessarily indicative of sugar fermentation, as proteolysis may be accompanied by evolution of gas bubbles.

The *Proteolytic Group* digest protein and liquefy gelatin and coagulated serum. Cultures in meat medium cause blackening of the meat, decomposing it and reducing it in volume with the formation of foul-smelling products.

Proteolysis is otherwise demonstrated by culture in gelatin or coagulated serum media, but evidence of proteolytic activity may not be forthcoming for several days. Strongly proteolytic clostridia will decompose gelatin, coagulated serum and cooked meat, whereas weakly proteolytic organisms may not attack the meat, and some not the coagulated serum. A glucose-gelatin medium containing 12 per cent. nutrient gelatin with 1 per cent. glucose and phenol red as an indicator has been recommended by Willis and Hobbs,¹ who have also incorporated milk in a complex medium (p. 232) in which proteolytic organisms show early zones of clearing due to the breakdown of the milk protein, as described by Reed and Orr (1941).² Alternatively, the method of Kohn (1953),³ employing formalinised gelatin cubes containing carbon granules, may be used.

Cl. welchii, *Cl. septicum*, *Cl. tertium* and *Cl. fallax* are examples of predominantly saccharolytic clostridia. *Cl. sporogenes*, *Cl. histolyticum* and *Cl. tetani* are proteolytic or predominantly so. There is, however, no hard and fast line of demarcation between the two groups. Thus, *Cl. welchii* has slight proteolytic activity, and *Cl. sporogenes* some saccharolytic properties.

Morphological and biochemical variations within strains of the same species, and indeed within subcultures of the same strain,

¹ Willis, A. T., & Hobbs, G. (1959), *J. Path. Bact.*, **77**, 511

² Reed, G. B., & Orr, J. H. (1941), *War Medicine*, **1**, 493.

³ Kohn, J. (1953), *J. clin. Path.*, **6**, 249.

render identification of the clostridia difficult. Whenever possible, the identity of a toxigenic species should be confirmed by specific toxin neutralisation tests, though it should be borne in mind that non-toxigenic strains of toxin-producing species occur.

Strains may be preserved by freeze-drying or by storage in various media. Cooked-meat broth containing chalk and minced cooked egg-white is a useful preservation medium. Germination of spores is sporadic, especially following heat-resistance tests, and this is considered to be due to fatty acids in the subculture medium. The inhibitory effect may be reduced by incorporating a little soluble starch or serum in the medium when subcultures are made from preservation media or from heat-resistance tests.

CLOSTRIDIUM TETANI

The causative organism of tetanus in man and animals.

Morphology.—A straight, slender, rod-shaped organism, 2–5 μ by 0·4–0·5 μ , with rounded ends; shorter forms and longer filaments are also noted; motile, with numerous long peritrichous flagella. Movement is not markedly active. The spores, early in development, may produce an oval and subterminal enlargement. The fully developed spore is characteristically terminal and spherical, two to four times the diameter of the bacillus, producing the drum-stick appearance which is a striking morphological feature of the organism. Strains vary in their tendency to produce spores. If sporing forms are scanty in normal culture media, the strain may be encouraged to produce spores by stab subculture in a tube of horse-flesh digest agar with 1 per cent. glucose, incubated for two to three days.

Staining.—Gram-positive, but there is considerable variation and Gram-negative forms are frequently encountered, especially in broth cultures. Only the periphery of the spore is stained by the Gram counterstain.

Cultural Characters.—An obligatory anaerobe; temperature range 14°–43° C.; optimum, 37° C.; grows on ordinary nutrient media but is more readily grown in cooked-meat medium or in Fildes' peptic blood broth (p. 232).

On solid media, surface colonies of the normal motile type of tetanus bacillus are characterised by their long branching projections. After forty-eight to seventy-two hours' incubation the central part of the colony, which rarely grows more than 1 mm. in diameter, becomes slightly raised and has a ground-glass appearance, while the edge shows a delicately filamentous appearance. A fine spreading growth may thus extend over the entire surface

of the medium and the spreading film of growth may not be apparent on cursory examination. On blood agar, haemolysis is evident in the region of initial confluent growth and may develop below individual colonies, but frequently does not appear below the spreading growth in young cultures.

Non-motile variants may produce quite isolated colonies lacking the characteristic feathery processes.

Agar stab culture.—No growth occurs on the surface; a white line of growth appears along the track of the inoculating wire but stops short of the surface, and lateral spikes, which are longest in the deeper part of the tube, develop from the central growth.

Biochemical Reactions.—Nutrient gelatin is slowly liquefied. Coagulated serum is slowly rendered more transparent and softened only. Litmus milk medium may show no coagulation or there may be delayed clotting. Cooked-meat medium shows slight digestion and blackening of the meat. Some gas is evolved due to breakdown of amino acids, but no carbohydrates are fermented by typical strains of *Cl. tetani*. Cultures have an unpleasant slightly pungent odour.

Viability.—The spores may be highly resistant to adverse conditions, but the degree of resistance varies. Many strains are killed by exposure to boiling water for five to fifteen minutes, but rarer, more resistant strains require boiling for up to three hours before being killed. They may resist dry heat at 150° C. for one hour, 5 per cent. phenol or 1 in 1000 perchloride of mercury for up to two weeks or more. Iodine 1 per cent. in watery solution and hydrogen peroxide (10 volumes) are said to kill them within a few hours.

Antigenic Characters.—Ten types are distinguishable by agglutination tests involving flagellar H antigens. Type VI consists of non-flagellate strains. All types produce the same neurotoxin, and toxigenic and non-toxigenic strains may belong to the same type.

Toxin.—The exotoxin, of which the neurotoxic component *tetanospasmin* is the essential pathogenic constituent, develops in broth cultures after five to fourteen days' growth at 35° C., the optimum time varying with the strain. Toxin yields from *Cl. tetani* cultures vary from strain to strain and also depend upon the culture medium used. One of the most satisfactory media is that of Mueller and Miller.¹

Tetanolysin is another constituent and this causes lysis of red blood corpuscles. It is oxygen-labile. *Tetanospasmin* has been

¹ Mueller, J. H., & Miller, P. A. (1954), *J. Bact.*, 67, 271.

separated as a pure crystalline protein with an estimated lethal dose for the mouse of 0.000001 mg. Tetanus toxin is thus an extremely powerful poison, second in potency only to the exotoxin of *Cl. botulinum*.

When tetanus toxin is injected into guinea-pigs or mice, the animals die within a day or two with the typical signs of tetanus. In animals, tetanic spasms may start in the muscles related to the site of injection ("local tetanus"). Experimental findings indicate that the toxin reaches the central nervous system by passing along the motor nerves, being absorbed probably by the motor end-plates and spreading up the spaces between the nerve fibres.¹ It seems to act as an excitant to the motor cells in the anterior horn of the spinal cord and may then diffuse to involve the whole central nervous system. The toxin may also interfere with the normal inhibition of motor impulses exercised by the upper motor neurone over the lower, producing early increase in tonus and tonic spasms localised initially to the musculature controlled by the spinal segment involved. This affords an explanation of local tetanus.

Antitoxin.—Tetanus antitoxin, often called antitetanus serum or A.T.S., can be obtained by immunising horses with toxoid. This serum is of value in the prophylaxis of tetanus, given immediately after wounding (p. 682). Its use as a curative agent after the development of tetanus is less effective than the corresponding antitoxin treatment of diphtheria. Tetanus antitoxin is standardised in Great Britain in terms of the *International Unit (1950)*, by comparison with a preserved standard serum.

Occurrence.—The tetanus bacillus occurs in the intestine of man and animals, but there is considerable variation in the frequency with which it is reported to have been isolated from their faeces. Tenbroeck and Bauer (1922)² isolated *Cl. tetani* from 34.7 per cent. of stools from 78 individuals in Peking. Kerrin (1928-29)³ examined more than 300 human stools in Scotland and none yielded *Cl. tetani*. The wide divergence in these figures may be partly related to the different ways of life of the communities investigated. The use of human faeces as fertiliser in the fields of China, whose population lives in more intimate contact with the soil, will play a part in the re-distribution of the bacillus.

It is uncertain whether this organism flourishes as a saprophyte in the soil or is derived entirely from the animal intestine. It is

¹ Wright, E. A., Morgan, R. S., & Wright, G. P. (1950), *J. Path. Bact.*, **62**, 569.

² Tenbroeck, C., & Bauer, J. H. (1922), *J. exp. Med.*, **36**, 261.

³ Kerrin, J. C. (1929), *Brit. J. exp. Path.*, **10**, 370.

especially prevalent in manured soil, and, for this reason, a wound through skin which may be contaminated with soil or manure deserves special attention. Whether derived from the soil or the faeces, however, tetanus spores occur very widely; they are commonly present, for instance, in street dust and may be present in the dust in hospitals and houses, on clothing and on articles of common use.

Tetanus is usually the result of contamination of a wound with *Cl. tetani* spores. The source of the infection may be soil, dirty clothing or dust, but spores may also be derived from the skin—especially in areas of the body that may be contaminated with intestinal organisms. Spores of *Cl. tetani* and other anaerobes may be embedded in surgical catgut (prepared from sheep's intestine), and this has been the source of infection in some post-operative cases of tetanus. The sterility of surgical catgut is now rigorously controlled in this country.

If washed spores alone are injected into an animal they fail to germinate, are phagocytosed and do not give rise to tetanus. It has been shown that the germination of spores of *Cl. tetani* is dependent on the reduced oxygen tension occurring in devitalised tissue and non-viable material in the wound. Infection, when it occurs, remains strictly localised in the wound and the tetanic condition is due to the effects of a potent diffusible exotoxin on the nervous system. Certain conditions favour the germination of the spores and the multiplication of the organisms in the tissues, e.g. deep puncture wounds; wounds accompanied by compression injury associated with devitalised tissues, necrotic tissue and effused blood; wounds contaminated with soil, the ionised calcium salts and silicic acid in which cause tissue necrosis; wounds containing foreign bodies such as pieces of clothing and shrapnel; infection by other organisms, such as pyogenic cocci and *Cl. welchii*. Thus, in war casualties, infection tends to occur when there are deep lacerated wounds caused by shrapnel which may carry in fragments of muddy clothing and particles of earth. Spores are then introduced under most favourable conditions for the development of the organism. Similar wounds may be sustained in civil life, notably as a result of gunshot injuries or following accidents on the roads and on farms. It should be borne in mind, however, that cases of tetanus have been reported, especially in children, in which the infection apparently derived from a superficial abrasion, a contaminated splinter or a minor thorn-prick. *Cl. tetani* infection may also occur in the uterus, as in cases of septic abortion. *Tetanus neonatorum* follows infection of the umbilical wound of newborn infants. Cases of post-

operative tetanus have been recorded due to imperfectly sterilised catgut, dressings or glove-powder, and some cases of post-operative tetanus have been attributed to dust-borne infection of the wound at operation.

Infection in Animals.—Many animals are susceptible to tetanus, young animals being more susceptible than adults. The horse is particularly susceptible. Infection may be acquired in lambs, calves, pigs and goats after castration or docking, especially if these operations are performed under dirty conditions. Young colts may develop tetanus following umbilical infection. In cows, tetanus is usually associated with parturition, especially after retention of the placenta. Carnivores are rarely affected and birds are almost completely resistant to the natural disease, although an occasional case has been described in geese and in a turkey. The guinea-pig and the mouse are susceptible to tetanus toxin, and mice are extensively used in the laboratory investigation of *Cl. tetani*.

The blood of most cattle contains neutralising antibodies, with small amounts in sheep and goats, which may account for the comparative rareness of tetanus in ruminants. The blood of horses, dogs, cats, pigs and humans does not normally contain antitoxin. Birds and other naturally resistant animals have no antibodies in their blood, so that their immunity cannot be attributed to the presence of neutralising antibodies similar to those found in artificially immunised animals.

Laboratory Diagnosis.—Films may be made from the wound exudate and stained by Gram's method; the appearance of "drum-stick" bacilli is suggestive evidence of the presence of *Cl. tetani*, but it is not conclusive as other organisms having terminal spores, which are morphologically indistinguishable from *Cl. tetani*, may be present (p. 706). Moreover, it is often impossible to detect the tetanus bacilli in wounds by microscopic examination.

The more reliable method for diagnostic purposes is to produce tetanus in mice by subcutaneous injection of an anaerobic fluid culture prepared from the wound. The injection, e.g. 0.2 ml. of a five- to ten-day cooked-meat broth culture, is made into the tissues to the right of the base of the animal's tail. Within a day or so in a positive test there may be stiffness of the tail and the hind limbs. The right hind leg is subsequently paralysed and the tail and spine of the animal tend to curve to the right. Thereafter, more generalised muscular involvement becomes increasingly evident, and tetanic convulsions may be elicited by trivial stimuli. Control animals should be included to which graded doses of

tetanus antitoxin have been administered thirty minutes previously as a prophylactic.

While significant results may thus sometimes be obtained with impure or mixed cultures from the wound, it is essential that the tetanus bacillus, if possible, should be obtained in pure culture so that it can be identified by its biological characters and its specific toxicity. In Fildes' method, which exploits the tendency of *Cl. tetani* colonies to spread and extend beyond the growth of other bacteria, the material is incubated anaerobically in 5 per cent. peptic-blood broth for two to four days at 37° C. The culture is then heated at 65° C. for thirty minutes to kill spreading non-sporing organisms such as *Proteus*. The condensation water of a peptic-blood agar slope is then inoculated from the heated culture, and the tube is incubated anaerobically. After twenty-four to forty-eight hours the edge of the culture is examined with a hand lens, when a growth of tetanus bacilli is seen as a mass of very fine filaments. Subcultures from the marginal growth frequently yield pure cultures of *Cl. tetani*. (It is advantageous to keep the bloodagar tubes prior to inoculation until the surface of the medium is dry at the top.)

This method of isolation will not be successful if a non-motile type VI strain is involved, and it is advisable to employ additional methods. It is also recommended, when possible, to vary the degree of preliminary heating of portions of the specimen under investigation. Thus, tissue from the wound may be ground up with sand under sterile conditions. One-quarter of this material may be extracted and used for direct animal inoculation. The remaining three-quarters is dispensed into six universal containers of freshly prepared cooked-meat medium. Two of these are heated at 80° C. for ten minutes, two at 70° C. for thirty minutes and two are not heated.¹ After several days' incubation at 37° C., subcultures from these may be made to blood agar, into shake cultures and into the condensation water of Fildes' peptic-bloodagar slopes as already described.

The Prevention of Tetanus²

Prompt and adequate wound toilet and proper surgical débridement of wounds are essential steps in the prevention of tetanus. Clean superficial wounds which receive prompt attention may not require specific protection against tetanus. Specific prophylaxis

¹ Robinson, D. T., McLeod, J. W., & Downie, A. W. (1946), *Lancet*, i, 152.

² See Parish, H. J., Laurent, L. J. M., & Moynihan, N. H. (1957), *Brit. med. J.*, i, 639.

is indicated in the case of deep wounds, puncture or stab wounds, ragged lacerations, wounds associated with bruising and devitalised tissue, and animal bite wounds. It is also advisable to regard all open wounds in children, farm workers and road-accident cases as potentially infected with spores of *Cl. tetani* and requiring protection.

The method of prophylaxis depends upon the state of immunity of the patient. While some degree of latent immunity is conferred even after only one injection of toxoid, for practical purposes it is wise to differentiate clearly between those likely to have a definite immunity and those who may not be immune. A patient may be regarded as *immune* for six months following the first two injections, or for five years following three injections (or a booster injection) of a planned course of tetanus toxoid (p. 683). Tetanus antitoxin should not be given to immune patients, but their active immunity should be enhanced by giving 1 ml. of tetanus toxoid intramuscularly at the time of injury.

A patient is considered *non-immune* if he has never had an injection of tetanus toxoid or if he has had only one such injection. If more than six months have elapsed after a course of two injections, or more than five years after a full primary course of three injections (or a booster injection) of tetanus toxoid, he may be regarded as non-immune. He is non-immune if more than one week has elapsed since a previous injection of tetanus antitoxin. He should be considered non-immune if there is any doubt about his immunisation history.

The *non-immune patient* is passively protected against tetanus by tetanus antitoxin. The usual prophylactic dose is 1500 units given by intramuscular or subcutaneous injection as soon as possible after injury. The dose is not reduced for a child. The injection may be repeated at weekly intervals as long as the risk of tetanus persists. Larger initial doses, e.g. 3000 to 10,000 units, may be given when the wound is a severe one. Antitoxin is never given intravenously as a prophylactic measure.

In the *treatment* of established tetanus, reliance is frequently placed on the intravenous injection of a large initial dose of antitoxin (30,000–200,000 units) followed by intramuscular injections. When intravenous antitoxin is prescribed, it should be preceded by a subcutaneous test dose, followed by an intramuscular test dose, at half-hour intervals (see below). The antitoxin should be diluted, warmed to room temperature and injected very slowly into the recumbent patient. All of the precautions listed below should be observed. Intrathecal administration of antitoxin may cause dangerous reactions.

Precautions to be Observed when giving Antitoxin.—In view of the risk of anaphylactic reactions following injections of antitoxin, routine precautions should be taken before antitoxin is administered. Information should be obtained from the patient regarding previous serum injections and any history of asthma, infantile eczema, urticaria or other allergic condition elicited. In the absence of any of these contraindications the full dose of antitoxin may be injected forthwith, but a sterile syringe and needle with adrenalin (1 ml. of 1 in 1000 solution) should be at hand. The patient should be kept warm before and after treatment and he should be under observation for at least thirty minutes after the injection.

If the patient has had a previous injection of serum, but gives no history of allergy, a subcutaneous test dose of 0·2 ml. antitoxin should be given, and a full dose of antitoxin may be given if no general reactions have occurred after thirty minutes. If the patient gives a history of allergy, the initial test dose should be 0·2 ml. of a 1 in 10 dilution of antitoxin subcutaneously. If no general symptoms develop within thirty minutes, this may be followed by 0·2 ml. of undiluted antitoxin subcutaneously. The full dose may be given if there are no general reactions after a further thirty minutes.

A careful record should be kept of all prophylactic injections given, and information should include the batch numbers of the preparations used and the nature of any reactions observed. It is especially important that a record should be given to the patient or his guardian. It is desirable that patients receiving passive protection with antitoxin should be actively immunised subsequently against tetanus with toxoid, because, apart from involving the risk of anaphylaxis, a second dose of antitoxin tends to be more rapidly eliminated than the initial dose and the passive protection afforded on the second occasion is reduced. The first dose of toxoid should not be given until three to four weeks after A.T.S., however, in view of the possibility of neutralisation and inadequate immunisation.

Many authorities consider that all persons should be actively immunised against tetanus in infancy and their immunity maintained by booster doses of toxoid at intervals of five to ten years. This is of particular value in the case of allergic patients, since the necessity of serum prophylaxis in the event of wounding, and the chance of allergic complications, is avoided.

Active Immunisation.—A course of three 1 ml. doses of tetanus toxoid (formol-toxoid, "F.T.") with intervals of six to twelve weeks between the first two, and six to twelve months between the

second and third injections, is of proved value in the prevention of tetanus. A reinforcing (booster) dose of 1 ml. toxoid should thereafter be given at intervals of five to ten years on at least two further occasions to maintain immunity.

Chemotherapy.—As tetanus is due to a toxin, antibiotics are only of use in the treatment or prevention of other infections, e.g. pneumonia, associated with clinical tetanus.

Although the prophylactic administration of antibiotics to all cases of open wounds is not recommended, there is justification for the prophylactic administration of an antibiotic such as penicillin in the case of a deep contaminated wound or an open wound associated with much devitalised tissue. Pyogenic infection is likely to occur in such wounds, and this favours the development of tetanus. Penicillin may be given at the time of injury and dosage maintained (either by repeated administration or by the use of a long-acting preparation such as benzathine penicillin) until healing is established. As in gas gangrene (p. 699), this additional safeguard cannot take the place of prompt and adequate surgical wound toilet.

GAS GANGRENE

Several members of the genus *Clostridium* are associated with rapidly spreading oedema, necrosis and gangrene of the tissues, and gas production, occurring as a complication of wound infection in man. The main source of these organisms is animal and human excreta. They were responsible for the gas gangrene which was so prevalent among the armies in Europe during the war of 1914-18 and, though much less frequent, was met with in the war of 1939-45. Of more than two hundred war cases of gas gangrene infected with a single *Clostridium* species, recorded in 1918¹ and 1943,² *Cl. welchii* occurred in more than 30 per cent., *Cl. oedematiens* in 5-17 per cent., *Cl. bif fermentans* (*sordellii*), *Cl. histolyticum* and *Cl. fallax* each in less than 1 per cent. In 42-60 per cent. of wounds in these two series, more than one species of *Clostridium* were involved. Data obtained in the two wars indicate that *Cl. welchii* occurred most frequently (about 60 per cent. of all cases) in gas gangrene, while *Cl. oedematiens* and *Cl. septicum* occurred in about 20-40 per cent. and 10-20 per cent. of cases respectively. The other clostridia mentioned occurred much less frequently.

¹ Weinberg, M., & Séguin, P. (1918), *La gangrène gazeuse*, Paris: Masson.

² MacLennan, J. D. (1943), *Lancet*, 2, 94.

The infection usually results from the contamination of a wound with soil (particularly that of manured and cultivated land), dirty clothing, street dust, etc., but may also be derived from the skin, especially in areas of the body that may be contaminated with intestinal organisms. As in the case of tetanus, the presence of foreign bodies, dead tissue and blood clot promotes the occurrence of gas gangrene in a wound, and the predisposing factors discussed regarding tetanus (p. 679) are equally applicable in the case of gas gangrene. Gas gangrene may develop as a result of extension of the organisms from the alimentary tract, as in cases of appendicitis or obstruction of the alimentary tract. In puerperal cases, and especially in cases of septic abortion, the organisms may gain access via the perineum to necrotic or devitalised tissues and set up a dangerous infection. It should be remembered, however, that a clostridial infection may also be located in subcutaneous tissue where there has been extravasation of blood or an accumulation of tissue fluids. Infection occurs, frequently in less severe form, in association with ischaemic conditions of the extremities, for example in diabetic gangrene. Other less severe forms of clostridial infection may occur without the typical toxæmia, such wounds having a foul odour and showing evidence of gas formation. Moreover, potentially pathogenic anaerobes may be cultivated from a wound which never shows any signs of gas gangrene. MacLennan¹ has classified anaerobic infections on clinical grounds and he recognises (*a*) simple contamination of a wound with clostridia; (*b*) anaerobic cellulitis, in which muscle is not involved; and (*c*) anaerobic myositis, which includes clostridial gas gangrene but may also be caused by anaerobic streptococci.

In wounds there is practically always a mixed infection, so that simple plating alone is frequently inadequate for diagnostic purposes. It must be emphasised that the separation and cultivation of anaerobes is more difficult than is the case with aerobes, but this is partly due to the fact that techniques for the isolation of anaerobes are not so well developed at present. While alternate culture on plates and in fluid media may sometimes be necessary before a pure culture is obtained, *direct* culture on selective agar media can yield more information regarding the importance of infection by any one pathogen. An account is given on p. 696 of the methods in general use for isolating and identifying the more important anaerobes, with particular reference to cases of gas gangrene.

¹ MacLennan, J. D. (1943), *Lancet*, **2**, 63.

CLOSTRIDIUM WELCHII**(*Cl. perfringens*)**

The organism most commonly associated with gas gangrene. There are six types designated A to F and distinguished by the various combinations of toxins they produce. The classical *Cl. welchii* of *gas gangrene* belongs to type A. The other types, with the exception of type F, are more commonly associated with disease in animals (p. 692).

Morphology and Staining.—A relatively large Gram-positive bacillus, about $4\text{--}6 \mu$ by 1μ , with square or rounded ends, occurring singly or in pairs, and often capsulated when seen in the tissues. In sugar media the bacilli are shorter, while in protein media they tend to become filamentous. The bacilli are non-motile. Spores are formed, but only in the absence of fermentable carbohydrates and abundantly only on special media such as Ellner medium (p. 233). They are typically oval, subterminal and not bulging, but many bizarre forms are seen.

Cultural Characters.—Obligatory anaerobe. Optimum temperature about 37° C . Grows best on carbohydrate-containing media, e.g. glucose agar or glucose-blood agar.

Surface colonies are large, round, smooth, regular and slightly opaque disks. Other types of colonies are observed, including one having a raised opaque centre and a flat transparent border which is radially striated. Rough flat colonies with an irregular edge resembling a vine-leaf also occur. On blood agar the colonies are usually surrounded by a variable zone of complete haemolysis, and a wider zone of incomplete haemolysis may occasionally develop.

Biochemical Reactions.—In litmus milk medium, acid, clot and gas production result; the gas breaks up the clot, producing the "stormy clot" reaction which is not specific for *Cl. welchii* but is produced by almost all strains of this organism. The culture has a sour, butyric-acid odour.

Gelatin is liquefied. Coagulated serum is usually not liquefied. In cooked-meat medium the meat is reddened and no digestion occurs.

Cl. welchii is actively saccharolytic and ferments, with gas production, glucose, lactose, sucrose, maltose, starch, and, in the case of some strains, salicin, glycerol and inulin. Mannitol and dulcitol are not fermented.

Viability.—*Cl. welchii* spores resist the action of the routinely used antiseptics and disinfectants. The spores of classical type A strains of *Cl. welchii* are only moderately heat-resistant and will

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not survive boiling for more than a few minutes. The spores of food-poisoning strains and type F strains, however, are markedly heat-resistant and may survive boiling for several hours.

Toxins.—Many different toxic and enzymic factors, distinguishable by *neutralisation tests* with antitoxic sera, have been demonstrated among the six types of *Cl. welchii*. The neutralisation tests may be performed by intravenous or intracutaneous injection of mice or guinea-pigs respectively, with mixtures of toxin and antitoxin. The intravenous test in mice involves the injection of 0·3 ml. of a five- to ten-hour culture in 1 per cent. glucose broth. If this proves lethal, mixtures of 0·9 ml. of test fluid and 0·3 ml. of the different type sera are held at room temperature for thirty minutes and then 0·4 ml. of each mixture is injected separately into groups of mice. Injections of the original test fluid without added antiserum are included as controls. A combination of *in-vivo* and *in-vitro* tests is recommended for the routine typing of *Cl. welchii*.¹

Antitoxin to the types of *Cl. welchii* commonly encountered in disease will neutralise the major lethal antigens of these types as follows:

- Type A antitoxin neutralises only the homologous toxin.
- Type B antitoxin neutralises the toxins of types A, B, C and D.
- Type C antitoxin neutralises the toxins of types A and C.
- Type D antitoxin neutralises the toxins of types A and D.

The types of *Cl. welchii* thus differ as regards their production of the several toxic factors. Several have haemolytic, lethal or necrotising properties and others have enzymatic activity against biological materials. The differences are indicated in the following table adapted from the work of Brooks, Sterne and Warrack (1957).² It is evident that, with the exception of type F, the types can be differentiated on the basis of their production of the four major lethal toxins, and, indeed, it has been argued that type F should be regarded as a subgroup of type C.

Alpha Toxin.—Produced by all types of *Cl. welchii* but notably by type A strains, this is the most important of the lethal toxins of the organism and is considered to be the main cause of the profound toxæmia associated with gas gangrene in man. The alpha toxin is lethal for laboratory animals and it is necrotising on intradermal inoculation. It is relatively heat stable, being only 50 per cent. inactivated after five minutes at 100° C. The toxin

¹ Oakley, C. L., & Warrack, G. Harriet (1953), *J. Hyg. (Lond.)*, **51**, 102.

² Brooks, M. E., Sterne, M., & Warrack, G. Harriet (1957), *J. Path. Bact.*, **74**, 185.

Type	Occurrence	Major Lethal Toxins								Minor Lethal or Non-Lethal Factors					
		α	β	ϵ	ζ	γ	δ	η	θ	κ	λ	μ	ν		
A	Gas gangrene. Puerperal infection: Septicaemia.	+++	-	-	-	-	(+)	+ -	+ -	-	+ -	-	+ -	+ -	
	Food-poisoning.	+++	-	-	-	-	-	(+)	+ -	-	+ -	-	+ -	+ +	
B	Lamb dysentery.	++ +	+++	+	-	-	(+)	-	+ +	-	+ +	-	+ +	+ +	
C	"Struck" in sheep. Enteritis in other animals.	++ +	+++	-	-	?	-	-	+ + +	+ + +	-	-	+ -		
D	Enterotoxaemia of sheep and pulpy kidney disease.	++ +	-	+++	-	-	-	-	+ +	+ +	+ +	+ -	+ -		
E	Doubtful pathogen of sheep and cattle. Enteritis necroticans in man.	++ +	-	-	+ + +	-	-	-	+ +	+ +	+ +	(+)	+ -		
F		++ +	+ -	-	-	+ +	-	-	-	-	-	-	+ + +		

++ = produced by all strains. ++ = produced by most strains. + - = produced by some strains.
 (+) = produced by very few strains. heavy type +++ = produced by large amounts.

is an enzyme, lecithinase C. In the presence of free Ca or Mg ions it can split lipoprotein complexes in serum or egg-yolk preparations with resulting opalescence. The reaction can be inhibited by specific antitoxin.

It appears that the lecithinase also attacks phospho-lipid constituents of the red blood cells of various animals, and the alpha toxin is thereby haemolytic for the red cells of most species with the exception of the horse and the goat. With the red cells of the sheep in particular this toxin provides an example of a "hot-cold" lysis.

Alpha toxin activity may thus be assayed by several methods, including turbidity tests using egg-yolk emulsion (lecithovitellin, L.V.) or human serum as indicator, and sheep red cell lysis tests incorporating antisera to other haemolytic toxins which may be produced by *Cl. welchii*.

Nagler's Reaction.—Several clostridia are able to produce opalescence in both serum and egg-yolk media, due to the production of lecithinases which cause visible precipitates in these media. The reaction was first demonstrated with the alpha toxin of *Cl. welchii*¹ and is specifically neutralised by *Cl. welchii* alpha-antitoxin (but the serologically related lecithinase of *Cl. bif fermentans* is also inhibited.)

In the test, equal parts of Fildes' peptic digest broth (p. 232) and sterile human serum (from clotted blood) are mixed, and 0.3 ml. of this mixture is placed in each of two small stoppered tubes, to one of which 0.03 ml. of a standard *Cl. welchii* antitoxin has been added. Both tubes are then inoculated with a drop of fluid culture or a colony picked from a plate culture. The tubes are incubated anaerobically at 37° C. and examined after sixteen, forty and sixty-four hours. A positive reaction is indicated by the development of pronounced turbidity in the serum with a yellowish curd on the surface, the effect being absent in the tube containing antitoxin.

This reaction has also been utilised for the rapid detection of *Cl. welchii* in direct plate culture, and allows a serologically controlled identification of the organisms to be made within twenty hours of inoculating the plate from the wound exudate. The method is described in a Medical Research Council Memorandum.² 2.65 ml. of human serum are mixed with 0.65 ml. of Fildes' peptic digest broth; the temperature is raised to 50° C. and 10 ml. of melted nutrient agar at 50° C. are added. A plate is poured,

¹ Nagler, F. P. O. (1939), *Brit. J. exp. Path.*, 20, 473.

² Medical Research Council War Memorandum No. 2, (1943), 2nd ed. (revised), London: H.M.S.O.

allowed to set and dry. On one half of the plate (which is appropriately marked) two or three drops of standard *Cl. welchii* antitoxin are spread and allowed to dry. The whole plate is then inoculated from the wound swab. On the section containing no antitoxin, *Cl. welchii* colonies show a surrounding zone of opacity, i.e. the Nagler reaction, while colonies of the organism on the remainder of the plate show no change. Further developments of this medium have included the incorporation of neomycin sulphate to inhibit aerobic sporing organisms and coliforms (Waksman *et al.*, 1949¹; Lowbury and Lilly, 1955²); lactose and neutral red to indicate lactose-fermenting organisms, and milk as an indicator of proteolysis (Willis and Hobbs, 1959³). The complex medium (p. 232) is of great use as an additional aid in the isolation and preliminary identification of all types of *Cl. welchii* and many of the important clostridia, but it should be used in parallel with other media as it may partially inhibit some members, notably *Cl. tetani* and *Cl. sporogenes*. *Cl. welchii* colonies produce a marked zone of opalescence with an acid (pink) reaction which is initially peripheral but, on oxidation, produces a pink colour within the colony.

Beta Toxin.—Types B, C and F produce this toxin, which is lethal and necrotising. Intradermal inoculation in the guinea-pig produces a purple-tinged necrotic area which, in the case of type C and F filtrates, is almost circular but, with type B filtrates, is more extensive and irregular in shape due to the associated hyaluronidase produced by type B strains (Oakley and Warrack, 1953).⁴

Epsilon Toxin.—This is produced by type B and D strains as a prototoxin which is thereafter activated by proteolytic enzymes. It is lethal and necrotising and its activity is largely dependent upon the presence of an activating enzyme and an alkaline environment. Filtrates may be trypsinised before assay.

Iota Toxin.—Only type E strains produce this toxin, which is also lethal and necrotising and, like epsilon toxin, is formed as a prototoxin which is then activated by proteolytic enzymes.

Gamma toxin is a minor lethal toxin.

Delta toxin is lethal. It is haemolytic for the red cells of even-toed ungulates (sheep, goats, pigs, cattle).

Eta toxin is said to be an insignificant lethal toxin.

Theta Toxin is an oxygen-labile haemolysin which may be related to this is active against mouse erythrocytes. Many rabbit, but is virtually inactive against the red cells of the horse, ox, sheep and lamb.
Food poisons

¹ Waksman, S. A., Lechevalier, H. A., & Harris, D. A. (1949), *J. clin. Invest.*, **18**, 934.

² Lowbury, E. J. L., & Lilly, H. A. (1955), *J. Path. Bact.*, **69**, 465.

³ Willis, A. T., & Hobbs, G. H. (1959), *J. Path. Bact.*, **77**, 511.

⁴ Oakley, C. L., & Warrack, G. H. (1953), *J. Hyg. (Lond.)*, **51**, 102.

animal sera inhibit theta toxin and, while this may be due to contained antibodies, it is known that tissue lipids and cholesterol inactivate it. Theta toxin is adsorbed on to meat particles so that strains assayed for theta production must be grown in medium free of meat particles. Culture supernatants (unfiltered, as the toxin is oxygen-sensitive) are then titrated for haemolysing activity against horse red cells in the presence of alpha antitoxin and under reducing conditions.

Theta toxin is also a lethal toxin.

Kappa toxin is a collagenase which attacks hide powder and gelatin. It may be this toxin which causes the softening of muscle connective tissue associated with gas gangrene.

Lambda toxin is a proteinase and gelatinase. It will decompose hide powder, but it does not attack native collagen.

Mu toxin is a hyaluronidase.

Nu toxin is a deoxyribonuclease.

Other Enzymes.—*Cl. welchii* cultures have been shown to possess other enzymatic properties. Enzymes are produced, particularly by some type B strains, which destroy blood-group substances. The organism also renders red blood cells inagglutinable to the myxoviruses (p. 756), probably by destroying virus receptors at the red cell surface. This is thought to be due to a receptor-destroying enzyme (R.D.E.) similar to that of *Vibrio cholerae* (p. 351). *Cl. welchii* renders red blood cells panagglutinable by exposing their T antigens so that they lose their specificity and react with any of the ABO antisera. A diffusible haemagglutinin elaborated by *Cl. welchii* causes agglutination of the red blood cells of man and most animals. It is produced by some strains after prolonged artificial subculture, but it is not produced by freshly isolated strains.

Animal Pathogenicity.—The virulence varies greatly with different strains. Some are markedly pathogenic to guinea-pigs by subcutaneous or intramuscular injection of 1 ml. of a twenty-four-hour culture in cooked-meat broth into the thigh, and the animal may die within twenty-four hours. A control animal may be protected by a prior injection of *Cl. welchii* antitoxin, e.g. 300–500 units. At autopsy, a spreading inflammatory oedema with gelatinous exudate and gas production is noted in the subcutaneous tissue; necrosis occurs in the underlying muscles which are sodden, friable and pink. Organisms from cultures washed with saline solution to free them from toxin and other soluble products are practically non-pathogenic. The products of growth of the bacillus increase its aggressiveness and, as toxin production occurs during early growth, young cultures should be used. The pathogenicity of a strain may be further enhanced by incorporating an equal amount of a sterile 5 per cent. solution of calcium chloride in the inoculum immediately before injection. Pigeons are exceedingly susceptible to experimental inoculation of *Cl. welchii*.

Occurrence.—Apart from its pathological relationships, *Cl. welchii* occurs normally in the large intestine of man and animals; its frequency in the human intestine approaches 100 per cent. It may invade the blood *ante mortem* and, multiplying in internal organs after death, produces the small gas cavities sometimes noted (e.g. in the liver) at *post-mortem* examinations.

In certain conditions the numbers present in the bowel are greatly increased, e.g. pernicious anaemia, intestinal obstruction. In pernicious anaemia the associated achlorhydria is probably a factor which allows this organism to flourish, and in this condition *Cl. welchii* may be found even in the duodenum and stomach.

Apart from wound infections, it may occur in uterine infections (e.g. septic abortion) and occasionally gains access to the blood stream from this site, producing intravascular haemolysis with subsequent oliguria or anuria. *Cl. welchii* also occurs in infections of the intestinal tract, gall-bladder and the urinary system.

Strains of *Cl. welchii*, conforming in most respects to type A but producing non-haemolytic or feebly haemolytic colonies on blood agar, are associated with a mild form of food poisoning (p. 391). Spores of these strains are markedly heat-resistant, surviving boiling for one to four hours. Food-poisoning strains of *Cl. welchii* are widely distributed, occurring in the faeces of healthy man and animals.

Cl. welchii, type F, has been reported as the cause of a condition occurring in Germany affecting man and named *enteritis necroticans*. The spores of type F strains are markedly heat-resistant.

Infections in Animals.—Type B strains cause *lamb dysentery*, and a synonym for this organism is *B. agni*. The disease is characterised by an enteritis, occurring within the first week of life, and death is due to toxæmia as a result of absorption of the toxins produced. Antitoxic serum has been utilised with great success in the prophylaxis of the disease, the lambs being injected as soon after birth as possible. Ewes may also be immunised (during pregnancy) with toxoid or with a formalised culture, a second dose being given fourteen days before lambing. The antibodies produced by such immunisation are conveyed to the lamb in the colostrum. A similar disease occurs in foals.

Cl. welchii, type C, originally named *Bacillus paludis*, is associated with an enterotoxaemic disease of sheep known in Kent as "struck" and occurring also in Wales. Enterotoxaemias associated with *Cl. welchii*, type C, have been reported in lambs and calves in U.S.A.; and in piglets in England.

A similar disease of sheep, *infectious enterotoxaemia*, is due to *Cl. welchii*, type D (*Bacillus ovotoxicus*). This is a sporadic disease

which is well known in Great Britain and has been reported in Australia and America. *Pulpy kidney disease* is also due to *Cl. welchii*, type D, and this is predominantly an acute and fatal disease of young lambs (three to eight weeks old), though older sheep may be affected. The disease is usually associated with overeating or high-level feeding, and is characterised by sub-endocardial haemorrhages with excess pericardial fluid. Appropriate methods of active or passive immunisation may be carried out against these diseases, as in the case of lamb dysentery. Type E strains cause enterotoxaemia of calves.

In veterinary medicine the diagnosis of *enterotoxaemia* is frequently substantiated by the demonstration of preformed toxin in the intestine of a dead sheep by *mouse toxicity tests*. The detection of a toxin of *Cl. welchii* in intestinal contents is not conclusive evidence that death was due to enterotoxaemia, as immune sheep are known to be capable of withstanding large quantities of such toxins. Results of these tests must therefore be considered along with other clinical and pathological evidence. For the test, intestinal contents or a ligatured segment of the terminal part of the small intestine should be submitted. Chloroform, 5 per cent., may be added as preservative if the specimen is to be sent by post. Approximately 20 ml. of contents, diluted with saline if too thick, are centrifuged at 3000 r.p.m. for thirty minutes. A small piece of cotton-wool is incorporated at the foot of the tube to assist clarification. The supernatant fluid is retained for the test. It is not necessary to prepare a bacteria-free filtrate as mice rarely succumb to intravenous injections of the unfiltered material free from toxin. Mixtures of 0·9 ml. supernatant fluid and 0·3 ml. of antitoxins A, C and D are prepared and allowed to stand at room temperature for thirty minutes. A batch of mice is injected intravenously with 0·3 ml. of the supernatant fluid alone. The mice of three further groups each receive 0·4 ml. of the appropriate supernatant-antitoxin mixture. Although the un-neutralised toxin usually kills in one to ten hours, the surviving mice should be observed for three days. Deaths occurring within five minutes of injection are usually ascribed to non-specific causes.

CLOSTRIDIUM SEPTICUM

(*Vibrio septique*)

Morphology and Staining.—Moderately large bacillus, with rounded ends, about 3–10 μ by 0·6–1 μ . Motile, with peritrichous

flagella. Tends to grow also in the form of long curved filaments. In the tissues it develops into large, swollen, Gram-positive "citron bodies". Spores are readily formed and are oval, central or subterminal, and "bulging". *Cl. septicum* stains Gram-positively as a rule, but degenerate forms are Gram-negative.

Cultural Characters.—Obligatory anaerobe. Optimum temperature, 37° C. Capable of growing on ordinary media. Glucose promotes growth.

Surface colonies are irregular, transparent, droplet-like colonies, later becoming greyish and opaque, with projecting radiations somewhat like those of *Cl. tetani*. On blood agar haemolysis is observed.

Agar stab.—A white line of growth with short, lateral processes.

Biochemical Reactions.—Litmus milk medium.—Slight acid is formed, and the milk is slowly clotted, but often the change is slight.

Gelatin is liquefied. Coagulated serum is not liquefied. In cooked-meat medium the meat is reddened and not digested.

Various sugars are fermented, e.g. glucose, lactose, maltose and salicin, but not mannitol. Sucrose is not usually fermented.

Antigenic Characters.—Agglutinations involving somatic antigens distinguish four groups which can be further subdivided on the basis of H antigens. There is considerable antigenic cross-relationship to *Cl. chauvoei*.

An exotoxin with lethal and haemolytic properties, the alpha toxin, can be demonstrated in cultures, and a specific antitoxin can be obtained by immunising animals. A deoxyribonuclease, the beta toxin, is also produced and a hyaluronidase has been described. Dafaalla and Soltys (1951)¹ considered that a haemagglutinin produced by *Cl. septicum* is non-diffusible.

Animal Pathogenicity.—Subcutaneous injection of cultures in laboratory animals produces a spreading inflammatory oedema, with slight gas formation in the tissues. The organisms invade the blood and the animal dies within a day or two. Smears from the liver show long, filamentous forms and also citron bodies.

Occurrence.—*Cl. septicum* may be associated with gas gangrene in man. It is responsible for braxy in sheep (see below) and for malignant oedema following wound infection in cattle and sheep. Gas gangrene due to *Cl. septicum* is quite common in pigs. The organism may also be the cause of some cases of blackleg in cattle and sheep.

¹ Dafaalla, E. N., & Soltys, M. A. (1951), *Brit. J. exp. Path.*, 32, 510.

Braxy is an acute fatal disease of yearling sheep characterised by a haemorrhagic inflammatory lesion of the abomasum. The disease is invariably associated with a *Cl. septicum* infection, and although it has not been reproduced experimentally the successful prophylaxis achieved with a formalised culture vaccine suggests that *Cl. septicum* is the organism responsible. The organism is thought to invade the mucous membrane of the abomasum, under conditions of stress, when it multiplies rapidly and gives rise to rapid death from a toxæmia.

Epidemiology.—The primary habitat is the soil. Areas with large numbers of spores of *Cl. septicum* appear to be associated with a higher incidence of *Cl. septicum* infections in man and animals than less heavily infected areas. The organism is an intestinal commensal of many animals and may therefore invade tissues *post mortem*. Braxy in sheep appears in the late autumn or winter and is almost entirely confined to lambs born in the previous spring which are outwintered on hill grazings or old lowland pastures. The disease does not occur if the flock is moved to new pastures on arable farms.

Laboratory Diagnosis.—The microscopic appearances, cultural characteristics and the results of pathogenicity tests in guinea-pigs allow of initial identification. Differentiation from *Cl. chauvoei* may present difficulties and is discussed below (p. 705).

Prophylaxis.—A vaccine consisting of formalised culture of this organism has been used with success in the prevention of braxy. Lambs are vaccinated in autumn with 5 ml. of formalised whole culture or 2 ml. of alum-precipitated toxoid.

CLOSTRIDIUM OEDEMATIENS

(*Clostridium novyi*)

This Gram-positive bacillus resembles *Cl. welchii* in morphology, but is somewhat larger and more pleomorphic. It possesses peritrichous flagella, but its motility is not active and is inhibited in the presence of oxygen. The spores are oval, central or sub-terminal.

Cultural Characters.—Surface colonies are transparent, flat, irregular and tend to fuse, forming a spreading film of growth. Cultures on blood agar produce slight haemolysis. Very small motile "daughter colonies" may move on the surface of the medium and break away from the edge of a large parent colony. Colonies on heated blood agar may produce a green halo and colonies on heated blood agar with benzidine become black after exposure

to air for an hour or so.¹ Some strains of *Cl. oedematiens* will not readily grow on the surface of solid media such as blood agar, especially if the medium is not freshly prepared. The addition of fresh brain infusion to the medium is recommended by Smith.² Primary isolation of such strains may only be accomplished by resorting to deep shake cultures in which colonies appear as small, irregular, "woolly" or "snow-flake"-like balls of growth.

Litmus milk medium—late clotting may occur. No digestion.

The organism is actively saccharolytic and ferments certain sugars including glucose and maltose.

Gelatin is liquefied. Coagulated serum is not digested. In cooked-meat medium the meat is reddened but not digested.

Type A strains produce a pearly layer or iridescent film on egg-yolk media (p. 232).

Antigenic Characters.—Four different serological types A, B, C and D, have been defined and these differ in the distribution of various soluble antigens.

Virulence.—Culture filtrates are highly toxic and possess haemolytic and lecithinase activity in addition to necrotising and lethal properties. The organism produces a natural infection in a wide range of animals. Type C strains are thought to be non-toxic.

Occurrence.—*Cl. oedematiens* is associated with a markedly toxic form of gas gangrene in man. The organism causes gangrenous infections in animals, e.g. "Big head" in rams in Australia. It also causes "Black disease" among sheep in Australia and New Zealand. This condition, which has been observed in Scotland, is activated apparently by the invasion of the liver fluke. A fatal necrotic hepatitis supervenes. Type D strains (*Cl. haemolyticum*) produce red-water disease (infectious icterohaemoglobinuria) in cattle.

Prophylaxis.—Recommended procedures for the prevention of Black disease in Great Britain include fluke eradication and the use of an alum-precipitated vaccine in late summer.

Bacteriological Diagnosis of Gas Gangrene

The bacteriological diagnosis of gas gangrene is usually combined with a general bacteriological examination of the infected wound with which this condition is associated. It is convenient here to give special reference to the recognition of the anaerobic bacilli.

¹ Gordon, J., & McLeod, J. W. (1940), *J. Path. Bact.*, 50, 167.

² Smith, L. de S. (1955), *Introduction to the Pathogenic Anaerobes*, Chicago, Illinois, U.S.A., University of Chicago Press.

Specimens of exudate should be taken from the wound, particularly from the deeper parts and from parts where the infection seems to be most pronounced. These may be obtained in capillary tubes, but sterile swabs (similar to throat swabs, p. 435), rubbed over the wound surface and soaked in the exudate, serve well for the purpose.

Two or three swabs should be taken from the wound, one of which is used for film preparations, the other for culture. If there are sloughs or necrotic tissue present in the wound, small pieces should be placed in a sterile screw-capped bottle and used for microscopic examination and culture.

Microscopical Examination.—Films are made in the usual way and stained by Gram's method. These give some general picture of the degree and nature of the infection and are of value in determining in a preliminary way if there is infection by the gas-gangrene anaerobes. Thus, if gas gangrene is present, Gram-positive bacilli predominate and are also fairly numerous, although *Cl. oedematiens* may appear to be relatively scanty in the wound exudate, even in an active infection. Thick, rectangular, Gram-positive bacilli suggest the presence of *Cl. welchii*, *Cl. fallax* or *Cl. bifermentans*; "citron bodies", boat- or leaf-shaped pleomorphic bacilli with irregular staining may indicate *Cl. septicum*; slender bacilli with round terminal spores suggest *Cl. tetani* or *Cl. tertium*; *Cl. oedematiens* occurs in the form of large bacilli with oval subterminal spores.

Cultures.—In addition to the media routinely inoculated for the detection of aerobes, the following media should be inoculated: (a) blood-agar plate to be incubated anaerobically; the surface should be well dried before inoculation to prevent spreading of colonies of certain anaerobes. If the agar content of solid medium is increased to 4·5–6·0 per cent., depending upon the brand of agar used and the other constituents of the medium, the spreading tendency of organisms is inhibited. Blood-agar plates containing 6 per cent. agar may be used for this purpose. Although the concentrated agar does not prevent the growth of any species, colonies tend to be smaller and morphologically atypical. (b) Plate of Willis and Hobbs' medium (p. 232). (c) Two tubes or bottles of cooked-meat medium; after inoculation, one is heated for thirty minutes at 65° C. to kill non-sporing organisms; for the cultivation of *Cl. oedematiens* it has been recommended that a third tube or bottle should be inoculated with a broth emulsion of exudate or tissue which has been heated for five to ten minutes at 100° C.

The anaerobic plates are examined after twenty-four and forty-

eight hours' incubation. It must be remembered that these may yield growths of various facultative anaerobes as well as the strict anaerobes. Comparison of the aerobic and anaerobic plates affords some indication of the presence of strictly anaerobic organisms in the wound exudate, but any suspected anaerobe must later be tested in subculture to ensure that it is unable to grow under aerobic conditions. (It may be noted that *Cl. tertium* and *Cl. histolyticum* can grow to some extent under aerobic conditions.) The colony characters of suspected anaerobes on the blood agar plate are carefully studied with the naked eye and plate-culture microscope, and films are made and stained by Gram's method; this may give some preliminary information as to the type of anaerobe. Each type present must be isolated in pure culture for further examination, e.g. fermentation tests and animal inoculation. A table of differential characters is given on pp. 706 and 707. The lactose-egg-yolk-milk-agar medium developed by Willis and Hobbs (p. 232) is most useful for the preliminary examination of pure cultures of clostridia. It indicates lactose-fermenting colonies; lecithinase-producing colonies produce a marked zone of opalescence extending beyond the colony (Nagler effect, p. 689); and zones of clearing develop around proteolytic colonies. Opalescence restricted to the medium underlying a colony and associated with an overlying iridescent "pearly-layer" appears to indicate lipase activity.

In the cooked-meat medium both aerobes and anaerobes flourish, but this growth is useful for later subculture should the plate cultures fail to yield successful isolation of organisms present in the wound. Film preparations also yield further information as to the morphological types of organisms growing in it.

Additional Methods.—Anaerobes may be isolated in deep agar-shake cultures. Four or five serial decimal dilutions of the exudate are prepared in broth, and each of these is used to inoculate melted agar kept at 45° C., which is then allowed to solidify in tubes. Following incubation, one of the dilutions will show colonies sufficiently separate to allow of single-colony subculture by means of a capillary pipette, after cutting the tube transversely. A convenient, alternative method is to take up the melted agar after inoculation in sterile capillary pipettes stoppered with cotton-wool, the capillary ends being then sealed and the pipettes incubated horizontally.

Certain reducing agents may be used for rendering fluid media anaerobic in the bacteriological examination of wounds, etc.—ascorbic acid (0.1 per cent.), sodium thioglycollate (0.1 per cent.), reduced iron and iron strips (p. 256), and these may

be adopted advantageously in routine work with the anaerobic organisms.

Prophylaxis of gas gangrene.—A polyvalent serum is available for prophylactic use and for treatment of cases in which the causal organism has not been determined. The prophylactic dose, given intramuscularly (or in urgent cases intravenously), is 10,000 international units *Cl. welchii* antitoxin, 5000 units *Cl. septicum* antitoxin and 10,000 units *Cl. oedematiens* antitoxin. The therapeutic dose, given intravenously, should be at least three times the prophylactic dose, and the administration should be repeated as necessary. Monovalent sera are also available for the treatment of cases after the causal organism has been identified. Reactions to antitoxin administered intravenously may be severe and precautions should be taken (p. 683).

Chemotherapy.—Much work on the antibiotic sensitivities of numerous clostridia isolated from wounds suggests that the order of activity of the common antibiotics is, in general, tetracyclines > penicillin > chloramphenicol.¹ Adequate clinical evidence is lacking, but in the treatment of gas gangrene, penicillin is widely used and may be administered together with a tetracycline. There is no evidence of antagonism. *Prophylactic* administration of penicillin (400,000 units each of potassium and procaine penicillin, repeated at intervals of six hours) in cases of serious, contaminated wounds, has largely replaced the prophylactic use of gas-gangrene antisera. The use of an antibiotic in this manner must never preclude prompt and adequate wound toilet.

CLOSTRIDIUM SPOROGENES

This Gram-positive motile bacillus is very widely distributed in nature and is generally regarded as a harmless saprophyte. It is about the same size as *Cl. welchii*, but more slender. Gram-negative forms are frequent in older cultures. It forms oval central or subterminal spores which may be highly resistant so that the organism is frequently encountered in mixed cultures in the laboratory, even after preliminary heating of these cultures to select heat-resistant pathogens. Its spores may survive boiling for periods of fifteen minutes up to six hours.

Cultural Characters.—Surface colonies may present a "medusa-head" appearance (cf. *B. anthracis*) if the plate is dry. Young colonies may be small, circular, raised and slightly opaque, but these soon produce outgrowths and the spreading margin of the

¹ Garrod, L. P. (1958), *J. Roy. Army Med. Cps.*, 104, 209.

colony becomes irregular with feathery outgrowths. On horse-blood agar the colonies are haemolytic, irregular and transparent with some central opacity where the colonies are raised. Shake colonies show as "woolly" balls of growth. A stab culture develops, like that of *Cl. tetani*, with lateral spikes.

Milk—the casein is precipitated and digested.

Gelatin and coagulated serum are liquefied.

Meat medium—the meat is blackened and digested.

The organism decomposes protein, producing amino acids, ammonia, sulphuretted hydrogen, etc., and cultures have an exceedingly putrid odour.

Acid and gas are produced from some sugars, including glucose and maltose. Lactose and sucrose are not fermented.

Occurrence.—*Cl. sporogenes* is a ubiquitous saprophyte and also occurs in the intestine of man and animals. It is frequently isolated from wound exudates in association with accepted pathogens. While its presence may accelerate an established anaerobic infection by enhancing local conditions, it is not regarded as a pathogen in its own right.

In pure culture it is virtually non-pathogenic to laboratory animals.

Clostridium histolyticum resembles *Cl. sporogenes* and is actively proteolytic. It is not a strict anaerobe. In meat medium, digestion occurs with the formation of white, crystalline masses consisting of tyrosine. When cultures are injected into animals, *in vivo* digestion of the tissues results. This organism is pathogenic and produces an exotoxin.

Clostridium bifementans.—Some workers consider this species distinct from *Cl. sordellii*, but the names are virtually synonymous. The latter title is frequently reserved for pathogenic strains. The organism produces spores readily and abundantly and these are usually oval and central or subterminal. The name *bifementans* refers to the organism's ability to decompose both sugars and proteins. It does not ferment lactose (cf. *Cl. welchii*). A lecithinase is produced which is serologically related to the alpha toxin of *Cl. welchii*. Pathogenic strains also produce a lethal toxin and are occasionally associated with wound infections in man and disease in animals.

Clostridium fallax resembles *Cl. welchii* in some respects, and has sometimes been mistaken for it (hence the name "fallax"). It is, however, shorter and more slender. The spores are usually subterminal. In milk the organism produces clotting and gas formation, but these changes take place slowly (as compared with *Cl. welchii*). It does not liquefy either gelatin or coagulated serum, and is non-proteolytic. It possesses saccharolytic properties. An exotoxin is formed, and when freshly isolated the organism is pathogenic on experimental inoculation in animals.

Clostridium tertium.—In morphology this organism tends to be long and slender. It is weakly motile. The spores are terminal and, when fully developed, oval in shape. It is not a strict anaerobe. The organism shows active saccharolytic properties. In milk, acid is formed with gas production and slow clotting. Meat is reddened, but not digested. Neither gelatin nor coagulated serum is liquefied. Its pathogenicity is doubtful, but when present in wounds, it may give rise to gas production. No exotoxin is produced.

CLOSTRIDIUM BOTULINUM

Botulism is a fatal form of food poisoning characterised by pronounced toxic effects mainly on the para-sympathetic system—*e.g.* oculomotor paralysis, pharyngeal paralysis, aphonia, etc. Animals are also subject to this disease, *e.g.* forage poisoning of horses, "Lamziekte" of cattle, sheep and goats in South Africa; "Loin disease" of cattle in Texas; "Limberneck" of fowls; "Duck sickness" in the western states of America. Sporadic outbreaks of botulism have been reported in mink in America, Scandinavia and England. Five main types of *Cl. botulinum* have been differentiated and designated A, B, C, D and E. Types A, B and E are associated with botulism in the human subject.

Morphology and Staining.—A sporng bacillus with rounded ends, about $4\text{--}6\ \mu$ by $0.9\text{--}1.2\ \mu$, occurring singly and in pairs. Spores are oval, subterminal and slightly "bulging". The bacilli are motile with peritrichous flagella, and stain Gram-positively unless degenerate.

Cultural Characters.—Strict anaerobe. The optimum temperature has been variously stated; earlier workers found growth occurred best at $20^{\circ}\text{--}30^{\circ}$ C.; more recent observations indicate that the optimum is about 35° C. Grows on ordinary media; meat medium yields abundant growth.

Surface colonies—large, greyish, irregular, semi-transparent, with a central "nucleus" and a reticular or fimbriate border.

Agar stab—a white line of growth, stopping short of the surface, with short lateral spikes or radiations; gas production is marked, especially in glucose-agar.

Gelatin is liquefied.

Coagulated serum is slowly liquefied, milk-casein is digested and meat is digested and blackened by type A strains and some type B strains; other strains of B, and types C, D and E are non-proteolytic.

The organism ferments glucose and maltose; type A also frequently ferments salicin and glycerol; type B ferments glycerol but not salicin; type C does not act on either of these substances.

Viability.—The spores of *Cl. botulinum* withstand moist heat at 100° C. for several hours. They are destroyed at 120° C. (moist heat) usually within five minutes. Insufficient heating in the process of preserving foods is an important factor in the causation of this form of poisoning, and great care is taken in canning factories to ensure that sufficient heating is achieved in all parts of the can contents.

Antigenic Characters.—In culture media and in contaminated foods, *Cl. botulinum* produces a powerful exotoxin which is responsible for the pathogenic effects in the disease. This toxin is destroyed when exposed to a temperature of 80° C. for thirty to forty minutes.

The toxin of type A has been isolated as a pure crystalline protein and quantitatively is probably the most potent toxic substance in nature, the estimated lethal dose for mice being 0.000000033 mg. In spite of its potency, the action of the toxin is slow and victims or experimental animals may die many days after receiving a lethal dose. The different types of the bacillus produce toxins which are immunologically different and neutralisable only by the appropriate antitoxin; thus, antitoxin produced from toxin A does not neutralise toxin B, and *vice versa*. Type C has been subdivided into two subtypes, C_α and C_β; C_α antitoxin neutralises C_α and C_β toxins, while C_β antitoxin fails to neutralise C_α toxin.

Diffusible haemagglutinins have been described in culture filtrates of *Cl. botulinum*.

Antitoxin can be prepared by immunising animals with toxoid preparations, and it is used therapeutically. In general, a bivalent serum containing antitoxins to the A and B types of toxin is employed, but its efficacy in the treatment of established botulism is doubtful.

Animal Pathogenicity.—Laboratory animals are susceptible to experimental inoculation and feeding with cultures. The resulting condition resembles the human disease in its symptomatology, the guinea-pig showing difficulty in breathing, flaccid paralysis of the abdominal muscles and salivation following intraperitoneal injection; at autopsy, marked congestion of the internal organs, extensive thrombosis and haemorrhages are noted.

Occurrence.—*Cl. botulinum* is a saprophytic organism and is widely distributed. Its natural habitat is soil, even virgin and forest soil. It may be found in vegetables, fruits, leaves, mouldy hay, ensilage and animal manure.

Botulism has been found to originate from a considerable variety of preserved foods—e.g. ham, sausage, canned meats and

vegetables, etc. It is due not to the formation of toxin by the organism in the intestine but to the absorption from the stomach and upper duodenum of *toxin preformed by the bacillus growing in the food*. Canned and bottled foods responsible for botulism frequently exhibit signs of spoilage.

In cases of botulism the bacillus may sometimes be demonstrated in the stomach contents and faeces, and *post mortem* in the intestinal contents and in the liver and spleen. It can also be isolated from the food responsible for the outbreak.

Rare cases of wound infection by *Cl. botulinum*, resulting in the characteristic signs and symptoms of botulism, have been recorded.

Botulism also occurs in such animals as horses, cattle, sheep and poultry, due to feeding on material in which the organism has been growing. Type C_α is responsible for a paralytic disease of chickens and botulism of ducks. Type C_β is responsible for forage poisoning in horses and cattle; lamziekte of cattle in South Africa is due to type D and results from eating the bones of decomposed carcases on the veldt. Limberneck of chickens is caused by types A and B. The ingestion of the larvae of carrion flies harbouring the organism is frequently responsible for botulism in birds.

Laboratory Diagnosis.—As botulism is essentially a food intoxication, the suspected food should be examined bacteriologically. It may occasionally be possible to demonstrate the presence of toxin in the patient's blood or in *post-mortem* material, e.g. blood, liver, by direct animal inoculation.

Gram-stained films of the food may first be examined for sporing bacilli. The food is then macerated in sterile salt solution and an extract cleared by centrifugation. This may be sterilised by filtration prior to animal inoculation. The extract is then injected intraperitoneally into guinea-pigs in 2 ml. amounts. Injections of heated extract (ten minutes at 100° C.) should be made in a group of control animals, and unheated extract plus the different type antitoxins, if available, may be inoculated into a third group. No deaths should occur in the second group if a specific botulinum toxin is involved and this may be indicated by the third group of tests.

Cl. botulinum may be isolated in pure culture from the food by preliminary heating of various samples at 65°–80° C. for thirty minutes to eliminate non-sporing bacteria. Cultures may then be made under anaerobic conditions on solid media, including a selective medium such as Willis and Hobbs' medium (p. 232), and in cooked-meat broth. Subsequent identification of *Cl. botulinum* is based upon its biological characters and its toxigenicity. Culture filtrates may be prepared from five- to ten-day cooked-meat broth cultures and tested for toxicity by animal inoculation tests as

described above. Faeces and vomit from a case of botulism may be similarly investigated for the causative organism.

Prophylaxis.—Home-canning of foodstuffs should be avoided and home preservation of meat and vegetables is not advisable. Acid fruits may be bottled safely in the home, heating at only 100° C., since a low *pH* is inhibitory to development of *Cl. botulinum*. A prophylactic dose of antitoxin (10 ml.) should be given intramuscularly to all asymptomatic persons who have eaten food suspected of causing botulism. Active immunity in man can be produced by the injection of three doses of mixed toxoid (types A and B) at two-month intervals, but the incidence of the disease under normal conditions does not justify this procedure. Similar immunisation of animals against the predominant type may be economically worth while and has been carried out in Australia on a small scale.

CLOSTRIDIUM CHAUVOEI

(*Clostridium feseri*)

The causative organism of most cases of quarter evil (blackleg, or symptomatic anthrax) in cattle and sheep, a disease characterised by a swollen and emphysematous condition of the subcutaneous tissues and muscles. The infection frequently affects the fore- and hind-quarters, which become dark or almost black in colour.

Morphology.—Resembles closely *Cl. septicum* and is 3–5 μ in length and 0.5–0.6 μ broad. Gram-positive in young cultures but older forms may be Gram-negative. Stains readily with ordinary dyes. Individual organisms tend to occur singly or in pairs, and not in long filaments. "Citron bodies" may be seen in the tissues. The bacilli are motile, with numerous peritrichous flagella. Spores are usually central or subterminal in position, elliptical in shape, and are broader than the bacillus.

Cultural Characters.—Strict anaerobe; optimum temperature, 37° C.; but grows at room temperature. Grows poorly on ordinary medium and a blood or meat medium is preferable. Liver infusion aids growth.

Colonies on agar—greyish-white, transparent, irregular, with radiating filamentous and branching processes.

Agar stab—growth only commences some distance below the surface; along the puncture a whitish line appears with lateral projections, the growth being luxuriant.

Milk—unchanged or may show acid formation with partial clotting.

Gelatin is liquefied slowly. Coagulated serum is not liquefied.

In cooked-meat medium the meat is reddened and is not digested.

Cl. chauvoei ferments glucose, lactose, sucrose, maltose, but not mannitol, salicin or inulin.

Antigenic Characters.—A somatic antigen is common to all strains.

Flagellar antigens distinguish two groups. Cross-reactions with *Cl. septicum* occur.

A lethal exotoxin is obtained in glucose-broth cultures, especially if calcium carbonate be added to neutralise the acid produced. Culture-filtrates are also haemolytic.

Occurrence and Pathogenicity.—The disease occurs in cattle and sheep. *Cl. chauvoei* infection has also been reported in pigs and in fresh-water fish. The organism is pathogenic for guinea-pigs and mice, these animals dying twenty-four to thirty-six hours after experimental inoculation. At autopsy there is an extensive blood-stained oedema round the site of inoculation and the muscles present a dark red or black appearance, while there is a considerable amount of gas produced; the bacillus is present in the heart blood in pure culture. It has been supposed that the natural disease results from infection of a wound by the spores which may be present in the soil of infected pastures. In cattle, infection usually takes place through the ingestion of spores with the fodder or drinking-water, and although the distribution of the lesions suggests a focus of infection in the musculature, the disease is seldom associated with a history of wounding. In sheep, on the other hand, most cases arise from wound infection (shearing, castration, docking, vaccination, and dog bites) and also in connection with parturition.

Laboratory Diagnosis.—*Cl. septicum* may also be responsible for a condition similar to blackleg.

In the differentiation of *Cl. septicum* and *Cl. chauvoei*, stress has been laid on the morphological elements seen in infected guinea-pigs: *Cl. chauvoei* exhibits "citron" and club-shaped forms, but no elongated filaments are observed on the peritoneal surface of the liver of inoculated animals, as in the case of *Cl. septicum*. *Cl. chauvoei* ferments sucrose but not salicin; *Cl. septicum* ferments salicin but not sucrose. The two organisms, however, are closely related.

Prophylaxis.—Various methods of prophylactic vaccination have been practised. A formolised whole culture of *Cl. chauvoei* in broth has been used as a vaccine with successful results. An antitoxic serum has been used for therapeutic purposes.

SOME DIFFERENTIAL CHARACTERS OF CLOSTRIDIA

	Morphology in culture*	Colonies on Blood-agar	Cooked-meat medium	Milk medium	Liquefaction of coagulated serum	Fermentation of				Pathogenicity to guinea-pigs and mice	
						Glucone	Lactose	Sucrose	Maltose	Salicin	
<i>Clostridium tetani</i>	Slender bacilli with round terminal spores	Transparent with long feathery spreading projections; usually haemolytic	Slight digestion, blackening and pungent odour	Unaltered	(but may be softened)	—	—	—	—	+	(tetanus produced)
<i>Clostridium tetanomorphum</i>	Resembles <i>C. tetani</i> ; found terminal spores	Small and transparent, with irregular outline	Gas; no digestion; no putrefactive odour	Unaltered	(but may be softened)	+	—	—	—	—	
<i>Clostridium welchii</i>	Large, thick, often rectangular bacilli; spores usually absent	Large, circular with regular outline; haemolytic	Gas reproduction, meat reddened	Acid, gas, rapid clotting, "stormy-clot"	—	+	+	+	+	(+)	+
<i>Clostridium septicum</i>	Large bacilli with central or sub-terminal spores†	Transparent, irregular with spreading projections; usually haemolytic	Gas, no digestion, meat reddened	Acid, gas, slow clotting	—	+	+	—	+	+	
<i>Clostridium oedematiens</i>	Like <i>C. welchii</i> , but somewhat larger and more pleomorphic; central or sub-terminal spores (not numerous)	Transparent, flat, tend to fuse and form spreading film; usually haemolytic	Gas, no digestion, meat reddened	Sometimes slow clotting	—	+	—	—	—	+	+

<i>Cl. botulinum</i>	Large pleomorphic bacilli; oval spores, usually subterminal	Large irregular colonies with raised centre; haemolytic	Gas; types vary in proteolytic activity (see text)	Casein precipitated and digested by some types; others produce no digestion	(+)	(-)	(+)	(-)	(+)	(-)	(+)
<i>Cl. sporogenes</i>	Somewhat slender bacilli; central or subterminal spores	"Medusa-head" formation or irregular, feathery projections; haemolytic	Gas, digestion, blackening and putrefactive odour	Acid, clot, digestion, later alkaline	(+)	(+)	(+)	(-)	(+)	(-)	(-)

See also table of characters of anaerobic bacilli in *Medical Research Council War Memorandum No. 2, Revised Second Edition, 1943.*

- All these organisms are Gram-positive, but Gram-negative forms are seen in older cultures; they are all motile, with peritrichous flagella, except *Cl. welchii*; but motility is not pronounced and has to be observed while the organisms remain in an anaerobic environment, e.g. withdrawn from a young anaerobic culture into sealed capillary tubes.

† Morphological forms seen in tissues are referred to in the text (*vide supra*).

Under fermentation signifies acid and gas production

+ Double symbols in brackets signify variability in reaction among strains.

For full details of the biological and other characters of the various members of the group, one of the larger works on bacteriology should be consulted.

CHAPTER XXXIV

SPIROCHAETES

THE pathogenic and commensal spirochaetes are slender flexuous spirals 4–16 μ in length and 0·1–0·6 μ thick. They are composed of regular tight coils or of loose irregular spirals of varying amplitude. As a group these organisms are more highly organised than other bacteria, for in addition to a cell wall and cytoplasm they contain from one to twelve long filaments applied to their bodies and it is to these structures that they owe their shape and in all probability their elasticity and characteristic movements. Some cells are only visible by dark-ground microscopy, many can be cultivated and the majority are parasitic in vertebrates. The three genera, *Treponema*, *Leptospira* and *Borrelia*, together constitute the family *Treponemataceae*.

TREPONEMA PALLIDUM

Syphilis is an infectious venereal disease caused by *Tr. pallidum*. Clinically the disease includes a sore on the genitalia which is followed by generalisation of the infection with protean clinical manifestations.

Morphology.—An exceedingly delicate, spiral filament 6–14 μ (average 10 μ) by 0·13 μ , with six to twelve coils which are comparatively small, sharp and regular. The length of the coils is about 1 μ and the depth 1–1·5 μ . The ends are pointed and tapering. The organism is feebly refractile, and in the unstained condition requires dark-ground illumination for its demonstration.

In electron micrographs *Treponema pallidum* is seen to be covered by an outer periplast which covers the whole organism. When this periplast is removed by digestion with pepsin or trypsin three fine filaments about 10 m μ in diameter are seen twisted around the organism and conforming to its coils. Fracture of these filaments results in the disappearance of the organism's coils; the filaments may then be spread out on the supporting film, in which situation they were mistaken by earlier workers for true flagella. *Tr. pallidum* is therefore not flagellate. The nature of the fibrils twisted around its protoplasm is uncertain, but it seems likely

that they are contractile and concerned in the maintenance of the characteristic shape and motility of the organism.

In addition to the typical form, as described, some variation in morphology may be observed: the number of coils to the unit of length may be more or less than normal, the filament may be thicker than normal in whole or part and the coils may be shallower and less regular than usual.

The spirochaete shows rotatory corkscrew-like motility and also movements of flexion. The coils remain relatively rigid, but there may be some expansion and contraction. Angulation, with the organism bending almost to 90° towards its centre is highly characteristic. Its progression is relatively slow as compared with many of the motile bacteria.

The organism divides by transverse binary fission. There is good evidence for the existence of a more complicated life cycle of reproduction in the case of cultivated non-pathogenic strains of treponemes such as the Reiter spirochaete, in which a granular, filterable phase has been described. Division into four and even more fragments has also been described. Some observers have claimed that granules or bud-like structures may be split off, remaining attached by pedicles or stalks before final separation. This budded form has also been regarded as a phase in the life history of the organism, and various supposed developmental bodies differing morphologically from the normal spirochaete have been described as originating from such structures.

Staining.—*Tr. pallidum* cannot be demonstrated by the ordinary staining methods. It can be stained by Giemsa's solution applied in a 1 in 10 dilution over a prolonged period (twenty-four hours) or in a 1 in 2 dilution for an hour, and appears faint pink in colour in contrast to the purplish colour of the coarser non-pathogenic spirochaetes. The organism may also be demonstrated by Fontana's silver or the India ink method using the exudate from the chancre (p. 711). In tissues, the spirochaetes can be stained by Levaditi's silver impregnation method (p. 137).

Cultivation.—It is generally agreed that pathogenic *Tr. pallidum* has not been cultivated in artificial media or in embryonated eggs or tissue cultures. The organism does grow in the testicles of experimentally inoculated rabbits and pathogenic strains can be maintained in this way (*e.g.* Nichol's strain). Certain other strains can be cultured under strictly anaerobic conditions in Smith-Noguchi medium (p. 238), or in digest broth enriched with serum. These strains, although originally isolated from syphilitic lesions, may have been contaminating saprophytes.

Viability outside the body is feeble under ordinary conditions.

This spirochaete is a strict parasite; it dies rapidly in water and is very sensitive also to drying. On the other hand it has been found that *Tr. pallidum* can retain its viability and virulence in necropsy material for some time at ordinary temperatures, and in serum kept in sealed capillary tubes it remains motile for several days. It is readily killed by heat (even at 41.5° C. in an hour) and dies out more slowly (in two to three days) if kept at 0°-4° C. Thus, the danger of transmitting syphilis by transfusion can be prevented by keeping the blood in the refrigerator for three to four days. *Tr. pallidum* remains viable in tissue slices of rabbit testis for long periods at temperatures of -55° to -65° C.

It may be noted here that *Tr. pallidum* is sensitive to penicillin, and this antibiotic has been extensively and successfully used in the treatment of syphilis.

Animal pathogenicity.—Monkeys have been infected experimentally by inoculation of a scarified area on the eyebrows and genitals, or by implanting tissue from a syphilitic lesion under the epidermis. The anthropoid apes are the most susceptible, and lesions typical of primary and secondary syphilis may result in these animals. Rabbits can also be infected in some cases by inoculation in certain sites: inoculation into the anterior chamber of the eye produces keratitis and iritis; intratesticular injection leads to a syphilitic orchitis; and inoculation of the skin of the scrotum may set up a chancre-like sore. Metastatic lesions may succeed the primary infection.

Inoculation of mice produces no lesions, and though infection takes place it is symptomless and apparently latent.

Occurrence.—In the primary stage, spirochaetes are present in large numbers in the chancre and in the exudate from it, but as the sore tends to heal they become less numerous, and may not be demonstrable in the exudate. They are present also in the lymph glands.

In the secondary stage, spirochaetes have invaded the blood stream and become widely distributed in the body. They are present in the roseolar skin lesions, mucous patches and condylomata, and have been demonstrated even in the blood.

In the tertiary stage, they are less easily demonstrated in lesions, but can be observed by suitable staining methods in the periphery of gummatæ, in arterial lesions, etc.

In general paralysis of the insane, *Tr. pallidum* has been demonstrated in the cerebral cortex.

In congenital syphilis, spirochaetes are found in certain internal organs, e.g. liver, often in very large numbers. They are present

also in the skin lesions, the blood, and the mucosa of the intestine and bladder, and have been demonstrated in the placenta.

LABORATORY DIAGNOSIS OF SYPHILIS

In the primary stage, when there is an ulcerated sore, *Tr. pallidum* can usually be demonstrated in the serous exudate from the lesion. The dark-ground illumination method is the most suitable technique for the purpose, and provides a convenient means of rapid diagnosis. Failing this, one of the staining methods can be used.

Tr. pallidum is recognised by its morphological features and movements, and must be carefully differentiated from other spirochaetes found in ulcerating sores, e.g. *Tr. gracile*, etc. (*vide infra*).

Obtaining a specimen of exudate from a syphilitic sore for microscopic examination.—The serous exudate should be obtained from the tissue, and should not include surface organisms, as other spirochaetes which may be confused with the *Tr. pallidum* are sometimes present. The presence of excessive numbers of red blood corpuscles in the specimen is also to be avoided, as they tend to obscure the spirochaetes. If a local antiseptic has been used, it may not be possible to find spirochaetes until a wet dressing of gauze, soaked in sterile saline-solution, has been applied to the sore for twenty-four to forty-eight hours. It is to be noted also that antisyphilitic treatment, initiated before the examination, diminishes the likelihood of successful microscopic diagnosis. The sore is cleansed with a swab soaked in warm saline solution, and the margin is then scraped lightly with some blunt instrument to abrade the superficial epithelium. On squeezing the base of the chancre, serum exudes, and if blood-stained, should be removed with dry gauze until clear exudate can be obtained. Some of this is then collected in one or two capillary tubes. Both ends of the tube are sealed in a flame, and the specimens are submitted for examination.

When the primary sore is in process of healing, microscopic examination of the exudate may yield negative results. At this stage, spirochaetes may be found in the fluid aspirated from the lymph glands by means of a syringe. The dark-ground microscopic examination of exudates must be repeated on at least three occasions at daily intervals before reliance can be placed on a negative finding.

In the secondary stage, spirochaetes may also be demonstrated in the serous exudate from the skin eruption, and from mucous

patches, etc. Exudate can be obtained from the skin eruption by scarifying and "cupping" with a test-tube.

After about two weeks from the onset of the primary sore, the *Wassermann reaction* can be employed for diagnosis. The reaction becomes progressively more pronounced with the advance of the disease, and is markedly positive in the secondary stage.

Owing to the fact that the reaction may be slow in developing, if at first a negative result is elicited in the primary stage, it is essential to repeat the test before excluding syphilis. A negative reaction in a case of suspected secondary syphilis is highly significant in excluding syphilitic infection, but in supposed latent or tertiary cases a negative result does not exclude the disease.

In cerebro-spinal syphilis both the blood and spinal fluid should be tested.

The *Flocculation reaction* may, for routine purposes, be substituted for the Wassermann test, and is simpler to carry out; but as the two reactions do not in all cases run parallel, the maximum amount of information on the serological state of a patient is obtained by applying both tests; moreover, one serves as a check on the other, and though the presence of one of these reactions, even in the absence of the other, may be of diagnostic significance, such discrepancy indicates the need for repeated testing and the use of the Kahn verification test as referred to on pp. 331, 332.

The possible occurrence of the Wassermann and flocculation reactions in diseases and conditions other than syphilis must be borne in mind, particularly when the reactions are used for diagnosis in tropical and subtropical regions. Yaws, bejel and pinta (pp. 714, 715) are due to spirochaetes closely related to *Tr. pallidum*, and positive reactions are to be expected in such conditions. Positive reactions have been recorded in spirochaetal relapsing fever, typhus, tropical ulcer and trypanosomiasis. The reactions are also present in a considerable proportion of cases of leprosy, especially of the nodular type. In malaria, positive reactions are sometimes present, though they are, as a rule, quantitatively weak and become weaker on repeated testing, disappearing within three months. During pregnancy the serum may give a weakly positive reaction which gradually diminishes in intensity on repeated testing and disappears after the birth of the child. Other conditions in which reactions have been recorded are scarlatina, lymphogranuloma inguinale, infectious mononucleosis, respiratory tract infections and primary atypical pneumonia, while smallpox vaccination, the administration of tetanus toxoid and foreign serum have been found to cause temporary positive reactions. Blood donation has been reported as responsible for such reactions in the donor.

Various other conditions have been reported as causative of positive reactions, but the evidence is inconclusive; and of course it must always be remembered that the occurrence of a positive reaction in a particular disease may in reality be due to associated or perhaps latent syphilis.

Positive reactions in conditions other than syphilis and closely related diseases are classified as "non-specific", "false positive" or "biologic" reactions. In most such cases the reaction is a weak or doubtful one and may pertain to one of the tests, Wassermann or flocculation, not to the other; on repeated testing it often shows quantitative variation or progressive weakening, and disappears within three months. Careful correlation with the clinical history and condition is essential in the interpretation of weak or doubtful reactions and especially when there is discrepancy between the results of the Wassermann and flocculation tests. The Kahn verification test has proved of value in such cases and should be used when there is any doubt; often this test clearly indicates a "syphilitic" on the one hand, or a "biologic" reaction on the other, though even this method may yield inconclusive results.

There is a second antibody which appears in the serum during the course of a syphilitic infection; it reacts specifically with *Tr. pallidum* or protein extracts of it, and is quite distinct from the antibody ("reagin") which unites with the lipoidal antigens of the Wassermann and the various flocculation reactions. The specific antibody develops more slowly than the Wassermann "reagin" and persists after the latter is no longer demonstrable. The following are two of a number of methods devised to demonstrate this antibody with the purpose of improving the specificity of the serological diagnosis of the treponematoses.

1. The Treponema Pallidum Immobilisation (T.P.I.) Test
has been studied the most extensively and results obtained with it accord closely with the clinical evolution of the disease.¹ Dilutions of the serum to be tested are mixed with a concentrated suspension of motile *Tr. pallidum* obtained from syphilomas in the testes of inoculated rabbits; an appropriate amount of fresh complement is then added and after incubation, the test is read by determining under the dark-ground microscope the proportion of spirochaetes which have been immobilised. This proportion is then compared with a similar estimate of the spirochaetes immobilised by normal control sera.² The test is complicated to perform, expensive in animals and reagents, and extremely time

¹ Wilkinson, A. E., & Sequeira, P. J. L. (1955), *Brit. J. vener. Dis.*, **31**, 43;
Wilkinson, A. E. (1957), *ibid.*, **33**, 25.

² Nelson, R. A., & Mayer, M. M. (1949), *J. exp. Med.*, **89**, 369.

consuming. For these reasons it can not be used as a routine test, but is reserved for specially selected cases.

In early syphilis the test has no advantage over conventional tests and in fact is of little value in suspected primary or secondary syphilis. In the later stages of syphilitic infection it affords strong confirmatory evidence of infection. The results of the test are particularly valuable in the verification or otherwise of possible false positive conventional tests. The main use of the test is in clarifying the problems which arise when positive or doubtful Wassermann and Kahn reactions are reported in patients who have no clinical sign of syphilis. The test is also valuable in cases suspected of latent or late syphilis in whom the conventional tests are negative.

2. The Treponema Complement Fixation Test employs an antigen prepared either from disintegrated *Tr. pallidum* (Nichols pathogenic strain) as in the method of Price and Whelan¹ or from a suspension of non-pathogenic spirochaetes of the Reiter strains obtained from cultures by the technique of Wilkinson.² Extracts containing spirochaetal proteins are also used as antigens.

Treponemal complement fixation tests have been in use too short a time for a full appraisal of their value to have been made. Nevertheless, the results that have been obtained accord well with those of the Treponema Immobilisation tests and there is the promise that the method will offer a higher degree of specificity than the Wassermann reaction. Since the antigens are stable for long periods and less complicated to prepare, it is to be hoped that the test will become a practical procedure for routine work in many laboratories.

TREPONEMA PERTENUE

The causative organism of Framboesia or Yaws, a tropical disease pathologically resembling syphilis, though differing in its contagious and non-venereal character, and in its clinical manifestations.

In morphology it is practically identical with the *Tr. pallidum*. When first described it was regarded as more slender than *Tr. pallidum*—hence the designation.

Its occurrence in lesions corresponds to that of the *Tr. pallidum*, and the diagnosis of the infection is carried out as in syphilis,

¹ Price, I. N. O., & Whelan, M. J. (1953), *Brit. J. vener. Dis.*, **33**, 18.

² Wilkinson, A. E., & Sequeira, F. J. L. (1955), *Brit. J. vener. Dis.*, **31**, 43.

by demonstrating the spirochaete in the papules or ulcers. The Wassermann reaction is positive.

The spirochaete is transmitted by contact, invading through abrasions of the skin, but it has been suggested that it may also be conveyed by insects. It has been found in Jamaica that a fly, *Hippelates pallipes*, feeds on the exudate of sores, and the spirochaetes persist in the diverticulum so that they are regurgitated when the insect again feeds, for example on a skin abrasion.

The infection is experimentally transmissible to monkeys and rabbits as in the case of syphilis. Certain differences have been noted in the experimental lesions as compared with those produced by *Tr. pallidum*. Penicillin therapy heals the lesions of primary and secondary yaws very rapidly.

Bejel, a disease of Arabia, is another non-venereal disease which is either related to syphilis or is a modified form of that disease.

Treponema carateum

This organism, which is indistinguishable morphologically from *Tr. pallidum*, is associated with a skin disease of the West Indies, Central and Tropical South America, often named "Pinta". The skin lesions are characterised by their pigmentation, grey, bluish-grey or pinkish, but eventually become white. The spirochaete is demonstrable in the lesions and lymphatic glands. The disease has been produced experimentally in the human subject. The Wassermann reaction becomes positive as the condition progresses. The disease responds to treatment with penicillin in the same dose and in the same manner as yaws and syphilis.

OTHER TREPONEMATA

Treponema calligyrum (or *gracile*).—This organism may occur in the secretions of the genitals, and morphologically resembles *Tr. pallidum*. Its differentiation from the latter is therefore of practical importance in syphilis diagnosis. It is not usually found if care has been taken to obtain serum from below the surface of the chancre (p. 711). It is thicker than *Tr. pallidum* and its spirals are shallower; by the dark-ground illumination method it appears "glistening", whereas *Tr. pallidum* is "dead white"; it stains more readily than *Tr. pallidum* by Giemsa's method.

Treponema genitalis, which is very similar to *Tr. pallidum*, has also been described as a commensal on the genital mucosa.

Treponema microdentium.—This organism flourishes in carious teeth, and may be found in the secretion between the teeth. It closely resembles *Tr. pallidum* in morphology, but is shorter (3–10 μ), and the coils are shallower. It is more easily stained by the ordinary methods than *Tr. pallidum*.

Treponema mucosum.—Similar to *Tr. microdentium* in morphology, but is stated to have the property of producing a mucin-like substance.

Treponema macrodentium.—Occurs in the mouth like *Tr. microdentium*. It bears some resemblance to *Tr. pallidum*, but is larger and thicker, with larger and less regular coils, usually two to eight in number. Its motility is also more active. It is more easily stained than *Tr. pallidum* and is coloured blue by Giemsa's method.

Treponema cuniculi.—Associated with an infectious disease of rabbits, which usually takes the form of a chronic local and superficial infection of the genitals. The spirochaetes can be demonstrated in the exudate from the lesions and in tissue sections. They are morphologically identical with *Tr. pallidum*.

LEPTOSPIRA ICTEROHAEMORRHAGIAE

This leptospire is representative of a large group of organisms which cause leptospirosis in man and animals and which are indistinguishable from one another, both morphologically and culturally. Typically, the infection in man occurs as a haemorrhagic jaundice (Weil's Disease); but a febrile anicteric syndrome is common; benign meningitis is sometimes the main syndrome.

Morphology and Staining.—A leptospire about 7–14 μ long by 0·1 μ broad. The coils are very numerous and so small and closely set together that they are difficult to demonstrate in stained preparations, though quite obvious by dark-ground illumination. In addition to these "elementary" spirals larger "secondary" coils may be seen, especially in stained films. Hooked ends are a characteristic morphological feature. Leptospires have a single, straight and somewhat rigid central axistyle around which is wound the cytoplasm of the spirochaete; the whole is contained within a clearly defined cell wall. The terminal hooks, where the spirals are looser, may be the site of some alteration of the influence of the axistyle. Active movement is observed in fresh preparations examined with the dark-ground microscope. The movement is mainly rotary but the organisms are also seen to glide rapidly across the field with either end foremost, occasionally bending and straightening again into the rigid form so characteristic of the genus.

The organisms can be stained by Giemsa's solution (as in the case of *Tr. pallidum*); the silver impregnation methods of Levaditi and Fontana also give satisfactory results.

Cultivation.—Leptospires are readily cultured provided they

are supplied with animal serum in the medium. Rabbit serum is usually employed but guinea-pig, sheep and cattle sera may be equally satisfactory provided no natural antibodies are present (it is advisable to test serum for the presence of leptospiral antibodies before incorporating it in the medium). For the primary isolation of leptospires and for maintaining stock cultures of the various serotypes for serological work, Stuart's and Korthof's media are recommended (p. 239). A semi-solid medium such as Dinger's modification of Noguchi's medium is useful for maintaining stock cultures. The constituents of this medium are as follows: tap water, 100 ml.; agar (3 per cent.) 6 ml. After sterilisation 10 per cent. inactivated rabbit serum is added. It has the advantage of evaporating less rapidly than fluid media and is thought to maintain the virulence of the organisms longer, since subculturing need not be done so frequently. Recently, solid culture media on which single colonies of leptospires can be obtained have been devised by Cox and Larson¹ and by Kirschner and Graham². Leptospires grow best between 28° C. and 32° C. but may also grow satisfactorily at 25° C. For primary isolation from animal tissues incubation at 37° C. is recommended followed by transfer to the lower temperature when growth is established.

Viability.—Unlike some saprophytic leptospires it is unlikely that the pathogenic serotypes multiply much outside the animal body although they may survive for many days if the external conditions are favourable. They require moisture for their survival and since they are particularly susceptible to acid, they seldom remain viable for long in localities where the pH of the water is less than 6.8. Salt water has a deleterious effect. They die out rapidly in acid urine, in sewage and in badly polluted water. They are susceptible to heat: 10 min. at 50° C. or 10 sec. at 60° C. kills them. They may survive for a time in infected animal tissue provided it is kept at a low temperature; thus guinea-pig liver has remained infective for up to 26 days at 4° C. and for 100 days at -20° C. They are rapidly dissolved by bile and bile salts and also by trypsin. Penicillin, streptomycin and the tetracyclines are moderately effective in cultures and in experimental infections and may have value as therapeutic agents in man if given early in the infection.

Occurrence.—The rat is the natural host of *L. icterohaemorrhagiae*. Most other types of pathogenic leptospires are carried by various species of wild rodents (although in some cases, other animals, e.g. dogs and pigs appear to act as the natural

¹ Cox, C. D., & Larson, A. D. (1957), *J. Bact.*, **73**, 4, 587.

² Kirschner, L., & Graham, L. (1959), *Brit. J. exp. Path.*, **40**, 57.

animal host). Each type has apparently a host of predilection, e.g. *L. icterohaemorrhagiae* is carried by the brown rat (*Rattus norvegicus*) and *L. hebdomadis* (the cause of seven-day fever of field workers in the Far East) by the field mouse (*Microtus mosebelloii*). The leptospire is usually well adapted to its host. It localises in the kidneys where it colonises the convoluted tubules without apparently causing any harmful effects. Periodically leptospires are shed in large numbers in the urine and in this medium may be transferred to more susceptible animals and to man. The organisms probably penetrate the skin and mucous membranes through cuts and abrasions. Certain occupations predispose to infection, e.g. workers in wet coal mines, sewage workers, fish curers, etc. are particularly liable to infection by *L. icterohaemorrhagiae* since the conditions in which they work frequently encourage rat infestation, while moist conditions allow the leptospires to survive for a time outside the animal body, and cuts and abrasions of the skin resulting from heavy manual work allow the organism easy access to the blood-stream.

Leptospiral infection may follow bathing or accidental total immersion in stagnant ponds, canals or rivers polluted by rodents and in these cases the organisms may penetrate the mucous membranes of the eyes and nasopharynx. Instances of infection through the consumption of contaminated food and water have also been reported.

Agricultural workers especially those engaged in work in the fields, e.g. rice-field workers, sugar-cane cutters, etc., are particularly liable to infection derived from the urine of rodents; in fact, this group of workers provides the highest proportion of all cases. *L. icterohaemorrhagiae* may infect dogs, especially those kept in kennels which are exposed to contamination by rats. The infection may produce a jaundiced condition known as "yellows" or a non-icteric disease characterised by severe nephritis and uraemia. Both forms have a high fatality rate. Man may become infected through handling sick animals. A similar condition has been reported in young pigs.

Saprophytic leptospires similar in morphology to the pathogenic serotypes are frequently present in water from taps and natural sources. They are referred to as *L. biflexa*. They differ from the pathogenic serotypes culturally and serologically.

Laboratory Diagnosis

Because of variability in the severity of the infection and the frequent absence of jaundice, leptospirosis should always be

considered in cases of undiagnosed pyrexia when the patient is likely to have been exposed to infection either through the conditions of his work or from some other cause (*vide supra*).

When attempting a laboratory diagnosis of suspected leptospirosis, the following points should be borne in mind:—(a) During the first week of illness, leptospires are present in the blood, but leptospiraemia is rare after the eighth day. The organisms may be demonstrated in the blood by dark-ground microscopy and by isolating the organism by direct culture or by animal inoculation. (b) Antibodies may generally be detected in the blood serum towards the end of the first week (although their production is occasionally delayed for longer periods) and increase in amount during the second and third weeks, after which they begin to decline. Residual amounts, however, may remain for many years after an infection. It is advisable to examine a specimen of serum during the early days of the illness and at 4 to 5 day intervals thereafter, since a rise in titre is not only highly significant but necessary to eliminate the possibility that the reaction may be due to either residual antibodies or to non-specific factors of normal serum sometimes present in low titres and also to elucidate paradoxical reactions in which the titres of heterogeneous antibodies may at first exceed those of the homologous ones. (c) Leptospires may be present in the urine during the second week of the illness and continue to be excreted for some 4 to 6 weeks after the onset (infrequently for longer periods). They are more readily detected during the second and third weeks than later. They may be seen by dark-ground examination of untreated urine and isolated by animal inoculation. Direct culture is not usually successful. Since leptospires are very sensitive to acid urine and may be lysed by antibodies present in the urine, the urine should be examined immediately after being voided and animal inoculation should be carried out as urgently as possible. To prevent acidity the patient should be given enough alkali by mouth to make the urine alkaline, provided this is clinically permissible.

(1) *Examination of Blood by Dark-ground Microscopy*.—During the first week leptospires may be detected by dark-ground microscopic examination of untreated blood. Only a small percentage of cases of leptospiraemia are likely to be detected in this way, but the technique of differential centrifugation of Ruys¹ may enhance the chances of seeing the organisms and thereby make an early diagnosis possible.

(2) *Cultivation*.—Bijou bottles containing 3 ml. fluid culture

¹ See ref. 1 footnote, p. 720.

medium are inoculated with 5 or 6 drops of the patient's blood, strict attention being paid to aseptic technique (leptospires will not grow in the presence of contaminants). Alternatively, the deposit after differential centrifugation¹ may be re-suspended in 2.0 ml. phosphate buffered saline (pH 8.1) and a few drops of it used to inoculate 4 to 6 bottles of culture medium. Daily culturing of the blood during the first few days of onset considerably enhances the chances of isolating the organisms.

(3) *Animal Inoculation.*¹—Laboratory animals, usually guinea-pigs (and hamsters if available), are inoculated intraperitoneally with whole blood during the first few days of the illness or with freshly voided urine at a later stage. Three days after inoculation and daily thereafter peritoneal fluid is withdrawn with a finely drawn-out Pasteur pipette introduced into the lower part of the abdomen while the animal is held in an upright position with stretched hind legs. As soon as leptospires are detected microscopically in the peritoneal fluid where they tend to localise during the early stages of infection, blood is withdrawn by cardiac puncture and a few drops introduced into several bottles of culture medium. Guinea-pigs are very susceptible to *L. icterohaemorrhagiae*, whereas the golden hamster (*Cricetus auratus*) is more susceptible than the guinea-pig to *L. canicola*. They should be used when about 6 weeks old, since older animals may be more resistant. In typical cases, the inoculated animals die in 8 to 12 days with jaundice, haemorrhages in the lungs, under the serous membranes and in the muscles.

With a suspension of the organism and its homologous antiserum prepared by immunising a rabbit with a living culture (see p. 325) an attempt can be made to identify a newly isolated strain as far as possible by comparing it with stock serotypes and antisera available in the laboratory by agglutination and agglutinin-absorption tests. The procedure of identification may be a lengthy one and require the use of stock antigens and antisera not available in the average diagnostic laboratory. In such cases, or whenever there is doubt about the identity of a newly isolated strain, it is recommended that help should be sought from one of the WHO/FAO Leptospirosis reference laboratories. For the addresses of these laboratories see WHO Report, p. 169.²

Serological Diagnosis.—Two methods are described below for carrying out a diagnostic serological investigation of the patient's serum.

¹ Wolff, J. W. (1954). *The Laboratory Diagnosis of Leptospirosis*. Springfield, Illinois, C. Thomas.

² World Hlth Org. Techn. Rep. Ser. (1959), 169.

1. *Agglutination-Lysis Test.*—The following technique is based on the standard procedure used in laboratories throughout the world and first developed by Schuffner (see Wolff, 1954, footnote p. 720).

For the test well-grown cultures of leptospires in Korthof's or Stuart's media are used. They should be from 7 to 10 days old and uniform in suspension. Separate tests are set up against each serotype likely to be responsible for the case under investigation. Dilutions of the patient's serum are made by the dropping method either in tubes or in depressions in porcelain plates. The procedure is summarised as follows:

Tube No.	(1)	(2)	(3)	(4)	(5)	(6)
First row:						
Saline	8	9	9			drops
Serum	2	1,	1,			drops
	from (1) from (2)					
Dilution of serum	1/5	1/50	1/500			
Second row:						
Culture	3	3	3	3	3	3 drops
Saline		2		2		2 drops
Serum 1/500					3	1 drops
Serum 1/50			3	1		drops
Serum 1/5	3	1				drops
Final dilution:	1/10	1/30	1/100	1/300	1/1000	1/3000

The mixtures are incubated at 32° C. (or 37° C.) for 3 hours and allowed to stand at room temperature for 1 hour before being read. Alternatively, if specimens are received late in the day the test mixtures are kept in the refrigerator (4° C.) overnight and read the following morning. Place a drop from each tube on a slide and examine with a 16 mm. objective using dark-ground illumination (p. 98). Wolff advocates the use of water instead of immersion oil between the condenser and the slide. It gives quite adequate illumination and is much cleaner than oil. It is not necessary to place a cover-slip over the drop and if each drop is quickly examined consecutively a large number may be included on one slide. Both agglutinating and lytic antibodies are detected by this test. Agglutination is more obvious in the lower dilutions and lysis in the higher. Lysis is indicated by a reduction in the number of live leptospires present in the serum-antigen mixture when compared with a non-serum control.

2. *Agglutination Test (Broom).*—This involves essentially the same technique as the agglutination-lysis test except that the

cultures are killed by adding formalin to give a final concentration of 0·2 per cent. The formalin should be neutralised with magnesium carbonate, since any traces of formic acid will cause non-specific agglutination.¹ The serum-antigen mixtures are kept in the refrigerator overnight (not incubated) before being examined for agglutination. Lysis does not occur. Killed antigens are more convenient and safe for routine work and stock suspensions of various serotypes may be stored until required. They have the disadvantage of tending to be unstable and liable to clump spontaneously. Titres of agglutination are slightly lower than those attained with living cultures.

Diagnostic Titres.—Since many serotypes are related serologically (e.g. *L. canicola* and *L. icterohaemorrhagiae* have common antigens) there may be a certain amount of cross-reactions between the strains used in one test. Titres of 1/300 and 1/1000 for a particular strain may be significant (they may rise to 1/30,000 or higher). Bearing in mind the possibility of non-specific agglutination and residual antibodies (*vide supra*) the significance of a single positive titre must remain in doubt unless a rising titre can be demonstrated.

A control must be included in both methods.

Other serological tests may also be employed but have certain disadvantages. In the *erythrocyte sensitisation test* (Chang and McComb, 1954),¹ human red blood cells are treated with an ethanol extract of leptospires and are rendered agglutinable by some serum antibodies. In the *sensitised erythrocyte lysis test* (Sharp, 1958),² the addition of complement to this system causes the lysis of sensitised cells. The latter test is the more sensitive, but in both a positive reaction is obtained with antigens prepared from all the leptospiral serotypes. The main value of these tests is in screening large numbers of sera such as those requiring examination for a serological survey. They are also of value as an additional test in distinguishing a specific response from an anamnestic reaction in the agglutination-lysis test. Complement-fixation tests have also been devised, but there is much difficulty in standardising the antigens employed.

Examination of Rats for Leptospiral Infection

Carcasses of rats dead only for even a few hours are unsatisfactory for examination. Whenever possible the live animal should be sent to the laboratory. It is then possible to anaesthetise the

¹ Chang, R. S., & McComb, D. E. (1954), *Amer. J. trop. Med. Hyg.*, 3, 481.
² Sharp, C. F. (1958), *J. Path. Bact.*, 76, 349.

animal and to obtain blood by cardiac puncture for examination by the methods described (p. 719). Indirect evidence of infection can be obtained by the agglutination test with serum; titres of 1 in 100 may indicate early infection. A suspension of the ground-up tissue of both kidneys should also be examined by both cultivation and animal inoculation methods.

In screening rats and other rodents for leptospiral infection satisfactory results have been obtained by inoculating culture media with small particles of kidney tissue punched out with a sterile Pasteur pipette after the surface of the kidney has been seared with a red-hot scalpel blade.

Examination of Water for Pathogenic Leptospires

This can be done by immersing a shaved and scarified area of skin of a young guinea-pig in the water for an hour at 30° C. Infection takes place through the skin with the resulting characteristic condition as described above.

CANICOLA FEVER

Leptospira canicola is closely related antigenically to *L. icterohaemorrhagiae*, but may be distinguished from it by serological tests. It is the cause of a common infection of dogs characterised by nephritis often chronic in nature and rather inconstantly by a variable degree of jaundice. The leptospires are excreted in large numbers and may invade through abrasions in the skin of people whose hands became contaminated with dog urine. Jackals and pigs may also harbour the organism, and piggery workers have contracted the infection through handling infected pigs.¹

Canicola fever in man is one of the milder forms of leptospirosis, in which meningeal symptoms predominate. Jaundice is only occasionally produced and then only in a slight degree. As with most forms of leptospirosis, renal involvement is a common feature, but the symptoms vary considerably in their intensity. The disease is rarely fatal. Laboratory diagnosis as for Weil's disease.

Other Pathogenic Leptospires

Over fifty different pathogenic leptospires have been identified, many of which are associated with disease in man. Individual members of the genus tend to differ in the degree of their patho-

¹ Coghlan, J. D., Norval, J., & Seiler, H. (1957), *Brit. med. J.*, **1**, 257.

genicity to man and animal and in their natural hosts, but the only reliable method of classifying them is based on their serological differences demonstrated by agglutination and agglutinin-absorption tests.

In Great Britain only two serotypes of leptospires have so far been isolated from human infections, viz. *L. icterohaemorrhagiae* and *L. canicola*, but recently other pathogenic serotypes have been demonstrated, i.e. *L. ballum* and strains related to *L. sejroe* and *L. saxkoebing*, which were found to be carried by wild rodents.¹ Hedgehogs too have been found to be infected by various serotypes.

The following exemplify the various serotypes which have been recognised in different parts of the world.

Leptospira hebdomadis.—This organism is responsible for "Seven-day fever" of the East, which is a non-icteric febrile illness with meningitis. It is carried by a field-mouse (*Microtus montebelloi*) and consequently, field workers are liable to the infection through becoming contaminated with infected mouse urine.

Leptospira autumnalis.—This organism has been found associated with a disease in Japan called Akiyami or harvest sickness clinically indistinguishable from "Seven-day fever". *L. autumnalis* can be distinguished from *L. hebdomadis* by its high infectivity to guinea-pigs, in which it produces typical haemorrhagic jaundice. It is carried by certain species of field-mice and rats. "Fort Bragg fever", which occurred among troops in North Carolina, U.S.A., was found by serological tests to have been caused by this serotype.

Leptospira grippotyphosa has been described as the cause of "Swamp fever" of Europe and certain parts of Asia, Africa, Israel and U.S.A. It usually attacks agricultural workers and produces a relatively mild illness resembling canicola fever with a low mortality rate. Various species of voles carry the organism. In the U.S.S.R. and Israel, cattle have been seriously affected.

Leptospira pyrogenes produces a febrile illness with or without jaundice and varying in its severity. It occurs among field workers in Indonesia and other parts of the Far East. Certain species of rats appear to act as reservoirs of infection.

Leptospira australis A and B.—These organisms are the causal agents of "Cane fever" in North Queensland. Sugar-cane workers are mainly affected, but *L. australis B* may also infect urban dwellers. The illness is comparatively mild but con-

¹ Broom, J. C., & Coghlan, J. D. (1958), *Lancet*, 2, 1041.

valescence is protracted. Lymphadenitis is a common feature. Certain species of rat are the carriers of the organism.

Leptospira pomona (syn. *L. suis*).—This organism was first isolated from cases of "Seven-day fever" among dairy farmers in North Queensland. It was later found to be the cause of "Swineherds'" disease in Switzerland. Pigs act as the reservoir hosts and usually suffer little effect; cattle are susceptible, especially calves and pregnant cows. In the U.S.A. the infection causes a heavy yearly loss of cattle due to jaundice and haemoglobinuria of calves and abortion of cows and pigs. The organism has been isolated in many parts of the world from human and animal sources. Certain species of field-mice may also act as carrier hosts.

Leptospira bataviae.—This organism causes leptospirosis of rice-field workers in Italy, where the field-mouse (*Micromys minutus sorcinus*) is the reservoir host. The disease in that part of the world is comparatively mild and jaundice is rare. In Indonesia, however, where the chief carrier host is the brown rat (*Rattus norvegicus*) cases are much more severe, jaundice is common and death may occur.

Leptospira sejroe was first observed in human infection in the island of Sejroe (Denmark). It has also been recorded in other parts of Europe. The disease is relatively mild. Certain rodents are carriers of the organism, and related strains have been isolated from rodents in Great Britain.

BORRELIAE

These are large, motile, refractile spirochaetes (about 10–30 μ by 0·3–0·7 μ) with irregular wide and open coils, which are relatively few in number. They are easily stained by the ordinary methods, and are Gram-negative. Some occur as commensals on various mucous membranes, e.g. mouth, and in gangrenous and ulcerative conditions on the surface of the body, the mouth and throat, and the genitals. This type of organism may also be found in the surface exudate of a syphilitic sore, and has to be differentiated morphologically from the *Tr. pallidum*. Other *Borreliae* are pathogenic and cause relapsing fever in man and the disease of fowls known as "spirochaetosis".

Borrelia buccalis is found in the healthy mouth; it is sluggishly motile with wavy serpentine movements.

Borrelia refringens occurs on the normal mucous membranes of the genital and anal regions. It is actively motile with lashing and rotating movements; under the dark field microscope it is refractile and appears brighter than other spirochaetes.

BORRELIA VINCENTI

Borrelia vincenti occurs in a pseudo-membranous condition of the throat or of the mucous membrane of the dental gum margins—Vincent's angina. It resembles *Borr. refringens*, but is sometimes described as smaller ($5-25 \mu$) and more delicate.

Under the electron microscope *Borr. vincenti* is seen to have a clear-cut cell wall within which some ten axial filaments are seen twisted spirally around the protoplasm of the body of the spirochaete.

Borr. vincenti is generally associated with a large fusiform bacillus—*Fusobacterium fusiforme* (p. 699). Films from the throat secretion, stained by dilute carbol fuchsin or methyl-violet, show large numbers of spirochaetes and fusiform bacilli.

A similar spirochaetal infection associated with *Fusobacterium* occurs in gangrenous balanitis, ulcerative stomatitis and gingivitis, and a chronic ulceration of the skin in tropical countries (tropical ulcer). The same type of infection has also been observed in putrid bronchitis and empyema, gangrene of the lung and pulmonary abscess.

Such infection by *Borr. vincenti* and *Fusobacterium* is generally associated with necrosis of tissue, pseudo-membrane formation and a putrefactive odour. Streptococci and vibrios are found frequently in close association with Vincent's organisms. Both spirochaete and fusiform bacillus are found constantly together in the lesions, but their exact rôle in the evolution of the disease remains uncertain. They may be primary pathogens in some circumstances and in others merely be secondary invaders in tissues damaged by some other disease process. *Borr. vincenti* is a strict anaerobe and can be cultivated in vaseline-sealed tubes containing infusion broth enriched with ascitic fluid. It grows abundantly in mixed primary cultures, but can be propagated in pure culture only with difficulty.

Borr. vincenti is highly susceptible to penicillin, and this antibiotic may be used locally in the treatment of Vincent's infection. The organisms are also sensitive to the tetracycline group of antibiotics.

BORRELIA RECURRENTIS (OBERMEIERI)

The causative organism of European Relapsing fever.

Morphology and Staining.—This organism is a spiral filament, cylindrical or flattened, with tapering ends, varying in length, as



a rule, from 10 to 20 μ , and about 0.3–0.5 μ broad, with about five to seven fairly regular coils 0.9–1.7 μ in amplitude. Active motility of a rotatory or oscillating type is noted in fresh preparations. Multiplication is by transverse fission. The structure of the organism as it is seen under the electron microscope is that of a bundle of some twelve filaments twisted spirally around the spirochaetal body external to the cell wall. These filaments are similar to those seen in *Tr. pallidum* and are probably concerned in the contractile movements of the organism. They are rather easily displaced during the manipulations of staining and may resemble flagella, for which at one time they were mistaken. The whole spirochaete is covered by a layer of slime-like material to a thickness of about 0.08 μ .

This spirochaete stains readily with Romanowsky stains (*e.g.* Leishman's), and may exhibit uniform staining or beading. It can be stained also with carbol fuchsin, and is Gram-negative. In fresh preparations of blood it can be seen with the ordinary microscope, but dark-ground or preferably phase-contrast illumination is more suitable for its demonstration in the living state. Silver impregnation methods may also be used for demonstrating the spirochaete in films or tissues (p. 136, 137).

Cultivation.—Artificial cultures were first obtained anaerobically in Smith-Noguchi medium, citrated blood containing spirochaetes from an infected animal, *e.g.* a white rat, being used as the inoculum.

Cultures have also been obtained in other media, but the organism does not readily adapt itself to artificial growth in the laboratory: (1) horse serum diluted with 2 parts of saline solution, and with 1 ml. of broth, containing 10 per cent. peptone, added to 10 ml. of the diluted serum; for subcultures, a drop of rabbit blood is also added; the medium is covered with a paraffin seal; (2) 20 per cent. rabbit serum with 80 per cent. Hartley's broth in tubes to each of which 1 gram of coagulated egg albumin is added; a vaseline seal is super-imposed and the cultures are incubated at 30° C.; (3) egg albumin is placed in a test-tube and coagulated by heat in the form of a slope (p. 247); 5 ml. of horse serum diluted 1:10 or rabbit serum diluted 1:5 are then added, the serum having previously been heated at 58°–60° C. for one hour; the medium is covered with a layer of sterile vaseline; before an inoculation is made, a drop of fresh rabbit or human blood is added.

Occurrence.—The organism is present in the peripheral blood during the pyrexial stage of the illness, and can be detected in blood films. When defervescence occurs it disappears from the

blood, but may still be present in considerable numbers in the spleen, where it is phagocytosed by large mononuclear cells.

It is transmitted from person to person by the body louse, *Pediculus humanus* var. *corporis*. After this insect has sucked blood from the infected individual the organisms are demonstrable in the stomach for a day, and then disappear. They reappear after about six days in the body cavity and become widespread throughout the body of the insect. Infection results either through the contamination of the bite-wound with the infective excreta of the louse, or by the crushing of the infective lice with the fingers in the act of scratching and by the simultaneous inoculation of the abrasions.

Experimental Inoculation.—Monkeys, white mice and white rats can be infected experimentally by subcutaneous injection of blood from a case of relapsing fever. The guinea-pig is not susceptible.

Borr. recurrentis is sensitive to penicillin and also to the tetracyclines.

Borrelia duttoni

The organism of West African Relapsing fever (African tick fever).

This organism is morphologically similar to *Borr. obermeieri*, but represents a separate species. Its distribution in the disease is also similar, but it is transmitted by ticks (*Ornithodoros moubata* and other species). Infection probably results from the contamination of the bite-wound by the infective excreta of the tick. It has been suggested that in the tick the organism goes through stages in a life cycle. Granules with the staining reactions of chromatin have also been observed in the spirochaete; these apparently separate from the spirochaete, and have been regarded as a phase in the life history of the organism. There is electron microscopical evidence that these granules contain coiled-up spirochaetes. It seems probable that they are formed under adverse physical conditions and that they represent a resting phase rather than a stage in reproduction. Such granules have been noted in the Malpighian tubules of infective ticks. Infectivity is transmitted from the female tick to a second generation.

Borr. duttoni is pathogenic to monkeys and certain laboratory animals (e.g. rat, mouse). It possesses a greater virulence for monkeys and other animals than *Borr. recurrentis*. Like *Borr. recurrentis*, *Borr. duttoni* is sensitive to penicillin and the tetracyclines.

Other Relapsing Fever Spirochaetes.—The originally described spirochaete of North American relapsing fever resembles

Borr. recurrentis, but has been regarded as a separate species on the basis of immunity reactions. It has been designated *Borrelia novyi*. It is louse-borne.

The organism of Indian relapsing fever also corresponds in its biology and pathogenesis to *Borr. recurrentis*. It has been named *Borrelia carteri*, but it is doubtful if the Indian strains can be differentiated from the European. This infection is also louse-borne.

Various specific names have been given to relapsing fever spirochaetes in different parts of the world, but it is questionable whether all these biological designations are justified.

Louse-borne spirochaetal relapsing fever, similar to the Indian form, occurs in various parts of Asia, but in Central Asia tick-borne relapsing fever is also present.

The common form of relapsing fever in North Africa is louse-borne. In tropical Africa the prevalent type is tick-borne (African tick fever, *vide supra*), though louse-borne infections occur in West Africa.

In the United States, Central and South America both louse-borne and tick-borne forms of the disease have been observed.

Immunity to the Relapsing Fever Spirochaetes.—Recovery from an attack is associated with the appearance of agglutinating and lytic antibodies in the blood serum, and in this way the general infection is temporarily checked, though spirochaetes may still persist in the internal organs. It would appear that the relapse is due to antigenic variation in the surviving spirochaetes. The variant strain uninfluenced by the antibodies produced towards the parent organisms is able to flourish and re-infect the blood. Multiple relapses, as in African relapsing fever, are apparently due to repeated antigenic variation. In Indian relapsing fever in which there are usually two attacks only, the relapse-strain transmitted experimentally to animals reverts to the serological characters of the original strain after producing a first attack in the animal.

DIAGNOSIS OF RELAPSING FEVER

During the pyrexial phases, the spirochaetes can frequently be demonstrated in the blood, but not during apyrexial intervals.

Thin or thick blood films are made as in malaria diagnosis, and stained by Leishman's method (pp. 130, 913).

Some workers prefer to stain the films with dilute carbol fuchsin.

If a drop of blood is mounted on a slide under a cover-slip and examined with the oil-immersion lens, the spirochaetes may be detected in the unstained condition and show active movement.

A more satisfactory method of demonstrating them, however, is by dark-ground, or phase-contrast illumination.

If spirochaetes are not detectable, inoculation intraperitoneally of white mice with 1·0–2·0 ml. blood drawn from a vein may reveal the infection, the organisms appearing in considerable numbers in the blood of the animals. A drop of blood from the tail of the inoculated animal is examined daily for a considerable period. An inoculum of 0·2 ml. of blood into the chorio-allantoic sac of the chick embryo may also be used.

Lice taken from a case can be examined for spirochaetes by keeping them in a test-tube for a day, then placing them in drops of distilled water on slides and piercing them with a needle so that the haemocele fluid becomes mixed with the water, which is then examined microscopically by dark-ground illumination. The spirochaetes can also be demonstrated in ticks by examining stained films from the stomach contents.

Borrelia theileri

This spirochaete is responsible for a blood infection occurring in cattle, sheep and horses in Africa. The disease is of a comparatively mild type. The organism appears as a spiral filament, 10–30 μ by 0·25–0·3 μ , and is actively motile when seen in fresh preparations of blood. It is transmitted by a tick (*Margaropus decoloratus*).

Borrelia anserina

This organism produces a general blood infection in geese and fowls. It can be seen in the blood of the infected bird both in unstained preparations and in films stained by a Romanowsky stain or dilute carbol fuchsin. It is a motile spiral organism, 10–20 μ in length by 0·3 μ in breadth, and exhibits several coils. Artificial cultures have been obtained in Smith-Noguchi medium. The disease is transmitted by ticks, e.g. *Argas persicus*, and a granular phase has been described analogous to that observed in the case of *Borr. duttoni* (*vide supra*). By experimental inoculation various species of birds may be infected, but mammals are not susceptible. Birds at the height of the infection can be cured by the use of penicillin.

SPIRILLUM MINUS

A causative organism of rat-bite fever. Though often described as a spirochaete, this organism conforms in its biological characters

to those of a spirillum, and the name *Spirillum minus* is generally used.

It is a short spiral organism about 2–5 μ in length and relatively broad, with regular short coils numbering one for each micron of the length of the organism. Longer forms up to 10 μ may also be observed. This organism is very actively motile, showing darting movements like those of a vibrio. Movement is due to terminal flagella, which are variable in number—from one to seven at each pole. In moving, the organism itself remains rigid and shows no undulation. It can be demonstrated easily by dark-ground illumination in fresh preparations, in which its active movement is seen and its flagella are also observed. It is most readily stained by a Romanowsky stain (e.g. Leishman's), but can also be stained by the ordinary aniline dyes. The organism has probably never been cultivated successfully.

In rat-bite fever the spirillum may be demonstrated in the local lesion, the regional lymph glands, and even in the blood, either by direct microscopic methods (*vide supra*) or by animal inoculation. Guinea-pigs, white rats and mice are susceptible to infection: the spirilla appear in the peripheral blood and can be detected by dark-ground illumination. Guinea-pigs develop a progressive disease and die of the infection. The intra-peritoneal inoculation of human infective material in mice is followed by no sign of disease; spirilla appear in the blood after five to fourteen days, but always in very small numbers. If the spirillum cannot be detected microscopically in the local lesion, or if the original bite-wound has healed, an enlarged lymphatic gland may be punctured by means of a hypodermic syringe; "gland juice" is aspirated, and investigated by direct methods or animal inoculation.

Spirillum minus occurs naturally in wild rats and certain other wild rodents, producing a blood infection. Conditions similar to rat-bite fever have also been reported following the bites of cats and ferrets.

It should be noted that at least two different specific infections may result from rat-bite and may be designated clinically "rat-bite fever": the condition due to *Spirillum minus* (described above) and that produced by *Streptobacillus moniliformis*, p. 666. The former presents a highly characteristic clinical syndrome: a relapsing febrile illness with a local inflammatory lesion, enlargement of regional lymph glands and a macular skin eruption, all these lesions fluctuating in parallel with the temperature. *Spirillum minus* has been demonstrated by direct examination in the local lesion and glands, and even in the blood of cases. Infection by *Streptobacillus moniliformis* is likewise an acute or subacute febrile

condition and may be associated with a skin eruption (e.g. erythema multiforme), but involvement of joints, even resembling the polyarthritis of acute rheumatism, is a feature of this illness. It seems likely that the two conditions have been confused with one another in the past.

Spirillum minus infections respond to treatment with the tetracyclines and penicillin.

CHAPTER XXXV

RICKETTSIAE; BARTONELLA

THE *Rickettsiae* occupy a biological position which is intermediate between that of the smaller bacteria and the larger viruses. They resemble the former in that they are visible with the light microscope, are known to divide by binary fission, and are susceptible to the action of antibiotics. It is, however, undisputed that, like the viruses, they are obligate intracellular parasites. A further distinctive property of rickettsiae is that they occur under natural conditions in the alimentary tract of such blood-sucking arthropods as lice, fleas, mites, ticks and bugs. Some have no relationship to human disease, but others when transmitted to unnatural hosts, such as man, cause severe diseases. The typhus fevers are due to infection by rickettsiae of various species and it is convenient to arrange these diseases according to the arthropod vectors which transmit them.

1. *Louse-borne typhus*

- | | |
|--|------------------------------|
| (a) Epidemic typhus | <i>Rickettsia prowazekii</i> |
| (b) Recrudescent typhus
(Brill's Disease) | <i>R. prowazekii</i> |
| (c) Trench fever | <i>R. quintana</i> |

2. *Flea-borne typhus*

- | | |
|-----------------------|---------------------------------------|
| Murine endemic typhus | <i>R. mooseri</i> (<i>R. typhi</i>) |
| Tabardillo | |

3. *Tick-borne typhus*

- | | |
|---|--|
| (a) Rocky Mountain spotted fever | <i>R. rickettsii</i> |
| (b) South African tick bite fever | <i>R. rickettsii</i> var. <i>Pijperi</i> |
| (c) Mediterranean fever
(fièvre boutonneuse) | <i>R. conorii</i> |

4. *Mite-borne typhus*

- | | |
|---------------------------------------|-------------------------|
| Scrub typhus
(Tsutsugamushi fever) | <i>R. tsutsugamushi</i> |
|---------------------------------------|-------------------------|

Morphology and Staining.—Cocco-bacilli measuring approximately $0.35\ \mu$ in length and $0.25\ \mu$ in width. They are usually described as Gram-negative though they are only faintly stained

by watery solutions of aniline dyes. They are, however, readily stained blue with Castaneda or Giemsa's stains (p. 140); with Macchiavello's stain (p. 141) they appear red against a blue background. Pleomorphism is frequent and the organisms may occur singly, in pairs or short chains or as filaments. *R. prowazekii* has a tendency to show slender filamentous forms whereas *R. mooseri* shows shorter and broader rod-like forms. Bipolarity with a suggestion of the presence of metachromatic granules is often evident in *R. tsutsugamushi*. In smears or sections of infected tissues, clumps and masses of rickettsiae are often seen lying within the cytoplasm of endothelial and serosal cells (*e.g.* in the cells lining the tunica vaginalis in experimentally infected guinea-pigs). Under the electron microscope they appear to have a mucoid envelope, a cell wall, and an electron-dense mass resembling a nucleus.

Cultivation.—The most satisfactory method for primary isolation is the inoculation of adult male guinea-pigs or mice. Whole blood or ground-up blood clot taken during the height of the patient's fever is injected by the intraperitoneal route. The first signs of infection in the guinea-pig are seen after nine or ten days, when the animal's temperature rises to 105° F. to 106° F.; it remains above normal for four to eight days. Usually the animals recover, but *R. tsutsugamushi* is highly pathogenic and often kills both mice and guinea-pigs within a few days. Some members of the group may produce transient and very mild infections. In the case of *R. mooseri*, however, well-marked swelling and oedema of the scrotum occurs with inflammation of the tunica vaginalis. Stained smears from the tunica show intracytoplasmic rickettsiae in the serosal and endothelial cells. Those members of the group causing epidemic and endemic typhus fevers are seen in clusters in the cell cytoplasm and those causing spotted fever are also seen within the nucleus. In human tissues the cells most frequently infected are the endothelial cells and the smooth muscle fibres around small blood vessels. It seems that rickettsiae grow best in cells whose metabolic activity is depressed by measures which reduce the oxidative processes of the cell (*e.g.* a lowering of the temperature or a reduction of the oxygen tension).

All strains of rickettsiae can be cultivated in the yolk sac of the chick embryo. Using this method and incubating the eggs at 32° C. big yields of the organisms can be obtained for vaccine production or for the preparation of serological antigens. The method is only of limited value in primary isolation. Tissue cultures of mouse lymphosarcoma cells and rat fibroblasts can also serve as hosts for these organisms.

Viability.—In general, rickettsiae are easily destroyed by heat, drying and chemical disinfectants. They die within a few hours at room temperature but may be preserved well in glycerol saline at 0° C. or by freeze-drying emulsions of infected tissue suspended in sterile skimmed milk.

Under certain circumstances they may survive drying for long periods and it is known that the dried faeces of lice and fleas may remain infective for months.

Rickettsial growth is inhibited by chloramphenicol and tetracyclines which are effective therapeutic agents. Sulphonamides accelerate the growth of the organisms, but para-amino-benzoic acid exerts a marked inhibitory effect and has been used with some success in treatment.

Antigenic Structure.—Each species of Rickettsia possesses its own specific antigens which provoke the production of homologous antibodies. These antibodies can be measured by agglutination and neutralisation tests, but are more conveniently demonstrated by the method of complement fixation. In washed and carefully purified rickettsial suspensions only the type-specific antigens remain, and by demonstration of these it is possible to differentiate epidemic from murine typhus, and both of these from the spotted fevers. Strains of *R. tsutsugamushi* are antigenically so heterogeneous that the complement fixation reaction is of doubtful value in the diagnosis of scrub typhus. There is also a soluble antigen which is group specific and is probably derived from the mucoid envelope of the organisms. It reacts with antibodies in the sera of cases of both epidemic and murine typhus, though not with those from spotted fever. It must be emphasised that the antigens required for the specific complement fixation reactions are both difficult and expensive to prepare and that these tests are of necessity only carried out in a small number of reference laboratories.

The Weil-Felix Reaction

The Rickettsiae of epidemic, endemic and mite-borne typhus and those of the spotted fevers contain in varying amounts an alkali-stable carbohydrate hapten which is shared by a non-flagellate strain of *Proteus* and some of its variants. This hapten is a somatic constituent of these bacilli, which are readily agglutinated by the sera of convalescent cases of typhus.

The reaction, first described by Weil and Felix, is very simple to carry out and of great practical importance in the diagnosis of rickettsial infections. By the end of the first week of the illness in epidemic typhus the titre of agglutinins for *Proteus OX 19* has

usually reached a figure of 200 and a peak of 1000-5000 is reached at the end of the second week. The agglutinins tend to disappear a few months after recovery and thus a positive Weil-Felix reaction is a useful indication of recent infection. Complement fixing antibodies persist for much longer periods and by providing evidence of past infection, their presence enables a retrospective diagnosis to be made.

The Weil-Felix reaction is performed in the same manner as the Widal test (p. 313) except that an O-agglutinable suspension of *Proteus X 19* is used as the agglutinins are of the O type. As this organism may revert to H form it should be grown on dry agar, and subcultures made from non-spreading separate colonies.¹ The tubes are incubated at 50° C. for four hours and then overnight at 37° C., after which final readings are made. The results of the Weil-Felix test should be interpreted strictly in relation to the clinical findings, for a positive reaction is occasionally observed in other diseases such as undulant fever, malaria, infectious mononucleosis and tuberculosis. The agglutination reactions with this and other *Proteus* suspensions in rickettsial infections are shown in the table below.

		<i>Proteus</i>			
			OX 19	OX 2	OX K
Epidemic	:	Louse-borne	+++	±	-
Brill's disease.	:	Usually		negative	-
Murine typhus	.	Flea-borne	+++	+	-
Tsutsugamushi fever	.	Mite borne	-	-	+++
Rocky Mountain spotted fever	.		+++	++	-
Fièvre boutonneuse.	.	Tick-borne	++	++	-
South African tick-bite fever	.		++	++	-

Epidemiology

Epidemic typhus fever, known also as Classical or European typhus fever, is caused by *R. prowazekii*. It is spread from man to man by the bite of the human body louse (*Pediculus corporis*) or more doubtfully by the head louse (*P. capitis*). Although potentially world wide, the location of the disease is now limited to the Balkans and the Middle East, North Africa, Asia, Mexico and the Andes. Lice become infected when they bite either patients

¹ A standard suspension can be obtained from the Central Public Health Laboratory, Colindale, London.

suffering from typhus fever or carriers in whom the infection has persisted in latent form for many years. When the infected blood reaches the intestine of the louse, the rickettsiae invade the epithelial cells and multiply until the host cells distend and rupture. As a result, the faeces become heavily laden with the organisms and when they are discharged on the skin, the rickettsiae are readily introduced into the human host through the abrasions caused by scratching or through fresh biting wounds. When standards of personal hygiene are low and when a considerable proportion of the population are infested with lice, typhus fever may assume epidemic proportions. Such conditions may arise in time of war, and if a reservoir of the rickettsiae in the form of a carrier of the latent infection is introduced into the community, typhus breaks out. The epidemic in Naples during the Second World War began in this way, but was quickly controlled by measures which were taken against the lice that were transmitting the infection. It was possible to delouse the persons of the affected community as well as their clothes, bedding and dwelling-places by the use of a residual insecticide DDT (dichloro-diphenyl-trichloro-ethane) which was readily applied by being blown forcibly through an insufflator. The result of prompt and thorough use of DDT was that the epidemic was cut short before it could spread throughout the whole city.

Rickettsiae may persist alive in the tissues of recovered patients for as long as twenty years without manifest symptoms. Occasionally, however, they become active once more and cause a recurrence of typhus fever often in an atypical clinical form. Recrudescence typhus fever is known as *Brill's disease*.

Murine typhus is caused by *R. mooseri* (*R. typhi*) and is primarily a disease of rats, amongst which it is spread by the rat flea (*Xenopsylla cheopis*) and the rat louse (*Polyplax spinulosus*). Occasionally the rat fleas carry the infection to man, and sporadic endemic typhus fever occurs. This disease is world wide and is most frequent in areas where rat infestation is high. In Mexico it is known as Tabardillo, from the cloak-like distribution of the rash. The disease has sometimes spread in epidemic form in man, but the vector responsible is not certainly known.

Spotted Fever. Rocky Mountain spotted fever was first recognised by Ricketts in western Montana in America. The causative organism is *R. rickettsii*. Spotted fever also occurs in North Africa along the shores of the Mediterranean, where it is named "fièvre boutonneuse" and is caused by *R. conorii*. In Kenya and South Africa similar infections are due to *R. rickettsii* var. *pipperi* and other rickettsiae. Spotted fever also exists in

Mexico, Brazil and Colombia. The organisms are transmitted by ticks usually of the Ixodidae family, e.g. *Dermacentor andersoni*, a vector of Rocky Mountain spotted fever. Rickettsiae enter the human host either by the bite of the tick or through minute abrasions such as may occur when an engorged tick is crushed in the fingers. Fresh tick faeces are infectious, but dried faeces, in contrast to those of lice in epidemic typhus fever, are non-infective. Normally *R. rickettsiae* inhabit healthy ticks and are passed transovarially throughout the life cycle. Vertebrate hosts such as horses and dogs are occasionally bitten by ticks and become for a time reservoirs of infection. After feeding on such animals, engorged ticks are a common source of infection for man.

Scrub typhus is caused by *R. tsutsugamushi* and is transmitted by the bite of the larvae of mites (*Trombicula akahaushi* or *T. deliensis*). The larvae become infected after biting an infected host and the rickettsiae are then carried to the adult mite and thence throughout the life cycle to the egg and to the larvae once more. The infection is transmitted by the young larvae which bite only once and which usually feed on wild rats and other rodents. The larvae are found on low-lying humid vegetation and on moist ground, whence they can readily attack man. Scrub typhus is very difficult to control because the mites and their larvae can only be eradicated by clearing away large areas of vegetation and drying the ground where camps or houses are to be sited. Miticidal chemicals such as dimethyl phthalate are used as an ointment to act as an insect repellent, and chemoprophylaxis with chloramphenicol is of considerable value.

Typhus Vaccines

Active immunisation against typhus is usually carried out with a formalised vaccine of the Cox type in which the rickettsiae have been cultivated in the yolk sacs of developing chick embryos. A vaccine of this type containing *R. prowazekii* is used against epidemic typhus and one containing *R. mooseri* against endemic typhus. A similar vaccine containing *R. rickettsii* is employed against Rocky Mountain spotted fever. The latter is given early in the summer months before the ticks are active.

Cox-type vaccines are administered in three 1·0 ml. doses at intervals of seven to fourteen days. Booster doses of 1·0 ml. are needed at yearly intervals or more frequently if the risk is great. Antibodies may persist for some years after inoculation. The booster dose results in a prompt rise of circulating antibodies reaching a peak in about ten days. A second type of vaccine prepared from "strain E", a living attenuated strain of *R.*

prowazekii, is at present under trial and shows considerable promise. Although reactions have followed the use of this vaccine it has caused no harm and has given a firm immunity against epidemic typhus for as long as five years.

In scrub typhus formalised vaccines of *R. tsutsugamushi* have proved to be ineffective. A vaccine of a living strain is under trial and the infection it establishes is controlled after some ten days by chloramphenicol therapy. The procedure is, however, not without risk, and on account of the antigenic heterogeneity of the *R. tsutsugamushi* its effectiveness is in some doubt.

Chemotherapy.—Tetracyclines, chloramphenicol and para-amino-benzoic acid all exhibit a rickettsiostatic effect. The tetracyclines and chloramphenicol are more or less equally effective against louse-borne, flea-borne, tick-borne and mite-borne typhus fevers.

Trench Fever, a form of louse-borne typhus fever, is caused by *Rickettsia quintana*. The disease occurred in both world wars and was shown to be transmitted from man to man by the human louse. *R. quintana* has been propagated in the intestinal epithelial cells of lice, but not in egg or tissue cultures. There are no specific laboratory tests for Trench fever.

Heartwater Disease of cattle, sheep and goats is caused by *Cowdria ruminantium*. It occurs in Central, East and West Africa and the infection is spread by the bite of the "bont" tick (*Amblyomma hebraeum*). Affected animals develop high fever, gastroenteritis, and oedematous swellings, the most characteristic of which is the hydropericardium, which gives the disease its name. Rickettsiae are found in large numbers in the endothelial cells of capillaries of many internal organs, especially those of the brain and renal glomeruli. It is thought that the serum from recovered animals can transmit a mild infection to young calves and it has been used in attempts to procure active immunisation.

Tick-borne fever of sheep in Great Britain has been regarded as a rickettsial infection and inclusion bodies are observed within the cytoplasm of granular leucocytes and monocytes. The organism is also found in cattle and goats.

Q FEVER

Q fever is an acute systemic infection usually characterised by an interstitial pneumonia; unlike other rickettsial infections it has no rash. The name of the disease is derived not from Queensland in Australia, where it was first recognised, but from the letter Q in "Query", because for some time its aetiology was

uncertain. The signs and symptoms are usually referable to the respiratory system but are very inconstant. Often the first indications of the diagnosis are the characteristic patchy areas of infiltration or the segmental consolidation seen on radiological examination resembling a "primary atypical pneumonia". More rarely the disease may declare itself with the prolonged fever and embolic signs of subacute bacterial endocarditis.

Coxiella burnetii is the causative organism. It is an obligate intracellular parasite, pleomorphic in appearance, and measuring $0.25\text{ }\mu$ by $0.5\text{ }\mu$ to $1.5\text{ }\mu$ in length. It is frequently seen as a diplobacillus and closely resembles the *Rickettsiae* in its morphology. The infective property of *Cox. burnetii* has been passed through a collodion membrane of an average diameter of $400\text{ m}\mu$. The organism infects guinea-pigs, hamsters, mice and the cells of the yolk sac of the developing chick embryo. It appears as clumps or masses within the cytoplasm of endothelial and serosal cells such as those lining the peritoneal cavity. *Cox. burnetii* is remarkably resistant to desiccation and may survive for long periods in the tissues or faeces of infected ticks. It remains viable for several days in water or milk. It can withstand heat at 60° C . for one hour, survives 1·0 per cent. phenol for one hour, and 1 in 1000 merthiolate for a week.

Cox. burnetii thus possesses properties which are distinct from those of all the other members of the family *Rickettsiaceae*. It is filterable, highly resistant to heat and disinfectants, does not produce a rash in infected persons and does not elicit the agglutinin to the *Proteus* X strains which characterises the typhus fevers. For these reasons the organism is no longer regarded as a true *Rickettsia* and has been designated the prototype of a new genus—*Coxiella*.

Laboratory Diagnosis.—This depends on the recovery of *Cox. burnetii* from the blood in the early febrile stages of the illness and on the results of serological tests for specific antibodies. The Weil-Felix reaction is negative.

Isolation of the Causative Organism.—Heparinised blood should be inoculated into as many susceptible hosts as possible, e.g. intraperitoneally into guinea-pigs and hamsters and into the yolk sacs of five- to six-day-old chick embryos. Guinea-pigs and hamsters do not die from the infection and seldom show any obvious sign of disease. The animals must be carefully observed for signs of illness and the rectal temperature should be recorded twice daily. When any definite fever or illness is detected, the animals should be killed and blood and tissue extracts from them should be injected into fresh animals. Smears from the spleen, liver and

from the yolk sacs of chick embryos of sluggish movement should be stained by Castaneda's and Macchiavello's stains (p. 140) and examined for the presence of rickettsia-like organisms. Animals which do not sicken should be bled by cardiac puncture after two weeks and the serum examined for antibodies to *Cox. burnetii*.

For *Complement fixation tests* killed antigens of egg adapted strains of *Cox. burnetii* are used. The Herzerling strain (Italian) and the Nine Mile strain (American) obtained from infected yolk sacs are in general use. Freshly isolated strains do not react well with sera in early convalescence and it is only after adaption to the egg that fully reactive antigens can be prepared. The methods used to make the antigen and to carry out the test are similar to those used in psittacosis (p. 346). Antibody titres rise from an initial low level to figures ranging from 64 to over 1000 in convalescence. Three samples of serum should be tested, the first taken at the onset of the illness, and the second and third at ten-day intervals thereafter.

Epidemiology.—*Cox. burnetii* is the cause of enzootic infections in domesticated animals such as cattle, sheep and goats. The disease is found throughout the world and has been reported in fifty different countries, including the United Kingdom. Apparently healthy animals may excrete coxiellae in their milk and also during parturition, when huge numbers of the organism are present in the placenta and the birth fluids. Man may be infected by drinking contaminated milk or by inhaling the infected dust from the straw and bedding soiled by the animals. There are many instances on record of the infection of workers by the inhalation of infected material in the laboratory. The infection is prevalent in many wild rodents, particularly in the bandicoot in Australia and in pigeons and other birds. *Cox. burnetii* is conveyed from animal to animal by the bite of ticks. More than twenty species of ticks mostly of the Ixodidae and Argasidae families are known vectors. It is uncommon for the organism to be carried throughout all stages of the life cycle of the tick and for the most part these arthropods are infected from the animal reservoir. Ticks seldom if ever transmit the disease to man. The risk of person-to-person transmission of Q fever is small, and strict isolation precautions are not needed.

Control measures for Q fever must include the adequate pasteurisation of milk. Coxiellae survive 143° F. for thirty minutes, but are killed at 145° F. for thirty minutes. The "flash" method of pasteurisation (162° F. for fifteen seconds) is effective and is recommended. In the laboratory great care must be taken with infected animals and eggs and they should always

be handled within special inoculation cabinets, where the risk of contamination of the air is eliminated. All carcases and eggs should be immersed in disinfectant solution and then incinerated. The use of a vaccine of the Cox type would seem to be indicated for laboratory personnel and for persons whose work brings them into contact with live or dead animals.

Both chloramphenicol and the tetracyclines are effective therapeutically in Q fever.

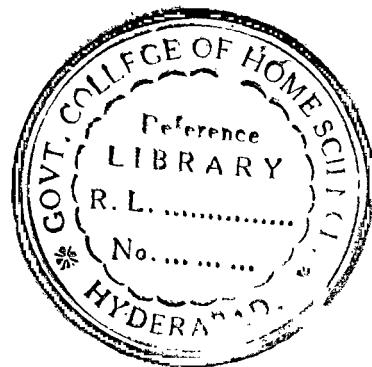
BARTONELLA BACILLIFORMIS

This organism is the cause of two quite distinct clinical conditions: Oroya fever, characterised by intermittent pyrexia, anaemia and high mortality, and Verruga peruana, a benign, nodular skin eruption. The organism under natural conditions grows on or in the erythrocytes of man or in the cytoplasm of his tissue cells. These infections are found only in mountainous districts in South America (Peru, Ecuador and Colombia). The disease is transmitted by the bite of sandflies, *Phlebotomus verucarum* in Peru and *P. colombianum* in Colombia.

The infective agent is seen in red blood cells as minute rod-shaped forms $0.3\text{--}2.5\mu$ in length; it is extremely pleomorphic and coccal forms are frequently found. The organism is actively motile, with 1-10 flagella attached at one pole of the bacillus. Flagella are not seen in blood films but are regularly present in cultures. The organism is Gram-negative, non-sporing, non-encapsulated and an obligate aerobe. It is best cultivated in semi-solid agar enriched with 10 per cent. rabbit blood at 28°C . It can survive many weeks in blood or blood cultures. Inoculated intracutaneously in monkeys, *Bartonella bacilliformis* produces Verruga regularly, but Oroya fever is manifest only occasionally unless the animals have previously been splenectomised. *Haemobartonella muris* is found in wild rodents and in many strains of laboratory rats. A considerable proportion of the animals show no sign of disease and react only mildly if inoculated with the organism. It has been observed that after splenectomy in rats, organisms which closely resemble *Bartonella moniliformis* frequently appear in the red cells; the infection is associated with marked anaemia and often has a fatal result. Presumably latent infection is prevalent in rats associated with a certain degree of natural immunity which is broken down by removal of the spleen. The infection is louse-borne.

Other species of *Haemobartonella* have been described in mice, guinea-pigs, squirrels, bovines and dogs.

It should be noted that these organisms are unusual in that they can be cultivated artificially and show a marked tendency in nature to multiply within the host's cells. *Bartonella* and *Haemobartonella* constitute two genera of the family *Bartonellaceae* and have been brought together with the *Rickettsiaceae* in the order *Rickettsiales*.



CHAPTER XXXVI

VIRUSES

GENERAL CONSIDERATIONS

AMONG the common contagious illnesses of man and animals there are many for which no bacterial cause has been found. These diseases have been known clinically throughout the centuries; smallpox has been recognised as a deadly infection since pre-Christian times and Hippocrates was perfectly familiar with the swollen neck in mumps. At the beginning of the present century it was realised that an agent present in the tissues or blood of such cases could transmit the infection in the absence of bacteria. Pasteur, when he failed to detect bacteria in infective material from rabid dogs, suggested that the agent responsible might exist in an invisible form.

In 1892 Pasteur's theory was confirmed, though in a different disease, by Iwanowsky, who showed that the mosaic disease of tobacco plants was caused by a minute agent which was so small that it was ultramicroscopic and would pass through the pores of a filter which would not permit the passage of any known bacterium. In 1898 the vesicle fluid from cases of foot and mouth disease in cattle was shown by Loeffler and Frisch to contain an infectious agent which was similarly filterable. In 1901 it was proved that yellow fever in man was caused by a filterable virus carried by mosquitoes. During the years that have followed very large numbers of filterable viruses have been obtained from widely different sources. The study of these minute micro-organisms and the hosts which they infect constitutes the new biological science of *Virology*, which has four main branches:

- (1) Human and animal virus infections.
- (2) Bacterial viruses (Bacteriophages).
- (3) Plant virus diseases.
- (4) Insect infections with polyhedral viruses.

In these pages we shall confine our attention to viruses associated with human and animal diseases (see p. 746). Consideration is given to the bacteriophages in Chapter XLIII.

Classification of Viruses

The present state of our knowledge of animal viruses is not sufficient to warrant the use of the Linnaean binomial nomenclature used for bacteria. It is, however, possible to group viruses together according to some of their outstanding properties and to distinguish the individual members of the groups. In the case of four of these groups the suffix *virus* is used and a descriptive adjective is added for each member. There are, of course, many viruses which do not fall into these groups.

<i>Group</i>	<i>Member</i>	<i>Common Name</i>
1. Poxvirus	Poxvirus variolae	Smallpox virus
	Poxvirus officinale	Vaccinia
	Poxvirus bovis	Cow-pox-virus
	Poxvirus varicellae	Chickenpox virus
	Poxvirus muris	Ectromelia virus
	Poxvirus avium	Fowl-pox virus
2. Myxovirus	Myxovirus influenzae A, B and C	Influenza virus A, B and C
	Myxovirus multiforme	Newcastle virus
	Myxovirus pestis-galli	Fowl plague virus
	Myxovirus parotidis	Mumps virus
	Myxovirus para-influenzae 1	(Sendai virus)
	Myxovirus para-influenzae 2	(Croup virus, C. A.)
	Myxovirus para-influenzae 3	(Haemadsorption virus)
3. Herpes virus	Herpesvirus hominis	(Herpes simplex virus)
	Herpesvirus suis	(Pseudorabies virus)
	Herpesvirus simiae	(Virus B)
4. Polio virus	Poliovirus hominis	Poliomyelitis virus
	Poliovirus muris	Mouse encephalo- myelitis virus (Theiler)

The polioviruses included in the fourth group are now regarded as a subgroup of a much larger group of *Entero viruses* which contains two other subgroups, the ECHO and Coxsackie viruses; all the entero-viruses inhabit the intestinal tract. A further large group contains arthropod borne viruses and its members are known as *Arbor viruses*; the members of this group are subdivided according to antigenic structure and binomials are not used.

Virus Diseases of Man

A. Generalised infections in which the virus is spread throughout the body in the blood-stream and in which many organs may be involved. Skin rashes are common.

- | | |
|------------------------------------|--------------------|
| 1. Smallpox | 6. Sandfly fever |
| 2. Varicella | 7. Dengue |
| 3. Measles | 8. West Nile fever |
| 4. Rubella | 9. Yellow fever |
| 5. Epidemic myalgia or pleurodynia | |

B. Infections which primarily affect specific organs or systems.

1. The skin and mucous membranes:

- | | |
|--|--|
| (a) Herpes simplex
(b) Warts
(c) Herpangina
(g) Molluscum contagiosum
(h) Inclusion cervicitis and urethritis
(i) Orf (contagious pustular dermatitis of sheep) | (d) Herpes zoster
(e) Vaccinia
(f) Cow-pox |
|--|--|

2. The respiratory tract:

- | | |
|---|---|
| (a) Influenza
(b) Acute respiratory disease due to adenovirus infection
(c) Common cold | (d) Croup in children
(e) Primary atypical pneumonia
(f) Psittacosis and ornithosis |
|---|---|

3. The central nervous system:

- | | |
|--|--|
| (a) Poliomyelitis
(b) Aseptic meningitis due to ECHO or Coxsackie viruses
(c) Rabies
(d) Lymphocytic choriomeningitis | (e) Encephalitis due to the mumps, measles, herpes simplex, virus B, and vaccinia viruses
(f) Encephalitis due to arthropod-borne viruses, e.g. louping-ill |
|--|--|

4. The eye:

- | | |
|---|--|
| (a) Trachoma
(b) Inclusion conjunctivitis
(c) Epidemic keratoconjunctivitis | (d) Pharyngo-conjunctival fever
(e) Newcastle disease |
|---|--|

5. The liver:

- | | |
|---|-----------------------|
| (a) Infective hepatitis
(b) Homologous serum hepatitis | (c) Rift Valley fever |
|---|-----------------------|

6. The salivary glands:

- (a) Mumps (b) Salivary gland disease

Virus Diseases of Animals

A. Generalised infections often with catarrhal symptoms:

Canine distemper	Pulmonary adenomatosis of sheep (Jaagziekte)
Hard pad disease	Enzootic abortion of ewes
Canine hepatitis	Nairobi sheep disease
Feline enteritis	African horse sickness
Feline pneumonitis	Equine influenza
Swine fever (hog cholera)	Equine infectious anaemia
African swine fever	Equine rhinopneumonitis
Atrophic rhinitis of swine	Fowl plague
Swine influenza	*Newcastle disease
Rinderpest	Infectious laryngotracheitis
Ephemeral fever of cattle	Hepatitis of ducklings
Malignant catarrhal fever of cattle	*Ornithosis
*Rift Valley fever	*Psittacosis
Blue tongue of cattle and sheep	Pneumonitis of mice
Calf pneumonia	

B. Infections characterised by skin lesions:

Foot and mouth disease of cattle	Sheep-pox
Vesicular stomatitis of horses and cattle	Horse-pox
Vesicular exanthema of swine	Swine-pox
Contagious pustular dermatitis of sheep (orf)	Rabbi-tpox
Lumpy skin disease in cattle	Fowl-pox
*Cow-pox	Pigeon pox
*Vaccina	Canary-pox
Infectious ectromelia of mice	

C. Diseases characterised by lesions of the central nervous system:

*Rabies	Infectious encephalomyelitis of pigs (Teschen disease)
Pseudorabies	Scrapie of sheep
Borna disease	*Virus B infections in monkeys
*Equine encephalomyelitis (eastern, western and Venezuelan)	*Yellow fever in monkeys
*Louping-ill of sheep	Theiler's mouse encephalomyelitis

* Diseases which are transmissible to man.

D. Diseases characterised by tumour formation:

Avian leucosis	Papillomatosis of rabbits,
Rous sarcoma of fowls	dogs, horses and bovines
Infectious myxomatosis of rabbits	Mammary tumours of mice
Infectious fibromatosis of rabbits	Polyoma disease of mice

E. Miscellaneous:

Silkworm jaundice	Sac brood of bees
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Fundamental Characteristics of Viruses

Human and animal viruses differ fundamentally from bacteria in three respects: they are small in size and consequently filterable; they can grow only in living cells and do not multiply on inanimate culture media; and they resist antibiotics, glycerol and some physical and chemical agents which destroy bacteria. They usually cause highly infectious diseases.

Small Size and Filterability

The unit used for the measurement of virus size is the millimicron ($m\mu$), *i.e.* a one-thousandth part of a micron (μ) or 0.000001 mm. In the following list the sizes of various representative viruses are given together with those of reference objects. The virus particles or *elementary bodies* vary in size from 500 to 10 $m\mu$; the largest are about the same size as small bacteria, and the smallest are about the size of large protein molecules. The larger elementary bodies, those over 200 $m\mu$ in diameter, are within the resolution of the light microscope and can be demonstrated in stained films or sections taken direct from the lesions of such virus diseases as smallpox and psittacosis.

Measurement.—Viruses may be measured by their capacity to pass through filters which are fine enough to hold back all but the smallest bacteria. Many types of filter have been used for this purpose, but the best are made from collodion (see p. 171) and have replaced the older diatomaceous earth filters of Berkefeldt and the Chamberland porcelain candles. Filtration methods, however, have no high degree of precision in virus measurement and have for the most part been replaced by newer techniques. Nevertheless, filtration does have a special use in measuring a very small virus when it is contained in a material contaminated with so much host cell protein that other methods cannot be used.

Another method by which virus size can be determined is by

Approximate Sizes of Viruses and Reference Objects

	Diameter or width x length in μ	
*Staphylococcus	1000	
*Serratia marcescens	750	
*Rickettsia prowazekii	475	
Psittacosis-lymphogranuloma group	330-490	
Pox viruses	300 x 250	
Mumps	170	
*Pleuropneumonia organisms (granular)	150	
Rabies virus	150	
Measles	140	
Herpes simplex	120	
Polyhedral virus	280 x 40	
Newcastle virus	115	
Influenza group of viruses	100	
Adenovirus group	100	
E.coli bacteriophage T2	95 x 65	
Rous sarcoma	65	
Lymphocytic choriomeningitis	50	
Tobacco mosaic virus	300 x 15	
Rabbit papilloma	45	
Yellow fever	40	
Arthropod-borne viruses - Japanese B encephalitis, etc.	40	
Poliomyelitis	28	
Coxsackie group	28	
ECHO group	28	
*Haemocyanin molecule	22	
Foot and mouth disease	10	
*Haemoglobin molecule	15 x 3	
*Albumen	10 x 2.5	

* For reference

Fig. 27

Micro-organisms above the upper line are visible with the optical microscope.

estimating the rate at which the particles fall in a suspending fluid; large particles being heavier fall faster than small ones. This relationship between particle size and rate of sedimentation follows a fixed law (Stoke's law) and holds good even when forces many times greater than that of gravity are applied to a virus preparation in a fast-moving centrifuge. From values for the density and viscosity of the medium, the distance of the filter paper from the axis of rotation and the speed in r.p.m., the diameter of the virus particle can be calculated (p. 295).

A third method of estimating virus size is by direct observation under the electron microscope. Viruses which are far beyond the limits of the resolution of the light microscope can be seen and photographed in this instrument, which is capable of resolving objects as small as $0.5 \text{ m}\mu$ in diameter (see p. 106). The usual method of making the measurements is to include in a suspension of purified virus some latex particles of known size (*e.g.* $250 \text{ m}\mu$); in electron micrographs the known and the unknown particles can be measured with accuracy and the size of the virus is determined with precision. One advantage of electron microscopical examination of viruses is that the shape as well as the size can be determined. In this way it has been found that the vaccinia virus particles are brick-shaped, that influenza viruses have a filamentous as well as a spherical form, and that bacteriophages have a sperm-like morphology, with a polyhedral head and a tail. The crystalline nature of plant viruses and the crystal-like lattice arrangement of the component sub units of some human viruses such as those of the adenovirus group are clearly seen in electron micrographs. In ultra-thin sections of infected cells it is possible under the electron microscope to follow the morphological changes *in situ* in the host cell as the virus multiplies.

Intracellular Habitat

A purified suspension of elementary bodies washed free of contaminating host material is biologically inert and has none of the respiratory or biochemical activities which characterise living cells. When such a suspension is injected into a susceptible host, however, the virus particles invade cells entering them through their walls by a mechanism which is only imperfectly understood. Once within the cell, virus multiplication begins rapidly, either in the cytoplasm or in the nucleus and continues until enough new elementary bodies are produced to invade neighbouring cells. In some cases the process completely destroys the host cells, while in others, the virus and host seem able to establish a state of symbiosis without any obvious harm to each other. When

gross damage to cells takes place, the virus infection is usually quickly recognised by the illness it causes and by the pathological lesions which follow. When little damage occurs and the host cells survive, the infection is latent and difficult to detect; often it is only revealed by the provocation of some external stimulus. The ultra-violet rays in strong sunlight, for instance, may provoke an exacerbation of a latent herpes virus infection in the lips or nose.

If virus multiplication at a local site continues long enough to reach a high peak, large numbers of elementary bodies and their toxins may be carried into the blood stream and thence to many distant sites. In such circumstances, at the onset of the viraemic phase, the host experiences the first feverish manifestations of an acute illness. The time required for the virus to multiply sufficiently to cause viraemia is the incubation period of the disease and varies for different viruses; in smallpox it is twelve days, in measles and poliomyelitis about ten days.

Reproduction.—Viruses are composed of a core of nucleic acid covered by a coating of protein. Influenza and poliomyelitis viruses contain ribonucleic acid (RNA) while the adenoviruses and the poxviruses contain deoxyribonucleic acid (DNA). In the earliest stages of infection the nucleic acid together with only a small part of the protein enters the host cell where it disintegrates and mingles with the intracellular materials. At this time the virus nucleic acid loses its identity and cannot be detected by infectivity tests. The term "eclipse phase" has been applied to this early stage in the reproductive cycle. The synthesis of viral nucleic acid and viral protein proceeds separately and in sequential steps; at the end of the process, infectious units are assembled which can survive outside the cell. The new virus particles may leave the cell as soon as they are formed, as does the influenza virus, or they may accumulate to be cast loose in groups when the cell undergoes a form of lysis, as in poliomyelitis. The continuing release of elementary bodies is characteristic of some virus species, including the adenoviruses, the influenza and herpes simplex viruses. The sudden bursting of the host cell and the release of large numbers of virus particles is associated pre-eminently with bacteriophages but also with the psittacosis and poliomyelitis viruses. The "eclipse phase" is a characteristic of the reproductive cycle of nearly all viruses other than those of the psittacosis-lymphogranuloma group, which multiply by binary fission.

Inclusion Bodies.—During the course of multiplication, many viruses are associated with the appearance of large distinctive

structures known as inclusion bodies. They may be situated either in the cytoplasm, or in the nucleus or, as in the case of measles, in both. The inclusion bodies are often acidophilic and usually appear as pink masses in smears or sections stained with Giemsa's stain or with Mann's eosin methyl-blue stain; basophilic inclusions are characteristic of the psittacosis-lymphogranuloma group of viruses. In size they vary from 1 to $30\text{ }\mu$ in diameter. In certain infections the inclusion body is intimately concerned with the reproductive cycle and can be seen to contain large numbers of virus particles when it is examined electron-microscopically. During the early stages of its formation, the elementary bodies are held in a jelly-like matrix.

Intracytoplasmic inclusion bodies are often so characteristic in their appearance that their presence in tissues is of diagnostic significance. The large Negri body is acidophilic and up to $20\text{ }\mu$ in diameter; its presence in the nerve cells of the hippocampus of the dog's brain justifies the presumptive diagnosis of rabies. Rather smaller multiple inclusions are found in the cells of hosts infected with the vaccinia virus and are known as Guarnieri bodies. Very large inclusions known as Bollinger bodies are characteristic of fowl-pox and those of molluscum contagiosum are so large ($20 \times 30\text{ }\mu$) that they are easily visible in sections of the skin seen under the low power microscope.

Intranuclear inclusions are acidophilic and may be of two types; Type A is granular in appearance and of variable size, and is found in herpes simplex, zoster, varicella and yellow fever; Type B, which is more circumscribed and sometimes multiple, is found in poliomyelitis, adenovirus infections and Rift Valley fever.

Infectivity

Viruses, as a group, are highly pathogenic in minute doses. Serum from a case of infective hepatitis injected in a dose of less than 0.1 ml. has been known to transmit the disease. A millionth part of 1.0 ml. of a 10 per cent. brain suspension from an infected monkey is sufficient to infect another animal with yellow fever. Probably there is considerable variation from virus to virus in the number of elementary bodies required to initiate infection in a susceptible host. In some circumstances the inoculation of a single particle is sufficient, but more usually ten or considerably more particles are required to establish a disease.

Epidemics are particularly characteristic of many virus diseases. Influenza assumes major epidemic proportions every second or third winter and at roughly forty-year intervals sweeps over the whole world, as is witnessed in the pandemic of 1918-19 and the

Asian influenza of 1957. Smallpox is notorious for the ease and rapidity with which it spreads and for the severity of the epidemics it causes. Amongst animals, foot and mouth disease and fowl pest have an amazing power to spread, not only from one host to the next, but from one geographical location to another.

Reaction to Physical and Chemical Agents

Outside the body, at room temperature, many viruses are extremely labile and may survive for only a few hours. Such is the case with the viruses of influenza, mumps and measles, and in these diseases great care must be taken to ensure that the specimens under investigation are frozen with a minimum delay. Other viruses, such as the smallpox and poliomyelitis viruses, are much hardier and may survive under ordinary atmospheric conditions for many days, weeks or even months.

Heat.—The viruses causing disease in man and animals are in general readily inactivated by moderate heat (56° – 60° C. for thirty minutes) though there are some notable exceptions—homologous serum jaundice and poliomyelitis viruses. Like bacteria, viruses are resistant to extremes of cold, and, in fact, freezing at -35° C. or -70° C. is a satisfactory method for their preservation much used in the laboratory. The majority of viruses are also well preserved by drying from the frozen state, using the method of freeze-drying (see p. 268). By this means, virus vaccines are preserved in an active form for long periods before use to immunise against such diseases as smallpox and yellow fever.

pH Variation.—Viruses remain viable as a rule within the range of pH 5 to 9, but are destroyed by extreme acidity or alkalinity. Certain of their properties, however, like haemagglutination (*vide infra*) may be profoundly disturbed by variations of a few tenths of a pH unit.

Ether and Sodium Desoxycholate Susceptibility.—The reaction of viruses to ether serves to differentiate two groups; some enteric viruses, for example the poliomyelitis and homologous serum jaundice viruses, are very resistant to its effects, while the arthropod-borne encephalitis viruses and the herpes simplex virus are destroyed by it. A second reagent which acts selectively on certain viruses is a 0·1 per cent. solution of sodium desoxycholate which readily inactivates the arbor viruses, the influenza and lymphocytic choriomeningitis viruses, while leaving enteric and other viruses unharmed. This property is so sharply defined that it serves to differentiate the group of encephalitis viruses from many others with which they may be confused.

Glycerol.—In a 50 per cent. solution of glycerol, ordinary non-

sporing bacteria are killed comparatively quickly, but many viruses remain alive in this fluid for several months or even years. The preservation of the vaccinia virus used prophylactically against smallpox is accomplished by means of glycerol. Other viruses which can be kept for long periods in glycerol at 4° C., or lower temperatures, are those of poliomyelitis, rabies and herpes simplex. On the other hand, some viruses, e.g. the rinderpest virus and the psittacosis group of viruses survive for less time in glycerol than certain bacteria.

Bactericidal Agents.—The most efficient disinfectants for use against viruses are oxidising agents such as hydrogen peroxide, potassium permanganate and hypochlorites, and organic iodine derivatives. Formaldehyde may also be used but is slower in its action. Phenol and certain cresol disinfectants such as lysol are active against only a few viruses and are not to be recommended for material contaminated by the poliomyelitis or smallpox viruses.

Antibiotics and Chemotherapeutic Substances such as sulphonamides, penicillin, streptomycin and the tetracyclines have no effect on true viruses. The fact that the agents of the psittacosis-lymphogranuloma group are susceptible to these drugs has led to their being considered as rickettsiae.

The Cultivation of Viruses

To cultivate viruses it is always necessary to provide living host cells; no inanimate culture medium can meet the growth requirements of a virus and there is no evidence that reproduction ever takes place outside a living cell. Host cells are usually provided in one of three forms:

- (1) The experimental animal.
- (2) Chick embryos.
- (3) Tissue cultures.

Animal Inoculation was at one time the only method available, but has now been largely replaced by newer methods. In general, animal inoculation techniques depend for their success on the demonstration of a recognisable disease or death after a defined time interval. The presence of inclusion bodies of characteristic morphology in the animal's tissues provides additional evidence of virus infection. The poliomyelitis virus, for example, after intraspinal or intracerebral inoculation causes typical paralytic disease in the monkey, while the variola or smallpox virus causes the formation of lesions in the scarified skin or cornea of the rabbit (Paul's Test) accompanied by the appearance of multiple eosino-

philic intracytoplasmic inclusions in the epithelial cells. Since these methods are slow and full of technical difficulties, they are employed only when no other means is available. When animals are suspected of rabies, brain tissue is taken at autopsy and is inoculated intracerebrally in mice and rabbits; if it is present the animals develop encephalitis and die seven to ten days later. Coxsackie viruses are identified by their unique property of causing severe myositis and paralysis in suckling mice. In yellow fever antibodies in the serum are detected and titrated by their power to protect mice against a lethal dose of the virus.

Chick Embryos provide more satisfactory hosts for they are clean and bacteriologically sterile; they also have the advantage that they lack any protective specific immune mechanism to counteract virus infection. Vaccinia and the other pox viruses together with the herpes simplex virus all produce lesions on the chorio-allantoic membrane which are highly characteristic and easily recognisable with the naked eye. Influenza viruses multiply in the cells of the embryo's lungs and in the cells lining the allantoic cavity. The psittacosis lymphogranuloma group of viruses and the *Rickettsiae* grow well in the yolk sac and usually kill the embryos within well defined time limits.

Tissue Cultures of human or simian cells are the most frequently used because they are relatively simple to prepare in the large quantities which are required by modern diagnostic virological methods. Suspensions of cells dispersed from tissue fragments or from bulk cultures of cell lines such as HeLa, are obtained by tryptic digestion (see p. 457). The cells adhere to the walls of the test-tubes and grow out to form a sheet, or *monolayer*, which is easily observed *in situ* under the low power objective of the microscope. Virus growth is determined by:

1. *The Cytopathic Effect (C.P.E.)*, i.e. the necrosis of the infected cells. Different viruses produce different effects and it is sometimes possible to distinguish one from another by the nature of the cell degeneration, e.g. giant cell formation by the measles virus. Poliomyelitis, ECHO, Coxsackie group B, varicella, zoster, herpes simplex, measles, adeno and dengue viruses, all produce a cytopathic effect in tissue cultures.

2. The appearance of haemagglutinin in the tissue culture fluids (e.g. mumps and influenza) or the presence of a complement-fixing antigen (adenoviruses and some enteroviruses).

3. The adsorption of erythrocytes to the infected cells (haem-adsorption) e.g. influenza and parainfluenza viruses.

4. The inhibition of cellular metabolism or the failure of virus infected cells to produce acid.

The details of the techniques of egg cultivation and tissue culture are given in Chapter XVIII.

Virus Haemagglutination

Some viruses, notably those of the influenza group, cause the agglutination of the red blood cells of man, fowls, guinea-pigs and other animals. Virus particles attach themselves to the surface of the cells by means of enzymatic groups which react with a mucopolysaccharide substrate. The reactive groups of this substrate contain neuraminic acid and the enzyme is known as neuraminidase.

When red blood cells are added to a virus suspension each cell becomes speckled with many adherent elementary bodies; often one particle is attached to one or more cells forming a bridge between them and fastening them together. In this way the cells are agglutinated into masses which fall rapidly in the suspending fluid to settle on the bottom of the tube in an irregular ragged pattern. Once the cells have been agglutinated, and when the substrate in the *receptor areas* has been exhausted, dissociation occurs and the virus is liberated. Red blood cells treated in this way are permanently damaged and can no longer be agglutinated by the virus, which, however, is unaltered by the process and retains its power to agglutinate fresh cells.

Haemagglutination is probably a model of the first stage of the natural infection of cells, because the mucopolysaccharide substrate which enables the virus to attach itself to the red cell envelope, is also found on the surface of the epithelial cells of the respiratory tract. In this way the influenza virus can be intimately applied to the surface of its host cell and has only to traverse the cell wall to initiate infection. The mucopolysaccharide substrate is also present in mucus from the intestine, ovarian cysts, milk, urine and serum; it is often referred to as a "non-specific inhibitor" of virus action. The virus enzyme is also found in some bacteria, notably the cholera vibrio and *Cl. welchii*; it is usually called the "receptor destroying enzyme" (R.D.E.). The property of haemagglutination resides in the virus particle itself; in the case of the influenza virus it is formed early in the reproductive cycle and can be demonstrated in immature, or "incomplete" virus, which is not yet infective.

The haemagglutination reaction is important because it provides a simple and rapid method by which virus can be detected in egg and tissue culture fluids. Furthermore infected cells in tissue cultures adsorb red blood cells to their surfaces and are thus shown to be harbouring virus. Haemagglutination is also the basis of a

method of virus purification; virus in crude infected fluids is adsorbed on red cells which are then sedimented; after removal of the supernatant the virus is eluted into a small volume of clean fluid and the erythrocytes are removed by slow centrifugation.

Haemagglutination is found in two other groups of viruses. Pox viruses give the reaction, but the haemagglutinin is distinct from the intact infective virus. The haemagglutinin of the vaccinia virus is destroyed by lecithinase (*e.g.* a toxin of *Cl. welchii*) and is a lipoprotein; it is a much smaller particle than the virus itself and can be separated from it by centrifugation or adsorption with red blood cells.

The arthropod-borne viruses which cause such infections as dengue, yellow fever, Japanese B and other types of encephalitis also possess haemagglutinins, as also does the mouse pneumonia virus (P.V.M., see p. 771). These haemagglutinins appear to have a reversible state of equilibrium with erythrocytes; they are inhibited by lipids and are very sensitive to slight variations of pH.

Haemagglutination reactions of all these three types are used in antibody estimations. Viruses when mixed with antibodies lose their power to agglutinate red blood cells; the extent to which a serum is able to inhibit haemagglutination is a measure of its antibody content. *Haemagglutination inhibition* tests are highly specific and are particularly valuable in serological work with the variants of the influenza viruses.

Interference and Interferon

When animals are inoculated with certain viruses they may become for a while resistant to the effects of a second and more virulent virus. The injection of Rift Valley Fever virus into monkeys, in which it causes only a mild illness, protects them against the challenge of a lethal dose of the yellow fever virus and similarly the benign lymphocytic choriomeningitis virus excludes the effects of infection by the poliomyelitis virus. The viruses in these examples of interference are unrelated and are distinct immunologically.

There are, however, many other instances of interference where the two viruses are more closely related and in these the phenomenon can be clearly demonstrated in experiments with chick embryo and tissue cultures. Thus previous infection with influenza A virus precludes subsequent invasion by the influenza A, mumps or Newcastle viruses. There is some evidence to suggest that interference may occur under natural conditions in man, for if patients convalescent from infection with dengue type I virus

are challenged with the immunologically distinct dengue II virus they can be shown to have an evanescent resistance.

Interference has frequently been observed to occur between viruses and their own mutants; the dermotropic vaccinia virus suppresses the growth of its neurotropic variant, and non-encephalitogenic strains of the herpes simplex virus protect the rabbit against virulent encephalitogenic strains.

The interfering action of living viruses may be due to a variety of causes. Enzymatically active virus may destroy or occupy all the receptor areas on the host cell and thus prevent access by the second virus; or the interfering virus may compete successfully for the control of enzyme systems and nucleic acid synthesis within the cell, leaving no metabolic processes available for the second virus. It is also possible that the mechanism of escape of the interfering virus from the cell is blocked and that this prevents invasion by the challenging virus. In short, if the cell is occupied by an interfering virus a second virus may be unable to enter.

It must be remembered, however, that there are some occasions when two viruses can invade and multiply in the same cell simultaneously without interference occurring. When one virus is situated in the nucleus and the other in the cytoplasm both can reproduce unhindered, e.g. herpes simplex and vaccinia, measles and poliomyelitis.

Killed virus as well as living virus possesses the power to interfere. Heat at 56° C. and exposure to ultra-violet light destroy the infectivity of a virus without affecting the property of interference. When heated influenza virus is incubated *in vitro* with chick chorio-allantoic membranes a substance is produced which possesses all the interfering activity of the original virus. This substance is known as "interferon"¹ and can be separated from the virus and can to some extent be purified.

Interferon differs from the original virus in many ways. It is much smaller in size, being a particle of about 0.034 μ in diameter as compared with 0.120 μ, the diameter of the virus particle. It is serologically distinct and is not neutralised by specific antiviral sera, it cannot be adsorbed on to erythrocytes, is non-dialysable and is not antigenic even when injected with adjuvants. It is stable over the pH range 2 to 11 and its ability to withstand pH 2 is useful in distinguishing it from inactivated virus. Several properties suggest that it is a protein; it is destroyed by peptic digestion and profoundly altered by trypsin, it is precipitated by ammonium sulphate and slowly denatured by shaking with an

¹ Isaacs, A., & Burke, D. C. (1959), *Brit. med. Bull.*, 15, 185.

amylalcohol-chloroform mixture. In general interferon resembles many other biologically active macromolecules.

Interferon inhibits the growth of a considerable number of viruses including influenza A and B, Newcastle, Sendai, cow-pox and vaccinia. It has no action on these viruses *in vitro*. Interferon has been produced by inactivated virus in a variety of host cells, including chick fibroblasts, monkey and calf kidney, and in pure lines of human cells. There is some evidence to suggest an element of specificity, for interferon produced in any one of these systems exerts its maximum effect in that system.

Interferon may have considerable potentialities as a therapeutic or prophylactic agent in human and veterinary medicine, as well as being a research tool for use in studying the problems of growth inside cells.

Virus Mutation

One of the most striking properties of animal viruses is their marked tendency to variation and mutation. In a growing virus population mutation occurs constantly and there is a continuously active process of selective proliferation and survival which determines the character of the dominant virus. Mutant viruses differ from the parent strains in many ways; they may have an increased rate of reproduction, altered haemagglutinating characters, increased or diminished pathogenicity or a modified antigenic structure. It is its extreme mutability and the plasticity of its antigenic structure that enables the influenza virus A to assume a new form about every third year and to initiate frequent epidemics.

In contrast, other mutants remain antigenically stable but lose the pathogenicity of the parent strain; such mutants are referred to as being "attenuated". Thus the vaccinia virus has retained the antigens of the variola virus but has lost its power to produce smallpox, and the 17D strain still contains the antigens of the yellow fever virus although it lacks its virulence. Both these mutants are of great importance because they can be used safely as highly efficient immunising agents.

The isolation of mutants of this type from other viruses is a matter of importance because vaccines containing live virus impart a full measure of durable immunity than do those containing killed virus. The selection of an attenuated mutant is usually accomplished by passing large doses of the original virus in a series of rapid transfers in an unfamiliar host, so that any fast-growing mutant will have optimal conditions for survival. When a mutant reproduces more rapidly than the parent strain it can

be separated in a pure clone from a single virus particle. This can be done by making limiting infective dilutions and transferring to a new host, or by plating on a monolayer of cells in tissue culture, using the plaque technique (p. 458).

By these methods, attenuated mutants have been obtained from the three poliomyelitis viruses and have been used in the living state as vaccines for oral administration. Vaccines of this type are at present under trial. Using egg cultivation methods, attenuated strains of the rabies virus (Flury strain), the canine distemper virus, and the rinderpest virus have been obtained and have provided efficient vaccines for veterinary use.

Recombination

If a host cell is confronted by two viruses simultaneously, there are three possible outcomes: (a) both viruses may enter and multiply without either influencing the other; (b) entry of one virus may inhibit the multiplication of the second (interference); (c) the presence of the first virus may influence the reproductive processes of the second so that the progeny develop characters derived from both the parent viruses. In the third eventuality a genetic transfer of material between the two growing viruses has occurred and is referred to as a process of *recombination*. It has been shown for example that the neuropathogenicity of a variant of influenza virus A (N.W.S.) can be transferred under these circumstances to another influenza virus (M.E.L.) which lacks this quality. Recombination has been most intensively studied in the group of influenza viruses, but has been induced between vaccinia and rabbit pox, and in strains of the psittacosis virus. For further reading on the subject of virus genetics the reader is referred to a review of the work of Burnet¹ and other workers.

Antiviral Immunity

Resistance to infection by viruses depends on the same defence mechanisms which operate against bacteria. Infection by viruses is consistently followed by the development of specific antibodies which can be measured by the usual immunological methods, such as complement fixation, neutralisation and precipitation. Natural immunity, together with species specific resistance, is as familiar in virus diseases as it is in bacterial infections. The rôle of antibodies in overcoming viral infections lies principally in their power to combine with the invading micro-organism and to prevent it gaining access to the host's cells. In this way they

¹ *The Viruses* (1959), Vol. 3, 275-305. New York: Academic Press.

are less efficient than antitoxins, which combine rapidly and avidly with bacterial toxins, but far more effective than the humoral antibodies in such infections as tuberculosis or typhoid fever. In the rare condition of congenital agammaglobulinaemia children do not develop antibodies after receiving inoculations of antigens nor after recovering from measles, mumps, chickenpox, or Jennerian vaccination. And yet these conditions are no more severe than in ordinary children and after recovery the children are clinically resistant to re-infection or re-vaccination. Viral immunity must rest on other factors besides humoral antibodies, and it seems probable that cellular resistance together with interference and interferon production by other viruses may be of importance. The rôle of phagocytic cells and the value to the host of non-specific factors such as complement and properdin is still undetermined.

The outstanding feature of all virus infections is the intracellular situation of the growing virus; here it is protected by the cell wall which is an impervious barrier to circulating antibodies. It is this circumstance which renders ineffective the therapeutic use of antisera once a virus infection is established; in paralytic poliomyelitis, for example, antibodies given in the form of convalescent serum or as gamma globulin do not benefit the patient, because they cannot reach the virus as it grows within the horn cells of the spinal cord. The real value in medicine of antibodies lies in their capacity to neutralise the virus *before* it reaches the host cells. Thus the early prophylactic use of human gamma globulin is highly effective in preventing the establishment of infection in persons recently exposed to measles or infective hepatitis.

Some virus infections are followed by a prolonged immunity which may persist for life, while in others it may last for only a few months. A prolonged immunity is characteristic of measles, mumps, smallpox, yellow fever and equine encephalomyelitis and second attacks of these diseases are extremely rare. It is significant that in all these diseases there is a phase of viraemia and often a long incubation period. After multiplication in a primary focus of infection for about ten days, the virus overflows into the bloodstream; it is then carried to fresh host cells at a distance where it may multiply to produce a rash and provide a powerful secondary stimulus to antibody production. Viruses which give long-lasting immunity are almost invariably antigenically homogeneous and ~~and~~ is rare for them under natural circumstances to vary or mutate.

One explanation of the reasons for the long persistence of a solid immunity may be that the virus remains alive in a modified

form within affected cells to act continually as a stimulus to antibody formation. It must, however, be stated that whenever such events have been proved to occur, as in herpes simplex infection around the lips in man, or in benign lymphocytic chorio-meningitis infections in mice, there is, instead of a solid immunity, a marked tendency to relapse with recrudescence of clinical lesions.

Limited immunity of short duration occurs most frequently in conditions where there is no evidence of a viraemic phase and where the infecting virus has a marked tendency to antigenic variation. Influenza, the common cold, and many other viral infections of the upper respiratory tract are followed by immunity of very short duration and second attacks of these illnesses occur very frequently. They are infections of surface mucous membranes and the viruses reach the host cells directly by the inhalation of infected particles without being exposed to antibodies or virucidal mechanisms in the bloodstream. Immunity to influenza is further limited by the existence of multiple immunological types of the virus. An attack of influenza is, of course, followed by the development of specific antibodies, but these are of limited effectiveness in protecting the individual, partly because the next virus may be of a novel antigenic type and partly because there is little opportunity for contact between virus and antibody. It is possible that the small measure of immunity that does follow an attack of influenza is mediated by antibodies present in the nasal secretions.

CHAPTER XXXVII

THE PSITTACOSIS LYMPHOGRANULOMA GROUP OF VIRUSES

THE group consists of a number of large viruses which are 250–500 m μ in diameter and which can be stained and seen with the light microscope. These viruses include the agents which cause psittacosis, lymphogranuloma venereum, trachoma and inclusion conjunctivitis in man, as well as those causing murine and feline pneumonitis and abortion in sheep. A number of similar viruses have been described in other animals.

The properties of these viruses are so different from those of other groups that some authors prefer to classify them with rickettsiae even though they have no arthropod vector. All the members of the group take Castaneda and Macchiavello stains in the same way as the *Rickettsiae*, and the inclusion bodies that they produce are basophilic. They have a complex cycle of reproduction which terminates in a phase of binary fission of the virus particles. All are sensitive to antibiotics and sulphonamides and all share a common heat stable antigen.

PSITTACOSIS

Psittacosis is an epizootic disease of birds which may affect man. Psittacine birds such as parrots, parakeets, cockatoos and budgerigars are natural hosts of the infection, and pet birds of this type have often been the source of human infections. Ducks, pigeons, turkeys, fulmars and certain species of gulls are also affected, and in these hosts the disease is known as ornithosis.

In birds the disease is characterised by diarrhoea, emaciation and a purulent nasal discharge. Man is infected by inhaling dust which is heavily contaminated by exudates from affected birds. The carrier state may persist in the birds for many months and human cases have not infrequently been traced to apparently healthy birds which were later proved to have harboured the virus. The resultant illness is pneumonic in type and in untreated cases may be severe with a 20 per cent. mortality. Often, however, the illness is mild and resembles an attack of influenza.

Properties of the Virus.—The mature elementary body is a sphere about 250 m μ in diameter; it stains blue with Castaneda's

stain. Under the electron microscope the particle is seen to be covered with a membrane like a cell wall, and to have an irregular surface; the appearance has been likened to that of a wrinkled pea. During reproduction the virus passes through a regular sequence of morphological changes. On entering the cell cytoplasm it gives rise, after a few hours, to a vaguely defined mass seen only in ultra-thin sections under the electron microscope. After eight or nine hours a basophilic inclusion appears and increases in size as time passes. Within the inclusion a few large particles about $1.0\ \mu$ in diameter appear and these divide and subdivide by binary fission to give rise to large numbers of mature elementary bodies. The final effect is that the parasitised cell disintegrates and the virus is liberated into the intercellular spaces.

The psittacosis virus is quickly inactivated by heat at 60° C . and by the ordinary disinfectants. Penicillin and the tetracyclines interfere with the growth cycle and cause the production of abnormal developmental forms within the cell. The virus is not sensitive to streptomycin and only a few strains are influenced by sulphonamides.

Culture.—The virus grows readily in the cells of the yolk sac of six- to eight-day-old chick embryos and in the peritoneal cavity and spleen of mice. Chick embryos die within four days of inoculation and mice in four to six days. Intranasal instillation of the virus in mice causes pneumonia, and intracerebral inoculation a fatal encephalitis.

The virus of ornithosis resembles that of psittacosis very closely and it is distinguished mainly by its pathogenicity for certain laboratory animals in which it is nearer to the lymphogranuloma virus. There is evidence to suggest that the ornithosis virus possesses a type-specific antigen.

The lethal effect of these viruses may be due in part to an endotoxin, for large doses may kill the host before there has been time for the virus to multiply. Latent infection in psittacosis and ornithosis may be common; it is known that the virus may fail to reproduce within the cells of tissue cultures grown in nutritionally poor medium. Multiplication resumes when a satisfactory medium is provided.

Antigenic Structure.—A heat-labile antigen is thought to be specific for the psittacosis virus itself; since it resists proteolytic enzymes and is destroyed by periodate, it is probably a carbohydrate. A second thermostable antigen is shared by all the members of the virus group and is employed in the diagnostic complement-fixation reactions in general use. The antibody to this common antigen is not protective.

Laboratory Diagnosis

Human Infections.—The sputum, which contains the virus during the first few days of the infection, is emulsified in Hanks' solution containing no antibiotics. Treatment of the specimen with sulphonamides or penicillin is contra-indicated. The suspension is spun in the centrifuge at 1000 r.p.m. to deposit cells and debris. Inoculate four to six mice intraperitoneally with 0·5 ml. of the supernatant. If the psittacosis virus is present, some or all of the mice will die seven to ten days later and will show a glairy peritoneal exudate, and enlargement of the spleen and liver. Films of the peritoneal exudate and impression smears of the spleen when stained with Giemsa's or Castaneda's stains show typical virus inclusions and clusters of elementary bodies. To confirm the diagnosis a 10 per cent. suspension of the infected mouse spleens in Hanks' solution should be passed to further mice and an attempt should be made to protect some of these with an immune serum.

If the mice inoculated with human material survive for ten days, they should be killed and the spleens examined microscopically for virus. If no virus is found, an emulsion of the spleens is injected intraperitoneally in four to six mice. If the animals of this second passage show no sign of disease the case is presumed negative; if they die, they are examined as above.

Eggs may be used as well as mice to isolate the virus, but often the sputum and the contained bacteria are so toxic that the embryo dies before the virus has time to multiply. Streptomycin added to the sputum suspension in concentrations up to 2000 µg. per ml. may be used to overcome this difficulty.

The sputum suspension in a dose of 0·1 ml. is inoculated into the yolk sac of six- to eight-day-old embryos. If the virus is present, the embryos will die three to four days later. Impression smears of the stalks of the infected yolk sacs should be stained by Castaneda's or Macchiavello's stains and examined microscopically for clusters of virus elementary bodies. Care is required to distinguish the virus from yolk sac granules which, however, are irregular in shape and size and are usually larger than the virus.

Serological confirmation of the diagnosis is obtained by the use of the complement fixation test (p. 346). Serum samples are taken early in the disease and ten to fourteen days later. A four-fold or greater increase in titre provides firm evidence of infection. The single observation on a convalescent case of a serum titre of 32 or higher is highly suggestive of recent infection.

Avian Infections.—Great care must be taken by laboratory

workers in handling dead birds and in performing the post-mortem examination, for the material is highly infective. The procedure is to remove the spleen and to prepare an emulsion from it. Mice and eggs are inoculated in the same way as for sputum from the human case.

If the birds have recovered, blood may be taken from the wing vein (see p. 423) and a complement fixation test carried out. Positive reactions should, however, be interpreted with caution because psittacine birds and pigeons frequently have low levels of antibodies.

LYMPHOGRANULOMA VENEREUM

(*Lymphogranuloma Inguinale or Climatic Bubo*)

This disease is transmitted by venereal contact. After an incubation period of three to twenty-one days a small primary sore appears; in the male it may be situated on the glans penis or in the urethra, causing urethritis; in the female the vaginal wall or the cervix uteri are the common sites. The virus spreads from the primary site to involve the regional lymph nodes, where an inflammatory reaction and suppuration develop. In the male, suppuration of the inguinal glands gives rise to a bubo, from which it is possible to aspirate pus. In the female, the lymphatic drainage carries the virus to lymph glands within the pelvis and inguinal buboes do not occur. As a late result of chronic inflammatory processes in the perianal tissues, rectal stricture, granulomatous involvement of the vulva, and elephantiasis of the genitalia may result.

Properties of the Virus.—The virus is a typical member of the psittacosis group. In morphology, viability and susceptibility to chemotherapeutic agents it resembles very closely the psittacosis virus.

Culture.—After it multiplies. Latent infection in ~~psittacosis~~ if the yolk sac of developing chick embryos. Usually the embryos do not die until after seven to nine days after inoculation. It will multiply in the mouse in seven to nine days after inoculation. It will multiply in the mouse in seven to nine days after inoculation and does not grow in the mouse.

Infectivity.—It does not infect birds. It does not infect birds. *Diagnosis.*—Isolation of the virus may be achieved by inoculation of the yolk sac of six- to eight-day-old chick embryos and by the inoculation of mice by the intracerebral route. If the pus is contaminated with bacteria it may be killed by streptomycin at a concentration of up to 2000 µg. per ml.; penicillin, tetracyclines and sulphonamides should not be used for the purpose for they may kill the virus.

Complement Fixation Tests are carried out using a heated antigen prepared from infected yolk sacs in the same way as for psittacosis (p. 346). The test becomes positive two to four weeks after the onset of the infection. A titre of 32 or higher in a clinically acceptable case suggests active infection, but better evidence is obtained if a significant rise in titre can be demonstrated during the course of the disease. False positive reactions may be given by syphilitic sera.

Frei Test.—Patients with lymphogranuloma venereum react allergically when inactivated virus is inoculated intradermally. The antigen is prepared by phenolising a purified virus suspension from infected yolk sacs.¹ Originally pus from the bubo suitably diluted and inactivated at 60° C. was used for the purpose. The test is carried out by injecting 0·1 ml. of the antigen intracutaneously into one arm and a similar amount of control material from normal eggs into the other arm. A positive reaction takes four days to reach its maximum and consists of an area of erythema with a central indurated papule at least 7 mm. in diameter. Minor reactions, especially if accompanied by any change in the control inoculation, should be disregarded.

The Frei test remains positive many years after the active infection has subsided. Like the tuberculin reaction it is an indication that infection has at some time taken place. The test is positive when infection has been due to other members of the group and depends on hypersensitivity to the heat-stable antigen. The Frei test, therefore, is not specific for lymphogranuloma venereum.

Tetracyclines and, to a lesser degree, sulphonamides are effective as therapeutic agents.

TRACHOMA

Trachoma is a disease which progresses through an early phase with follicles in the conjunctivae, to bleb formation and papillary hypertrophy, and then to fibrous tissue formation with scarring and anatomical distortion. Corneal pannus results.

The disease is prevalent in Eastern Europe, the Middle East, North and Central Africa and parts of South America and is the cause of much blindness. Infection is due to a virus and is spread from eye to eye directly by the fingers and also by flies. Secondary bacterial infection is present at all stages of the infection and plays an important accessory part in tissue damage.

¹ Available commercially as "Lygramum" (Squibb).

Properties of the Virus.—The elementary bodies are 0·3–0·4 μ in diameter, and electron microscopically have the “wrinkled pea” appearance of the psittacosis-lymphogranuloma group. Larger forms 0·7 μ in diameter are also seen. They are stained blue by Castaneda's stain and red by Macchiavello's stain.

The virus multiplies within the cytoplasm of the epithelial cells of the conjunctiva and passes through a cycle of morphological changes closely similar to that of the psittacosis virus. The first sign of infection is the appearance of an “initial body”, a basophilic inclusion 0·3–0·8 μ in diameter. This body divides by fission and as the particles increase in number they become smaller and more acidophilic. The mature inclusion consists of a mass of elementary bodies which are stained reddish by Giemsa's stain; they are set in a matrix composed of glycogen. In the final stage of development the inclusion bodies often entirely replace the cytoplasm.

Many different shapes and sizes of inclusions may be seen; they are usually known as Halberstaedter-Prowazek bodies and on their detection depends the confirmation of the clinical diagnosis.

The infection has been transmitted to human volunteers and to baboons. Only recently has it been found possible to cultivate the virus in the laboratory.¹ Infected corneal scrapings are treated with streptomycin at a concentration of 2500 $\mu\text{g}/\text{ml}$. to destroy bacterial contaminants and inoculated into the yolk sac of five- to six-day-old chick embryos. Virus cultured in this way has the typical morphology of the group and is infective when introduced into the cornea of human volunteers.

Laboratory Diagnosis.—Smears of scrapings of the conjunctival epithelium are made on glass slides. They are fixed for ten minutes in methyl alcohol and stained overnight with a 1 in 10 solution of Giemsa's stain. A search is then made for the basophilic Halberstaedter-Prowazek inclusions.

Sulphonamides and tetracyclines are very valuable in the treatment of trachoma.

INCLUSION CONJUNCTIVITIS

A virus close to the newbo, the trachoma virus causes inclusion blenorhoe² and urethritis in ad inclusion conjunctivitis as well as cervicitis. *Properties of the virus* are thos.

The trachoma group; the inclusions formed the psittacosis-lymphogranuloma group; the epithelial cells are

¹ See Collier, L. H. (1959), *Brit. med. Bull.*, 15,

basophilic with a glycogen matrix and are indistinguishable morphologically from those of trachoma. When transmitted to human volunteers a severe conjunctivitis results, but there is no subsequent scarring or pannus; when inoculated into apes, a severe mucopurulent conjunctivitis is produced. Recently the virus has been isolated from infected epithelium by the inoculation of the yolk sac of chick embryos and has been cultured in this way in series and in quantity.¹

Laboratory diagnosis is made by the examination of smears of corneal scrapings in the same way as for trachoma.

Epidemiology.—The reservoir of the infection is the human genital tract and the virus is spread by venereal contact. The virus may be present in cells without apparently causing any symptoms, for it has been detected in smears of the transitional epithelial cells just within the *os uteri* of apparently healthy women. In the male it has been found to be associated with a mild purulent urethritis. A small proportion of cases of non-gonococcal ("non-specific") urethritis (about 3 per cent.) are due to infection with this virus. The disease has been known in the past as "swimming bath conjunctivitis" from the fact that many cases have occurred in bathers. It is probable that the water may have been infected by the discharges from the genital tracts of infected persons.

The virus may be transmitted from the cervix uteri to the infant conjunctiva at birth, giving rise to a severe, acute, mucopurulent conjunctivitis. There is, however, no corneal ulceration and the lesions slowly heal without scarring.

Tetracycline and sulphonamide therapy is effective.

ENZOOTIC ABORTION IN EWES

Abortion and premature lambing is frequent in flocks on tick-free pastures in south-east Scotland and has been reported in New Zealand and Australia. The causative organism is a virus of the psittacosis-lymphogranuloma group.²

Properties of the Virus.—In size, morphology and staining reactions the ewe abortion virus is indistinguishable from other members of the group. It can be cultivated readily in the yolk sac or on the chorio-allantoic membrane of the developing chick embryo. When injected intravenously into pregnant ewes abortion results, and when instilled intranasally in mice a

¹ See Collier, L. H. (1959). *Brit. med. Bull.*, 15, 231.

² Stamp, J. T., McEwen, A. D., Watt, T. A., & Nisbett, D. L. (1950), *Vet. Rec.*, 62, 251.

pneumonia is produced. Cattle may be infected experimentally and abort, but the disease does not appear to occur naturally in bovines. The virus possesses the same heat-stable complement-fixing antigen that is found in all the other members of the group.

Laboratory Diagnosis.—The gross appearances of the aborted placenta and membranes are similar to those seen in the bovine as a result of infection with *Brucella abortus* (see p. 649). Films should be made from the diseased cotyledons, from the chorion, and from purulent discharges. On staining with Macchiavello's or Castaneda's stain, many clusters of virus elementary bodies can be seen within the cytoplasm of cells. The virus may be isolated by the egg cultivation methods used for psittacosis; usually seven to eight days pass after inoculation into the yolk sac before the embryos die.

Epidemiology.—Abortion and premature lambing are the only striking features of the infection and usually take place late in pregnancy, often in the last two or three weeks of the gestation period. The infected membranes and discharges contain huge amounts of the virus and are able to contaminate the pastures and lambing pens very heavily. Probably infected pastures are the common source of infection. However, lambs born of infected ewes may survive and carry the virus in their tissues until maturity. When such animals are themselves fertilised they are frequently liable to abort and in one experiment 27 per cent. lambed with infected foetal membranes.¹ It is possible that the ewe abortion virus occasionally infects man; there is one case recorded of a laboratory worker who contracted the infection.

Control.—A vaccine prepared from formolised yolk sac cultures of the virus,² precipitated with alum and suspended in mineral oil, has been shown to stimulate the production of virus-neutralising antibodies. The use of this vaccine by subcutaneous inoculation before service has proved highly effective in reducing the incidence of ewe abortion.

Feline Pneumonitis

The aetiological agents responsible for the common forms of respiratory infections in the domesticated cat are ill understood. "Cat flu" is a very common and highly contagious illness, and much remains to be done in the study of this condition. From some types of cases American workers have isolated a virus which has all the morphological and cultural characteristics of the psittacosis-lymphogranuloma group. The feline pneumonitis virus has a similar host range

¹ McEwen, A. D., Littlejohn, A. L., & Foggie, A. (1951), *Vet. Rec.*, **63**, 489.

² McEwen, A. D., Dow, J. B., & Anderson, R. D. (1955), *Vet. Rec.*, **67**, 393.

to the other members of the group and after infection neutralising and complement-fixing antibodies appear. The significance and the incidence of infections with this virus in felines in Great Britain has yet to be determined.

Cat Scratch Disease

Cat scratch disease is characterised by fever, malaise and adenitis. The primary lesion is usually a cat scratch or bite often developing into a cutaneous pustular lesion and followed by a marked adenitis and bubo formation. Mere contact with a cat, however, has sometimes been thought to cause the infection and has been followed by marked adenitis. Clinically the disease resembles many other infections of lymph glands especially lymphogranuloma venereum.

The disease is thought to be due to a virus and elementary bodies of the psittacosis type are said to have been found in stained preparations of pus. Monkeys have been infected by the inoculation of the pus and develop nodules at the site of injection and a lymphadenopathy.

In man the diagnosis depends on the use of an intra-dermal test with antigen prepared from pus from a lymph gland of an undoubted clinical case. A positive reaction consists of a firm papule 0·5-1·0 cm. in diameter within forty-eight hours of inoculation. Although the results of such tests appear to indicate that the antigen is specific, positive reactions should be interpreted with caution, since they may occur in normal people presumably as the result of previous undiagnosed infection. It is not yet clear whether cat scratch fever is a feline disease transmitted to man (e.g. feline pneumonitis) or is an infection of which the cat is merely a vector.

Murine Pneumonitis

Several viruses associated with pneumonitis in mice have been described. Nigg's mouse pneumonitis was isolated from a healthy colony and has all the morphological and general characters of the psittacosis, lymphogranuloma group. Horsfall and Hahn's pneumonia virus of mice (P.V.M.) has been isolated from healthy mice as well as from patients with pneumonia. This virus agglutinates mouse and hamster red blood cells. The natural occurrence of mouse pneumonitis viruses must be borne in mind when human material is inoculated. The instillation of sputum suspensions into the nares of mice under ether anaesthesia may well activate a latent pneumonitis virus being carried by the animal. Furthermore, it is probable that mouse pneumonitis viruses occasionally cause disease in man.

CHAPTER XXXVIII

SMALLPOX AND VACCINIA

THE POX VIRUSES; VARICELLA AND ZOSTER VIRUS

THE characteristic feature of the diseases caused by the pox group of viruses is the formation of papules, vesicles and pustules in the skin; generalised manifestations of illness may be very severe or entirely absent. In man these viruses cause smallpox, alastrim, vaccinia, chickenpox, herpes zoster and molluscum contagiosum. In animals they give rise to cow-pox, swine-pox, mouse-pox (ectromelia) and to similar diseases in all domestic animals except the dog and the cat. Myxomatosis in rabbits is the result of infection with a virus which has many of the characters of a pox virus. Avian pox viruses cause fowl-pox and similar infections in turkeys, pigeons, canaries and a wide variety of other birds. In avian pox diseases the lesions tend to be proliferative rather than pustular with the formation of multiple tumour-like masses.

Pox viruses are within the size range $200 \times 300 \text{ m}\mu$ to $264 \times 332 \text{ m}\mu$ and are large enough to be visible with the light microscope. They have a predilection for infecting epithelial cells, in which they produce characteristic eosinophilic intracytoplasmic inclusions. The great majority of pox viruses can be cultivated in the chorio-allantoic membrane of chick embryo, where they give rise to pock-like lesions which are easily recognised with the naked eye. Under natural conditions, however, most of these viruses are restricted to a single host, although notable exceptions are the cox-pox and vaccinia viruses which can infect man, cattle and a number of other animals. Animal pock-producing viruses are closely related antigenically to each other, but are distinct from the avian pox viruses.

SMALLPOX AND VACCINIA

Smallpox virus (*Poxvirus variolae*) and vaccinia virus (*Poxvirus officinale*)

Morphology and Staining.—The elementary bodies of both viruses are $200-300 \text{ m}\mu$ in diameter as seen by dark-ground microscopy or in stained preparations and roughly spherical in shape. They can be stained with aniline dyes, as in Gutstein's or

Paschen's methods (see p. 139) but not by Castaneda's method. When dried films of purified virus preparations are examined electron-microscopically, the appearance is that of brick-shaped particles measuring $210 \times 260 \text{ m}\mu$, in which the central area is a mass of material especially opaque to the electron beam. This brick-shaped appearance, however, may be an artefact because in ultra-thin sections of infected cells the virus particles are oval in shape and have a multi-layered covering membrane. The central electron-dense material in the vaccinia virus contains DNA and this virus is known also to contain phospholipids, neutral fat, carbohydrate, flavin and biotin in proportions similar to those of bacteria and mammalian cells.

Host Range.—The host range of the smallpox virus is limited to the primates; apart from man, monkeys are the only animals susceptible to natural infection. Other animals are only slightly susceptible, and of these the rabbit is sometimes used in Paul's test for the smallpox virus; here the virus is inoculated into the scarified cornea and produces a keratitis. Intracytoplasmic inclusions (Guarnieri bodies) in epithelial cells are characteristic of infection with variola and vaccinia viruses; they are round or oval, eosinophilic and there may be one or more in an infected cell. Guarnieri bodies consist of masses of elementary bodies set in a matrix. The vaccinia virus has a much wider host range than the variola virus; calves, rabbits and sheep are all used regularly for the propagation of the virus for vaccine lymph, and monkeys, mice rats, hamsters and guinea-pigs can also be infected, though they are rather less susceptible.

Both viruses grow well on the chorio-allantoic membrane of the ten-day-old chick embryo, each producing its own characteristic pocks. The variola virus gives rise to white circular plaques of epithelial hyperplasia which are visible to the naked eye forty-eight hours after inoculation and reach 1-2 mm. in diameter in seventy-two hours; these lesions are uniform in size and often lie near the blood vessels of the membrane. The virus from cases of alastrim may grow more slowly and the pocks may not be visible until four to six days after inoculation. The pocks of the vaccinia virus are generally much larger and more variable in size than those of the variola virus; after seventy-two hours' incubation the majority are 4-5 mm. in diameter with a definite yellowish coloration. Small seedling pocks beside the larger pocks of vaccinia are characteristic.

Vaccinia virus grows readily in many types of tissue culture; minced tissue suspensions of the Maitland type, explants in plasma clot of chick embryo, and rabbit kidney tissues are all highly

susceptible. In monolayer cultures of trypsinised human or monkey kidney, or in human amnion or HeLa cells the vaccinia virus produces a marked cytopathic effect within forty-eight hours of inoculation. In suitably prepared monolayer cultures of monkey kidney the virus gives rise to plaques.

Viability.—Variola virus is very stable and survives in exudates from cases for many months; living virus has been recovered from crusts kept at room temperature for over a year. It can be preserved in sealed ampoules at 4° C. for many months and indefinitely by freeze drying. Vaccinia virus in calf lymph stored in the dark at -10° C. retains its activity for at least six months. Between 0° C. and 10° C. vaccine lymph retains its potency for at least fourteen days, and at room temperature (22° C.) for seven days. Freeze dried vaccine kept under an atmosphere of nitrogen at 37° C. maintains its activity for several weeks and at 4° C. for years. The virus is destroyed by moist heat at 60° C. in ten minutes, but in the dry state can resist 100° C. for five to ten minutes. Both viruses withstand 10 per cent. phenol at 4° C. for several weeks, but at 37° C. are killed by it within twenty-four hours. Ultra-violet light, X-rays and gamma rays are rapidly lethal, 0.01 per cent. potassium permanganate and 50 per cent. ethyl or methyl alcohol and acetone kill the virus within one hour. Acids at a pH value of 3 destroy the virus within an hour.

Antigenic Characters.—It is almost impossible to distinguish the three viruses of variola major, alastrim and vaccinia by serological methods because they all share major common antigenic components. Purified suspensions of variola and vaccinia viruses can be shown to contain two antigens; one, the LS antigen, dissociates from the elementary bodies on standing at refrigerator temperature; the other, the nucleo-protein (NP) antigen, is associated with the virus particles themselves. The LS antigen is a loose combination of the L and S components in an elongated protein molecule which is known to have a molecular weight of about 240,000. The L component is inactivated by heat at 60° C., but the S component is stable at 90° C. or above. Antibodies to the LS antigen precipitate and also fix complement with their homologous antigens, but they do not protect an animal from the effects of the vaccinia virus nor will they neutralise the virus in laboratory tests. The NP antigen can be extracted from elementary bodies with dilute alkali; it is known to contain 6.0 per cent. desoxyribonucleic acid and it is a second serologically specific component of the vaccinia virus.

Haemagglutination.—Preparations of the vaccinia and variola viruses agglutinate the red blood cells of mammals, but erythrocytes

from only about 60 per cent. of fowls are sensitive to the virus. The haemagglutinin is smaller than the virus particle and can be separated from it by centrifugation; it is $65\text{ m}\mu$ in diameter and is mainly composed of lipoprotein. When the haemagglutinin is removed from virus preparations there is no loss of infectivity. The haemagglutinin is heat stable and able to withstand boiling for ten minutes; it is distinct from the virus particles and from the LS and NP antigens. Antibodies to the haemagglutinin are developed after smallpox and after vaccination; they are not related to either neutralising or LS antibodies.

Occurrence.—Smallpox virus enters the body through the upper respiratory tract; it first infects the mucosal cells and soon afterwards is thought to reach the regional lymph nodes. At this stage the patient is not infectious and it is improbable that there is an open lesion in the respiratory mucosa. A transient viraemia may follow with the infection of reticulo-endothelial cells throughout the whole body; multiplication of the virus in these cells leads to a second and more intense viraemia which heralds the onset of the clinical illness. The virus can be isolated from the blood in a proportion of cases of smallpox, but the phase of viraemia is short-lived and by the end of the second day of the fever the virus can no longer be detected. During the first three or four days of the fever the virus multiplies in the epithelial cells of the skin; focal lesions are formed which give rise to the rash and maculae appear in typical centrifugal distribution and progress to papular, vesicular and pustular stages. Smears made from the early papular lesions show very large numbers of elementary bodies and in the later stages crusts from the pustules are heavily laden with virus.

Classical smallpox (*variola major*) has a case mortality which varies from 5 per cent. in patients with a discrete rash to 40 per cent. in fulminating cases with a confluent rash. *Variola minor* (*alastrim*) is usually less severe than variola major at all stages of the illness, the rash is less profuse, the fever of shorter duration and the fatality rate is below 1 per cent. Variola minor may be indistinguishable from a mild case of variola major in a well vaccinated person. In other vaccinated contacts the infection may give rise only to fever and symptoms similar to those of the pre-eruptive phase without progressing further, a condition known as *variola sine eruptione*.

Epidemiology.—The origin of infection in smallpox is a patient suffering from the disease. Infected particles may be inhaled directly by the susceptible contact. The virus may also be transmitted indirectly by clothing, bed-linen, utensils or dust, and there

have been many occasions when workers in hospital laundries have contracted the infection from contaminated bed-linen. Patients are not infective during the incubation period of the disease, but from the time of the first appearance of the rash until the desquamation is complete they may be a profuse source of the virus. The clinical picture of variola may be considerably modified by previous vaccination and persons who develop only minor symptoms of the disease provide a dangerous source of infection.

Variola major is endemic in India, Pakistan, Burma, the Middle East, and in Latin America and Africa. In Britain the disease is no longer endemic and outbreaks when they have occurred have been traced to importation of the infection from abroad. Although smallpox is a highly infective disease it has not so great an epidemic potential as measles or chickenpox. When the disease has been introduced from abroad into Britain, extensive epidemics have not occurred and spread has usually been limited to close contacts of cases.

Laboratory Diagnosis.—Often there is great urgency in confirming the clinical diagnosis of smallpox. The collection of the necessary specimens has already been described (see p. 438). A quick presumptive diagnosis can be made in about 60 per cent. of cases by the direct microscopic examination of smears from the skin lesions. The films are stained preferably by Gutstein's alkaline methyl violet stain (see p. 139) and in cases of smallpox large numbers of elementary bodies uniform in size and about $0.3\text{ }\mu$ in diameter can be recognised; in chickenpox elementary bodies are usually scanty or absent. A report on this finding can be given within two hours of the specimen reaching the laboratory.

The presence of the virus can be detected rapidly in material from the skin lesions by preparing suspensions of vesicle fluid or crusts and using them as antigens with a high titre antivaccinal serum in complement fixation¹ or agar gel diffusion precipitin reactions.² The results of these tests are available within twenty-four hours. The complement-fixation test gives a positive reaction in over 90 per cent. of cases of smallpox, but does not distinguish between the vaccinia and variola viruses.

The virus can be isolated from the skin lesions or from the blood in the pre-eruptive phase of the illness. A suspension of vesicle fluid or ground-up crusts or serum from the blood is inoculated on to the chorio-allantoic membrane of ten- to twelve-

¹ Craigie, J., & Wishart, F. O. (1936). *Canad. Publ. Hlth J.*, 27, 351.

² Dumbell K. R. & Nizamuddin, M. (1959). *Lancet*, 1, 916.

day-old chick embryos; after forty-eight hours small white pocks are present and by seventy-two hours they are 2 mm. in diameter and identifiable as specific smallpox lesions. This procedure detects the virus in over 90 per cent. of cases of smallpox and has replaced the older method of inoculating the rabbit's cornea (Paul's test) which is not sufficiently reliable for routine use.

The three methods described should be used in combination so that the tests may serve to check each other. The combined use of these tests also helps to overcome the occasional difficulties which may arise from anticomplementary activity by the crust suspension or the appearance of non-specific lesions on the chorio-allantoic membrane. The results of the tests provide a very sensitive indication of the presence of the variola virus and give reliable confirmation or exclusion of the clinical diagnosis.

Antibody tests on the patient's serum can be carried out with the complement-fixation technique, using antigen prepared from vaccinia lesions in rabbits. Antibodies do not appear in the serum in smallpox until the eighth day of the disease, and the test is seldom of diagnostic value. As 30-40 per cent. of persons vaccinated within six to twelve months give a positive result, the test is only of value in a limited number of cases.

Vaccination may provide a useful diagnostic procedure because less than 10 per cent. of cases can be successfully vaccinated on the first day of the rash in smallpox, and none after the sixth day.

Prophylaxis.—General measures: When a case of smallpox is diagnosed the patient must be removed to a hospital or unit specially reserved for variola cases and after admission strict isolation precautions must be observed. The patient's clothing, bedding, personal possessions and his house should be disinfected with steam or formaldehyde vapour. All persons who could possibly have been in contact with the patient or his possessions during the feverish phase of his illness must be traced and placed under supervision for sixteen days.

The source of the infection must be sought and the chain of contacts followed back to the first notified case. In this work the results of laboratory tests may often be of great value in the diagnosis of doubtful cases.

Close contacts of smallpox patients should be vaccinated as soon as possible, but this measure may only be effective if used within two to three days of exposure. There is evidence to suggest that gamma-globulin prepared from hyper-immune serum in a dose of 1.5 g. affords some protection and its use should supplement vaccination of close contacts.

VACCINATION

The practice of vaccination stems from 1796, when Jenner inoculated a boy on the arm with the exudate which he obtained from a cow-pox lesion on the hand of a dairy-maid. When two months later the boy was inoculated with material from a case of smallpox no illness resulted and there was no local lesion. This simple and safe measure for protection against so serious an illness was soon taken into general use all over Europe. At first arm-to-arm vaccination was practised, but this was replaced by the inoculation of vaccinia lymph obtained from the skin of calves infected with the virus. The strains of vaccinia at present used in vaccine lymph manufacture are avirulent mutants of obscure parentage.

The preparation of Vaccine Lymph.—Vaccinia virus for vaccination has usually been obtained by inoculating the scarified shaved skin of the calf with pustular material from the rabbit similarly inoculated with the virus. Such scarification of the calf has consisted of lineal scratches half an inch apart on the skin of the abdominal area. (The virus is maintained by alternate inoculation in calves and rabbits.) About the fifth day, when well-developed vesicular lesions are present, the contents are scraped off with a Volkmann spoon, and mixed in a mortar with four times their weight of 50 per cent. glycerol. After storage at -10° C. for some time, the number of extraneous organisms diminishes, and the resultant product, which contains the virus, is called "glycerinated calf-lymph". Bacterial counts are made from the lymph at intervals, and when the number of cultivable bacteria is reduced to a certain figure as prescribed in the Regulations under the Therapeutic Substances Act, the material is passed for issue, provided it is free from haemolytic streptococci, Clostridia and other pathogenic bacteria, and conforms to a prescribed standard of potency. It should be noted that if the potency of the lymph is to be maintained it should be kept at below 0° C. or alternatively it should be freeze dried (Collier¹).

A modification adopted at the Lister Institute (McClean²) is briefly as follows: the sheep is used and the scarification is made on the flank; one part of the material collected from the lesions is ground up with two parts of 1 per cent. phenol and kept at 22° C. for forty-eight hours; this generally lowers the bacterial count

¹ Collier, L. H. (1955), *J. Hyg. (Lond.)*, 53, 76.

² McClean (1949), *Lancet*, ii, 476.

to the requirement of the Therapeutic Substances Regulations though if necessary the treatment may be prolonged for a further twenty-four hours; after this two parts of glycerol are added and the lymph is stored at -10° C .

Recently vaccines have been developed from chick embryo and bovine embryo tissue cultures. They have the advantage that they can be conveniently prepared under sterile bacteriologic conditions; if they prove potent they may replace the calf or sheep lymph now in general use.

Technique of Vaccination.—The multiple-pressure method is recommended. The skin is first cleansed with soap and water and allowed to dry. A drop of lymph is then placed at the site of inoculation and with the side of a Hagedorn needle held parallel to the skin multiple "pressures" are made to the skin through the lymph (figs. 28 and 29). In this way the inoculum is forced into the deeper layers of the epidermis. The area inoculated may be only one-eighth of an inch in diameter. The number of "pressures" varies from ten to thirty, e.g. thirty for primary vaccination of infants, ten for primary vaccination of children of school-age. This procedure involves less risk of septic infection

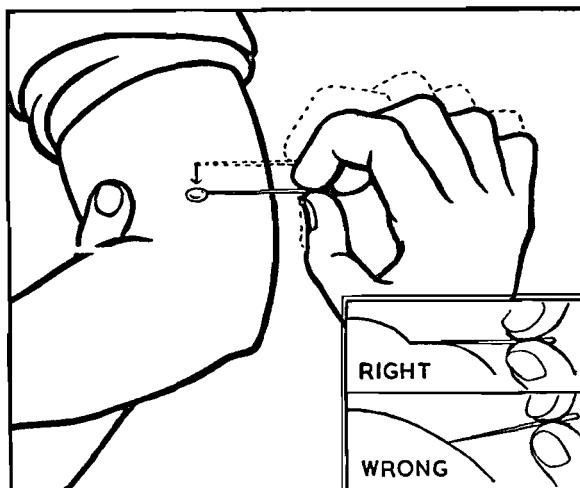


FIG. 28

The multiple pressure method of vaccination against smallpox.
Notice the up and down motion of the needle and the angle at
which it should be held.

Reproduced by permission of the Controller of Her Majesty's Stationery Office
from Memo on Vaccination against Smallpox. Minist. of Health (1948).
London: H.M. Stationery Office.

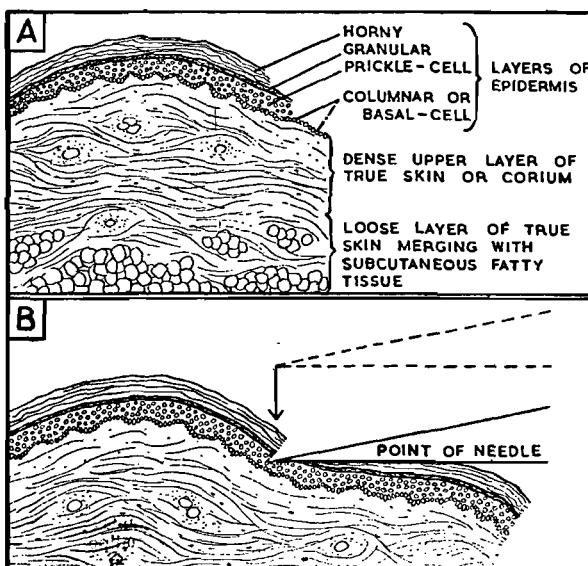


FIG. 29

The multiple pressure method of vaccination against smallpox. Diagram of a section of the skin of the arm. The motion of the needle and its final position penetrating only as far as the basal cell layer.

Reproduced by permission of the Controller of Her Majesty's Stationery Office from Memo on Vaccination against Smallpox. Minist. of Health (1948). London: H.M. Stationery Office.

and less severe reactions than may occur with the older scarification cross-hatched method.¹

Alternatively a single scratch $\frac{1}{4}$ in. in length may be made through the drop of lymph. Care should be taken to avoid any bleeding. No dressing is required until four to five days after vaccination, and the lymph should simply be allowed to dry *in situ*.

In a person lacking immunity a papule forms at the site of inoculation in three to four days and this becomes vesicular in five to six days; in eight to ten days the vesicle becomes pustular with a zone of surrounding inflammation; finally the pustule heals with the formation of a crust which is desquamated about the twenty-first day, leaving a depressed scar. In persons who have been recently vaccinated and possess a satisfactory immunity, there may be no reaction, or a papule may appear more rapidly than in the non-immune subject and resolve without the development of a

¹ Parish, H. J. (1952), *Bacterial and Virus Diseases*. Edinburgh, Livingstone.

vesicle. (Such reaction, however, cannot be accepted in all cases as indicating an effective immunity.) In those who have been previously vaccinated but have lost their original degree of immunity, an accelerated "vaccinoid" reaction is noted: a papule appears quickly, becoming also vesicular and pustular more quickly than in the completely non-immune person.

Before a vaccination can be regarded as successful it is essential that the stage of vesiculation should have been reached. Primary vaccination in children should be carried out within the first two years of life. It should not be done on a child with eczema or other skin lesions. There is a rather greater risk of generalised vaccinia and of neurological complications following vaccination in infancy (0-1 years) than in children 1-4 years old. Revaccination in non-epidemic countries should be done at regular intervals of five to seven years, for example on entering school and again on leaving, as well as in circumstances when there is a risk of exposure to smallpox. A certificate on a special internationally accepted form showing vaccination in the previous three years is required for entry into many countries. Between the ages of 5 and 18 years primary vaccination carries a risk of post-vaccinial encephalitis (*vide infra*); the incidence varies from 1 in 8000 to 1 in 70,000 vaccinations with a case mortality of up to 50 per cent. For this reason the vaccination of young adolescents should only be carried out when there is a grave risk of variola.

Vaccination provides a powerful defence against the risk of contracting smallpox and is an effective protection against the risk of dying if the disease is contracted. Immunity can be demonstrated eight to nine days after vaccination and antibodies reach their peak within two to three weeks. Protection lasts for a variable period according to the individual; in general five to seven years pass before it is possible to revaccinate successfully. Experience has shown that mortality in smallpox is four times greater in unvaccinated than in vaccinated persons.

Complications of Vaccination.—Septic infection at the site of vaccination may be caused by the introduction of pathogenic bacteria either at the time of vaccination or later when the vaccinal lesion has developed. *Generalised vaccinia* is a rare complication characterised by the appearance of discrete vaccinal lesions in crops over the surface of the body; the condition has an incidence of 1 in 1,000,000 vaccinations and a case mortality of 30-40 per cent. Persons with chronic skin diseases and especially young children with eczema are prone to develop widespread vaccinal lesions—*Eczema vaccinatum*—the result of implantation of the vaccine virus on the open skin lesion. *Eczema vaccinatum* is very

similar to *Eczema herpeticum* (Kaposi's disease or varicelliform eruption) in which herpes simplex is a causal virus.

Post vaccinial Encephalitis.—Within a fortnight of vaccination an acute disseminated encephalomyelitis may supervene in a very small percentage of cases, mostly older children who have not been vaccinated as infants. A disease identical in its clinical and histological characters has been recorded following other infective diseases, such as smallpox and measles, and occasionally in non-exanthematous cases.

Clinically there is paralysis, at first flaccid and later spastic, while meningeal symptoms are frequently noted in children. Histologically the outstanding characteristic is the demyelination of the areas round the blood vessels.

It was originally thought that the encephalitis might be due to the vaccinia virus affecting the central nervous tissue, but it is now considered that post-vaccinial encephalitis may be a different disease, due possibly to a separate virus or toxic agent, activated by the vaccinial lesion. It has also been suggested that the condition is due to allergy, with resultant vascular thromboses which produce the characteristic areas of demyelination round the blood vessels.

COW-POX VIRUS

In cow-pox the eruption appears on the teats as small papules which later give rise to vesicles and pustules. The cows themselves are not seriously affected and no generalised symptoms occur. Friction during the milking process generally causes the lesions to break and raw tender areas are formed. Crusting follows and the dried scabs fall off in about ten days, leaving an unscarred surface. This disease was first described by Jenner, who realised that the infection could be transferred to the hands of milkers with vesicle formation. In man the lesions may occur in the interdigital clefts, or the back of the hands and also on the fore-arms and face. The lesions resemble those that follow primary vaccination, although they may be more indurated and the vesicle fluid is often blood-stained.

Cow-pox is a separate disease entity in bovines and is caused by a virus which, although related to the vaccinia virus, is distinct from it.

*Properties of the Cow-pox Virus (*Poxvirus bovis*)*.—In size, morphology and resistance to heat and chemical agents the cow-pox virus is identical with the vaccinia virus. It can be distinguished readily from vaccinia virus by the characteristic red

haemorrhagic pocks to which it gives rise in cultures on the chorio-allantoic membrane of the chick embryo. The intracytoplasmic inclusions of the cow-pox virus are much larger than the Guarnieri bodies of vaccinia and have a denser matrix; they have also a tendency to distort the shape of the host cell. Serological studies indicate that there is a quantitative difference in the minor antigenic components of the two viruses.

Other Animal Pox Diseases

Sheep-pox is an extremely serious disease when it occurs in epizootics and has a case mortality varying from 5 to 50 per cent. The virus is quite unrelated antigenically to any other member of the pox group. Goats also may be infected.

Swine-pox is clinically similar to cow-pox and sheep-pox. The disease may be severe in form with a mortality of about 20 per cent. or, as in Britain, mild with ill-defined pock-like lesions, mainly in young pigs. The milder form, however, may be due to infection with the cow-pox virus. The swine-pox virus from the severe disease is not related to other pox viruses.

Infectious Ectromelia of Mice.—This virus disease occurs in a chronic or acute form. In the chronic disease one foot becomes oedematous with serous exudation on the surface and crusting; later necrosis and sloughing results and spread may take place to another foot or the tail. In the acute disease necrotic lesions occur in the liver and other viscera. In the epithelial cells of the skin and intestine large acidophilic cytoplasmic inclusions are found, and elementary bodies about 260–300 $m\mu$ in diameter have also been demonstrated in lesions by ordinary microscopic examination of suitably stained preparations. The virus can be cultivated in the chorio-allantoic membrane and produces plaques similar to those of vaccinia. A serological relationship exists between the virus and the variola-vaccinia viruses. Epizootics of ectromelia in mouse colonies can usually be prevented or controlled by vaccinating the tails of the susceptible animals with vaccinia lymph.

The ectromelia virus has been studied extensively by Fenner,¹ who used it as a model to elucidate the pathogenesis and epidemiology of pox diseases.

Avian Pox Viruses

These viruses affect many different species of birds, including the domestic fowl, canary, pigeon, turkey, sparrow and magpie.

¹ Fenner, F. (1948), *Lancet*, ii, 915.

The viruses are adapted to their own hosts and when transferred to birds of different species cause only minor signs of disease. The canary-pox and fowl-pox viruses have been more thoroughly investigated than the remainder.

The Properties of the Fowl-pox Virus.—In morphology, size, cultural characters and in resistance to physical and chemical agents the fowl-pox virus is closely similar to the vaccinia virus. In the infected bird the virus gives rise to large spherical inclusions (Bollinger bodies) in the epithelial cells of the cornified layer of the skin. These inclusions are highly characteristic of fowl-pox; they are eosinophilic and larger than the nuclei of the cells, which are often displaced by them and pushed to the side. Within the Bollinger bodies there are many elementary bodies of the virus.

The fowl-pox virus can be cultivated on the chorio-allantoic membrane of the developing chick embryo. Fowls can be immunised against fowl-pox by inoculation with the pigeon-pox virus, which has a low pathogenicity for the fowl.

Contagious Pustular Dermatitis of Sheep (Orf)

The manifestations of this disease are pustules on the lips and round the mouth and on the mucosa of the mouth, the cornea, the feet and legs and other parts of the sheep's body. The infection is transmissible experimentally in lambs by inoculation of the skin with filtrates from the pustules. Human infections with the virus are sometimes seen as granulomata on the hands of those who handle diseased animals, their skins, or their carcasses. The causative virus has the same size and morphology as the vaccinia virus, but little is yet known of its other characters. It has not been cultivated in the chick embryo or in tissue cultures and the only susceptible host at present available is the lamb. The disease has been controlled under field conditions by means of a living vaccine consisting of finely ground fully virulent scabs suspended in a 1 per cent. concentration in glycerol saline.

CHICKENPOX AND ZOSTER

These two diseases differ greatly in their clinical manifestations and yet there can be little doubt that they are caused by a single virus.

Chickenpox (varicella) is a mild and extremely infectious disease occurring principally in young children. Papules which rapidly become vesicles appear on the first day of the illness; they occur in

successive crops and spread from the face and scalp to the trunk and limbs but do not involve the hands or feet.

In zoster a very similar vesicular eruption is confined to an area of skin corresponding to the distribution of a sensory nerve. These lesions may be very painful and are usually unilateral; the most frequent sites are the trunk and an area on one side of the face supplied by one of the divisions of the trigeminal nerve. Zoster is a sporadic condition in adults and seldom occurs in children. It is not highly infectious.

Properties of the Virus.—Elementary bodies are abundant in vesicle fluid collected within twelve hours of the appearance of the chickenpox lesion; by twenty-four hours, however, they have become very scanty. They can be demonstrated by the staining methods used for the smallpox virus. In films of vesicle fluid examined electron microscopically the virus is brick-shaped and measures $210 \times 240 \text{ m}\mu$, but in ultra-thin sections of infected cells it has a rounded appearance.

Cultivation.—The virus in vesicle fluid from cases of chickenpox and also from zoster can be cultivated in a variety of cells of human origin and in some from monkeys. The virus will multiply in growing explants of human prepuce or in human embryonic skin-muscle in plasma clots.¹ Trypsinised cultures of human amnion cells have been used successfully for the isolation of the virus from vesicle fluid, and monkey kidney and HeLa cells support the growth of virus strains in infected fluids from tissue culture.²

The cytopathic effect in all types of tissue cultures is characteristic and is identical whether the inoculum is obtained from chickenpox or zoster material. Foci of infection appear and enlarge slowly as contiguous cells are infected; these lesions are first seen six or seven days after inoculation and reach their maximum in about three weeks. Characteristic acidophilic inclusions develop within the nuclei of the cells at the centre of the lesion and later syncytial cytoplasmic masses are formed. The supernatant fluid from tissue culture usually contains very little infective virus since elementary bodies are retained within the host cells. To propagate the virus it is necessary to detach infected cells from the glass and to transfer them to fresh cultures. Infective virus can be released from infected cells by ultra-sonic disintegration.

Laboratory animals and the chick embryo are not susceptible to infection with the virus and the only susceptible host (apart from

¹ Weller, T. H., Witton, H. M., & Bell, E. J. (1958), *J. exp. Med.*, **108**, 843.

² Taylor-Robinson, D. (1959), *Brit. J. exp. Path.*, **40**, 521.

tissue cultures) is man. When vesicle fluid from a case of zoster was inoculated experimentally into the skin of young susceptible children a local lesion at the site of inoculation appeared nine to twelve days later. One of seventeen such children developed a generalised rash on the fourteenth day. Contacts of the inoculated children developed chickenpox after the usual incubation period. It has been repeatedly observed that natural infection of children with chickenpox may follow contact with a patient with zoster and it is established that varicella contracted in this way is epidemiologically and immunologically typical.

Antigenic Characters.—Vesicle fluid or extracts of crust from chickenpox or zoster behave identically in the presence of sera from convalescents from either disease. There is no difference between the two in agglutination tests nor in complement-fixation reactions.¹ In precipitin tests using the Ouchterlony technique no difference can be detected between the antigens of zoster and varicella or in the antibodies in the sera.² The virus is unrelated to the virus of herpes simplex.

Occurrence.—It is thought that chickenpox is contracted by inhaling infected particles and that the virus enters the body through the respiratory mucosa. The rash is presumed to be caused by the localisation of the virus in the skin from the blood stream; a viraemic phase is postulated at or just before the onset of the illness. The incubation period is twelve to sixteen days. Histologically the vesicles in chickenpox and zoster are identical. The lower layer of the epidermis is ballooned and there is degeneration of the epidermal cells with the formation of multi-nucleate giant cells. In the cells at the base of the lesions there are eosinophilic intranuclear inclusions similar to those seen in infected tissue culture cells.

Zoster has, as a rule, a prodromal period of three or four days of fever and malaise before the appearance of the skin eruption. At this time there is commonly pain and tenderness over an area of skin supplied by nerves from one of the dorsal nerve roots. In this area papules appear and rapidly become vesicles; during the succeeding days the vesicle fluid becomes cloudy, and then after rupture of the vesicles the lesions dry up and healing slowly follows. The lesions are unilateral and may be very painful; in some cases especially when the trigeminal nerve is involved there may be residual paralysis. In 2-3 per cent. of cases there is a generalised rash. In some cases there may be a meningeal reaction

¹ Taylor-Robinson, D., & Downie, A. W. (1959), *Brit. J. exp. Path.*, **40**, 398.

² Taylor-Robinson, D., & Rondle, C. J. M. (1959), *Brit. J. exp. Path.*, **40**, 517.

with an increased number of mononuclear cells in the cerebro-spinal fluid. Encephalitis is a rare complication.

The primary site of infection in zoster is in the dorsal root ganglion where there is an acute inflammatory reaction; a monocytic inflammatory exudate is seen with haemorrhage and necrosis of the ganglion cells. Degenerative changes follow, passing down the nerve fibres to reach the skin and in some cases centrally to reach the posterior column where they involve the anterior horn cells and give rise to paralysis.

The incubation period in zoster is difficult to determine; it is shorter than in varicella and estimates range from three to seven days and from seven to fourteen days.

Laboratory Diagnosis.—In chickenpox there is seldom any need for laboratory help in diagnosis unless the possibility of smallpox has to be considered. Confluent chickenpox in a young adult may resemble smallpox very closely, and in such patients specimens must be collected and examined by the methods described for smallpox on p. 438. In direct smears from the skin lesions elementary bodies may be seen, but they appear smaller than the variola virus and are much less numerous. Vesicle fluid and extracts of crusts from patients with zoster and varicella fail to fix complement when mixed with anti-vaccinial serum.

The virus in material from either disease may be identified in complement-fixation reactions with antisera from convalescent cases of chickenpox and zoster. Antibodies in the serum can be measured in complement-fixation and neutralisation tests using as antigen vesicle fluid or tissue culture fluids.

Epidemiology.—Chickenpox is a highly contagious disease of childhood. Its maximum incidence is between the ages of two and six years, but it is not uncommon in the first year of life. About 20 per cent. of cases may occur in adult life. The disease has a seasonal prevalence in the winter and spring; the case mortality is negligible.

Zoster is a sporadic disease uncommon in children. The disease is not highly infectious and there is no prevalence at any particular season. The mode of infection in zoster is unknown. Contacts with zoster do not usually develop zoster themselves, but are more liable to suffer from chickenpox.

The Relationship of Zoster to Chickenpox.—The pathogenesis of these two diseases and laboratory studies of the viruses isolated from them leave little doubt that they are different responses to infection with the same virus. It has been shown that high antibody titres are present in zoster in the early stages after the onset of the infection and that in chickenpox such levels are not attained

until late in convalescence.¹ This, together with the shorter incubation period of zoster, has led to the suggestion that zoster is the manifestation of infection in the partially immune subject. Thus varicella may represent dissemination of the virus in the blood stream of the susceptible person, whereas zoster represents the invasion of nerve pathways in a patient who has a small measure of immunity remaining from a childhood infection. Alternatively, zoster may represent the reactivation of a latent virus dormant for many years in the patient's tissues. There is, however, little evidence to indicate in which tissue the latent virus lies.

Molluscum Contagiosum

The lesions of this disease are small copper-coloured warty tumours on the trunk, buttocks, arms and face. It is spread by direct contact or by infected fomites. In the epithelial cells very large inclusion bodies, mainly acidophilic in their staining reaction can be observed. The inclusions may reach 20–30 μ in diameter and crowd the host cell nucleus to one side, eventually filling the whole cell. When material from the lesions is crushed some of the inclusions are burst open and from them large numbers of elementary bodies escape. These virus particles have the size and morphology of the vaccinia virus. The infection has been transmitted with filtrates to human subjects but as far as is known laboratory animals and tissue cultures are not susceptible to the virus.

Warts

There are several different types of warts. The common wart (*verruca vulgaris*) is a discrete hard rounded papilloma occurring usually on the back of the hands, around the nail-beds on the fingers, and on the wrists. Plantar warts are particularly common and are infectious; they are frequently seen on the soles of the feet and are often spread from person to person in bathing-pools. Genital warts are small, rough, fungating, outgrowths on the coronal sulcus of the penis and prepuce, or on the labia and around the vulva. Laryngeal warts may be small and flat or pedunculated. Warts have been transmitted to human volunteers by the injection of filtrates made from ground-up papillomata. The agent passes through all grades of Berkefeld candles. Eosinophilic intra-nuclear inclusions are present in 43 per cent. of plantar warts and in about 4 per cent. of common warts. Electron microscopical studies of ultra-thin sections of warts have shown virus-like

¹ See footnote, p. 786.

particles 52 m μ in diameter arranged in a crystalline manner in the nuclei of the rete cells. Attempts to transmit the infection to laboratory animals, the chick embryo, and tissue cultures have so far been unsuccessful.

If warts are made to bleed new warts appear on the skin where it was contaminated by the blood. They are spread in swimming-baths, barbers' shops, and in chiropody and hairdressing establishments. Warts usually disappear spontaneously and there is evidence to suggest that there may be some measure of immunity after recovery.

Myxomatosis

Myxomatosis is a highly infectious and almost invariably fatal disease of rabbits; it is characterised by mucopurulent discharges from the eyes, nose and genital openings and by the presence of tumour-like masses of tissue involving the head, neck and many other parts of the body. The disease was originally endemic in Brazil and affected only the native wild rabbit (*Sylvilagus brasiliensis*) in which it was manifested as a single localised tumour of the skin. It is spread amongst the animals by the bite of the mosquito, but there is no evidence that the virus ever multiplies in the body of the vector. Myxomatosis has now spread over the whole of the North and South American continents and has been introduced into Europe and Australia. The European rabbit (*Lepus europeus*) is highly susceptible to the infection, and sweeping epizootics during the last ten years have decimated the rabbit populations of Australia and Great Britain.

Properties of the Virus.—Morphologically the virus is identical with the vaccinia virus and electron-microscopical examination shows that it measures 290 \times 230 \times 75 m μ . It is related antigenically to the rabbit fibroma virus of Shope.

Host Range and Cultivation.—The only susceptible animal is the rabbit and all other animals including the hare, monkeys and man are resistant. The virus can be cultivated in the developing chick embryo and produces pocks on the chorio-allantoic membrane. The virus will also grow in the brains of newborn mice and a variety of mammalian cells in tissue culture.

Myxomatosis is mechanically transferred from the skin of the infected animal to the susceptible rabbit by the mosquito, whose mouth parts are contaminated with the virus. In Great Britain the main vector is not a mosquito but the rabbit flea. The viraemia which follows infection is not of direct importance in providing a source of the virus for the arthropod vectors, but, of course, is the means whereby the disease is spread throughout the rabbit's body.

Tumourous masses with a rubbery consistency are found in the lymph nodes of the head and neck and throughout the body and the spleen is enlarged. Sections of myxomatous tissue show large stellate cells embedded in a homogeneous mucinous material. Haemorrhages and inflammatory changes are present and in the epithelial cells are large acidophilic inclusions which resemble the Bollinger bodies of fowl-pox.

The complex subjects of the mechanisms of transmission of myxomatosis in Australia and Europe and of the emergence of genetically resistant rabbits have been reviewed by Fenner.¹

In wild rabbits myxomatosis is virtually an uncontrollable disease. Domestic rabbit and laboratory stocks can be protected by insect-proofing of their breeding quarters. Vaccination of the animals with the related rabbit fibroma virus (Shope) offers a high but not absolute degree of protection.

¹ Fenner, F. (1959), *Brit. med. Bull.*, **15**, 240.

CHAPTER XXXIX

MYXOVIRUSES; ADENOVIRUSES; OTHER RESPIRATORY VIRUSES

THE infections discussed in this chapter are the common maladies which make up the bulk of the respiratory diseases occurring every winter. They vary in severity from a simple cold in the head to a feverish and prostrating attack of influenza or to severe bronchitis and bronchopneumonia. Although the pharynx, larynx and the upper air passages are most frequently involved, the conjunctiva and salivary glands may also be affected. Most of these diseases are highly infectious and many commonly occur in epidemics. Our knowledge of their aetiology is far from complete, but two main groups of viruses, the Myxoviruses and the Adenoviruses, are responsible for a considerable proportion of respiratory illnesses. In addition, the common cold viruses, the ECHO viruses, and a number of newly described viruses also cause respiratory infections.

MYXOVIRUSES

The myxoviruses are named from their affinity for mucins. As a group they are usually within the size range 80–150 m μ , are destroyed by ether, cause the haemagglutination of the red blood cells of fowls and some other vertebrates, and grow in the amniotic cavity of fertile hens' eggs. Their normal habitat is the respiratory tract of mammals or birds.

Myxovirus Influenzae A, B and C

Influenza is an acute febrile infectious respiratory disease of man commonly encountered in epidemic form.

Morphology.—In infected allantoic fluid the virus particles are roughly spherical and mostly 80 to 100 m μ in diameter. Filamentous forms of about this diameter and up to several microns in length are often present in strains of virus A recently isolated from man; they can be demonstrated by dark-ground microscopy and in stained preparations.

Host Range and Cultivation.—The ferret is the most susceptible animal; after intranasal inoculation it develops fever and a nasal

discharge due to inflammation of the ciliated epithelium over the turbinate bones. Ferrets recover quickly from the infection and develop high titres of antiviral antibodies in their serum. Mice are less susceptible, but with repeated "blind passage" by intranasal inoculation under ether anaesthesia, strains of the virus can be adapted to give rise regularly to pulmonary consolidation. The developing chick embryo at the age of thirteen days provides in its trachea and lung buds, cells which are highly susceptible to small doses of the myxoviruses. Although free growth of the virus takes place, the embryos do not die from the infection and no obvious lesions can be detected. The eggs, after inoculation (see p. 447), should be incubated for forty-eight to seventy-two hours at 36° C. The presence of the virus is detected by the demonstration of haemagglutinating activity in the embryonic fluids. Once adapted to the amniotic cavity, influenza viruses A and B can be transferred to the allantoic cavity where a rapidly growing mutant emerges to outgrow the original virus inoculum. The original, or "O," virus grows better in the amniotic than the allantoic cavity and its variation to the derivative, or "D" phase, is accompanied by altered haemagglutination properties (see p. 798). Influenza virus C grows only in the amniotic cavity. Influenza viruses can also be grown in tissue culture monolayers prepared from chick or human embryonic tissues, human amnion cells or monkey kidney cells. Their cytopathic effect may not be obvious and they are detected by the demonstration of haemagglutinating activity in the tissue culture fluids or of the adsorption of guinea-pig erythrocytes to the surface of the monolayers (haemadsorption).¹

Haemagglutination.—Virus is adsorbed to receptor areas on the red-cell surface and agglutination of erythrocytes results from the elementary bodies acting as bridges which join adjacent cells. Bivalent cations (*e.g.* Ca) must be present in the suspending fluid for the reaction to take place. After prolonged contact at room temperature the virus is released, or *eluted* from the red cells. The eluted virus is unaltered and remains capable of agglutinating fresh red cells. The treated red cells, however, are permanently altered and can no longer be agglutinated by the same virus although they still may be agglutinated by some other kinds of viruses. Myxoviruses have a varying ability to remove or destroy receptors from red cells and some, which are placed high on the so-called "receptor gradient", remove receptors not only for themselves but for all other viruses placed lower on the gradient. The order

¹ Vogel, J., & Shelokov, A. (1957), *Science*, **126**, 358.

of viruses on the receptor gradient starting with those with the weakest action on red cell receptors is: mumps, Newcastle, influenza A (MEL strain), influenza B (Lee), influenza A (Swine), influenza B (Mil). A series of events similar to the virus reaction with red cells occurs with the cells of the respiratory tract. The receptor destroying reaction is enzymic in nature, and the cell surface receptor is a neuraminic acid-containing mucopolysaccharide which is attacked specifically by the virus enzyme (neuraminidase).

The Virus Growth Cycle.—Infection of the cell begins when the virus particle becomes attached to the receptor area on its surface; for a short time the attachment is loose, but it soon becomes irreversible and either the whole or part of the particle passes through the cell wall to enter the cytoplasm. Once within the cell, the virus loses its identity for a period of two or three hours and cannot be detected by infectivity experiments or by haemagglutination or complement-fixation reactions. This stage of the reproductive cycle is known as the "eclipse phase" and it is thought that at this time the virus particle has broken down to sub-units, each of which multiplies separately. Fluorescent antibody staining and isotope studies suggest that replication of viral nucleoprotein takes place in or very close to the nucleus.

Three or four hours after infection of the cell, the newly formed viral nucleoprotein becomes antigenically active and can fix complement in the presence of antiserum. The antibodies developed against this antigen are group-specific; *i.e.* they can react not only with the original infecting strain but also with all members of the same type.

The properties of this complement-fixing or soluble antigen can be studied when cells are broken open by rapid freezing and thawing a few hours after infection. The antigen is found to consist of small particles $12\text{ m}\mu$ in diameter, *i.e.* about one-tenth the original size of the virus of the elementary body. It has a molecular weight of approximately 600,000, and contains 5.3 per cent. ribonucleic acid; it is non-infective and non-haemagglutinating. On account of its small size the antigen is usually referred to as the *soluble* antigen, a term which is to be preferred since other constituents developed later in the influenza virus particle are also able to fix complement. Antibodies against the soluble antigen are induced during infection, but not by the inoculation of inactivated virus; they are not protective and do not neutralise live virus.

Following the formation of the soluble antigen, the haemagglutinin appears in the cells; it is not infective and is considered to be

a mucoprotein which contributes about 13 per cent. of the weight of the mature particle. In the haemagglutinin resides the enzymatic activity of the virus which gives it a special affinity for the host cell. The completion of the mature infective particle can be seen in ultra-thin sections of cells under the electron microscope. The process takes place at the periphery of the cell cytoplasm and the newly formed virus particles are extruded through the cell wall, acquiring as they go an outer coating of lipoid material. The extrusion of the virus continues over a period lasting some thirty-six hours without apparent damage to the cell. A single infected cell releases between 60 and 120 mature infective particles. With some virus strains, filaments rather than spherical elementary bodies emerge and this process may be a stage in the reproductive cycle modified so that normal segmentation does not occur. Filamentous virus has normal haemagglutinating and antigenic properties, but may not be fully infective.

Structure of the Virus Particle.—There is considered to be a central core of type-specific ribonucleoprotein surrounded by strain-specific mucoprotein and held together in a layer of lipoprotein. The mature elementary body contains 0·9 per cent. RNA, all of which is carried into the host cell and there gives rise to about 70 sub-units which later form the soluble antigen. Figure 30¹ gives a schematic representation of some present views on the structure of the influenza virus. Ether treatment of infective elementary bodies denatures the lipoprotein covering and disrupts the virus; as a result, two immunologically distinct particles are liberated—the soluble antigen and the haemagglutinin.

Viability.—The influenza virus withstands slow drying at room temperature on articles such as blankets and glass; it has been demonstrated in dust after an interval as long as two weeks. When contained in allantoic fluid, or in infected tissues immersed in glycerol saline it will survive for several weeks at refrigerator temperature. It can be preserved for long periods at -70° C. and remains viable indefinitely after freeze drying.

Exposure to heat for thirty minutes at 56° C. is sufficient to inactivate most strains; the few which survive this treatment are killed by a ninety-minute exposure at the same temperature. These viruses are also inactivated by phenol, formalin in a concentration of 1 in 5000, salts of heavy metals, detergents, soaps, and many other chemicals. Iodine in the form of vapour or as a solution is particularly effective. Propylene glycol vapour is active against the virus present in airborne droplets.

¹ Modified from Frisch-Niggemeyer, W. (1959), *Z. Naturw.*, 146, 168.

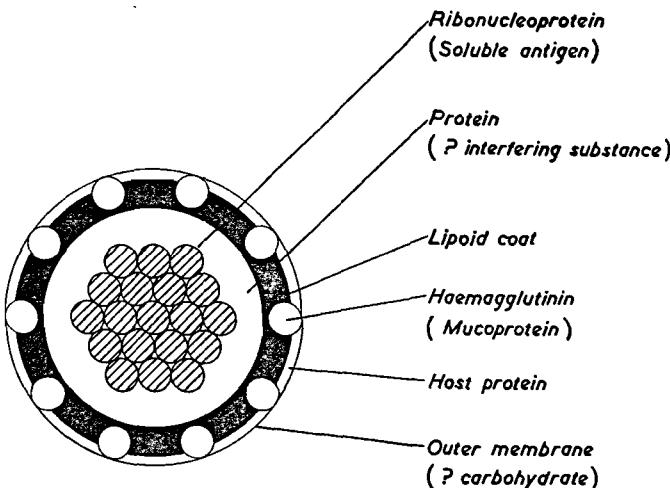


Diagram to indicate the arrangement of subunits and constituents of an influenza virus elementary body.

(W. Frisch-Niggemeyer)

FIG. 30

Toxicity.—Influenza virus elementary bodies are toxic to such laboratory animals as mice and rabbits. After the intravenous inoculation of highly purified virus preparations the animals may die in eighteen to forty-eight hours with gastro-intestinal haemorrhages and necrotic lesions in the spleen and liver. Immunised animals do not suffer these effects when inoculated with the virus intravenously.

Antigenic Characters.—There are three immunologically unrelated influenza virus types, A, B and C, and in addition there is the swine influenza virus which is related antigenically to type A. Influenza virus A has more than 18 different antigenic components which are shared by the strains in varying proportions. The antigens composing the strain-specific patterns are situated in the mucoprotein at the surface of the elementary body. They are distinguished by haemagglutination inhibition tests or by a complement-fixation technique developed by Lief and Henle,¹ in which they are freed from the soluble antigen by ether disintegration of the virus. The soluble complement-fixing antigen which contains the RNA of type A is common to all strains and is

¹ Lief, F. S., & Henle, W. (1956), *Virology*, 2, 753.

used as the antigen in routine serological tests for the influenza group as a whole (see p. 374).

Antigenic Variation and Immunity.—Antigenic variation is highly characteristic of influenza virus A and the process is one of a continuing evolution of new subtypes which replace the older strains from which they are derived possibly as a result of the selective action of host antibodies. Many different antigenic patterns emerge by mutation and selection, some antigens advancing to a dominant position and others receding into the background. Strains isolated at the beginning of an epidemic usually have an antigenic structure differing appreciably from that of previous strains. These changes in the antigenic mosaic occur constantly in the course of endemic and epidemic infection in man and can also be induced deliberately in the laboratory. Major mutation with the emergence of completely new dominant antigens is very infrequent and has probably only occurred at intervals of thirty to forty years. When it happens, the new strain is highly infective and world-wide epidemics (pandemics) are thought to have been due to the spread of what is in effect a new virus in a highly susceptible population. The disastrous pandemic of 1918-19 in which ten million people died and the pandemic of Asian influenza in 1957-58 were caused by new mutants of type A virus.

Major epidemics of influenza involving whole countries or large parts of a continent are frequent events and occur at intervals of three or four years. Since 1932, when the first human strain of the influenza virus was isolated, characteristic strains from each epidemic have been collected and compared, using strain-specific absorbed sera in haemagglutination inhibition tests. According to Burnet¹ these virus strains have shown an immunological drift with the successive emergence of seven new antigenic patterns. Thus the original strain of the 1932 epidemic (labelled the WS strain) was replaced in 1934 by an antigenically different strain which was isolated in Puerto Rico (PR8 strain). During the following years the PR8 strain was replaced by a succession of new types. In 1946 in Australia another new strain known as CAM emerged and proved to be the precursor of yet another change in the antigenic make-up of current influenza viruses. This CAM strain proved to have such slight relationships to previous standard strains that it was classified in a subgroup of influenza A and designated as A prime (A¹). The A¹ viruses replaced the A strains and remained dominant throughout the world until the

¹ Burnet, F. M. (1960), *Principles of Animal Virology*, p. 359. New York, Academic Press.

Asian virus appeared in 1957. This latter possessed a new major antigen and differed so much from the A¹ strains that it is designated A².

The antigenic drift of the influenza virus is reflected in the immunity of the general population. As the individual grows older he experiences an increasing number of epidemics of influenza. With each infection by a new type of virus he acquires in his serum a new specific antibody. Thus in the early years of life the antibody spectrum is narrow, but gradually broadens as age increases. The serum of a person of mature years will often neutralise any of the known standard viruses of previous years, although, of course, that individual will be susceptible to infection with the new virus which appears in the next epidemic. It is these factors which make it difficult to produce an efficient prophylactic vaccine against influenza.

Antigenic variation in type B virus occurs in the same manner as in type A, but the changes are less pronounced and occur more gradually. Type B does not cause the large epidemics that characterise type A; typically it is associated with endemic disease and small epidemics in institutions. It has, however, been recognised as the cause of considerable localised epidemics involving a whole large city. Type C virus appears to exist as a single stable antigenic type.

P-Q-R Variation.—Influenza viruses may vary in the avidity with which they combine with antibody and are neutralised by it. Their behaviour in haemagglutination inhibition tests with convalescent ferret antisera has led to their classification into P, Q and R phases. P phase virus has a high avidity for antibody and is inhibited to a high titre by its homologous antiserum only. Q phase virus is poorly neutralised, even by its own homologous serum, and R phase virus is inhibited to high titre not only by homologous antiserum but by antisera to heterologous but related strains. Freshly isolated strains are commonly in the P phase, but the Q phase is not infrequently encountered. Variation from the P to the Q phase may be induced in the laboratory by growing the virus in the presence of homologous antiserum and a change in the reverse direction from Q to P follows one or two mouse passages. In the Q phase the virus with its low combining power for antibody would be expected to survive in a partially immune population, and variation to the P phase might be accompanied by the emergence of a new antigenic pattern characteristic of an epidemic. There is some evidence to suggest that this type of variation does in fact occur under natural conditions.

Type A strains also show considerable differences in their

sensitivity to non-specific inhibitors such as serum mucopolysaccharides. Highly sensitive strains have a greater avidity for antibody and are therefore preferred as antigens in haemagglutination inhibition tests.

O-D Variation.—Yet another type of variation on the part of the influenza virus occurs in the laboratory soon after its primary isolation. Some two to five days after the inoculation into the amniotic cavity of throat washings from a case of influenza the amniotic fluid develops the power to agglutinate red blood cells. With rare exceptions the virus at this stage can agglutinate guinea-pig or human Group O cells but not fowl erythrocytes. If, however, the fluid is passed by the allantoic route to another set of embryos the infected fluid of this second passage will agglutinate fowl cells to almost or quite the same titre as guinea-pig cells. This change in agglutinative power is referred to as O (original)→D (derivative) variation and is the result of mutation which occurs at a rate of about 1 in 10,000 as the virus grows in the amnion.

It has been suggested that the influenza virus is infective for man only when it is in the O phase. If susceptible human volunteers are inoculated, relatively enormous doses of atomised D phase virus are needed to induce a typical infection whereas far smaller doses of O phase virus have been shown to be capable of setting up a febrile reaction with a specific antibody response.

Further evidence of the instability of the influenza viruses is provided by the phenomenon of hybridisation or recombination. When two virus strains, each with distinctive characters, are inoculated simultaneously in eggs, there results on subsequent passage under suitable conditions, a single strain which has properties derived from both the parent strains.

Swine Influenza Virus.—This virus is associated with sudden epizootics of acute respiratory disease in pigs. By itself the virus causes only mild symptoms in the animal, but when combined with infection with *Haemophilus suis* it causes a severe disease. During non-epizootic periods the virus is maintained in earthworms and lungworms. The sequence of events is that lungworms living in the bronchi of pigs ingest the virus, which then is carried throughout the life cycle of the parasite. Larvae from the lungs are coughed up, swallowed and finally reach the ground in the faeces excreted by the animal. Here they are ingested by earthworms, in which they persist for long periods. When such earthworms are fed to pigs, no ill effects result, but if the animals are also given an intramuscular injection of *H. suis*, or stressed by exposure to cold, they develop typical swine influenza.

The swine influenza virus has the general properties of myxo-viruses and is related antigenically to type A virus. Since swine influenza was first noticed as a disease in 1918, when the pandemic of human influenza was rife, it has been suggested that both diseases were caused by the same agent. Support for this theory is given by the fact that many people of the generation who experienced the 1918-19 pandemic still possess neutralising antibodies for the swine influenza virus, while those born after 1923 have no antibodies for the strain.

Laboratory Diagnosis.—Virus isolation can be carried out by inoculating throat washings or material from throat swabs into the amniotic cavity of the developing chick embryo (see p. 447 and p. 792).

Serological tests can be performed with the complement-fixation test (see p. 347) and the haemagglutination inhibition test (see p. 350).

Epidemiology.—Influenza is spread by the discharge of infected secretions and secretion droplets. The usual means whereby the virus is conveyed into the respiratory tract is still uncertain. Patients are probably infectious for a short time before the onset and for a few days thereafter. Healthy carriers occur and may disseminate the infection, but it is not known how important a part they play.

The periodicity of outbreaks of influenza is characteristic, and in general there would appear to be a pandemic at roughly forty-year intervals with major epidemics of type A infections at about three-year intervals; endemic foci of infection with type B occur yearly and at three- to six-year intervals moderate epidemics of type B infections are recognised. The epidemiological picture is complicated by the existence of upper respiratory infections due to other viruses such as the para-influenza viruses and the adenoviruses which also occur in epidemic form. Epidemics of virus influenza occur with an abrupt rise in the incidence of cases and a very rapid spread over an irregularly defined region. The course and extent of an epidemic is controlled partly by the nature and antigenic novelty of the prevailing virus and partly by the pre-existing immune state of the community. The cold weather in early spring and increased overcrowding are factors which facilitate the spread of the virus. In major epidemics it has been possible to trace the virus from point to point along travel routes and to record the advance of the epidemic from place to place and country to country.

The age distribution of influenza shows the highest incidence in the age-group 5-9 years, a sharp decline in the 15-24 age-group,

a rise between the ages of 25 and 34, and hereafter a downward trend to a low level in older people.

Attack rates in epidemic influenza are high and incidence rates of 20-40 per cent. have often been noted. The disease is rarely fatal, but there is a marked feverish reaction with inflammation of the bronchial mucosa. Some cases, however, are complicated by bacterial pneumonia, and in these the outcome may be rapidly fatal. The invasion of virus-damaged tissue by *Staphylococcus aureus* is followed by the formation of multiple abscesses and severe tissue destruction with necrosis of the trachea, bronchi and bronchioles. Fulminating staphylococcal pneumonia is the cause of many influenzal deaths, especially in the elderly. Secondary invasion with pneumococci, *H. influenzae* or haemolytic streptococci is also associated with influenzal pneumonia. It was this complication which was responsible for the very high mortality in the 1918-19 pandemic.

Immunity to influenza is generally believed to be short-lived and in experiments with type B virus human volunteers could be reinfected with the same strain four months after recovery from this first infection. However, epidemiological evidence suggests that immunity to the natural infection may last for three to four years.¹

Influenza virus type C does not cause epidemics but gives rise to widespread subclinical infections.

Prophylaxis.—As influenza has an incubation period of only one or two days, control measures such as isolation and quarantine are scarcely applicable. Intensive efforts have been made to develop vaccines to protect key personnel such as medical and nursing staffs and public service employees. Egg-cultivated vaccines containing formol-inactivated strains with adjuvants (alum, oil emulsions) have been under extensive trial and have yielded results which appear to be disappointing. However, there are considerable difficulties in the interpretation of the results of trials of influenza vaccines because the position is blurred by infections with other respiratory viruses. On some occasions, as in the case of the Asian strain, it has been possible to prepare a vaccine containing the current strain and to administer one or two doses of it before the epidemic reached the community. In such circumstances, the attack rate was reduced to about one-third of that in control groups.² Polyvalent vaccines have been prepared from a mixture of strains of types A and B known to contain all

¹ Pickles, W. H., Burnet, F. M., & McArthur, M. (1947), *J. Hyg. (Lond.)*, 45, 469.

² Medical Research Council Report (1958), *Brit. med. J.*, 1, 415.

the major antigenic groupings and the effect of these is being studied.

A living attenuated strain of the influenza virus has been developed by workers in the U.S.S.R. and is administered intranasally. The virus multiplies locally in the nasal mucosa and it is claimed that it affords protection in the face of an epidemic. It is stated that it produces sharp reactions in children under seven years of age and that antibodies do not necessarily appear in the blood after inoculation.

Para-influenza Viruses

In recent years a number of new viruses have been isolated from mild respiratory tract infections and cold-like illnesses. Some of these viruses have the general characters of myxoviruses in that they multiply in the amniotic cavity of eggs and in monolayer tissue cultures, are ether sensitive and agglutinate avian and mammalian red blood cells. They possess a receptor-destroying enzyme which attacks the same area on the surface of the red cell as influenza viruses A and B. They differ from influenza viruses in their somewhat larger size and in their ability to lyse as well as agglutinate red blood cells. In this respect they resemble the Newcastle and mumps viruses and with them may form a subgroup of the myxoviruses. One of them, the Sendai virus, shares antigenic components with the mumps and Newcastle viruses.

Myxovirus Para-influenza 1 (Influenza D, Sendai or Japanese haemagglutinating virus; haemadsorption virus type 2, HA₂ of Chanock¹).—The Sendai virus was originally isolated from mice inoculated with material from newborn children with pneumonitis. It is undoubtedly endemic in some stocks of laboratory mice and possibly is pathogenic for pigs. Its role in the causation of human disease is not yet known and it has not been isolated in Great Britain or the U.S.A.; it has been stated to be the cause of outbreaks of influenza-like illnesses in the U.S.S.R. However, complement-fixing antibodies to the virus are widespread, and rising titres of antibodies have been noted in respiratory tract infections in Scotland.

The para-influenza 1 virus is closely related antigenically to the Sendai virus. It was isolated in the first instance from throat swabs taken from children with croup and was detected by the clumping of added guinea-pig red cells on the surface of infected monkey kidney cells in tissue culture. This reaction is known as

¹ Chanock, R. M., et al. (1958), *New Engl. J. Med.*, **258**, 207.

haemadsorption. Para-influenza 1 can be cultivated in eggs only with difficulty. Patients infected with para-influenza 1 develop complement-fixing antibodies against the Sendai virus but not against the mumps virus. The contribution of para-influenza virus 1 to the total of human respiratory disease is unknown. When it was inoculated into 32 human volunteers, 24 of them developed respiratory symptoms after an incubation period of five to six days.

Myxovirus Para-influenza 2 (CA or croup associated viruses).—This virus was isolated from the throats of cases of acute laryngo-tracheobronchitis in children and rising antibody titres to the virus were observed during convalescence. The virus can be cultivated in tissue cultures of HeLa and monkey kidney cells, and in human amnion cells and human embryonic lung. The cytopathic effect produced shows the formation of syncytial masses. The virus agglutinates the red blood cells of newly hatched chicks and to a lesser extent human red cells at 4° C. There is a serological relationship between the para-influenza 2 virus and the mumps virus. Serological surveys suggest that infection with the para-influenza 2 virus is widespread and that it is acquired early in life.

Myxovirus Para-influenza (Haemadsorption virus type 1, HA₁, Chanock¹).—This virus was first isolated from children with febrile respiratory illness.² This virus has also been isolated from outbreaks of respiratory disease in cattle and calves ("shipping rattle and calves isolated from outbreaks of respiratory disease in cattle and calves"). It is been cultivated in the amniotic cavity of developing chick embryos. Culture fluids from the fifth tissue culture passage of the virus have produced colds in volunteers after a short incubation period. A minor serological relationship exists between this virus, para-influenza 1, and the mumps virus.

Mumps Virus

Mumps is an acute contagious disease whose most constant and characteristic feature is a large painful swelling of one or both parotid glands. The disease is one of great antiquity and was one of the first infections to be recognised, for it was accurately described by Hippocrates in the fifth century B.C. The name is derived from the mumbling speech which is the result of the pain on moving the jaws. Usually there is a constitutional reaction and not infrequently other glands as well as the parotids are involved.

¹ Chanock, R. M., et al. (1958), *New Engl. J. Med.* 258, 207.

*Properties of the Mumps Virus (*Myxovirus parotidis*).*—The size of the elementary bodies varies from 80 to 340 m μ in diameter. Filaments have not been described.

Cultivation.—The virus grows rather slowly in the amniotic and allantoic cavity of the chick embryo. The inoculated eggs should be incubated for five days at 35° C. After two or three amniotic passages, most strains can be transferred to the allantoic cavity where they adapt themselves and grow very readily. A few strains, however, remain incapable of being transferred in this way. When inoculated directly into the opening of the parotid duct in monkeys the virus causes typical clinical mumps; the parotid glands contain much virus and emulsions of them can be used as complement-fixing or skin-testing antigens.

The mumps virus agglutinates fowl, human and other red blood cells, fixes complement in the presence of specific antibody, and elicits a delayed allergic reaction in the skin of persons who have previously been infected. Like the Newcastle virus it causes the lysis of erythrocytes. Infective particles can be concentrated by centrifugation at 20,000 r.p.m. for twenty minutes but the particles of the complement-fixing antigen require 30,000 r.p.m. for sixty minutes.

Viability.—Infectivity is rapidly lost at room temperature. The virus is well preserved in skimmed milk at -50° C. or -70° C. or by freeze drying. The haemagglutinin, haemolysin and infective properties are destroyed by heat at 56° C. for twenty minutes. The complement-fixing and allergic skin antigens withstand 65° C. for an hour. Exposure to 0.2 per cent. formalin at 4° C. for twenty-four hours and intense ultra-violet light irradiation for 0.28 seconds destroy the infectivity of the virus without impairing the haemagglutinin or complement-fixing antigens. Ether treatment at 4° C. completely destroys infectivity. The virus is most stable between pH 5.8 and pH 8.0.

Antigenic Characters.—The mumps virus is a single antigenic entity distinct from other myxoviruses; it is not subject to variation and there are no marked differences between the strains. There are two components of the virus which are capable of fixing complement, the V or viral antigen and the S or soluble antigen. The V antigen is associated with the virus elementary body and the S antigen, which is analogous to the soluble antigen of the influenza virus, is a smaller particle extracted from cells in the early stages of infection.

Occurrence.—The incubation period of mumps is one of the longest, eighteen to twenty-one days. The common manifestations are fever with unilateral or bilateral parotitis. The virus is

excreted in the saliva for about three days after the onset and transmission is by the inhalation of infected particles or by fomites contaminated with saliva. Recent evidence suggests that the virus may also be present in urine. Patients are thought to be infectious for about three days before the onset and to remain so for about six days thereafter.

In young children convalescence is uneventful and recovery is complete in about ten days. In about 30 or 40 per cent. of cases infected with the virus the disease is inapparent. In males over the age of thirteen years mumps may be complicated by orchitis which appears some four to seven days after the onset of parotitis. Up to 20 per cent. of male cases are affected.

In almost every case of mumps there seems to be some involvement of the central nervous system with an increase in the lymphocytes in the cerebrospinal fluid. Occasionally the condition proceeds to a frank meningoencephalitis which presents four to seven days after the onset of parotitis; in these cases the mumps virus can be isolated from the cerebrospinal fluid. Rather uncommonly mumps meningoencephalitis may occur without any sign of involvement of the salivary glands and is indistinguishable clinically from aseptic meningitis caused by other viruses such as those of the ECHO and Coxsackie groups (see p. 824). In some cases submaxillary gland involvement is encountered, but pancreatitis and oophoritis are rare complications.

Epidemiology.—Mumps is world-wide in its distribution, and the only reservoir of infection is man. The disease is predominantly one of children aged five to fifteen years, but adults who have escaped infection in childhood are often attacked. Epidemics of mumps are not uncommon in young soldiers in army camps. Although mumps is one of the common diseases of childhood, it is not as highly infectious as measles. Amongst adults the history of a past mumps infection is obtained in about 60 per cent. of people, whereas 90 per cent. have had measles.

Laboratory Diagnosis.—Virus isolation is carried out by inoculating saliva or cerebrospinal fluid into fertile eggs. Saliva can conveniently be collected by placing dental cotton-wool rolls over the openings of the parotid ducts and leaving them in the mouth for about twenty minutes. The rolls are then removed and placed in screw-capped vials containing Hanks' balanced salt solution with added antibiotics. The containers should be transported to the laboratory frozen at -70° C. in insulated boxes containing solid carbon dioxide. On arrival at the laboratory, the saliva is expressed from the rolls, added to the transport fluid and

clarified in the centrifuge at 2000 r.p.m. for ten minutes. The supernatant is inoculated into the amniotic cavity of eight 11-day-old chick embryos; the eggs are then incubated for five days at 35° C. The virus may be detected in the amniotic fluid by the haemagglutination of fowl red blood cells and its final identification is established serologically with specific antiserum in complement-fixation or haemagglutination inhibition tests. The primary isolation of the virus is sometimes a matter of difficulty because saliva may have toxic properties for the chick embryo, and because only a small proportion of inoculated eggs show signs of infection.

Serological Tests.—Antibody rises can be detected in paired acute and convalescent sera. The complement-fixation test using both soluble and viral antigens is recommended. Antibodies to the S antigen develop early and are present in significant amounts within two or three days of the onset of the infection and they may reach their peak titre before the appearance of antibody to the V antigen. Antibodies to the V antigen appear on the eighth or ninth day of the disease, reach their maximum by the end of a month and thereafter decline very slowly. Subsequently the anti-S antibodies disappear relatively rapidly and are seldom detectable nine months later. Antibodies to the V antigen persist for several years at very low, barely detectable levels. The haemagglutination-inhibiting antibody is similar in its duration to the anti V antibody. The skin test for mumps is of little practical value in diagnosis because the hypersensitive state is not developed until three to four weeks after the onset.

Prophylaxis.—Gamma globulin *prepared from mumps convalescent serum* may be of value in conferring passive immunity to exposed persons and if given immediately after the onset of parotitis reduces the incidence of orchitis. Gamma globulin from normal adults contains only traces of specific antibody and has little protective value.

Vaccines prepared from egg-grown strains inactivated by formalin or ultra-violet light have been used with some success in reducing the incidence of mumps in army camps. Although antibody formation is stimulated by the vaccine, booster doses are needed after six to nine months to maintain their antibody level. A living attenuated egg-adapted strain of mumps has also been used and is sprayed into the mouth; antibody production reaches the same proportions as with the inactivated vaccine. No clinical case of mumps has been reported as a result of the use of the live vaccine, and this method is at present on trial for prevention of the infection.

Newcastle Disease Virus

Newcastle disease (a form of fowl pest) is an extremely infectious epizootic condition of domestic poultry. Fowls, turkeys, pheasants, guinea-fowl as well as many other avian species are affected. Cormorants may suffer inapparent infections and act as a source of infection for poultry in coastal regions. The disease has a world-wide distribution and constitutes a major economic hazard. In Europe the infection is characterised by hyperpyrexia, severe diarrhoea, and a thick mucopurulent nasal discharge. The incubation period is three to ten days. The mortality rate is 90-95 per cent. In America the disease is milder, having a mortality of 5-50 per cent., in which signs of tremor, incoordination and wing and leg paralysis predominate.

The disease can be transmitted experimentally to susceptible poultry by the inoculation of discharges from infected animals and also with blood and tissues, e.g. spleen and bone-marrow.

The Newcastle virus which causes this disease has recently been named *Myxovirus multiforme*. The elementary body is roughly spherical with a diameter varying from 80 to 200 $\text{m}\mu$ (average 115 $\text{m}\mu$). Filamentous forms occur, but are found only infrequently. The strains grow readily in the fertile hen's egg and kill the embryo with haemorrhagic lesions in forty-eight hours. In HeLa cell monolayer tissue cultures they produce a characteristic cytopathic effect with syncytium formation. The virus agglutinates red blood cells from fowls and a variety of mammalian species and elutes from them rather rapidly and often incompletely at room temperature; in high concentrations it has marked powers of haemolysis. The virus is immunologically distinct from other myxoviruses, although there are minor relationships between it and the mumps and Sendai viruses in complement-fixation tests. All strains are closely similar to each other in antigenic structure and there is little or no tendency to variation. Laboratory diagnosis may be accomplished by isolation of the virus in fertile hen's eggs and also by demonstration of specific antibodies in the blood by means of a haemagglutination inhibition test.

Occasionally the Newcastle disease virus causes inflammation of the conjunctiva in man due to an accidental infection in the laboratory or during the handling of infected birds. There is a severe conjunctivitis which may be accompanied by a painful swelling of the pre-auricular gland and, more rarely, by a severe generalised influenzal type of illness.

A vaccine containing a living attenuated egg-adapted strain of

the Newcastle virus has been developed for poultry flocks and is given by intranasal instillation. Its use in Great Britain, however, is forbidden, since it may give rise to paralysis and other complications and may also initiate dissemination of the virus.

Fowl Plague Virus

This is a highly contagious infection of chickens, turkeys, pheasants and some wild birds. The disease is rapidly fatal; and nearly 99 per cent. of infected birds die within twenty-four to thirty-six hours of the onset. The incubation period is three to five days. A mucopurulent nasal discharge and oedema of the head and neck are the usual signs and at *post mortem* small petechial haemorrhages are present in the serous membranes. There is histological evidence of a diffuse encephalitis.

Properties of the Fowl Plague Virus (Myxovirus pestis-galli).—The size of the elementary bodies varies from 80–120 m μ and filamentous forms which may be up to 6 μ in length, are common. The virus grows readily in all the tissues of the developing hen's egg and kills the embryo in about thirty-six hours. Intranuclear lesions, probably inclusions, have been described in the tissues. The virus is readily adsorbed to fowl red blood cells and causes their agglutination. Antigenically the virus is distinct from other myxoviruses and no serological races have been described. The blood of infected birds contains the virus in high concentrations and can be used to transfer the infection experimentally.

ADENOVIRUSES

Adenoviruses derive their name from the fact that they were first isolated from fragments of adenoid tissue removed surgically and grown in tissue culture. They are able to live in both tonsillar and adenoid tissue in a latent or masked form without any apparent harm to the individual. In other situations, however, adenoviruses cause a wide variety of clinical syndromes including coryza, pharyngitis, sporadic or endemic acute respiratory tract infections, pneumonia, acute conjunctivitis, and epidemic keratoconjunctivitis. The adenovirus group consists of 23 serological types, 18 of them of human and 5 of simian origin.

Properties of the Viruses.—The elementary bodies are roughly spherical and approximately 70 m μ in diameter. When seen in electron microscopical preparations stained by uranyl acetate they have a hexagonal outline and a central electron dense mass. The external structure of the virus particle has been studied by a

negative staining technique in which the virus is treated with phosphotungstic acid and sprayed on to electron microscope grids. By this method the elementary bodies are revealed as regular icosahedra each with 252 spherical surface subunits 7 m μ in diameter.¹

As the virus grows in the nuclei of host cells the elementary bodies are disposed in a crystalline array to form a cubic, body-centred, packing of identical spheres. Electron micrographs of ultra-thin sections of infected cells show that the virus particles are aggregated in sharp-edged rhomboidal crystals composed of a regular lattice of spheres with a centre to centre spacing of 60–65 m μ . The crystals are 3–7 μ long and can be studied with the light microscope; they are basophilic and Feulgen-positive. Treatment with desoxyribonuclease destroys the Feulgen-positive characteristic and suggests that the virus contains DNA.

Cultivation.—All the adenoviruses can be cultivated with a cytopathic effect in tissue cultures. Human strains grow most abundantly in continuous lines of human malignant cells such as HeLa or KB, or in primary cultures of human amnion, while simian strains grow best in monkey kidney cells. Strains of either origin can be adapted to grow in heterologous cells in tissue culture; although on primary isolation the cytopathic effect may be quite obvious, repeated passages with large inocula may be required to establish a human strain in monkey kidney cells or vice versa. Most strains can also be adapted to grow in rabbit or pig kidney cells or in bovine embryonic tissues. The range of susceptible tissue culture host cells varies for different types of adenoviruses and although types 1 to 9 are readily propagated in HeLa cells, types 10, 12, 13, 15 and 17 require several serial passages with large inocula before they can be obtained in reasonable quantity. Inhibitors of the growth of adenoviruses are sometimes present in the calf, ox or horse serum commonly used as an ingredient of the tissue culture medium. For work with adenoviruses, 20–40 per cent. human serum in Earle's balanced salt solution is recommended as the growth medium for the host cells. Before inoculation, this medium should be removed and the monolayers should be washed three times with balanced salt solution to remove antibodies. The cells may then be maintained with medium 199 containing up to 7·5 per cent. chicken serum.

The cytopathic effect with some adenoviruses is first seen some four days after inoculation and with others is not obvious for

¹ Home, R. W., Brenner, S., Waterson, A. P., & Wildy, P. (1959), *J. molec. Biol.*, **1**, 84.

seven or eight days or even longer. The appearance is that of aggregates of rounded cells with areas denuded of cells. Infected cells are not lysed and do not die, although they become rounded and fall off the glass surface; they retain the ability to take up neutral red in the same way as normal cells. Inoculated cultures must be observed daily for eighteen days before being regarded as negative.

Growth Cycle.—The virus is absorbed slowly by the cell before it begins to multiply in the nucleus. With type 5 there is a latent period of twelve hours during which the virus goes into an eclipse phase. This is followed by a period of rapid increase which lasts some eight hours and thereafter multiplication proceeds at a slower rate for over thirty hours. Liberation of the virus from the host cell is slow and incomplete; only about 6 per cent. of the virus is released spontaneously. The rate of viral multiplication varies for the different types of adenoviruses; types 3, 4 and 7 have a shorter growth cycle than types 1, 2, 5 and 6.

Viability.—Adenoviruses are more stable than the myxoviruses. They remain viable after seven days at 36° C., fourteen days at room temperature (22°–23° C.), and seventy days at 4° C. They are totally inactivated by two and a half to five minutes at 56° C. They are not inactivated by ether and are stable with the pH range from 6 to 9.5, though at pH 3 and pH 10 or above partial but not complete loss of infectivity results.

Antigenic Characters.—There are 23 established serotypes which are distinguished by serum neutralisation tests performed with rabbit antisera. Cross-reactions between the different types are rare. Type 7 strains have been subdivided into 7 and 7a. All strains of adenoviruses have the power to fix complement in the presence of specific antibody. The complement-fixing antigen is a small soluble particle which can be separated from the elementary bodies by filtration or centrifugation. Results of complement-fixation tests are group-specific and suggest that the soluble antigen is shared by all adenovirus types.

Occurrence.—In man, infection with adenoviruses results in catarrhal inflammation of the mucous membranes of the eye and the respiratory tract, sometimes with enlargement of the regional lymph nodes.

Acute pharyngitis is probably the most frequent manifestation of adenovirus infection. About half the cases are febrile, and coryzal symptoms and cough are frequent. Young children of pre-school age are commonly infected, often on repeated occasions. Most children have antibodies to types 1, 2, 3 and 5 by the time they begin school life.

Pharyngoconjunctival Fever.—The triad of fever, pharyngitis and conjunctivitis lasting for about one week is a characteristic of infections with types 3, 7a and 14. This syndrome is encountered more frequently in the summer months, when it may spread rapidly amongst the members of a family and be associated with outbreaks in schools or day-nurseries.

Acute Respiratory Disease (A.R.D.) is a feverish coryza which, unlike virus influenza, has a gradual onset. Headache, sore throat and cough are common but not severe. This illness seldom has clear-cut characters in civilian practice, and the diagnosis is usually only made when epidemics occur in such communities as large military camps.

Pneumonia.—The illness resembles primary atypical pneumonia (see p. 812). In children, type 7a is a frequent cause of the condition and in adults it occurs as a complication of A.R.D. due to types 4 and 7.

Acute Follicular Conjunctivitis occurs principally in adults. The disease begins with a unilateral non-purulent inflammation of the conjunctiva with enlargement of the submucous lymphoid follicles and swelling of the pre-auricular lymph node. Fever and systemic effects are usually absent. After a few days the other eye shows a similar involvement and the condition usually clears up within a week. Types 3 and 7a have been isolated from these cases.

Epidemic Keratoconjunctivitis.—This condition is due to infection with the single adenovirus type 8. Factory workers are principally involved, especially those whose trade exposes them to the risk of small corneal abrasions from dust or metal particles such as are disseminated in arc welding and riveting. The acute phase of the infection may last for several weeks and healing is slow. Type 8 virus can easily be spread by contaminated towels to other members of a patient's family. Epidemics have been recorded where the virus was spread amongst patients in an ophthalmic clinic by means of contaminated eye solutions and instruments.

Epidemiology.—Adenoviruses are widespread in the continents of Europe and North America. In respiratory disease the viruses are spread by the inhalation of infected particles and possibly through contamination of the conjunctiva by infected fingers or droplet-spray. The seasonal incidence is maximal in the winter. Pharyngoconjunctival fever has a maximal incidence in the summer months, and types 3 and 7a viruses have been thought to be spread in swimming-bath water. In addition to being present in the exudates of the oropharynx and the eye, adenoviruses have been

recovered from faeces and from the mesenteric lymph nodes. The significance of the presence of adenoviruses in the intestine is still in doubt.

Clinical Syndromes due to Adenoviruses

Disease	Associated Adenovirus Type
Acute febrile pharyngitis	1, 2, 3, 5.
Pharyngoconjunctival fever	3, 7a , 14, 1, 2, 5, 6.
Acute respiratory disease (A.R.D.) . . .	4, 7, 3, 14.
Virus pneumonia:	
(a) in infants	7a , 1, 3.
(b) in adults	4, 7, 3.
Acute follicular conjunctivitis	3, 7a , 2, 6, 9, 10.
Epidemic keratoconjunctivitis	8.

The most common types are given in heavy figures.

Laboratory Diagnosis

Virus isolation is carried out by the inoculation of monolayer tissue cultures of human cells, preferably HeLa amnion, or thyroid, in which adeno-viruses produce a characteristic cytopathic effect. They can be identified as members of the adenovirus group by their capacity to fix complement with a known positive human or rabbit antiserum. The type is determined in neutralisation tests with type-specific rabbit antisera.

Serological Tests.—Infection with any one type of adenovirus stimulates a rising titre of complement-fixing antibodies to the group soluble antigen. This provides a simple and practical test for the detection of infections with the adenovirus group although it does not identify the type of the infecting strain. Complement-fixing antibodies often rise from very low levels to titres of 1 in 128. The technique of the complement-fixation test is that described on p. 348. The antigen used is prepared from a heavy culture of HeLa cells infected with a large inoculum of an undiluted seed stock of any adenovirus type and incubated for five days (*i.e.* for at least two days after the completion of the cytopathic effect). After thorough homogenisation in a blender and clarification by centrifugation at 2500 r.p.m. for twenty minutes the antigen is titrated against a positive serum and is then ready for use.

Rises of type-specific antibodies to the adenoviruses can be

measured in neutralisation tests against serotypes in HeLa cell tissue cultures.

Prophylaxis.—A trivalent killed vaccine containing types 3, 4 and 7 grown in monkey kidney cells has been on trial. This vaccine has been found useful in preventing A.R.D. in army recruits in the U.S.A.

PRIMARY ATYPICAL PNEUMONIA

This condition is in effect a syndrome of variable symptoms and signs which usually are less prominent than would be expected from the appearances of the soft patchy areas of consolidation that are seen at the bases of the lungs on radiological examination. It is characteristic that there is no response to sulphonamide and penicillin therapy.

Aetiology.—Although there is evidence to associate a wide variety of infective agents with the syndrome, it is true to say that the cause of the great majority of cases is yet to be discovered. Pneumonia of this type is found in Q fever, due to infection with *Cox. burnetti*, and a similar illness is caused by the psittacosis and ornithosis viruses. The influenza viruses and the Sendai virus, together with certain members of the group of adeno-viruses (especially type 4), are thought to be able to cause pneumonia of this type. The evidence, which suggests that 5–15 per cent. of cases of primary atypical pneumonia is due to these agents, is based on serological observation. Whether the rising antibody titre reported can be accounted for by specific infection or whether it is due to an anamnestic reaction is not decided.

In 30–40 per cent. of cases of primary atypical pneumonia, a rising titre of antibodies to a non-haemolytic streptococcus (*Streptococcus M.G.*) may be demonstrated (see p. 319). Titres of 20 or over are regarded as significant. *Streptococcus M.G.* is not thought to be related aetiologically to the syndrome and it is possible that the reaction is due to a heterogenetic antigen possessed by the streptococcus.

Another unexplained serological finding in this condition is that an antibody is evoked which is responsible for the agglutination of human red blood cells at low temperatures. A rising titre during convalescence from the illness or a titre of 1 in 20 or over is regarded as significant (see cold agglutination reaction, p. 320).

The work of Liu¹ has suggested that the virus originally described by Eaton² may be an important cause of primary atypical

¹ Liu, C. (1957), *J. exp. Med.*, **106**, 455.

² Eaton, M. D. (1942), *Science*, **96**, 518.

pneumonia. This virus has been isolated by the amniotic inoculation of sputum into ten-day-old chick embryos. It causes no damage to the developing chick embryo, but five to six days after amniotic inoculation it can be detected in the cells of the trachea and bronchi by staining with fluorescent antibody (see p. 141). Hamsters and cotton rats are also susceptible to infection. Eaton's virus has been isolated most constantly from those patients having positive cold agglutination reactions. Using the indirect fluorescent antibody test rising tides to Eaton's virus can be demonstrated in cases of atypical pneumonia with or without cold agglutinins or *Streptococcus M.G.* antibody. A recent survey in the U.S.A. indicates that Eaton's virus is the cause of common and widespread infections.¹

Laboratory Diagnosis.—Paired samples of serum should be tested for cold agglutinins, *Streptococcus M.G.* agglutinins, antibodies to *Cox. burnetii*, psittacosis, the influenza viruses A, B and C, the Sendai virus and the group of adeno-viruses. The sputum should be examined bacteriologically, and virologically as in psittacosis. Blood cultures and animal inoculations may be required if Q fever is suspected.

Miscellaneous Viruses

Respiratory Syncytial Agent (Chimpanzee coryza agent, C.C.A.).—This virus was isolated from two children suffering from croup and bronchopneumonia respectively. After inoculation into chimpanzees the animals developed coryza. The virus is sensitive to ether and is 90–130 m μ in diameter. It has not been cultivated in eggs and does not agglutinate erythrocytes; it cannot therefore be regarded as a myxovirus.

Coe virus has been isolated in Great Britain and in the United States of America from patients suffering from febrile colds and sore throats. Judged by filtration experiments, the virus is about 20 m μ in diameter and it is ether-resistant. It is readily isolated in cultures of HeLa cells but not in monkey kidney cells. In many respects Coe resembles the enteroviruses. A serological survey in Great Britain has shown that the incidence of infection with the Coe virus is low in children under the age of ten years and rises steadily with age. By the age of fifty years, 50 per cent. of individuals have antibodies to the virus and there is a significantly higher proportion of infection in males.

¹ Cook, M. K., Chanock, R. M., Fox, H. H., Huebner, R. J., Buescher, E. L., & Johnson, R. T. (1960). *Brit. med. J.*, 1, 905.

J.H. and 2060 viruses were isolated from feverish colds and have been thought to be aetiologically related to the common cold. The two viruses are related antigenically but are not identical. They grow with difficulty and very slowly in monkey kidney cells. The J.H. strain may take as long as twenty-five days to produce its cytopathic effect. It is possible that these viruses may belong to the ECHO group. The ability of these two viruses to produce coryza when inoculated into human volunteers is in doubt.

ECHO viruses types 11 and 20 have been recovered from children with respiratory infections such as coryza and croup. There is some evidence also that ECHO virus type 10 can cause coryza in monkeys.

The Common Cold

There is little doubt, even on epidemiological evidence alone, that the common cold is due to infection with a virus. It is a commonplace that when a susceptible person comes into contact with someone with a cold he is liable to be infected and forty-eight hours later develop a cold himself. It is known that thermal chilling alone does not cause colds, though their incidence is certainly greatest at those times of the year when outdoor temperatures are falling. Although some of the viruses already described can cause coryza, it is probable that they are not responsible for the majority of ordinary common colds.

There have been repeated attempts over many years to cultivate the virus or viruses of the common cold, but until recently the results were disappointing. It was known that a scanty growth of the agent could be obtained in tissue cultures of human embryonic tissues, but there was no means of detecting the virus in the laboratory; always it was necessary to inoculate the tissue culture fluids into human volunteers to determine the presence of infectivity.

A considerable advance in our knowledge of the common cold virus has recently been made by D. A. J. Tyrrell¹ and his colleagues. By cultivating the virus at 33° C. instead of 37° C. and by using a culture medium slightly more acid than previously, they have carried cold-producing viruses through ten serial passages without undue loss of infectivity. Moreover, they have found that some strains of the virus are cytopathogenic and cause foci of degeneration in monolayer tissue cultures of human and monkey kidney cells.

¹ Tyrrell, D. A. J., Bynhoe, M. L., Hitchcock, G., Pereira, H. G., & Andrewes, C. H. (1960), *Lancet*, i, 235; Hitchcock, G., & Tyrrell, D. A. J. (1960), *ibid.*, 237; Tyrrell, D. A. J., & Parsons, R. (1960), *ibid.*, 239.

Haemagglutinating viruses such as the Sendai virus or ECHO virus type 11 are not able to multiply to their full extent in human embryonic kidney cells previously inoculated with cold viruses. This interfering action of the cold viruses provides another means whereby they can be detected in the laboratory.

The following data about strains of common cold viruses have been obtained using these new methods. They pass through a collodion membrane of an average pore diameter of $100\text{ m}\mu$, and survive overnight exposure to 20 per cent. ether. They are inactivated by heat at $56^\circ\text{ C}.$ for thirty minutes and by exposure to acid at pH 2. Convalescent serum from a person recently recovered from a cold inactivated his own virus strain. Whether the common cold is due to a single virus or a group of viruses remains to be determined.

Infectious Laryngo-Tracheitis

This is a highly contagious respiratory infection of domestic fowls, turkeys and pheasants. The incubation period is less than forty-eight hours, and after entering the flock infection does not stop until almost every bird has been attacked. The symptoms are those of cough and severe dyspnoea due to respiratory obstruction. The principal pathological lesion is a thick blood-streaked inflammatory exudate over the surface of the larynx and trachea. Intranuclear inclusions are found in infected epithelial cells in the trachea.

The virus of infectious laryngo-tracheitis is $45\text{--}82\text{ m}\mu$ in diameter. It can be propagated on the chorio-allantoic membrane of the developing chick embryo, where it produces white plaques. The virus can be cultivated in minced chick tissue cultures of the Maitland type and also in monolayers of chick cells. The virus survives at room temperature for periods up to ninety days but it is readily inactivated by heat at $55\text{--}75^\circ\text{ C}.$ and by the usual antiseptics. No haemagglutinating activity has been described. The relationship of this virus to other respiratory viruses is not determined.

Recovered birds may carry the virus for as long as two years and thus form the reservoir from which new outbreaks are derived.

Control of the disease usually necessitates the complete eradication of infected flocks and the vigorous disinfection of contaminated premises. In some countries vaccination is practised; an unattenuated strain is used for the purpose and is introduced by painting on the cloaca.

CHAPTER XL

ENTEROVIRUSES; VIRAL HEPATITIS AND INFECTIOUS MONONUCLEOSIS

THOSE viruses which multiply predominantly within the cells of the intestinal tract of man are grouped together in a family known as the Enteroviruses. In the family are three smaller groups; the poliovirus subgroup consisting of the three poliomyelitis viruses; at least twenty-four ECHO viruses; and thirty Coxsackie viruses. Most are within the size range 25-33 m μ and are antigenically distinct from each other. They can be isolated from human faeces in tissue culture, and in the case of Coxsackie viruses also by the inoculation of infant mice. As well as causing poliomyelitis these viruses give rise to a wide variety of acute feverish illnesses in man.

POLIOMYELITIS

Usually infection with the poliovirus is quite inapparent and the individual, although at the time excreting the virus, is unaware of any ill effects. Occasionally he may have a minor illness with, at most, symptoms of fever, headache and vomiting. In a smaller proportion of infections there are signs of a meningeal reaction of some severity and these may subside or proceed to involvement of the central nervous system with localisation of the virus either in the anterior horn cells of the spinal cord or in the region of the respiratory and vasomotor centres in the medulla. The clinical diagnosis of poliomyelitis based on the signs of paralysis is made in less than one in a thousand infections.

The Properties of Poliovirus hominis.—The poliovirus is a spherical particle 27 m μ in diameter; it is the smallest known human pathogen. The virus was the first animal virus to be purified and obtained in crystalline form; it contains 25-30 per cent. ribonucleic acid (RNA).

Two outstanding characteristics of the virus are its affinity for nervous tissue and its narrow animal host range. The only animals readily susceptible are the primates, though it has been possible to adapt some strains to grow in small rodents and chick embryos. Cynomolgus and rhesus monkeys can be infected by the oral route and develop paralysis; in chimpanzees, however, the infection is often asymptomatic. Under the influence of cortisone,

monkeys become more susceptible to small parenteral doses of the virus. The animals develop a viraemia which is suppressed when antibodies appear, and later they excrete the virus in their faeces. Poliomyelitis viruses are most easily isolated and cultivated in *in vitro* tissue cultures of monkey kidney or in HeLa cells where their cytopathic effect becomes rapidly apparent. They can also be grown in a wide variety of human cells in tissue culture explants, e.g. embryonic skin, muscle, kidney, tonsil, prepuce, testis and uterus; monkey testis or lung can also be used.

Viability.—The poliovirus is one of the most stable known. In aqueous suspensions of human faeces at 4° C. it survives for many months, and in pieces of spinal cord in 50 per cent. glycerol in normal saline it remains viable for periods of eight years or more. It can be preserved for many months or years at -20° C. or -70° C. Unlike most other viruses its infectivity is not well preserved by freeze drying. In human stools the virus may survive at room temperature for as short a time as one day or for as long as several weeks, depending on the amount of virus present, the pH, the amount of faecal moisture and other environmental conditions. The virus is readily killed by moist heat at 50°-55° C. but milk, cream and ice-cream exert a protective effect so that the virus in these foodstuffs may survive exposure to heat at 60° C. It is destroyed by the process of pasteurisation of milk at 62° C., but the safety margin is not sufficient for certain inactivation and the flash method at 72° C. is to be preferred.

In infected human spinal cord the virus is rapidly inactivated at pH values below 2 or over 11; it survives for ten days at 4° C. in 1 per cent. phenol, eighteen hours at 4° C. in ether, and 0.1 per cent. sodium desoxycholate. Inactivation of poliovirus in tissue culture fluids is complete after seven days exposure to 0.025 per cent. formaldehyde at 37° C., but its antigenicity is retained so that it can be used as an immunising agent. The most active disinfectants are oxidising agents such as potassium permanganate and hypochlorites. In the absence of organic matter free chlorine in a strength of 0.05 parts per million will inactivate the virus, but higher concentrations than this are needed to disinfect swimming-bath water or materials contaminated by faeces.

Antigenic Characters.—Three immunological types of the virus have been identified by neutralisation tests carried out in the monkey or in tissue cultures. The prototype strains of type I are the Brunhilde and Mahoney strains; type II, which includes the rodent adapted strains, the Lansing and M.E.F.1 strains; and type III, the Leon and Saukett strains. The three types are immunologically distinct, but overlapping in neutralisation tests

is not infrequent. Type I is the common epidemic type, type II usually associated with endemic infections, and type III occasionally causes epidemics. The size, chemical and physical properties, and the resistance of the three types are all identical.

In partially purified preparations of polioviruses there are several particles which differ in size, density, chemical composition, infectivity and antigenic characters. These particles can be separated by sedimentation in a sucrose density gradient, and two of them, the D and C particles, can be distinguished according to their predominant antigens. D particles have the characteristics of infective virus, are well defined, and are of uniform appearance in electron micrographs; they contain 25-30 per cent. RNA and about 70 per cent. protein. The C antigen configuration is associated with particles which are less electron dense and which are structurally impaired so that they have a ring or "doughnut" appearance; they are devoid of RNA and have the power to react with antibodies which appear very early in the serum of acute cases of poliomyelitis. If D particles are exposed to heat or to ultra-violet light, C antigen is produced. D particles in concentrated preparations give type-specific reactions in complement-fixation tests and in precipitin reactions carried out by the agar gel diffusion method. C particle preparations also react specifically but are liable to react heterotypically with antibodies to all the three virus types. The preparation of purified suspensions of D particles for use in diagnostic serological work, although highly desirable, is still a matter of considerable technical difficulty.

Occurrence.—The only natural source of the virus is man; the virus is spread from person to person, and no intermediate host is known. The human reservoir of infection consists of persons who excrete the virus in their faeces and perhaps less commonly in their oropharyngeal secretions. The great majority of these people have no paralytic manifestations of the infection and suffer no illness; the virus they excrete enters the new host by ingestion or inhalation.

In paralytic poliomyelitis, the virus can be found in the faeces for a few days preceding the onset of acute symptoms and is present in over 80 per cent. of cases in the stool during the first fourteen days. After three weeks some 50 per cent. of patients still excrete the virus and at five to six weeks 25 per cent. Only a few cases continue to excrete the virus after the twelfth week. No permanent carriers are known. The virus can be isolated from the oropharynx of many cases for a few days before and after the onset of the illness.

There is still some doubt as to the route by which the virus is disseminated throughout the body. One suggestion is that there is a primary focus of viral multiplication and that from this site

the virus is disseminated in the blood stream. It is probable that on entering the body the virus first invades the lymphoid tissues in the upper respiratory tract or the Peyer's patches of the small intestine and the associated mesenteric lymph glands; during the next seven days large amounts of virus are produced locally in these extraneuronal sites until finally it spills over into the lymphatics and is carried into the blood stream. In those cases which die of an overwhelming infection within a short time from the onset, the Peyer's patches and the mesenteric lymph nodes are found to be greatly swollen and inflamed and to contain large amounts of virus.

The viraemic phase marks the end of the incubation period and is manifest in the patient by the fever and generalised toxic symptoms; it is followed by a period of about forty-eight hours of relative well-being (the disease is biphasic) while the virus is invading nerve tissue, and then, in serious cases, the signs of paralysis appear. Viraemia has been proved to occur after experimental infection of monkeys and has been demonstrated on several occasions in man. It is probable that the process can be arrested at various stages so that the virus may multiply in the intestine without ever reaching the blood stream, or once in the blood stream the virus may be overcome by the patient's natural defence mechanisms before it can reach nerve cells. Even if the virus destroys nerve cells, it is only when certain critical areas are involved that paralysis results. In this way it is possible to explain abortive and non-paralytic forms of poliomyelitis. It must be stated, however, that it is not yet known whether viraemia is a constant feature of the disease and that no adequate explanation has yet been offered of the manner whereby the virus enters the nerve cells in paralytic poliomyelitis.

An alternative explanation of the pathogenesis of poliomyelitis is that the virus is first deposited on the mucous membranes of the mouth or intestine where it enters the peripheral nerve endings. The virus then ascends along the axons to reach the peripheral ganglia and then the central nervous system. Virus multiplication follows with the production of lesions which may be small and heal quickly or which may progress to involve vital areas and cause paralysis. The evidence to support this view depends on the finding of lesions in the regional ganglia. It is, however, possible that such changes are caused not by the ascending spread of the virus but by its effect as it spreads outwards from the central nervous system. In general, it may be stated that there is considerable doubt as to whether the poliomyelitis virus can enter through intact nerve endings. In the experimental monkey,

however, it is known that the virus can enter a nerve which has been deliberately cut and that it can travel along the proximal fibres to reach the central nervous system; such a sequence of events may occasionally occur in man and is thought to happen after tonsillectomy.

Factors Predisposing to Infection.—There are a number of factors which are known to shorten the incubation period, enhance the severity of the infection, and promote the localisation of the virus in the central nervous system, thus predisposing to paralysis. Muscular activity during the pre-paralytic phase of the illness leads to paralysis of the limbs used. Pregnant women are more susceptible than non-pregnant women. Poliomyelitis occurring near full term is apt to be severe and may assume the bulbar form. Tonsillectomy carries an increased risk of bulbar poliomyelitis, and this risk persists for several months or even years after the operation.

Paralytic poliomyelitis may also occur in children who have received immunising injections of alum-containing diphtheria toxoids, particularly when combined with pertussis antigens. It is probable that the irritant properties of the alum or other adjuvants used in these vaccines is more important than the nature of the antigens. A similar effect has followed the use of penicillin, arsenicals and heavy metals in mass campaigns against yaws. Paralysis occurs in the limb which receives the inoculation, and its incidence is approximately 1 in 37,000 injections. There is much doubt about the manner in which the paralysis is precipitated; the irritant inoculum in the muscles may provide a site for the local proliferation of the virus circulating in the blood or it may perhaps render the anterior horn cells of the corresponding segment of the cord more susceptible to virus invasion.

In paralytic poliomyelitis it sometimes happens that the patient has a double infection and that both the poliomyelitis virus and a Coxsackie virus (see p. 827) can be recovered from the faeces. Viruses of Coxsackie group A occur with significantly greater frequency than those of group B in paralytic poliomyelitis. The constancy of the association of the poliomyelitis and Coxsackie group A viruses has been sufficient to suggest that infection with the latter predisposes to paralysis. Coxsackie B viruses are not associated with paralytic poliomyelitis and it is of interest to note that, in contrast to group A viruses, they have an interfering action which spares the mouse from the effects of experimental poliomyelitis.

Epidemiology.—Poliomyelitis occurs throughout the world, but in temperate climates assumes an epidemiological pattern quite

different from that found in the tropics. In Europe and North America the disease is endemic with periodic epidemic increases, usually in the late summer; in the tropics it occurs uniformly throughout the whole year without any tendency to seasonal variation. As hygiene improves, however, the epidemic pattern changes.

At the beginning of the century, paralytic poliomyelitis was known as "Infantile Paralysis" because it most frequently attacked children under 5 years old. Since the Second World War, however, the position has changed and only one-third of the patients are under 5 years of age and one-third are in the age-group 5-15 years. The remaining third are over 15. No age is exempt, and poliomyelitis has been recorded at the age of 70 years. In places where overcrowding and poor sanitation permit rapid dissemination of the virus, poliomyelitis remains a disease of infancy, and by the age of 4 years practically all the children have acquired immunity to all the three virus types. It is established that infants are less likely to develop paralysis than older children, but the peak incidence is still in the 1-3 year-olds: in recent epidemics over 60 per cent. of fatal cases have been in patients over 15 years old in whom the infection tends to be more severe.

The higher standard of hygiene in some countries impedes spread of the virus over wide areas and individuals or whole communities may escape infection for many years. In this way it may happen that a large part of a population lacks immunity to the polioviruses and that when infection is introduced it spreads so rapidly among susceptible persons that an epidemic results. The introduction of more virulent strains may also be a factor in epidemic spread. It is difficult to know which of these factors was more important when in this country in 1947 the incidence of poliomyelitis rose from the usual figure of 4 per 100,000 to 18 per 100,000 of the population. A preponderance of the clinical infections has been caused by type 1 virus.

Poliomyelitis is often contracted during a period of quite close proximity to an infected person; the virus may be inhaled in infected particles or ingested. It is thought that the virus is most frequently transferred by the hands of persons who are excreting the virus or by those who have touched contaminated fomites.

Faeces provide a rich and persistent source of the virus; it has been calculated that one gram of stool may contain several million infective doses of the virus. Sewage in urban populations contains the virus throughout the summer and early winter months, while poliomyelitis is prevalent. Water supplies may occasionally be contaminated by sewage, and in rural areas this

may be a means by which infection is spread. There is little evidence, however, that the virus can survive the purification processes used for a piped water supply, and urban water-borne epidemics have not been described. Faecal pollution of swimming-baths has often been thought to spread the infection, but there is little direct evidence on this point and with adequate chlorination the risk is small. Flies may carry the virus to food on their feet or by regurgitating after feeding on exposed faeces or sewage.

Immunity is permanent to the virus type causing the infection. Although the three virus types are antigenically distinct there is some evidence to suggest that prior infection with type II virus may confer a measure of resistance to the paralytic disease caused by type I.

Virus neutralising antibodies are formed early during the disease (often before the seventh day) and persist for several decades. Complement-fixing antibodies are of much shorter duration.

Laboratory Diagnosis

Virus Isolation.—The virus may be recovered from faeces or throat swabs taken early in the disease. Two such specimens should be collected on successive days as suspension is made in course of the disease. A 10 per cent. faecal smear of penicillin and Hank's balanced salt solution containing 100 units. There is to remove 100 µg. of streptomycin per ml. and is then centrifuged in a coarse particles. Throat swabs are treated in monolayer tissue. The material is then inoculated into HeLa cells. If the cytopathic effect is usually seen in the cells within 48 hours. Identification of the virus type is within 4 weeks by a neutralisation test in which a measured dose of virus (approx. 100 T.C.D. 50) is exposed to the action of standard specific antisera. It is convenient to dilute the virus isolate 1 in 100,000 and 1 in 100,000 and to mix these two dilutions with a suitable amount of the standard antisera. In fatal cases specimens obtained at autopsy should include the cervical and lumbar enlargements of the spinal cord, the medulla, mesenteric lymph nodes and portions of small intestine and colon with their contents. After homogenising these tissues are treated in the same way as faeces. All types of infected material can be preserved in the refrigerator at 4° C. but better results are obtained when storage is at -30° C. Tissues should be placed in 50 per cent. glycerol saline before storage.

Serological Tests.—Paired samples of serum are required; the

first must be taken as soon as possible after the onset of the disease and the second after an interval of three to four weeks. Neutralisation tests (see p. 352) are usually employed with all the three virus types. If the first sample has been taken sufficiently early it is often possible to show a significant rise of antibodies to the infecting virus type, but in practice this may not always be achieved because of the insidious nature of the onset. Antibody tends to rise rapidly, and titres of 1000 or higher are usual by the end of the third week of the disease. In type I infection some type II antibody may develop as well. Complement-fixation and flocculation tests are not yet in general use for routine diagnostic work.

Prophylaxis and Control.—Since it has proved impractical to prevent the widespread dissemination of the virus and because it is impossible to recognise the trivial infections that the poliomyelitis viruses cause, it has become obvious that the disease can only be controlled by raising the immunity of the population to a high level.

Active immunisation with a killed vaccine has been advocated by Salk for this purpose. The vaccine contains strains of the three types of virus inactivated by exposure to formaldehyde. In the vaccine used in America type I is represented by the Mahoney strain, type II by M.E.F.1, and type III by Saukett. A second vaccine of this type was developed in Britain and for greater safety less virulent strains were employed; the type I virus used was Brunenders, an attenuated variation of Brunhilde, type II was a strain of M.E.F.1 adapted to suckling mice and the type III virus was again Saukett. Both vaccines have been used extensively and many millions of people have received them without ill effect; they cause no local or general reactions and each has been proved to lower significantly the incidence of paralytic poliomyelitis. Initially two doses of 1.0 ml. of the vaccine are given intra muscularly at an interval of three weeks. Two booster doses are required, one six to nine months after the second dose and the last a year or two later. Lately in North America the poliomyelitis vaccine has been included in a quadruple vaccine which also contains diphtheria toxoid, tetanus toxoid and pertussis vaccine.

Vaccines containing living attenuated viruses of all three types are also under trial; they can be given by mouth, and since the strains multiply in the intestinal tract there is no need for repeated doses. These viruses are mutants which have lost practically all their capacity to invade the tissues of the monkey and cause paralysis, while retaining their power to grow in the alimentary tract. No cases of paralytic poliomyelitis in man have been reported as a result of the use of living vaccine. Preliminary results

of their immunizing capacity are encouraging; it has also been suggested that the living vaccine could be recommended to produce an enduring immunity in persons who have already received the Salk vaccine.

Passive immunity can be conferred by injecting gamma globulin. The doses recommended are as follows: for persons over the age of 7 years 1.5 g., for the age-group 1-6 years 1.0 g., and for infants under 1 year old 500 mg. To afford protection gamma globulin must be used as soon as possible after exposure or when possible, before the risk is taken. As it is in short supply and of rather doubtful value, gamma globulin is reserved for special occasions; it is used for pregnant women after exposure, for children after tonsillectomy at epidemic periods, and for laboratory workers accidentally contaminated by the virus.

ECHO VIRUSES

When tissue cultures began to be used for the isolation of poliomyelitis viruses from stools it was soon found that many other viruses also could be recovered from the intestinal tract. These viruses can only be isolated in tissue cultures, optimally in those from monkey kidneys. Originally they were called "Orphan" viruses because they seemed unrelated to known diseases, and since they were present in faeces they were named "Enteric, Cytopathogenic, Human, Orphan i.e. ECHO viruses".

Properties of the Viruses.—By definition ECHO viruses are (1) cytopathogenic for monkey and human cells in tissue culture; (2) non-pathogenic for suckling mice and other laboratory animals; (3) unrelated to other known cytopathogenic viruses, e.g. poliomyelitis, Coxsackie B, herpes simplex, influenza, mumps, measles, varicella, and the adeno viruses; (4) neutralised by human gamma globulin; (5) ether resistant; (6) possessed of complement-fixing antigens; (7) characterised by plaques (see 458) different from those of the poliomyelitis viruses; (8) in the size range 25-35 m μ . These criteria, however, can only be considered as tentative, for there are a number of anomalies. ECHO type 10 virus, for example, is 75 m μ in diameter, produces a strikingly different cytopathic effect and can infect a much wider range of host cells, including those of the pig, dog, cat, rabbit and calf as well as those of three simian species. For these reasons it is now regarded as the prototype of a new group, the Reo-viruses.¹

The ECHO viruses are in general similar to the poliomyelitis viruses in size, stability and resistance. They survive long periods

¹ Sabin, A. B. (1959), *Science*, **130**, 1387.

at 4° C., are not inactivated at 37° C., and are stable at pH3 and pH11. They contain at least 25–30 per cent. RNA. Continuous cell lines, *e.g.* HeLa, are not, however, suitable for attempts at ECHO virus isolation, as the cytopathic effect may be absent or very slow to appear.

When ECHO viruses are seeded in dilute inocula on to the surface of a sheet of tissue culture cells, each single elementary body sets up an area of cell lysis or a plaque (see p. 458) which is easily recognised. Types 7, 8 and 12 produce large circular plaques with clearly defined edges and a diameter after a week's growth of 1 cm. Types 1, 3, 4, 6, 9, 11, 13, 14 and 16 produce irregularly shaped plaques which develop slowly and seldom reach 0·5 cm. in diameter.

Haemagglutination is caused by types 3, 6, 7, 10, 11 and 12. These strains react with a receptor on human group O cells which is distinct from that of the myxoviruses.

Antigenic Characters.—Twenty-four distinct antigenic types have so far been distinguished by neutralisation tests in tissue cultures. Cross-reactions occur between types 1, 8 and 12. Antigenic variation is known to occur in types 6, 9 and 10, and may be a common occurrence under natural conditions. The specificity of strains may be altered by cultivation in the presence of a heterologous antiserum.

Occurrence.—ECHO viruses are not infrequently found in the stools of apparently healthy young children, but the significance of this finding is not clearly understood. There is little evidence to suggest that they are normal commensals in the intestine and it seems more probable that they are carried for indefinite periods after mild or inapparent infections. The clinical manifestations which follow infection are not the same for all the ECHO viruses and each of the various types may cause a variety of illnesses. Aseptic meningitis, diarrhoea, mild respiratory tract infections and colds have all been found to be associated with the presence of ECHO viruses in the host and are accompanied by a rising antibody titre to the infecting strain.

Clinical Syndrome	Associated ECHO Types
Aseptic meningitis	2, 3, 4, 5, 6, 7, 9, 12, 14, 16, 21.
Aseptic meningitis with rash	4, 6, 9, 16.
Diarrhoea	2, 7, 8, 10, 11, 12, 14, 18, 19.
Mild respiratory illness	8, 10, 11, 20.
Not yet associated with illness	1, 13, 15, 17.

ECHO virus type 9 is constantly associated with illness and has been isolated in Great Britain from cases of aseptic meningitis and from mild undifferentiated fevers. Both types of infection may be accompanied by a macular or maculopapular rash which is found very frequently in young children and decreases in incidence in the older age-groups. ECHO virus type 9 appears to be able to invade nervous tissue and infection with it is sometimes characterised by a mild and transient paralysis; it has been recovered from the medulla of an infant who died in coma. This virus together with types 4, 6 and 16 has caused epidemics of a feverish illness, sometimes with meningitis and occasionally with a rash. ECHO virus type 18 has been closely associated with epidemics of acute diarrhoea in infants under one year old and many other types have been recovered from children with diarrhoeal illnesses. ECHO virus type 20 has been recovered from the throats of children suffering from cold-like illnesses and mild respiratory tract infections.

Epidemiology.—ECHO viruses occur in all parts of the world. They are found more frequently in children than in adults and are more prevalent in the summer and autumn months in temperate climates. The viruses are spread by the same route as the poliomyelitis viruses and are widely and rapidly disseminated when hygiene and sanitation are poor. The epidemics they cause mainly affect children and take the clinical form of undifferentiated fever, aseptic meningitis or acute diarrhoea.

Laboratory Diagnosis.—ECHO viruses are readily isolated from throat swabs, stools or cerebrospinal fluid. The procedure is that described for poliomyelitis and involves the inoculation of monolayers of monkey kidney tissue cultures. Human amnion cells may be used, but HeLa and other continuous lines of human cells are unsuitable. Strains are identified by testing first against pools of known antisera and then by neutralisation by a single type specific antiserum. Infections with more than one ECHO type can sometimes be revealed by the different types of plaques produced in monolayer cultures. Serological tests are burdensome, and unless there is some indication of the prevailing type it is often impractical to set up the many neutralisation tests required.

Other Enteroviruses.—ECHO-like viruses have been frequently isolated from animals. Monkeys appear to carry them asymptotically and their presence is frequently detected by a cytopathic effect observed in uninoculated monkey kidney tissue culture cells. Often the cells show marked vacuolation and the viruses responsible have been known as "foamy agents". There are at least 25 enteric cytopathogenic monkey orphan (ECMO viruses). Similar

agents have been recovered from bovines (ECBO viruses) and from swine (ECSO viruses). Further investigation is required into the relationship of these animal viruses to human enteric viruses and the possibility of their transfer from one host to another.

Teschen Disease

Teschen Disease is an acute encephalomyelitis in pigs. The animals suffer from flaccid paralysis and develop lesions in the central nervous system which closely resemble those of human poliomyelitis. The causative virus is 10–15 m μ in diameter and has many of the general properties of the poliomyelitis viruses. It is, however, completely distinct antigenically and represents a unique type. The virus is excreted in the faeces and infection follows ingestion of contaminated food. Teschen disease occurs on the continent of Europe but has not been reported elsewhere. Sporadic cases of a similar though milder disease due to the related Talfan virus have been reported in Great Britain.

COXSACKIE VIRUSES

The third group of the Enterovirus family contains the thirty Coxsackie viruses which cause such diverse illnesses as aseptic meningitis, epidemic myalgia or pleurodynia, herpangina and neonatal myocarditis.

Properties of the Viruses.—The virus particles are 25–35 m μ in diameter. One strain (group A type 10) has been obtained in a highly purified state from the carcasses of infected mice by a combination of the processes of salting out and ultra-centrifugation; its elementary bodies when stored at 4° C. formed dodecahedral crystals about 100 m μ in diameter. The crystals have a high infectivity titre and contain a mixture of infective and non-infective units. About 4 per cent. of the virus particle is made up of nucleic acid, probably of the ribose type. In their reactions to chemical and physical agents Coxsackie viruses do not differ materially from other enteroviruses.

The most outstanding characteristic of the Coxsackie viruses is their pathogenicity for newborn mice and hamsters. It is during the first forty-eight hours of life that these animals are fully susceptible to infection; thereafter they acquire a natural resistance and after the age of five days they can no longer be infected. By definition Coxsackie viruses are unable to infect adult mice. Two broad groups of the viruses have been made according to the

histological nature and the situation of the lesions they produce in mice.

Group A viruses, of which there are twenty-five, cause a single lesion, a widespread severe myositis involving skeletal muscle throughout the whole body. The principal muscles to be involved are those of the hind limbs, and in life the mice appear to have a flaccid paralysis. Usually the signs of infection appear four or five days after inoculation and progress until the animal dies four or five days later.

Group B viruses, of which there are five, cause widespread lesions in many organs. The myositis produced is characterised by focal lesions and gives rise to tremors, incoordination and a paralysis resembling the spastic type. The viruses also cause areas of necrosis in the foetal fat lobules, especially those in the interscapular and cervical pads of brown fat. They also cause meningoencephalitis and pancreatitis. The incubation period of group B infections in mice is prolonged and symptoms may not be obvious until the tenth day after inoculation. Inoculated mice must be kept under observation for three weeks.

Tissue cultures are of limited value in isolating the viruses. Types A 9 and B 1, 2, 3, 4 and 5 grow well in monkey kidney monolayers and produce a cytopathic effect resembling that of the poliomyelitis viruses. Types A 11, 13, 15 and 18 will grow in HeLa but not in monkey kidney cells. In general, however, the cytopathic effect in HeLa cells is variable and slow to develop. Explants of human uterus in plasma clot cultures are susceptible to those strains which grow in monkey kidney cells. The majority of group A strains do not grow in tissue culture and to isolate them it is necessary to inoculate infant mice.

In monkeys Coxsackie viruses do not produce clinical diseases, but after inoculation a viraemia is developed and later the virus is excreted for several weeks in the faeces. Group A types 7 and 14, however, possess the power to cause a mild paralysis in monkeys with lesions in the central nervous system resembling those of poliomyelitis. Group A type 7, which has caused paralytic disease in man, is identical with the Russian AB IV strain which was at first thought to constitute a fourth type of the poliomyelitis virus.

Antigenic Characteristics.—Thirty antigenic types have been defined by cross-neutralisation tests in mice and cross-complement-fixation reactions. Twenty-five have the pathogenicity of group A and five the characters of group B. Each of the five group B types are subject to antigenic variation. Group B type 3 has an agglutinin for human group O cells.

Occurrence.—Coxsackie viruses are found in the oropharyngeal secretions and faeces of children and young adults and are disseminated in the same way as the other enteroviruses. Their association with human infection is as follows:

Clinical Syndrome	Associated Coxsackie Virus	
	Group A	Group B
Aseptic meningitis	4, 7, 9.	1, 2, 3, 4, 5.
Aseptic meningitis with rash . .	9.	—
Fever with rash	16.	1.
Epidemic Myalgia (Bornholm disease)	—	1, 2, 3, 4, 5.
Herpangina	2, 4, 5, 6, 8, 10.	—
Neonatal myocarditis . . .	—	2, 3, 4.

Clinically aseptic meningitis has the same manifestations whether it is caused by the Coxsackie, ECHO or poliomyelitis viruses. It is discussed further on p. 830. Epidemic myalgia or Bornholm disease, so-called because it was first described on the Danish island of Bornholm, is characterised by fever and the sudden onset of agonising stitch-like pains in the muscles of the chest, epigastrium or hypochondrium. Although the disease is most frequently recognised in its epidemic form, many sporadic cases also occur. Herpangina is a sudden feverish illness of young children; the lesions in the mouth are highly characteristic and consist of papules on the anterior pillars of the fauces which soon become vesicles and finally shallow ulcers with a greyish base and a punched-out edge. In newborn infants severe and often fatal myocarditis has been reported and the causative virus has been found in high concentrations in the myocardium at autopsy. It may be that the newborn baby acquires the virus from its mother, and that, like the infant mouse, it is highly susceptible to infection.

Epidemiology.—Aseptic meningitis, epidemic myalgia and herpangina all occur characteristically as epidemics. The peak incidence is usually in the summer months and large epidemics may occur. In the summer of 1951 a widespread epidemic of Bornholm disease occurred in Great Britain and in 1959 a high incidence of aseptic meningitis due to Coxsackie viruses was reported in Scotland. Usually half the persons involved in epidemics are under the age of ten years and three-quarters under twenty. Herpangina is seen in very young children under five

years of age in day nurseries and kindergarten schools, where it spreads rapidly. Sporadic cases of all the clinical forms of infection occur and often the patients are young adults who have acquired the infection presumably from family contacts.

Laboratory Diagnosis

Virus Isolation.—The virus can readily be isolated from throat swabs or faeces during the first two weeks of the infection and can also be recovered from the cerebrospinal fluid from cases of aseptic meningitis. Stools and throat swabs are treated with antibiotics and clarified by slow centrifugation in the manner used in isolation of the poliomyelitis viruses. The first step is to inoculate monkey kidney tissue cultures and HeLa cells. Cytopathogenic agents growing in these cultures will include all the group B types and group A type 9. If this procedure yields no virus the specimens should be inoculated into mice no older than forty-eight hours by the combined intracerebral and intraperitoneal routes. Subsequently viruses will be placed in their appropriate groups according to the histological appearances of the lesions they produce. The causal relationship of a newly isolated virus to the illness should be confirmed by demonstrating a rising titre of homologous antibodies in the patient's serum. The final identification of the numerical serotype is carried out by neutralisation tests in tissue cultures or, if this is not possible, in infant mice. The procedures are burdensome, for there are thirty possible type-specific antisera to set against the unknown virus and, unless some information is available as to the prevalent infecting type, it may require much time to complete the task.

Serological Tests.—Neutralisation and complement-fixation reactions may be of value provided that the first serum has been taken within three days of the onset of the disease. Again the large number of viruses in the group often makes these tests impractical unless there is some clue as to the nature of the prevailing virus.

Aseptic Meningitis

Physicians are familiar with an acute feverish illness in which all the classical signs of meningeal irritation are present and yet the cerebrospinal fluid is bacteriologically sterile. The condition has been known as "abacterial", "serous", "virus", "lymphocytic" and "benign", but no one term is precisely accurate and the name "aseptic" is retained on the ground of common usage. The cell count in the cerebrospinal fluid is raised and figures between 50 and 2000 per cu. mm. have been recorded. The cells

are predominantly lymphocytes and no bacteria can be seen in stained smears or obtained in culture. The protein content is always raised, usually to 80 or 100 mg. per ml.; the figure may, however, be as low as 50 mg. per ml. and the increase may not be apparent until several days after the onset of infection.

It must be remembered that this clinical syndrome may be associated with bacterial as well as viral infections. Tuberculous meningitis, middle ear disease, subdural abscesses, leptospiral meningitis, and even pyogenic meningitis under antibiotic therapy can all be associated with such a clinical picture.

Primary virus infections giving rise to aseptic meningitis are caused by (1) Poliomyelitis viruses (non-paralytic poliomyelitis); (2) Coxsackie viruses especially group B; (3) ECHO viruses; (4) Lymphocytic choriomeningitis virus; (5) Herpes simplex virus; (6) certain Arbor viruses, e.g. Western equine encephalitis. In addition aseptic meningitis occurs as a complication of the following: (1) mumps, (2) chicken pox, (3) measles, (4) herpes zoster, (5) infective hepatitis, (6) sandfly fever and (7) Jennerian vaccination.

VIRAL HEPATITIS

Viral hepatitis includes two human diseases whose relationship to each other is still in doubt. Infective hepatitis and serum hepatitis (homologous serum jaundice) are both characterised by the same clinical features of fever, nausea, malaise and jaundice. In both diseases severe degenerative changes occur in the parenchymal cells of the liver and marked impairment of hepatic function results. Usually, healing is remarkably complete and normal function is restored, but in a small proportion of patients relapses occur and occasionally the end-result is multilobular cirrhosis of the liver.

Infective hepatitis, or to use its older name "catarrhal jaundice", is a disease of children and young adults. It occurs endemically and more characteristically in epidemics in institutions, schools and in military camps. The virus is excreted in faeces and infection follows the ingestion of contaminated food; thus under conditions of communal living spread is facilitated and epidemics break out.

Serum hepatitis is contracted only when the virus is injected into a susceptible person; it most frequently occurs after the injection of human serum.

Properties of the Viruses.—Every attempt to isolate these viruses has so far failed and all the usual cultural methods for viruses are of no avail. The only known susceptible host is man, and much

of the knowledge that we have of these viruses has been obtained from studies on human volunteers.

Virus A. Infective Hepatitis.—The size of the virus particle is not known although it has been proved to be able to pass through a Seitz E.K. filter. The virus can be preserved for long periods in the frozen state—*e.g.* eighteen months at -20° C . It survives heat at 56° C . for thirty minutes and withstands chlorine at a concentration of one part per million for thirty minutes and 10 per cent. ether at 4° C . for twenty-four hours. When given orally or parenterally to human volunteers the disease develops in seventeen to thirty days and the virus is present in the blood and faeces in the pre-icteric and early icteric phases of the illness.

Virus B. Serum Hepatitis.—The virus, according to filtration experiments, is $26\text{ m}\mu$ or less in diameter. It survives storage at room temperature for six months and can be kept at -20° C . for over four years; it is also well preserved by freeze drying. It has survived heat at 60° C . for four hours. Ultra-violet light has failed to inactivate the virus in plasma. The virus is not affected by 0.25 per cent. phenol, 0.05 per cent. merthiolate, nitrogen mustard in a concentration of 50 mg. per litre, or 10 per cent. ether. It is inactivated by beta-propiolactone in a concentration of 4 g. per litre, and by sulphur mustard in a 0.005 M concentration.

Occurrence.—The only source of infective hepatitis is man; the virus is present in his blood and intestine during the incubation period as well as in the pre-icteric and icteric phases of the acute illness. It is probable that most patients excrete the virus in the faeces for about one month after the onset but some, especially infants, may be faecal carriers for several months. The virus is also present in the blood stream, where it is known to have persisted for as long as eight months.

The only known source of *serum hepatitis* is human blood or serum or their products, and transmission occurs solely after the injection of these substances. When the donor of the blood or serum is traced it is seldom possible to elicit a history of previous jaundice. Sometimes the donors suspected as being the original source of infection develop jaundice many months later.

Minute amounts of blood or serum, as little as 0.01 ml., have often been known to have transmitted serum hepatitis. The disease develops after a remarkably long incubation period, which on an average is about eighty days but may be as short as forty days or as long as one hundred and sixty days after the injection. The duration of infectivity following the jaundice is not known. In many cases the virus is in the blood for many months after recovery and long after the liver function tests have again given normal

results. The longest recorded period for a person to have carried the virus in the blood is five and a half years. Patients with serum hepatitis do not seem to excrete the virus and their immediate contacts are not liable to contract the infection.

Epidemiology

Infective Hepatitis exists throughout the world. It is essentially a disease of children and 65 per cent. of cases occur between the ages of five and fifteen. There is a seasonal trend with increased prevalence in the autumn and winter. Outbreaks in institutions, schools, mental hospitals and orphanages are common; often there is a characteristic interval of three to four weeks between the appearances of small crops of cases and this sequence may continue for months or even years, involving new members as they join the community. A similar sequence of infections is a familiar occurrence in large families. The disease has a special tendency to widespread epidemics amongst troops under wartime conditions. In the Mediterranean theatre in the Second World War, many thousands of young soldiers suffered from a severe form of infective hepatitis. Communal living, poor sanitation and transfer by flies were all factors which aided the spread of infection. There are many examples of explosive waterborne epidemics, and many others have followed the contamination of foodstuffs by carriers. Oysters from a river bed contaminated by sewage were responsible for one epidemic. It must also be remembered that, like serum hepatitis, the infection can be transmitted by the use of syringes and needles contaminated with blood.

Serum Hepatitis.—On repeated occasions serum hepatitis has occurred in hospitals and clinics where syringes and needles were used without sterilisation between each inoculation. Since the introduction of proper cleaning and sterilising methods such outbreaks are rare and occur only when faulty technique prevents the destruction of the virus. In venereal disease clinics so-called "arsenical jaundice" in patients receiving courses of intravenous injections for syphilis used to be a common complication; for many years it was thought to be a toxic manifestation of the arsenic treatment, but when regular routine daily sterilisation of all syringes was introduced the condition virtually disappeared. Serum hepatitis has often followed blood transfusions and the use of plasma and serum injections. Human serum has sometimes been used as a constituent of suspending or preservative solutions for yellow fever virus vaccines and on at least two occasions big epidemics of hepatitis occurred in service personnel receiving the vaccine.

A Comparison of Infective and Serum Hepatitis.—The very considerable differences between these two diseases are shown in the table, and they are sufficient to suggest that the causative viruses A and B are separate entities. The fact that a patient who has recovered from infective hepatitis can still contract serum hepatitis and *vice versa* would support this view; but whether this represents two distinct viruses or antigenically dissimilar variants of the same virus is quite unknown.

A Comparison of Infective Hepatitis and Serum Hepatitis

Disease	Infective Hepatitis	Serum Hepatitis
Virus . . .	A	B
Infectiousness . .	Contagious.	Not contagious.
Incubation period . .	15–40 days.	40–160 days.
Onset . . .	Acute.	Insidious.
Fever . . .	Over 100° F.	Low-grade.
Age . . .	Children and young adults.	Any age.
Virus in blood . .	Three days before onset and in acute phase.	In incubation period and acute phase.
Virus in faeces . .	In incubation period and acute phase.	Not present.
Longest known duration of carrier state:		
(a) Blood . . .	8 months.	5 years.
(b) Faeces . . .	16 months.	Not present.
Route of successful experimental transmission . .	Oral and parenteral.	Parenteral only.
Seasonal incidence . .	Autumn and winter.	All the year round.
Prophylactic value of gamma globulin	Good.	Ineffective.
Immunity:		
(a) Homotypic . .	Present.	Uncertain.
(b) Heterotypic . .	None.	None.

Infective hepatitis is an example of the familiar pattern of the natural history of the many infectious virus diseases of man. Serum hepatitis, however, is quite different, for it is obviously an

artificially induced condition; it cannot be produced by the ingestion of icterogenic serum and follows only after needle puncture. The condition is not contagious and the virus is not found in the stools. If serum hepatitis is a distinct entity there should be some means whereby it is spread naturally but this so far has eluded observation. Burnet¹ has suggested that the virus may be transmitted from parent to offspring transplacentally and that as a consequence the new host has an immune-tolerance to the virus but remains a persistent carrier.

Both types of hepatitis have indistinguishable clinical manifestations which may vary from the severe to mild and silent forms. In both the virus has been shown to be present in the blood in the presymptomatic period. Virus B, for instance, was demonstrated in the blood of one patient no less than eighty-seven days before he developed the first symptoms of illness. It is therefore apparent that blood from supposedly normal donors who have never had hepatitis, may yet contain Virus A or Virus B, or both.

Prophylaxis and Control

Infective Hepatitis.—An outbreak of the disease in schools and institutions is often largely due to poor personal hygiene and inadequate sanitary arrangements. All the hygienic measures which are required to control the spread of pathogenic intestinal bacteria should be enforced. It is of importance to make every effort to ensure that possible virus carriers do not act as food handlers. All needles, syringes, blood-counting pipettes and lancets that may have been in contact with patients with infective hepatitis should be autoclaved. Passive protection may be conferred up to six days before the onset of symptoms by the use of gamma globulin. The dose recommended for persons of the age of seven and over is 0.5 g. and for children below this age 0.25 g.

Serum Hepatitis.—People who have had jaundice should not be used as blood donors. Persons without a history of hepatitis, however, may have the virus in their blood and the problem of making blood and blood products safe for transfusion has not yet been completely solved. Exposure to ultra-violet light and treatment with nitrogen mustard have been recommended but are not always effective in killing the virus. Beta-propiolactone and sulphur-mustard are at present on trial and offer better prospects of success. When pools of plasma are made and stored for use later, the risk of contaminating large volumes should be reduced by taking into each pool the plasma of not more than five donors.

¹ Burnet, F. M. (1960), *Principles of Animal Virology*, p. 254. New York, Academic Press.

All transfusion apparatus, syringes, needles, blood-counting pipettes, lancets, etc., must be autoclaved before use. Such sterile precautions are required not only in transfusion work but in haematological, diabetic and venereal disease clinics, and indeed should be employed whenever the skin of a patient is punctured. Gamma globulin has been found to give no protection against serum hepatitis.

Laboratory Diagnosis.—There are no specific laboratory tests that can be used to detect the virus or its antibody. Some help, however, may be obtained from biochemical tests of liver function. Many unusual serological manifestations are found in infective hepatitis and false positive Kahn and Wassermann tests are frequent. Probably the altered processes of protein production in association with severe liver damage result in the production of globulins which react with a wide variety of different bacterial and viral antigens.

INFECTIOUS MONONUCLEOSIS

Infectious mononucleosis or glandular fever is an acute feverish illness characterised by malaise, sore throat, enlargement of the lymph glands, a mononuclear blood picture, and heterophile antibodies in the blood. Liver function tests indicate that liver damage is constantly present. In about 40 per cent. of cases the spleen is palpable, and in about 10–20 per cent. a rubelliform rash is seen; jaundice and meningeal symptoms are infrequent complications. Three clinical forms, anginose, glandular and febrile, have been distinguished according to the predominant clinical features. The infection occurs most commonly among school children and young adults. The disease is often mild and probably ambulant undetected forms are frequent; it is rarely fatal. It is not highly infectious, although epidemics have occasionally been reported.

Aetiology.—The nature of the infective agent is unknown, but has been thought to be a virus on account of the infectious nature of the disease. In general, no laboratory animals can be infected and attempts to transmit the disease to man have usually failed. On one occasion when 250 ml. blood was transfused from a clinical case to a human volunteer the typical clinical picture of severe mononucleosis resulted. The cultivation of *Listeria monocytogenes* (see p. 535) from the blood of a few cases has suggested that this organism may cause the infection in man, but a constant association with this or any other bacterium has never been demonstrated.

Laboratory Diagnosis.—Some 50–80 per cent. of patients develop heterophile antibodies which can be demonstrated by the Paul-Bunnell reaction (see p. 320). The characteristic blood picture also helps to establish the diagnosis; a relative or more usually an absolute increase in the number of lymphocytes, together with the presence of abnormal monocytes, is diagnostic. A proportion of cases whose clinical illness and blood picture conform to those of infective mononucleosis do not have a positive Paul-Bunnell reaction and are referred to as “sero-negative infectious mononucleosis”.

Canine Hepatitis

Infectious canine hepatitis or Rubarth's disease is widespread in Britain and in the continents of Europe and North America. The disease at one time was confused with canine distemper, but is a distinct entity caused by a different virus. Young dogs and puppies soon after weaning are most susceptible; infection also occurs in foxes, in which it takes the form of an acute encephalitis. After an incubation period of six to nine days, dogs suffer from high fever, vomiting, diarrhoea and abdominal pain. Petechiae of the gums and inflammation and engorgement of the tonsils are common. Icterus is uncommon. The disease has a rapid course and 25 per cent. of the animals infected die within a few days of the onset. At autopsy the predominant features are subcutaneous oedema and a haemorrhagic peritoneal exudate. The liver is pale and swollen; histologically the endothelial cells of the sinusoids and the Küpffer cells show marked degeneration and the presence of acidophilic intranuclear inclusion bodies. The gall-bladder mucosa is markedly oedematous and the wall thickened.

The virus is excreted in the urine for many weeks after full recovery and it is from this source that the dissemination of the virus is maintained. The virus may also be spread on the hands of animal handlers contaminated by infected saliva.

Properties of the Virus.—The size is not known, but the virus can pass through a Berkefeld N filter. It can be cultivated in monolayer tissue cultures of dog kidney cells and produces a recognisable cytopathic effect. It can also be cultivated in the developing chick embryo.

Vaccines.—Several vaccines which contain living attenuated strains of the canine hepatitis virus are available. One is combined in a single prophylactic dose with an attenuated strain of the canine distemper virus. Another reinforces the antigenic stimulus of the attenuated hepatitis virus with two doses of inactivated virus.

Mouse Hepatitis

Weaned mice doubly infected with the mouse hepatitis virus and *Eperythrozoon coccoides* (a micro-organism related to *Haemobartonella*) develop a fatal hepatitis.¹ Neither agent, however, can alone cause evident illness. In the presence of *E. coccoides* the virus multiplies more abundantly, but the nature of this synergism is not understood. A proportion of normal mice carry *E. coccoides* and are thereby rendered susceptible to virus infection.

Hepatitis of Ducklings

This is an acute epizootic disease of young ducklings. It is a scheduled disease in Great Britain and is a rapidly fatal infection. Only young ducklings two to three weeks of age are affected; adult ducks, fowls and turkeys are resistant. Infected ducklings often die within a few hours of the onset of the disease; at autopsy the liver is enlarged with fatty change and shows subcapsular petechiae and larger haemorrhages. No inclusions have been described in the infected tissues. The virus can be isolated from the liver and other tissues by egg cultivation. The chick embryo is not killed by the virus, but five days after inoculation a greenish discolouration of the liver and a viscid condition of the yolk-sac is characteristic of the action of the virus. The virus will pass through a Berkefeld W filter; it does not agglutinate erythrocytes and is not related to the Newcastle virus. Ducks recovered from the disease are resistant to re-infection and their serum possesses neutralising antibodies. Active immunisation with vaccines of egg fluids or embryo livers has not proved satisfactory.

¹ Gledhill, A. W., Dick, G. W. A., & Niven, J. S. F. (1955), *J. Path. Bact.*, 69, 299.

CHAPTER XLI

ARTHROPOD-BORNE (ARBOR) VIRUSES

THE term *arbor viruses* is a convenient abbreviation which is used as the name of a large group of viruses transmitted by arthropods. Members of the group infect man and many other mammals and birds. Arthropods take in the virus when they feed on the blood of an infected animal and transmit it to a new host when they take their next meal. The virus multiplies within the body of the arthropod without damaging its tissues or producing disease. Those viruses which, like the rabbit myxomatosis virus, are merely conveyed mechanically on the soiled mouth parts or legs of the arthropods are by definition excluded from the arbor virus group.

Arbor viruses are most prevalent in those parts of the world where tropical heat and rain encourage the abundant breeding of arthropods near to susceptible animals. Many of these viruses have complex infection cycles involving several vertebrate and invertebrate hosts.

The arbor viruses have been divided into three groups on immunological grounds. At present group A contains 8 types, group B 23, and group C 5; in addition there are at least 44 ungrouped types.

Properties of the Viruses.—The viruses are within the size range 20–50 m μ in diameter.

Host Range and Cultivation.—The viruses in nature infect a wide range of animals, including monkeys, horses, sheep, cattle, deer and pigs, as well as domestic poultry and wild birds. The suckling mouse is the most susceptible of all laboratory animals; adult mice are usually resistant to infection by all routes of inoculation except the intracerebral. The viruses multiply in the chick embryo after inoculation into the yolk-sac or on to the chorio-allantoic membrane. They grow readily in tissue cultures of chicken and mouse embryo, or in pure cell lines often with marked cytopathic effects. It is characteristic of arbor viruses that they all possess haemagglutinating activity.

Viability.—In aqueous suspensions arbor viruses are unstable at room temperature; at –20° C. they survive better but there is a slow progressive loss of infectivity over several months until it is lost at the end of about nine to twelve months. In solid CO₂ at

—70° C. the viruses survive for several years provided that they have been placed in containers sealed so that the CO₂ does not gain access to them. Suspended in fluids containing serum or bovine albumen these viruses can be well preserved by freeze drying.

Formaldehyde and ether even in very low concentrations inactivate the viruses and heat at 60° C. for ten minutes or ultra-violet light for ten to thirty minutes kills them. Sodium desoxycholate in a 0·1 per cent. concentration inactivates arbor viruses but fails to affect enteroviruses. This is considered to be an excluding property in that no arbor virus has yet been shown to resist this chemical.

The effect of the pH of the suspending fluid is of the greatest importance in maintaining the infective titre of the virus. The majority of these viruses should be kept within the pH range 7–8, and for haemagglutination work pH 9 is obligatory.

Antigenic Structure.—There is a considerable degree of antigenic sharing amongst the arbor viruses, and they have been classified in three groups A, B and C according to cross-reactions in haemagglutination-inhibition and complement-fixation reactions. Antigens for serological reactions are usually obtained from the infected brain tissue of newborn mice, but occasionally infected liver or serum may be used. The details of the techniques of haemagglutination and haemagglutination-inhibition are described by Clarke and Casals.¹

Occurrence.—The great majority of arbor viruses give rise in man to a relatively severe form of encephalitis. After an incubation period which may vary from four to twenty-one days there is a sudden onset of fever with signs resembling those seen in aseptic meningitis. Marked drowsiness or stupor is characteristic and the mortality, which varies with different viruses, is between 5 and 25 per cent. In elderly people the case fatality is much higher, reaching 50–80 per cent.

The pathogenesis of these infections in man has not been well studied, but it is thought that the virus multiplies first in an extraneurial situation and that a viraemic phase follows some two to three days before the onset of the clinical illness. In fatal cases there is a severe inflammatory reaction most pronounced in the cerebral cortex but involving all parts of the central nervous system.

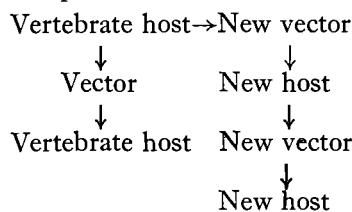
Epidemiology.—To understand the epidemiology of the arbor viruses a knowledge is necessary not only of the organisms themselves but also of the ecology of the arthropod vectors and non-

¹ Clarke, D. H., & Casals, J. (1958), *Amer. J. trop. Med. Hyg.*, 7, 561.

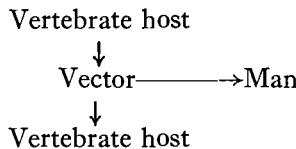
human hosts of the viruses. Human infection is usually only an unimportant incident in the perpetuation of the virus in nature. Transmission of the virus from one vertebrate to the next may depend on more than one arthropod species, and several species of animals may be infected by the vector's bite. The infection chain may simply be:

Vector→Vertebrate host→Vector→Vertebrate host

or it may be more complex:



or again:



When the tick is the vector, the virus may be carried transovarially through several life cycles before the infection is again transmitted.

For further reading on this subject the reader is referred to larger works.^{1, 2}

Laboratory Diagnosis.—The virus may be isolated from the blood in the early stages of infection; it is often difficult to recover it from the cerebrospinal fluid. The virus may also be found in infected brain tissue of fatal cases. Suckling mice should be inoculated intracerebrally and intraperitoneally and eggs by the chorio-allantoic method. Tissue cultures may also be used. When the virus is recovered from the inoculated mice or embryos it is identified in suitable complement-fixation or haemagglutination-inhibition reactions. Adult goose erythrocytes are useful in haemadsorption tests in tissue cultures.

Serological tests for antibodies can be carried out using the haemagglutination-inhibition technique but the results are usually

¹ Horsfall & Rivers (1959), *Viral and Rickettsial Infections of Man*. London, Pitman.

² Smith, C. E. J. (1959), *Brit. med. Bull.*, **15**, 235.

Arthropod-borne Viruses Pathogenic for Man

Group	Virus	Prevalent Location	Human Disease	Vector
A	Encephalomyelitis: Eastern Equine . . .	Eastern U.S.A. and Canada, Central and South America	Encephalitis	M
	Western Equine . . .	Western U.S.A. and Canada, Argentina	Encephalitis	M
	Venezuelan Equine Chikungunya . . .	S. America East and South Africa	Encephalitis Dengue-like	M M
B	Encephalitis: Japanese B . . .	Far East	Encephalitis	M
	Murray Valley . . .	Australia and New Guinea	Encephalitis	M
	St. Louis . . .	U.S.A., Trinidad	Encephalitis	M
	West Nile . . .	Egypt, India, Israel, Uganda	Dengue-like	M
	Zika . . .	Nigeria, Uganda	Systemic	M
	Yellow Fever . . .	Equatorial Africa and America	Hepatitis	M
	Dengue, Type I . . .	Hawaii, India, Far East	Fever, rash, muscle pains, lymphadenopathy	M
	Dengue, Type II . . .	India, New Guinea, Trin- idad, Uganda	Encephalitis	M
	Louping-ill . . .	Britain, Czechoslovakia, U.S.S.R.		T
	Russian spring - summer encephalitis.	Central and Eastern U.S.S.R.	Encephalitis	T
C	Omsk haemorrhagic Fever	U.S.S.R.	Haemorrhagic	T
	Kyasanur Forest Fever .	India	fever	T
	Apeu	Brazil	Systemic	M
Ungrouped	Marituba	Brazil	Systemic	M
	Oriboca	Brazil	Systemic	M
	Bwamba	Uganda	Systemic	M
	California encephalitis . . .	Western U.S.A.	Encephalitis	M
	Rift Valley Fever . . .	Kenya, Uganda, South Africa	Systemic	M
	Colorado Tick Fever . . .	Western U.S.A.	Systemic	T
	Sandfly Fever: Naples strain . . .	Southern Italy	Systemic	P
	Sicilian strain . . .	Egypt, Sicily	Systemic	P

M = Mosquito; T = Tick; P = Phlebotomus.

only group specific because of the common antigens shared by these viruses. For general diagnostic use the complement-fixation test using infected chick embryo antigens is satisfactory.¹

¹ Lenette, E. H., Wiener, A., Neff, B. J., & Haffman, M. N. (1956), *Proc. Soc. exp. Biol. (N.Y.)*, 92, 575.

Complement-fixing antibodies develop more slowly than neutralising or haemagglutination-inhibiting antibodies and thus they offer a better chance to demonstrate a significant rise of titre.

YELLOW FEVER

Yellow fever is primarily a disease of monkeys and possibly of some other forest animals. In nature the infection is transmitted from monkey to monkey by forest mosquitoes of the genus *Aedes*; man is only involved incidentally when he is bitten by an infected mosquito. The human disease may vary from an almost symptomless infection to a severe jaundice with haemorrhage and death. Yellow fever is a truly tropical disease and is endemic in large areas of equatorial South America and Africa.

Properties of the Virus.—From measurements by filtration the diameter of the virus is 17–25 m μ .

Host Range.—The yellow fever virus is pathogenic to all the species of African and South American monkeys. Most African strains of the virus are highly pathogenic and kill by causing acute necrosis of the liver. Other strains have predominant neurotropic qualities and some may cause only a mild feverish illness. Adult mice can be infected by the intracerebral route, but not by intraperitoneal inoculation. Infant mice are highly susceptible and can be infected by any route of inoculation. Guinea-pigs are susceptible after intracerebral inoculation, but different strains of the animals vary in their response to intraperitoneal inoculation. The rat and the rabbit are completely insusceptible. The virus can be readily cultivated in the chick embryo and in tissue cultures of chick or mouse embryos. Serial transfer of the virus in mice and other hosts readily modifies its pathogenicity for monkeys.

There is conclusive evidence that yellow fever multiplies in the mosquito. *Aedes aegypti* becomes infected when it bites a host during the first three days of the disease and then after an interval of twelve days it is able to transmit the infection. The insect remains infective for life, but the virus is not transmitted through the egg to the next generation. Mosquitoes are the only blood-sucking arthropods known to play a part in the spread of yellow fever.

Viability.—The virus is extremely labile and is readily inactivated by heat and antiseptics. It is also rapidly inactivated if it is diluted in normal saline and to prevent this 10 per cent. normal serum or 0·75 per cent. bovine albumen should be added to the diluent. It survives in 50 per cent. glycerol saline at 0° C. for three months but is best preserved by freeze drying.

Antigenic Characters.—After infection with the yellow fever virus, neutralising, complement-fixing and haemagglutination-inhibiting antibodies appear. The first two of these antibodies are not identical and neutralisation appears some days before complement-fixation. In mild infections and after vaccination complement-fixing antibodies may be absent although the serum has definite neutralising qualities. The antigen used in the complement-fixation test is prepared from infected mouse brain or monkey liver and there is some evidence to suggest that it is not the virus itself which reacts but some product derived from the infected tissues. From cross-reactions in complement-fixation and haemagglutination-inhibition tests the virus has been placed in group B of the arbor viruses. It is related immunologically to dengue, Uganda S, and Zika viruses (see table, p. 842).

Occurrence.—After the virus has been injected through the skin by the mosquito it spreads to the local lymph glands, where it multiplies. After an incubation period of three to four days it invades the blood stream and is carried to the liver, spleen, bone marrow and kidneys, where it sets up necrotic processes. In fatal cases the pathological changes seen at autopsy are those of jaundice with haemorrhage into the gastric mucosa near the pylorus, and fatty change of the liver and kidneys. Histologically there is a characteristic coagulative hyaline necrosis (the "Councilman lesion") scattered diffusely throughout the liver. The necrotic changes are often most marked in the mid-zone of the lobules and are preceded or accompanied by cloudy swelling and fatty degeneration.

Yellow fever virus infection is sometimes accompanied by the formation of acidophilic intranuclear inclusion bodies (Torres bodies); they do not contain nucleic acid and probably do not consist of virus particles. These inclusions partially surround the nucleolus, are variable in size, granular in appearance and irregular in outline. Torres bodies are frequently found in the liver of the infected monkey but are uncommon in human tissues.

Epidemiology.—At one time yellow fever was prevalent in urban communities and the virus was transmitted from person to person by *Aedes aegypti*. This mosquito is essentially of a domestic type and breeds close to human dwellings; antimosquito measures have now eliminated the insect in most towns and urban yellow fever has been virtually eradicated.

Yellow fever, however, still occurs in people who live in remote rural parts in the tropics. It is referred to as "Jungle" or "Sylvatic" yellow fever and is perpetuated amongst the monkey population by different species of mosquitoes.

Tree-dwelling mosquitoes of the genus *Haemagogus* are responsible for the persistence of the disease in Central and South America and *Aedes africanus* and *Aedes simpsoni* in equatorial Africa.

Laboratory Diagnosis.—The virus may be recovered from the blood during the first five days of the illness. The serum is injected intracerebrally in mice (preferably infant mice) and when the signs of encephalitis appear the animals are killed and an emulsion from their brains is prepared. Identification of the virus is carried out by neutralisation tests with a specific antiserum.

Serologically the diagnosis can be confirmed with neutralisation tests in adult mice on serum taken early in the disease and again during convalescence. A standard dose of a mouse-adapted strain of yellow fever virus is required in these tests. Neutralising antibodies develop early in the infection and are present on the fifth day. The antibody response in yellow fever is of two types, depending on whether the condition is primary or whether it occurs in an individual who has previously been infected with another agent belonging to group B of the arbor viruses. In primary infections the antibodies produced are specific and enable the diagnosis to be made with certainty. In superimposed infections antibodies with wide cross-reactions with other arbor virus appear and a specific diagnosis is virtually impossible. Complement-fixing antibodies appear later than the neutralising antibodies and persist for only a few months; in mild infections and after vaccination with the 17D egg-adapted virus they are not developed. The complement-fixation test, however, provides satisfactory evidence that infection is due to yellow fever virus infection, especially when used on convalescent sera from known cases of primary infection.¹ In fatal cases the diagnosis of yellow fever may be made from the characteristic histological lesions seen in the liver (*vide supra*).

Prophylaxis.—Vigorous mosquito control measures have virtually eliminated urban yellow fever. For protection against jungle yellow fever or for prevention of spread from endemic areas prophylactic vaccination is effective. The vaccine in general use contains a living attenuated strain of the virus (17D) and affords excellent protection; it is prepared from chick embryos inoculated with 17D and incubated at 37° C. for four days. The infected embryos are ground-up and freeze-dried in ampoules under dry nitrogen gas. The vaccine should be stored in the coldest part of

¹ Theiler, M. (1959), in *Virus and Rickettsial Infections of Man*, p. 351. London, Pitman.

a refrigerator (the ice chamber) and is reconstituted immediately before use with ice-cold physiological saline. Once made up, the virus must be used within thirty minutes and an ice-cold syringe should be used for the injections. As the virus is alive only one inoculation is required; the minimum dose for immunisation has been set at 500 lethal mouse doses and this is contained in a volume of 0.5 ml. It is most unusual for any local reaction to follow yellow fever immunisation; but general reactions with symptoms of encephalitis have occasionally been reported in infants, all of whom have recovered without sequelae. It is usually advised that children under the age of one year should not be vaccinated unless there is a great risk of yellow fever. Immunity is effective by the ninth day after inoculation. Full protection is maintained for at least six years and in 70 per cent. of persons for nine years.

It is recommended that the vaccine should be given at least 4 days before smallpox vaccination or not until three weeks after a successful vaccination, for fear of encephalitis. In West Africa, however, French workers have combined dried yellow fever vaccine (mouse neurotropic strain) with dried vaccinia virus in a suspending solution of gum arabic and have applied it to the scarified skin. Satisfactory levels of immunity against both diseases are achieved simultaneously, but the incidence of reactions was about 15 per cent.

DENGUE FEVER

Dengue is a mosquito-borne virus disease widespread in the tropics. The disease is benign but extremely prostrating with high fever, intense headache and very severe pain in the muscles, joints and behind the eyes. The fever may continue or may have a remission after the third day with a secondary rise three or four days later giving rise to the characteristic "saddleback" temperature chart. A maculo-papular or scarlatini-form rash appears on the third or fourth day and persists for about three days.

Properties of the Virus.—The dengue viruses have the general properties of the family of arbor viruses. They are members of group B and are immunologically related to the yellow fever virus and to the Uganda S and Zika viruses. They do not infect the guinea-pig, rabbit, or cotton rat, and only cause inapparent infections in monkeys. Some strains have been adapted to adult or suckling mice and produce a flaccid paralysis in the animals. Other strains have been cultivated in the chick embryo and monkey kidney tissue culture. There are at least two distinct immuno-

logical types and recently two more related strains have been isolated from a haemorrhagic feverish condition seen in children in Thailand.

Occurrence.—Dengue follows the introduction of the virus by the bite of the mosquito *Aedes aegypti*. At the site of the bite, a red papule 1–4 cm. in diameter is always present; the lesion is characteristic and is the site of local viral multiplication. Within twenty-four hours of the onset of the first fever the virus is in the blood stream.

Dengue occurs in the eastern Mediterranean, Africa, India, the Far East and on many islands in the Pacific Ocean. The infection is transmitted by certain species of *Aedes* mosquitoes and is perpetuated in a cycle of infection which includes monkeys or man. The mosquito becomes infective some seven to ten days after biting a dengue patient and remains so for the rest of its life. The virus is not, however, transmitted transovarially to the progeny of the insect. When cold weather renders the mosquito inactive, outbreaks of dengue cease, and it is only in those parts of the world where warm humid conditions persist throughout the year that dengue is constantly maintained.

Laboratory Diagnosis.—The virus may be isolated by the intracerebral inoculation of suckling mice with blood taken during the first three days of the illness. When the mice die the virus is identified by neutralisation tests with type-specific antisera. If the mice survive, the presence or absence of the virus can be determined by challenging the animals when they are four to six weeks old with an intracerebral inoculation of 100 LD₅₀ of a mouse-adapted strain. If the mice are unaffected by the challenge they are presumed to have acquired a specific resistance from dengue virus in the original inoculum.

Neutralising antibodies appear within seven days of the onset of fever and complement-fixing antibodies about two weeks later. The haemagglutination-inhibition test is, however, the most useful because when the convalescent phase serum obtained within two days of defervescence is compared with the acute phase serum, a clear-cut rise of antibody titre can usually be demonstrated. Antigens of both type 1 and type 2 dengue viruses must be employed in these tests. Great care is required in the interpretation of serological findings because of the possibility of heterotypic responses following infection by other group B viruses. ■

Prophylaxis.—Control depends upon the eradication of the mosquito vector. The yellow fever (17D) vaccine gives no protection against dengue. There is no dengue virus vaccine available for use at present.

SANDFLY FEVER

Sandfly fever is also known as phlebotomus fever because the virus which causes it is transmitted by the sandfly *Phlebotomus papatasii*. The disease has an abrupt onset with high fever, severe headache, muscular aching, and pain behind the eyes. Photophobia, stiffness of the back and neck, and leucopenia are characteristic signs. There is no encephalitis and the cerebrospinal fluid is normal. Recovery is complete within a few days and fatality is almost unknown. The disease occurs commonly in countries bordering the Mediterranean and is seen also in Kenya, Egypt, Russia, China and India.

The sandfly fever virus is introduced by the female phlebotomus and an itching papule appears at the site of the bite. After an incubation period of three or four days the virus is liberated into the blood stream, where it may be found a day before and a day after the onset of the clinical illness.

The Properties of the Virus.—The diameter of the elementary bodies is 17–25 m μ . It can be propagated in the brains of suckling mice by intracerebral inoculation. Laboratory animals including monkeys and the chick embryo are not susceptible to the infection. Two distinct antigenic types, the Naples and the Sicilian strains, have been distinguished by Sabin and are not related to groups A, B or C of the arbor viruses.

The diagnosis of sandfly fever is usually made on clinical and epidemiological grounds. Specific proof of infection with the sandfly fever virus can be obtained by isolation of the strains in suckling mice inoculated with blood taken on the first day of the fever. Further confirmation may be obtained by the demonstration of rising antibody titres in neutralisation, complement-fixation and haemagglutination-inhibition tests.

Phlebotomus papatasii is only about 2 mm. long and is able to pass through the mesh of ordinary screens and mosquito nets. Control measures are directed against the vector and comprise the regular and systematic application of D.D.T. to the breeding grounds of the phlebotomus. Insect repellents such as dimethyl phthalate used at night are useful in preventing the disease.

RIFT VALLEY FEVER

This disease, which is also known as enzootic hepatitis, was first described by Daubney and Hudson in 1931 in Kenya, where there was an extremely severe epizootic amongst lambs. Most of the

lambs died, but many of the ewes recovered. Native shepherds and European investigators of the epizootic developed a dengue-like fever with severe back pains.

The Rift Valley fever virus is about $30\text{ m}\mu$ in diameter. It can be grown in fertile eggs and in tissue cultures of the suspended cell type; sarcoma cells of rat and mouse origin and fibroblasts from human and murine sources are susceptible. The infection can be transmitted by blood, liver or spleen tissue from infected sheep to cattle, mice, field voles, dormice and squirrels. Rabbits, guinea-pigs, birds and reptiles are not susceptible. The virus has the property of haemagglutination and has been classified as an arbor virus which is distinct from those in groups A, B and C.

The disease in lambs is characterised by marked necrosis of the liver with haemorrhages into other organs. Inclusion bodies are numerous in the liver. In man the disease is mild and resembles dengue with a typical "saddleback" temperature chart.

Rift Valley fever is transmitted by the bite of mosquitoes; three species of *Aedes* and several species of *Eretmapodites* transmit the virus to sheep and bovine, murine and human hosts.

The diagnosis may be made by the inoculation of suckling mice with blood taken from a case during the first twenty-four hours of infection. When the infected animals die, the virus is identified by the characteristic lesions it produces in the liver and by neutralisation with specific antisera. In convalescence the diagnosis can be made by demonstrating the development of neutralising, complement-fixing, and haemagglutination-inhibiting antibodies. The results of these tests are remarkably specific and there is very little tendency to overlap with other arbor viruses. Antibodies to the virus appear as early as four days after the onset of the clinical illness and they persist for as long as twelve years after recovery.

LOUPING-ILL

This is primarily a disease of sheep characterised by an encephalomyelitis giving rise to cerebellar ataxia and disorder of nervous functions. It is common in Scotland and in the North of England and owes its name to the peculiar atactic leaping movements of the animal ("louping"). The disease is spread through the bite of the tick *Ixodes ricinus*.

The virus is about $20\text{ m}\mu$ in diameter and is related antigenically to the virus of Russian spring-summer encephalitis; it is placed in group B of the arbor viruses. It can be propagated in mice and the developing chick embryo. Cattle, pigs and monkeys can also be infected.

Louping-ill has been described in laboratory workers, shepherds and agricultural workers in Northern England. The disease takes the form of a mild encephalitis, but subclinical forms may also occur. The diagnosis may be confirmed by isolating the virus from the blood by the intracerebral inoculation of mice or by neutralisation or complement-fixation tests on the patient's serum.

Other Tick-borne Virus Infections

The louping-ill virus is not an important cause of human disease, but the closely related virus of Russian spring-summer encephalitis is widespread in Central Europe and the U.S.S.R., where it gives rise to meningo-encephalitis of varying severity sometimes accompanied by flaccid paralysis. Russian spring-summer disease is endemic in goats and probably other animals; it is transmitted from animal to animal by ticks in infected pastures. Two other tick-borne viruses are placed in group B of the arbor viruses and are the cause of Kyasanur Forest fever in northern India and of Omsk fever in the U.S.S.R. The result of infection in man with these viruses is an illness in which haemorrhages dominate the clinical picture. Fever and mild involvement of the central nervous system are followed by epistaxis and severe gastrointestinal and uterine bleeding. A generalised lymphadenopathy is also common. This group of infections is known as the Russian tick-borne complex and much remains to be learned of their pathogenesis and epidemiology.

CHAPTER XLII

MISCELLANEOUS VIRUS INFECTIONS OF MAN AND ANIMALS

MEASLES

MEASLES is probably the most infectious of all the common fevers. The clinical diagnosis of the disease is usually not a difficult matter because a characteristic macular rash develops after a prodromal period of fever with catarrhal symptoms, conjunctivitis and the appearance of Kopliks spots on the buccal mucosa. The incubation period is ten to fourteen days and the rash often appears fourteen days after exposure.

Properties of the Virus.—Filtration experiments suggest that the diameter of the elementary body is 140 m μ .

Cultivation.—The virus can be isolated from the blood or throat washings of a patient during the first twenty-four hours after the onset of fever. For this purpose primary cultures of human amnion or chorion cells or a continuous line of amnion cells provide the most suitable susceptible tissue culture cells. Monkey kidney cells are less satisfactory.

Once established in tissue cultures, the measles virus will grow in a wide range of primate tissue cultures. Human kidney obtained at operation or at autopsy and human embryonic lung or kidney all provide highly susceptible cells. The virus also grows in continuous lines of cells derived from human normal tissues such as heart, kidney, amnion and bone marrow, as well as in lines of carcinoma cells such as HeLa, K.B., and Hep 2. Monolayer cultures of dog kidney are also susceptible. In monolayers of monkey kidney cells and of some strains of HeLa cells small plaques are produced.

The cytopathic effect of the measles virus is characteristic, with the formation of large multinucleate giant cells and syncytial masses in which many vacuoles give a lacework appearance. After continued passage of the virus in human amnion cells the nature of the cytopathic effect alters, and in addition to giant cells, increasing numbers of refractile stellate cells appear. Variation of the constituents of the culture medium may modify the cytopathic lesions. With glutamine deficiency, for example, more giant cells are formed, but when the glutamine is restored the number of

giant cells is diminished and the appearance of the cytopathic effect is delayed, while the virus yield is increased. The most constant feature of cells infected with measles virus is the late appearance of type A eosinophilic intranuclear inclusions (see p. 752).

Measles can be reproduced in rhesus monkeys by the parenteral inoculation of blood or catarrhal secretions from patients or infected tissue culture fluids. The disease is usually mild and about one-third of the inoculated animals develop fever, conjunctivitis and a macular rash. No mammals other than monkeys have been infected successfully with the measles virus. Some strains of the virus have been adapted to the amniotic cavity or chorioallantoic membrane of the chick embryo after their primary isolation in tissue culture.

Viability.—The virus survives at room temperature for up to thirty-six hours at 4° C. for several weeks, and for many months in the frozen state in the temperature range -15° to -72° C. Freeze drying preserves the virus well, though with some loss of infectivity. Formaldehyde in a concentration of 0.025 per cent. at 37° C. for four days brings about complete loss of infectivity without alteration of the complement-fixing activity. The virus withstands 10 per cent. ether at room temperature for forty minutes and pH 6 at -70° C. for sixty hours. At pH values below 6 the virus is inactivated.

Antigenic Characters.—There would appear to be one stable antigenic type, but information on this point is as yet incomplete. A complement-fixing antigen is present in infected tissue culture fluids; it can be separated from elementary bodies by centrifugation and has a particle size of 11 m μ .

The measles virus is related antigenically to the virus of canine distemper. It is neutralised specifically by the serum of ferrets recovered from distemper, but not by normal ferret serum. Conversely, ferrets immunised by the measles virus are partially protected when challenged with distemper virus.

Occurrence.—Young children in the prodromal phase, when the catarrhal symptoms are prominent, are the main source of the measles virus. They discharge infected particles which are inhaled by the new victim and the virus reaches the respiratory tract, where it grows silently for some days in lymphoid tissue. When multiplication has continued to the point when many infected cells break open, the virus floods into the circulation and causes the prodromal illness. During the following two or three days the virus is localised in the skin and there produces the rash. This viraemia is quenched when antibodies appear in the blood.

It is of interest that multinucleated giant cells similar to those occurring in tissue cultures can be found in the organs of persons who have died during an attack of measles. It is probable that the giant cells begin to be formed about seven days before the appearance of the rash.

Measles is most infectious in the two or three days before the appearance of the rash; thereafter infectivity rapidly wanes and after a few days is lost.

Epidemiology.—Measles is endemic throughout most countries of the world. The disease has a characteristic tendency to epidemicity every second year so that in Great Britain and North America there are "measles years" which alternate with years in which only a few cases are encountered.

The greatest incidence in measles is in the age group 1–5 years, and by the age of 20 years 90 per cent. of persons have had an attack of the disease. After the first six months of life passively acquired maternal immunity disappears and susceptibility is universal. Only about 1 per cent. of these susceptibles fail to contract measles on their first close contact with it. Although measles is usually benign in young children it may cause severe or even fatal illnesses in infants under the age of one year or in elderly people. Secondary invasion with such pathogenic bacteria as haemolytic streptococci, staphylococci and *H. influenzae* cause the complications of bronchopneumonia or otitis media. Another serious complication of measles is encephalomyelitis developing two to six days after the rash; the features of this condition may resemble those of aseptic meningitis (see p. 830), but there is an average case fatality of 15 per cent. Encephalitis occurs in about 1 in 700 cases of measles, is commoner in girls than boys, and is more frequent in the 5–9 years age-group than in young children.

When measles is introduced into an area where previously it was not endemic, a sweeping epidemic of great severity follows amongst a virgin population of highly susceptible persons. In these circumstances there is a high incidence of complications and the mortality is increased. Epidemics of this type have occurred in isolated communities, especially on islands; one particularly severe outbreak occurred in Greenland in 1951. When the infection was first introduced into the Fiji Islands in 1875, it carried off 20–25 per cent. of the entire population.

Prophylaxis.—Measles is so infectious and susceptibility to it so high that there is no point in isolating cases to stop the spread of the infection in the population. Hospital patients with measles, however, should be isolated to protect them from the risk of cross-

infection with pathogenic bacteria, and to stop the spread of infection to patients ill with other diseases.

Passive immunity can be conferred on contacts by the subcutaneous inoculation of convalescent or adult serum, or gamma globulin prepared from them. The protective effect is complete if an adequate dose is given within five days of exposure; after this time the disease may not be prevented, but its severity is usually modified and the risk of complications reduced. The use of gamma globulin prepared from pooled normal adult sera is effective; the dose is 15 mg. per pound body weight.

Active immunisation with live or inactivated vaccines is not yet available.

CANINE DISTEMPER

Distemper is a highly infectious disease which causes more deaths and permanent disability in young dogs than any other infection. After an incubation period of four days the common manifestations are fever, coryza and mucopurulent discharges from the eyes and nose. Bronchopneumonia is a frequent complication and inflammation of the gastric and intestinal mucosa is not uncommon. In their pathogenesis there are many resemblances between canine distemper and measles. Other animals such as ferrets, silver foxes and mink together with wolves and some small rodents are also susceptible to distemper.

Distemper is caused by a virus 20–30 m μ in diameter. The virus can be cultivated in the chorio-allantoic membrane of the developing chick embryo where it produces greyish white lesions. It produces cytopathic changes in monolayer tissue cultures of dog and ferret kidney cells with giant cell formation.

Cross-neutralisation tests with convalescent sera from cases of distemper and measles suggest that the two viruses may share major antigens. Distemper convalescent serum, however, fails to fix complement with a measles virus antigen and it is likely that differences as well as similarities will be revealed when the antigenic structure of these viruses has been fully investigated. There is also evidence which suggests that an immunological relationship between the distemper and rinderpest viruses may exist.

The virus is present in the blood and discharges of infected animals during the acute phase and has also been recovered from the urine and faeces. The disease can be transmitted with filtrates of these materials to dogs and ferrets by subcutaneous inoculation. In the ferret the incubation period is nine to eleven days. Intracytoplasmic eosinophilic inclusions are characteristically present;

they are found principally in the epithelial cells of the respiratory tract and the urinary tract, particularly in those of the bladder. They can, however, be found in many other situations. Intracellular inclusions may also be present.

Active immunisation of dogs can be carried out with a vaccine containing a living attenuated strain of the distemper virus cultivated in eggs. This vaccine has to a large extent replaced other methods of immunisation in which living virus was combined with a protective dose of hyper-immune serum or with the previous use of a killed vaccine.

Hard pad disease in its initial stages is indistinguishable from distemper. There is fever, conjunctivitis and diarrhoea, followed by a painful hyperkeratosis of the pads of the feet and the nose. Later, nervous symptoms appear, followed by convulsions, and death occurs four to six weeks after the onset. The condition is due to a virus which is probably related to the distemper virus, being infective to ferrets after an incubation period of twenty-three days (cf. distemper).

RUBELLA

Rubella (German measles) is a mild infective disease characterised by a macular rash and enlargement of the cervical lymph glands.

The virus has been found in the blood and nasopharyngeal secretions in the early phases of the illness. It is able to pass through Seitz and Berkefeld W filters, but few other data about it are available. Monkeys are the only susceptible laboratory animals and suffer a mild slightly feverish illness after inoculation. Claims to have cultivated the virus in eggs or tissue cultures have not yet been confirmed.

No serological tests are available for diagnostic purposes and isolation of the virus is not a practical procedure.

Rubella is a mild disease with a low mortality; it is probably transferred by the inhalation of infected particles. Epidemics of rubella are less frequent than those of measles and as a consequence rubella occurs more commonly in young adults. Rubella, nevertheless, is of great importance, since congenital abnormalities affecting the ear, eye, heart and brain may result in children born to mothers who have suffered from rubella in the first three months of pregnancy. There is no agreement on the assessment of the chances of an abnormal child being born to a woman who contracts rubella in pregnancy. Estimates have ranged between 25 and 70 per cent, of the babies having one or more congenital abnormalities,

Prophylaxis.—In women, in the first trimester of pregnancy, gamma globulin in a dose of 5 mg. per kilo body weight may be used within five days of exposure to infection. It is advocated by many that young girls should be exposed deliberately to rubella before they reach the child-bearing age.

HERPES VIRUS

The most common form of this infection is a vesicular eruption at the mucocutaneous borders around the lips and nose. The lesions somewhat resemble those of herpes zoster both clinically and histologically, and in the past there has been some confusion between the terms *Herpes simplex* and *Herpes zoster*. For clarity the prefix *Herpes* is now retained only in *herpes simplex* and is no longer used in conjunction with *Zoster*, which is a different disease caused by an unrelated virus (see p. 784).

Properties of the Virus.—The *herpes simplex* virus is spherical with a diameter of 100–150 m μ .

Cultivation and Host Range.—A wide range of laboratory animals are susceptible; they include rabbits, guinea-pigs, rats, mice and hamsters. The reactions produced depend upon the route of inoculation. When introduced into the scarified cornea of the rabbit, keratoconjunctivitis results between twelve hours and seven days after inoculation. The conjunctiva and nictitating membrane become intensely inflamed and a mucopurulent discharge develops. After intracerebral inoculation there is encephalitis with fever, convulsions and muscle weakness. Death is the usual termination. A similar encephalitis follows the intra-corneal inoculation of certain neurotropic strains. Suckling mice 1–3 days old are particularly susceptible to intraperitoneal and intracerebral inoculation with the virus. Many strains produce an inflammatory reaction when injected into the skin of the pads of the guinea-pig or intracutaneously in the rabbit. The fertile hen's egg is susceptible to infection, and inoculation of the virus on to the chorio-allantoic membrane of 11–13-day-old chick embryos gives rise to characteristic pocks. These lesions appear twenty-four hours after inoculation as small white heaped-up plaques 1–2 mm. in diameter. Inoculation of the virus into the yolk-sac or amnion kills the embryo after an incubation period of forty-eight to seventy-two hours. Human amnion, rabbit kidney and HeLa cells in tissue culture are readily infected with the *herpes* virus and a typical cytopathic effect is seen often within twenty hours of inoculation. Cytoplasmic granulation appears and the cells become rounded and balloonized; the formation of multi-

nucleate giant cells is characteristic. The cells of choice for tissue culture studies of the herpes virus are trypsinised kidney cells obtained from 6-week-old rabbits.

The reproductive cycle of the herpes virus has been studied in HeLa cells. The particles are slowly adsorbed to the surface of the host cells over a period of two or three hours and there follows an eclipse phase lasting at least nine hours. By the twelfth hour it is possible to detect with the electron microscope virus particles 30–40 m μ in diameter in the nucleus. These particles gradually enlarge to a diameter of 70–100 m μ and acquire a single covering membrane, whilst progressive changes in the nucleus incorporate them in an eosinophilic inclusion body which fills the whole nucleus. By fifteen hours the particles begin to leave the nucleus and as they pass through the nuclear membrane they seem to acquire a second covering membrane. They then pass on through the cytoplasm and are liberated through breaches in the cell wall as the fully mature elementary bodies, 120–130 m μ in diameter and covered by a double membrane. The first infective virus is liberated about fifteen hours after infection and by twenty-six hours many mature particles can be seen on the cell surface. The release of the virus is by a slow leak rather than by rupture of the cell.

Viability.—In infected egg membranes the virus is somewhat unstable; activity is lost after twenty hours at 30° C. and after five hours at 37° C. If egg-yolk or 10 per cent. normal rabbit serum is included in the suspending fluid, the virus may, however, be preserved in the frozen state for many months. In whole animal tissues in 50 per cent. glycerol saline at 4° C. the virus remains virulent for years. Herpes virus is killed by moist heat at 52° C. in thirty minutes, but it may survive dry heat at 90° C. for the same period. It is inactivated by ether, 0·5 per cent. formaldehyde and 1 per cent. phenol, but is more resistant than non-sporing bacteria to 1·0 per cent. gentian violet.

Antigenic Characters.—Antibodies in human sera can be demonstrated by neutralising and complement-fixing methods. The antigen associated with complement fixation can be obtained in a soluble form by high speed centrifugation of extracts of herpes infected tissues.

Occurrence.—Infections with the herpes virus are extremely common and very variable in their clinical manifestations. There are two forms of herpetic disease; (1) primary infections of susceptible persons who have no circulating antibodies and (2) recurrent localised disease in patients who are partially immune. Primary infections are common early in life and probably take place soon after the child has lost its maternal antibodies; in about

90 per cent. of cases the disease is subclinical but in the remainder aphthous stomatitis or a systemic feverish illness occurs. Serological surveys have shown that many more adults have circulating antibodies than can be accounted for by a history of clinical infection.

The commonest recurrent infection is *Herpes simplex* (*Herpes labialis* or *Herpes febrilis*). Reddish papules appear at mucocutaneous junctions and quickly vesiculate. They are especially common around the lips and nostrils. When fully developed thin-walled vesicles are closely grouped on an erythematous base; later the lesions become pustular, crust over and some finally heal without a scar. After healing the infection commonly remains latent for long periods and the lesions recur often at precisely the same site, and at frequent intervals. They seem to be provoked by such non-specific stimuli as cold, exposure to sunlight, menstruation, a variety of common bacterial and viral infections (e.g. lobar pneumonia) and artificial fever.

Eczema herpeticum (*Kaposi's varicelliform eruption*) is a primary manifestation of infection with the herpes virus in a patient already suffering from eczema. The condition is characterised by vesicles widespread over the eczematous skin and is very severe, especially in children under the age of 1 year.

Aphthous stomatitis, or acute herpetic gingivo-stomatitis, is the commonest type of primary infection with the herpes virus. The condition is usually seen in children 1-3 years old and is characterised by fever with red swollen gums and a vesicular eruption on the oral mucous membranes. Submaxillary and cervical lymphadenopathy is very common.

Herpetic meningo-encephalitis.—Herpetic involvement of the central nervous system may give rise to a clinical syndrome resembling aseptic meningitis (see p. 830) or may cause a more severe encephalitis with coma and cranial nerve palsies.

Herpetic keratoconjunctivitis may involve the superficial structures of the eye in acute inflammatory reactions and may also cause ulcers of the conjunctiva and small dendritic ulcers of the cornea. Keratitis and iridocyclitis may complicate the infection. Herpetic infections of the eye are a serious hazard to sight and it must be remembered that steroids, used locally or generally, facilitate the spread of the virus in the tissues, causing an increased severity and a higher incidence of the deeper forms of the disease.

Genital herpes occurs in both sexes with lesions on the genitalia resembling *Herpes simplex*. In the male the infection may occasionally take the form of a "non-specific urethritis".

Epidemiology.—The herpes simplex virus is probably more

widely distributed in man than any other virus. Over 60 per cent. of people are infected with the virus and remain carriers for life. In such persons antibodies are constantly present in the blood, but the virus remains in their tissues in a state of latency. Primary infection occurs most commonly in infants between the ages of 1 and 5 years. The virus is transmitted by direct contact in kissing or by saliva contaminating drinking vessels and utensils.

Laboratory Diagnosis.—The virus may be isolated from the vesicle fluid from lesions on the skin or mucous membranes and may also be found in the throat, saliva and faeces. Material should be inoculated into suitable host systems of which the chorio-allantoic membrane of 12-day-old chick embryo, 2-day-old suckling mice and rabbit kidney tissue cultures are recommended (*vide supra*). The lesions developed in these hosts should be examined histologically for intranuclear inclusions and the virus identified by neutralisation tests with specific antiserum.

Antibodies in the patient's serum may be measured in complement-fixation reactions and in neutralisation tests. The presence of antibody in the serum of an adult, however, is of doubtful significance because 60–90 per cent. of people have antibodies continually present in their serum.

No specific preventive or therapeutic measures are available for these conditions.

Virus B

Virus B has achieved in monkeys the same state of successful parasitism that obtains in man with the herpes virus. Two per cent. of rhesus monkeys carry the virus in a latent form, but the incidence may rise to 75 per cent. when the animals have been housed together for periods of six weeks or more. Virus B is thus a natural parasite of monkeys; it has properties closely similar to the herpes virus to which it is antigenically related.

Human infections with this virus are almost invariably fatal but are fortunately infrequent. The virus is introduced through the bite of an infected monkey; vesicles develop at the site of the bite after an interval of about three days and on about the seventh day the virus reaches the central nervous system by way of the peripheral nerves. An ascending paralysis follows with involvement of the respiratory centres and death.

Infection with this virus present a hazard to laboratory workers who handle monkeys or who use their tissues in cultural work. Bites and wounds require prompt disinfection and it has been suggested that a large dose of gamma globulin may be of value by virtue of the herpetic antibodies that it should contain.

LYMPHOCYTIC CHORIO-MENINGITIS

Benign lymphocytic chorio-meningitis is primarily an enzootic disease of mice. The infection is transmissible to man, in whom the commonest manifestation is that of the syndrome of aseptic meningitis.

The virus is 40–60 m μ in diameter, and is pathogenic for mice, guinea-pigs and many other small rodents. Rabbits are not susceptible. When inoculated intracerebrally in mice a severe encephalitis results in which the animals exhibit marked spasms and convulsions. In guinea-pigs the disease takes the form of a generalised systemic infection. The virus can be cultivated in the chorio-allantoic membrane of 11–12-day-old chick embryos, but no specific lesions are produced and the embryos do not die. Tissue cultures of suspended minced fragments of chick or mouse embryos also provide susceptible host cells.

The virus is well preserved by freeze drying or storage at –70° C. In infected whole tissue stored in 50 per cent. glycerol at 4° C. the virus survives for several years. The virus is inactivated by exposure to 10 per cent. ether overnight, and by 0.5 per cent. formalin. Antibodies in the serum may be measured by complement-fixation and neutralisation tests.

In man the commonest clinical picture is that of aseptic meningitis, but other clinical forms that have been reported include severe influenzal illness, and primary atypical pneumonia.

The lymphocytic chorio-meningitis virus is essentially an inhabitant of mice and in these animals exists in a latent form which is transmitted by the mother to her young *in utero*. The virus is excreted by the mice in urine, faeces and nasal secretions. Most human infections are contracted from material contaminated by mice, and the most likely route of infection is thought to be through the respiratory mucosa.

Laboratory Diagnosis.—The cerebrospinal fluid shows a marked cellular response; 200–1000 cells per c.mm. are present and 90 per cent. of them are lymphocytes. The protein is raised to 60–100 mg. per ml., but normal values are found for the chloride and sugar estimations; these findings help to distinguish lymphocytic chorio-meningitis from tuberculous meningitis. The virus may be isolated from the cerebrospinal fluid by intracerebral inoculation of mice. Virus identification is carried out by neutralisation tests or complement-fixation reactions with specific antisera.

Serological tests may give a retrospective diagnosis if virus

isolation tests have not been carried out. Complement-fixing antibodies appear by the fourteenth day, but are slow to reach a peak. Three samples of serum are usually required to establish the existence of a rising titre of antibodies; the first should be taken as soon as possible after the onset of the illness, the second fourteen days later and the third after an interval of four to five weeks.

There are no specific measures to control infection with this virus. Extermination of mice will usually eradicate the disease. Those whose work as laboratory assistants or rodent exterminators obliges them to come into contact with mice must take precautions to avoid inhaling infected material.

SALIVARY GLAND VIRUS DISEASE

This condition is also known as Generalised Cytomegalic Inclusion Disease from the inclusions which are characteristic of the infection. The disease affects man and a number of animals, causing great enlargement of epithelial cells in the liver, bile ducts, renal tubules and the bronchial epithelium as well as in other organs. The nuclei of cells contain large amphophilic inclusions and smaller multiple inclusions are found in the cytoplasm.

The Properties of the Virus.—Virus particles of two sizes have been described in ultra-thin sections of infected fibroblasts examined in the electron-microscope. The smaller particles at 80–100 m μ are seen at an early stage of infection in the nucleus and larger particles 150–500 m μ in diameter are found in the cytoplasm.

Host Range and Cultivation.—The disease occurs naturally in man and there are more than a hundred recorded fatal cases in children mostly under the age of 2 years. Monkeys, guinea-pigs and mice are also affected. The virus can be cultivated in roller-tube tissue cultures of fibroblasts from explanted fragments of adult human uterus or prepuce. Strains of the virus are species specific, even in tissue culture, although the similarity of the cellular changes they produce suggests that they are biologically closely related.

Viability.—At 4° C. the virus loses its infectivity within a week but can be preserved in 50 per cent. glycerol for long periods at this temperature. It is destroyed by heat at 56° C. in thirty minutes, and is inactivated by exposure to 20 per cent. ether for two hours, and by storage at pH values below 5.

Occurrence.—In infants the disease takes the form of a general-

ised blood dyscrasia associated with enlargement and damage of the liver. Cerebral calcification and chorio-retinitis have also been described and at this age the disease is usually fatal. In older children, diarrhoea, severe hepatic and renal destruction, with cerebral lesions, are commonly observed. The disease seems to be rare in grown persons; but subclinical infections may occur, because the virus has been recovered from the urine and adenoid tissue of healthy persons and a high incidence of complement-fixing antibodies has been reported in adults.¹

Laboratory Diagnosis.—The typical inclusions can be detected in cells in urinary sediments or in biopsy tissues. The virus can be isolated from throat swabs, urine or tissue fragments by the inoculation of tissue cultures of human explanted tissues. A complement-fixing test using as an antigen infected tissue culture fluids is valuable for diagnostic purposes and for serological surveys. Neutralisation tests are used to identify the virus after isolation and to detect antibodies.

RABIES

This is a disease of all warm-blooded animals and especially of dogs. The virus is excreted in the saliva of the animals and is transmitted by biting.

Properties of the Virus.—The diameter of the virus particle measured by filtration is 100–150 m μ .

Host Range and Cultivation.—There is a wide range of susceptible animals, including man and almost all mammals; the outcome of infection is nearly always fatal, with the exception that vampire bats are known to be able to transmit the virus for several months without showing any sign of the disease themselves. The virus invades the blood and the central nervous system of the host and is excreted in the saliva, milk and urine. These materials can be used to transmit the infection to laboratory animals, including rabbits, mice and guinea-pigs. Freshly isolated strains of the virus are known as "street virus" and will kill laboratory animals with a severe encephalitis after an incubation period which varies from one to twelve weeks according to the species inoculated. Multiple eosinophilic inclusions known as Negri bodies are found in the nerve cells especially in the hippocampus, and measure 0.5 μ to 20 μ in diameter. Negri bodies are specific in rabies infection and their detection in nerve tissue enables a diagnosis of rabies to be made.

Serial brain-to-brain passages of the virus in rabbits yields an

¹ Rowe, W. P., et al. (1956), *Proc. Soc. exp. Biol. (N.Y.)*, 92, 418.

attenuated or "fixed" strain of the virus which is no longer able to multiply in extraneurial situations. Negri bodies are scanty in the brains of animals inoculated with the fixed virus. The virus grows in the tissues of 7-day-old chick embryos inoculated by any of the usual routes; the maximum yield of virus is obtained nine days after inoculation. The virus can also be grown in monolayer tissue cultures of hamster kidney and in minced mouse embryo cultures of the Maitland type.

Viability.—The infectivity of the virus in tissues kept at room temperature is gradually lost in seven to fourteen days. At 4° C. the virus survives several weeks and in 50 per cent. glycerol saline at 4° C. for many months. The best diluent for the preservation of the virus is 2·5 per cent. normal guinea-pig serum in saline.

At 37° C. the virus survives four to five days, at 50° C. one hour, at 60° C. five minutes, and at 100° C. two to three minutes. Rabies virus is more resistant than vegetative bacteria to disinfectants; 0·5 per cent. formaldehyde fails to kill it in two months at 4° C., but 1·0 per cent. formaldehyde, 3 per cent. cresol and 0·1 per cent. mercuric chloride inactivate in fifteen minutes.

Antigenic Characters.—All strains of the virus are antigenically similar. Antibodies in the serum after vaccination can be demonstrated by neutralisation and complement-fixation methods; after vaccination an antibody response may or may not occur.

Occurrence.—The portal of entry is the bite and the rabies virus in the infected saliva is introduced into the depths of the wound. Occasionally the virus may gain access through a pre-existing scratch or abrasion if this has been contaminated by saliva. The virus multiplies locally in the tissues, invades damaged nerve fibres, and spreads centrally to reach the brain and spinal cord. It is not yet decided whether the virus travels along the axons of nerves or along perineurial lymphatics. There is no evidence that the virus is spread through the blood stream or lymphatic channels, and it has only rarely been isolated from the cerebrospinal fluid.

The incubation period varies from ten days to two years after the bite of a rabid animal; its duration may depend on the distance the virus has to move from the point of entry to reach the brain. Average figures are: for bites on the leg sixty days, on the arm forty days and on the head thirty days. The incubation period is shorter in children than in adults.

The first symptoms of the disease are headache, fever, a profound sense of apprehension, and a feeling of irritation at the site of the bite. The patients complain of a dry throat and thirst, but they will not drink. High fever, difficulty in swallowing and a fear of water (hydrophobia) become the dominant symptoms, and the

patient passes into delirium with generalised convulsions. The outcome is invariably fatal and at autopsy there is a severe encephalitis, characterised by generalised hyperaemia and a pronounced cell destruction throughout the brain especially marked in the medulla. Demyelinisation is widespread and the cells of the posterior horn are severely damaged.

Epidemiology.—The epidemiology of rabies is determined by the animal sources of the infection. Dog and cat rabies constitute the most important source because 95 per cent. of human infections are derived from these animals. In the dog the incubation period is as a rule two to eight weeks, but it may be as long as eight months. The disease is characterised by two clinical forms. In "furious" rabies the animals become aggressive, vicious and excited; they snap and bite at the approach of any other creature. In "dumb" rabies paralysis of the muscles of the head and neck occurs and the dog cannot chew its food; its owner, believing it has some object lodged in its throat, may attempt to remove the obstruction and contaminate his hands with infected saliva. About 50 per cent. of rabid dogs excrete the virus in their saliva. They are infectious for only about ten days before their death.

Many species of wild animals suffer from rabies. One of the most important vectors is the fox, which may transmit the infection to farm animals. In Canada many cases of cattle rabies have been attributed to this means of spread. In Europe, Iran and the Middle East wolves and jackals have transmitted the infection, and in India the mongoose. Other species which are able to transmit rabies are rats, badgers, opossums, musk-rats, racoons, skunks and squirrels.

Rabies may infect all the usual domestic animals, including the horse, cow, sheep, goat or pig. Often the animals are infected by the bite of rabid foxes, or other wild life.

In Trinidad and South America rabies is transmitted by the vampire bat *Desmodus rotundus murinus*. The bat is the only species so far recognised as being latently infected with rabies virus. Fruit- and insect-eating bats are also known to harbour the virus, and when rabid are able to transmit the infection to other bats, animals and man.

Control.—It is only by the complete eradication of the animal reservoir that rabies can be eliminated. This has been possible in Great Britain, where the enforcement of strict quarantine regulations has prevented the entry of infected animals for many years. The result has been that there is no longer any source of the virus to infect animals and there has been no case of rabies in the country since 1921.

In other countries it has not been possible to eliminate the animal reservoir so completely, and rabies still presents a major problem to many public health authorities. When the dog is the principle reservoir, the disease can be brought under effective control by the active immunisation of pet dogs and by the destruction of all strays and wild dogs. An effective vaccine in general use contains the living avirulent egg-adapted Flury strain of the rabies virus. It is given in a dose of 3·0 ml. intramuscularly into the hind limb of the animal and an effective protection lasting three years is developed.

The Flury virus vaccine has been used experimentally with promising results in man. The virus, like the "fixed" virus, does not multiply in human tissues and it is therefore necessary to give a series of injections comprising a total dose of about 20 gm. of infected chick embryo to obtain the required antibody response. The hazard of allergic encephalitis may perhaps be reduced with a chick embryo vaccine, and if this hope materialises the method may replace the use of animal brain suspensions.

Laboratory Diagnosis.—In man the clinical findings are so characteristic that laboratory confirmation is not usually necessary. The virus, however, is present in the saliva and may also be isolated from specimens of the nervous tissue and salivary glands obtained at autopsy. The material is first treated with antibiotics and then inoculated intracerebrally in mice; the animals develop a flaccid paralysis of the limbs and die within six to eight days.

In the dog the diagnosis depends on the demonstration of Negri bodies in the brain tissue and the isolation of the virus; there is no satisfactory serological test that can be used in life. If there is the slightest reason to suspect a dog of rabies, the animal should be kept in strict isolation and observed for a period of ten days; if it survives for this period, rabies may safely be excluded. If unmistakable symptoms of rabies are observed, the animal is killed (preferably with chloroform) and the diagnosis is confirmed by laboratory examination. If the laboratory is at some distance, the head is removed, wrapped in a cloth soaked in 50 per cent. glycerol saline, and forwarded in cracked ice. In the laboratory the scalp is reflected, the skull is opened by means of sterile bone forceps and the brain removed with aseptic precautions. The hippocampus, which is situated in the floor of the lateral ventricle, is dissected out, smears are made by squeezing a portion of the tissue between two slides, and pieces are also fixed for histological examination. In addition, a suspension is prepared for animal inoculation. The smears are fixed in methyl alcohol for five

minutes, and stained by Giemsa's method (p. 132) or by Mann's method (see p. 139). For section, the tissue is fixed in Zenker's fluid (p. 142) and stained as above. The animal's salivary glands should also be removed and homogenised for mouse inoculation. The diagnosis depends on the finding of the characteristic Negri bodies in the cytoplasm of the nerve cells. These bodies are best seen when the animal has reached the paretic stage of the disease. Intracerebral inoculation of mice with brain emulsion is also carried out, and if the virus is present, paresis and death occur as described above.

Prophylaxis.—When a person is bitten by a dog suffering from or suspected of rabies, the first step is the thorough cleaning of the bite wound with soap and water. The puncture wounds should then be thoroughly dried and cauterised with concentrated nitric acid applied with a capillary pipette; after a few moments a solution of sodium bicarbonate should be applied to neutralise the acid.

Rabies hyper-immune horse serum is used to confer passive immunity and is most effective if given within twenty-four hours of the infliction of the bite; some protection, however, is afforded if the serum is given as late as seventy-two hours after exposure. The recommended dose is 0·5 ml. per kg. body weight given by intramuscular injection.

Exposed persons should also receive a course of active immunising injections of a phenolised virus vaccine (Semple's vaccine) or of an ultra-violet light inactivated vaccine. The inoculations comprise a series of fourteen to eighteen daily subcutaneous injections of 2·0 ml. of the vaccine; care should be taken to place each injection in a different situation to avoid local reactions. The dose of vaccine may be increased or the course prolonged when there has been a serious risk of infection.

The vaccines used contain crude suspensions of the brain tissue of infected rabbits in *corrus murinus* varying between 4 and 20 per cent. The result of this type of vaccine is not fully known, case fatality in this type of persons being about 1 in 10,000. Mild neuritis of muscles in the face and limbs may occur, but can be eliminated.

In Great Britain the use of this vaccine is only necessary for returning travellers who have been bitten while abroad or for workers in quarantine establishments. Its use is contraindicated for patients who have not been exposed to a real risk of rabies.

Pseudo-Rabies (Aujeszky's Disease—Mad Itch)

This is a disease which affects the central nervous system, causes paralysis, and occurs in dogs, cattle, horses, pigs, sheep, rats and certain other animals. It is rapidly fatal in all these species except the adult pig. In pigs the disease closely resembles Teschen or Talfan disease (see p. 827). An important point of differentiation from rabies is that the animals suffer from intense itching of the hindquarters. It also differs in that the animals are not aggressive. The virus is not strictly neurotropic, for it may be demonstrated in the oedematous subcutaneous tissue in the local skin lesion and in the lungs. It is not found in the saliva. Histologically the virus causes severe and widespread neural damage and characteristic intranuclear inclusions have been described in the most severely affected neurons. Antigenically it is distinct from the virus of rabies with which it has no relationship whatever. The size of the virus is about 120 μ . The principal natural reservoir of the virus is the adult pig, but the rat is also latently infected. There is evidence that the disease may spread from rats to pigs and from pigs to cattle. The virus of Aujeszky's disease has been isolated by Lamont from cattle, pigs, cats and rats in Northern Ireland. It can be cultivated in the chorio-allantoic membrane of the chick embryo, where it produces white plaques after four to five days' incubation and also by tissue culture methods. Many strains cause a haemorrhagic encephalitis and death of the embryo.

FOOT-AND-MOUTH DISEASE

Foot-and-mouth disease is a highly contagious condition of cattle, pigs, sheep, goats and deer. It is characterised by vesicles on the feet and in the mouth along with constitutional symptoms of infection. The disease may also occur naturally in the hedgehog. The infection spreads rapidly among farm animals, and large epizootics are the cause of much economic loss in many parts of the world.

*The Properties of the Virus.*¹—Estimates of the size of the virus vary from 8–12 μ according to filtration experiments to 22 μ from electron-microscopical measurements. A small component of the virus about 6 μ is associated with complement-fixing activity.

Host Range and Cultivation.—The virus is present in the vesicles

¹ For further reading, see Brooksby, J. B. (1958), *Advances in Virus Research*, V, p. 1. New York.

on the mouth and feet and also in the blood, saliva, milk and excreta of infected animals. The disease may be transmitted by the inoculation of these materials into the scarified mucous membrane of the tongue or lips of cattle, sheep and pigs. The disease can also be produced experimentally by the intracutaneous inoculation of the skin of the pads of guinea-pigs' feet. Suckling mice and rats are susceptible to the disease by experimental inoculation and the virus multiplies in the skeletal muscle, causing a flaccid paralysis; adults cannot be infected. It has been found in comparative infectivity titrations that cattle and suckling mice are almost equally susceptible and that guinea-pigs are approximately 100 per cent. more resistant to the virus.

The virus can be readily cultivated in bovine tongue epithelium tissue cultures of the suspended type. It can also be cultivated in monolayer cultures of trypsinised pig kidney cells, in which it is capable of producing plaques 2-3 mm. in diameter. Strains have also been adapted to grow in the chick embryo and in newly hatched chickens.

Viability.—If dried slowly in albuminous material, the virus retains its infectivity for many months and it is thus possible for the infection to be transmitted after long intervals by infected fodder and other contaminated materials. In infected fragments of tongue epithelium and in vesicle fluid the virus survives at room temperature for two months or more; immersion in neutral glycerol saline and storage of the material at 4° C. preserves its infectivity indefinitely. The virus is resistant to alcohol, ether and chloroform, but is rapidly inactivated at pH values below 6 or above 10. For the disinfection of contaminated premises, a 4 per cent. solution of sodium carbonate is recommended.

Antigenic Characters.—There are seven recognised antigenic types of the virus which have been differentiated by cross-immunity experiments and by serological methods. The first two types to be described were designated O (Oise) and A (Allemagne) according to their areas of origin and to these a third, type C, was added. Three further types have been isolated in various parts of South Africa and are known as S.A.T.1., S.A.T.2 and S.A.T.3; the seventh was found in Eastern Asia and is known as Asia 1. Animals that have recovered from one type are not immune to another type. There is some evidence to suggest the existence of a group antigen shared by the different types. In addition to seven basic types a large number of variants have been recognised.

Occurrence.—Foot-and-mouth disease is spread from animal to animal by means of infected saliva. The incubation period is twenty-four to forty-eight hours and the disease spreads rapidly

until the whole herd is involved. The importance of the disease, which is notifiable in Great Britain, is not on account of its power to kill, for the mortality is not high, but by virtue of the morbidity and the consequent loss of flesh and reduction of the yield of milk. The disease is characterised by fever, drooling of saliva and the appearance of vesicles on the mucous membranes of the mouth and the skin of the muzzle. The animal is lame because of the foot lesions and lies down as much as possible; it is unable to feed and as a result rapidly loses flesh. Within twenty-four to forty-eight hours the virus invades the blood stream and reaches the muscles, where it causes degenerative lesions, especially in the heart. The mouth lesions usually heal promptly, but those on the feet require much longer before recovery is complete.

Epizootiology.—The foot-and-mouth disease virus is often introduced to a herd from far-distant parts. The viability of the virus makes it possible for it to be carried alive long distances in infected meat or on contaminated fomites. Migrating birds (especially starlings) have been thought to carry the virus, and there is some evidence to suggest that from time to time they have brought the infection to Great Britain from Belgium and the Low Countries. Pigs have frequently contracted the infection from eating swill containing infected fragments of meat imported from South America.

Control.—When foci of infection occur in Great Britain the movement of all animals out of an infected area (usually a 5-mile radius around infected premises) is prohibited. Animals are admitted to the area only on licence and must be taken to a farm or slaughter-house not less than 2 miles from the infected place. Movement within the area is permitted only on licence. In Great Britain all infected and exposed animals are slaughtered and their carcases incinerated or buried. The contaminated premises and all contaminated material are thoroughly disinfected with 4 per cent. sodium carbonate solution. The premises are kept unstocked for six weeks after disinfection and at the end of this period only a portion of the new herd is allowed to enter the farm. If no cases occur during the next three weeks the quarantine is lifted and complete re-stocking is permitted.

Inactivated vaccines prepared from the blood or tissues of experimentally infected cattle have been used to immunise animals. The immunity conferred by such vaccines lasts for six months to a year and at the end of this time a booster dose is required. The use of vaccines is not permitted in Great Britain, but on the Continent and in Mexico they have been used in an attempt to build up a barrier of immune animals around an

endemic area of foot-and-mouth disease and thus to contain the infection. Vaccines containing living attenuated strains of the virus are now on trial in Africa.

Foot-and-Mouth Disease in Man.—The disease is very rarely transmitted to man and there are very few authenticated records of human infection. Laboratory workers and those handling infected animals have been known to develop a mild fever with vesicles on the hands and stomatitis. Recovery took place within a week and neutralising antibodies were developed in the serum.

Laboratory Diagnosis.—Infected tissues, if possible tongue epithelium, should be sent to the laboratory in neutral glycerol saline. The specimen is triturated in saline and the virus in it is detected by setting up the suspension in a complement-fixation reaction against hyper-immune guinea-pig sera. The virus may be isolated from the suspension by the inoculation of suckling mice or primary monolayer tissue cultures of pig kidney cells. Final identification of the infecting type is carried out with cross-immunity tests, using guinea-pigs, suckling mice and cattle.

VESICULAR STOMATITIS

The disease occurs naturally among cattle, horses and swine. The disease is not a serious one, but because of the vesicles that occur in the mouth and on the tongue is easily mistaken for foot-and-mouth disease. The virus is antigenically distinct from the foot-and-mouth disease virus and is larger, being 60–90 m μ in diameter. It can be cultivated without difficulty in the chick embryo and in monolayer cultures of chick, ox and pig tissues, where it has a cytopathic effect. The vesicular stomatitis virus gives plaques in monolayer culture of ox embryo kidney. There are two immunologically distinct strains. The virus has the same degree of resistance to physical and chemical agents as the foot-and-mouth disease virus. The disease is mild, and drastic control measures are not required.

VESICULAR EXANTHEMA

This disease occurs naturally only in pigs and, like foot-and-mouth disease, is characterised by vesicles on the mouth and feet. Cattle are resistant to the infection and the virus is only mildly pathogenic to horses. No lesions are produced in the foot-pad of the guinea-pig, a finding which differentiates the vesicular exanthema virus from those of vesicular stomatitis and foot-and-mouth disease.

The virus is 14–22 m μ in diameter and there are probably at least seven immunological types. It can be cultivated in monolayer tissue cultures of pig kidney cells, in which it produces a cytopathic effect and plaques similar to those of the foot-and-mouth disease virus.

The Susceptibility of Animals to the Vesicular Viruses

Susceptible Animal	Foot-and Mouth Disease	Vesicular Exanthema	Vesicular Stomatitis
Pig	+	+	+
Horse	—	±	+
Cow	+	—	+
Guinea-pig	+	—	+
Adult mouse . . .	±	—	—
Suckling mouse . . .	+	—	+

RINDERPEST

Rinderpest or cattle plague is an acute and highly infectious disease, especially of cattle but also of all ruminants and swine. It is characterised by fever, severe haemorrhagic catarrh from the mucous membranes of the nose and eyes and profuse diarrhoea followed by weakness. Death usually occurs within four to seven days of the onset. The disease is endemic in all parts of Asia and Africa and is of great economic importance.

The Properties of the Virus.—The virus is present in the blood, body fluids, intestinal mucous membranes and other tissues and is discharged in the secretions and excretions of sick animals. It has a particular affinity for lymphoid tissue and is often located within leucocytes; it is not found free in the blood or tissue fluids and for this reason it has been difficult to measure its size by filtration. It is known, however, to pass Berkefeld and coarse Chamberland filters, and is probably 80–120 m μ in diameter. The virus in dried secretions outside the body does not survive more than forty-eight hours and is easily inactivated by heat, sunlight,

putrefactive changes and the usual disinfectants. Antigenically, the virus is stable; there is no evidence to suggest a multiplicity of types. There is no evidence that the virus causes haemagglutination.

Host Range and Cultivation.—Cattle, goats, sheep, camels, pigs and many species of game suffer the natural infection. Experimentally, strains have been adapted to multiply in rabbits, guinea-pigs and the chick embryo. Regular passage in these laboratory hosts encourages the production of a mutant which becomes increasingly pathogenic for the new host while losing its virulence for cattle. The virus can be cultivated in tissue culture monolayer cells from chick and ox embryos and from those of calf testis. Multinucleate syncytial masses containing type B intranuclear inclusions can be seen in the infected monolayer.

Occurrence.—The disease is spread by direct contact between infected and susceptible animals. The exact route of transfer of the infection is not established, but is thought to be through contaminated food and drinking water. Biting arthropods have not been incriminated as vectors. The incubation period is three to eight days and the affected animals usually die within a week of the onset. Oedema, haemorrhagic ulceration of the intestine with inflammation of the Peyer's patches are the characteristic *post-mortem* findings. Pneumonia is found in many cases.

Rinderpest is enzootic in many parts of Africa and Asia, and there is always a risk that the infection may be conveyed to new areas. The movement of herds and the importation of beasts from distant endemic areas has often introduced the virus into a susceptible animal community with the result that a devastating epizootic has broken out.

Control.—The disease has been excluded from Great Britain, most of Europe, and from North and South America by a rigid embargo on the importation of animals or meat from endemic areas. In countries where rinderpest is endemic, the principal means of control is prophylactic vaccination.

Several vaccines containing live attenuated strains of the rinderpest virus have been developed and their use is rapidly followed by an effective immunity which lasts from one to two years. These vaccines have been prepared from goat-adapted, rabbit-adapted and egg-adapted virus strains.

Live vaccines have the advantage that they rapidly induce resistance by virtue of the interference phenomenon and they have been effective in the field in checking an outbreak. Care, however, is needed in selecting the type of vaccine to be used because some of the strains are of sufficient virulence to cause

severe reactions and some mortality in certain breeds of cattle. Milder strains, on the other hand, may induce only a short-lived immunity. The goat-adapted (caprinised) virus should not be used on stocks of European cattle, which are usually highly susceptible to rinderpest. It is used successfully on indigenous cattle in Africa and confers an immunity lasting four to six years. The rabbit-adapted (lapinised) virus is less virulent and is used throughout Africa and Asia; the immunity given lasts about two years. The egg-cultivated virus has not been extensively used. Calves born of immune dams lack rinderpest antibodies at birth but receive them in colostrum in the first few hours of life. The calf retains this immunity for several months and cannot be actively immunised satisfactorily until the age of 8 months.

Laboratory Diagnosis.—The virus is isolated by the inoculation of tissue cultures or susceptible cattle with suspensions of infected tissue. It can be identified in complement-fixation and agar gel diffusion tests and in neutralisation tests in which immune rabbits or cattle are challenged with the freshly isolated strain.

CHAPTER XLIII

BACTERIOPHAGES

THE phenomenon of transmissible lysis of bacteria was first observed independently by Twort and d'Herelle. It was demonstrated by the latter in the following way: a few drops of liquid faeces from a case of bacillary dysentery were added to a tube of broth which was incubated overnight; filtration of the culture through an earthenware filter yielded a filtrate which, added in small quantities to a young culture of *Shigella shigae*, produced lysis of the bacteria after a period of incubation; further, a filtrate of this lysed culture possessed a similar lytic property towards a fresh culture and the lytic property was thus shown to be transmissible indefinitely from culture to culture. D'Herelle suggested that this phenomenon is due to a minute organism, Bacteriophage (generally abbreviated to "phage"), which is parasitic and destructive to bacteria, propagating and multiplying at the expense of the bacterial cells. This view has now been amply confirmed and the phages are undoubtedly closely related to other viruses.

The presence of a phage attacking a host bacterium can be shown by two general methods: (a) the ability to lyse a young culture in a suitable liquid growth medium and so to cause clearing of the medium (*i.e.* the method used by d'Herelle); (b) the ability to produce a clear area when spotted on to an agar plate inoculated with the host bacterium. This clear area is known as a *plaque* and can be produced by a single phage particle. It is in this latter case analogous to a bacterial colony. Using these methods, it has been shown that there are phages active against most bacteria, each having a marked degree of specificity for its host species. This has been made use of in the identification and typing of several groups of bacteria, *e.g.* staphylococci and salmonellae (pp. 471, 575). Phages can be distinguished by means of their host range, morphology of the particles as shown by the electron-microscope, morphology of plaques of lysis produced on indicator strains of bacteria growing on solid media, biochemical and immunological characters and susceptibility to various environmental factors.

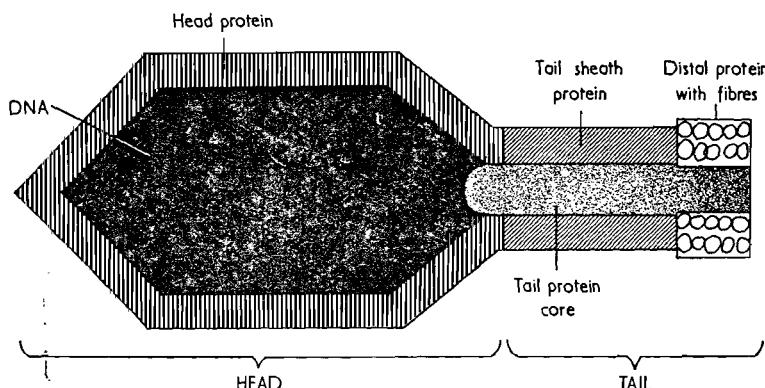
*Properties of Bacteriophages.*¹—Most of the available information on phage has been obtained from extensive studies on the *Esch*

¹ Adams, M. (1959), *Bacteriophages*. New York, Interscience Publishers.

coli phages, T1-T7, but many other phages that have been studied appear to have the same general properties.

Phages can readily be seen in suitable preparations with the electron-microscope. A phage particle consists of a head which may be spherical, ellipsoidal or hexagonal, about 50 m μ by 100 m μ in diameter. Attached to the head is a tail which is in the form of a hollow tube or sheath with a central core, and at its end a tail-piece bearing fibrils by which the phage adsorbs to the host cell wall. The length of the tail varies; in some phages it is very short, in others about 100 m μ long and 25 m μ broad.

Phages consist solely of specific proteins and desoxyribonucleic acids (DNA). The protein forms a coat to the phage head and also the substance of the tail. The DNA is contained within the head (Fig. 31).



A DIAGRAMMATIC REPRESENTATION OF A TYPICAL VIRULENT PHAGE PARTICLE

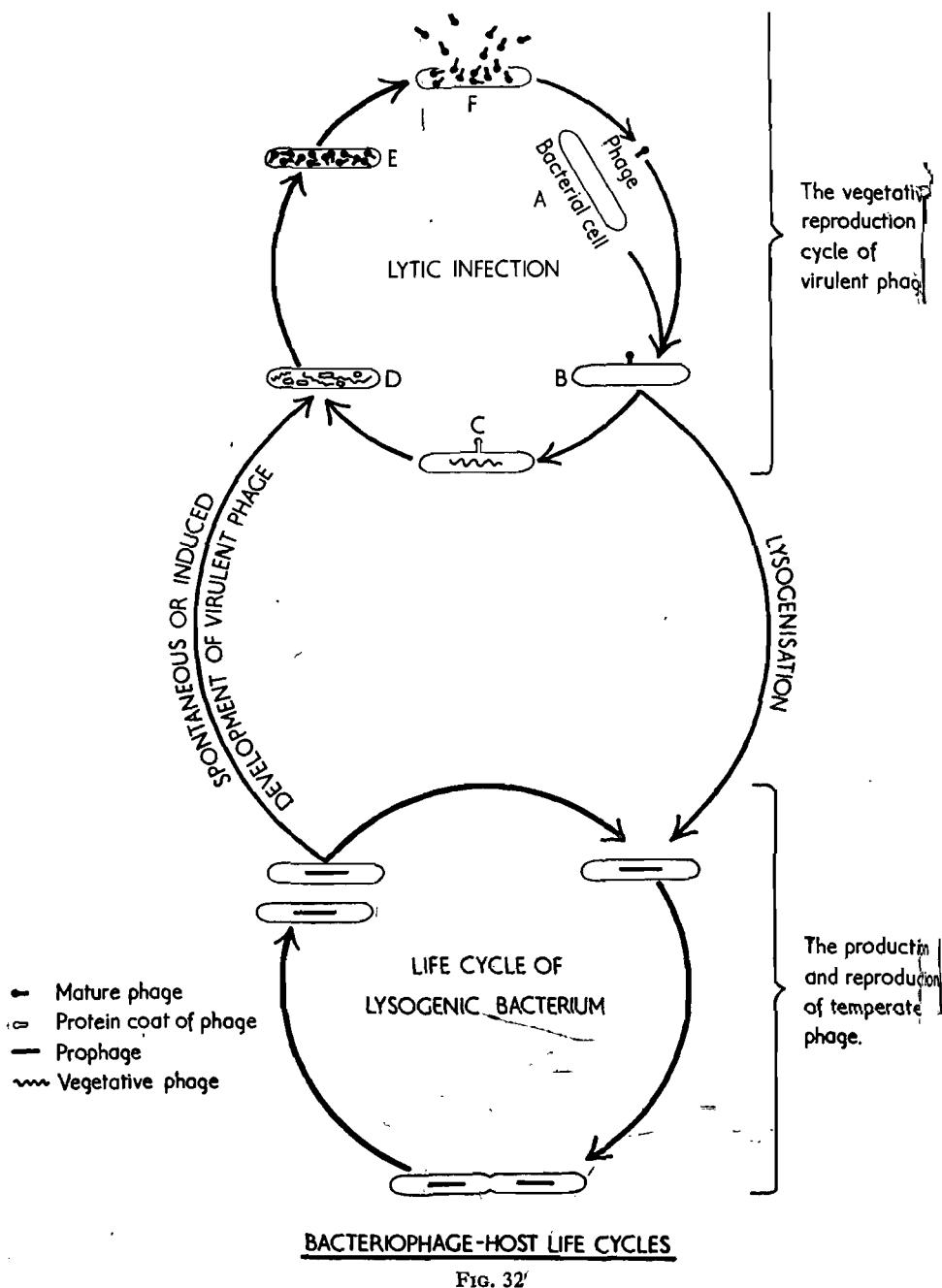
FIG. 31

Phages have considerable resistance to physical and chemical agents; they may withstand heating at 75° C. for thirty minutes and resist drying for long periods. They can be propagated only in living and growing cultures of bacteria.

Phages are antigenic, containing a number of different antigenic components, and exhibit a high degree of serological specificity. Antisera will agglutinate phage particles, but it is easier to demonstrate antibody by the neutralisation of phage activity.

Phage particles exhibit some of the genetic properties of more organised cells and their characters can change as a result of *mutation* or by *genetic recombination* when two different phages enter the same host cell. In addition, some properties of phage

VIRUSES



may be temporarily modified by the host cell; passage through one host thus temporarily decreases the ability of the phage to infect another host.

Phage Reproduction.—If infection of a bacterium by a phage results in lysis of the host cell, the phage is said to be *virulent*. We may divide the cycle of the vegetative reproduction of virulent phage into various stages (see Fig. 32).

1. *Phage Adsorption and Penetration*

Stage A.—Phages, as a result of Brownian movement, collide with bacterial cells (phages are non-motile, the tail being an attachment device).

Stage B.—As a result of this collision, adsorption may occur between the complementary surfaces at the tip of the phage tail and the superficial layers of the bacterium. The cell wall of the host bacterium contains receptor sites which receive the phage particles and in some cases these receptors may be associated with, or identical to, the somatic antigens. This adsorption process is a specific one and the surface constituents of a bacterium may exhibit more specificity in this respect than in serological tests. Hence it is possible to subdivide a bacterial species into a number of phage types according to the nature of the phages capable of producing lysis. With some phages, adsorption may further depend upon the presence of specific cofactors, particularly inorganic ions and amino acids.

Stage C.—As a result of adsorption, a lysozyme-like enzyme which is a component of the phage tail is activated. This destroys a localised portion of the cell wall. (If sufficient phages are adsorbed on to a single bacterial cell, leakage through the holes may produce immediate lysis—so-called “lysis from without”.) The tail sheath of the virus then contracts and the DNA inside the head is forced through the bacterial cytoplasmic membrane into the cytoplasm. Thus the protein component of the phage can be considered as a disposable micro-syringe causing the injection of DNA into the host cell.

2. *Phage Multiplication*

Stage D.—After infection of the bacterium, there is a period amounting to about half the total division cycle, when no phage is detectable within the host cell (the eclipse phase). During this phase, bacterial protein and nucleic acid synthesis are halted and the activities of the host are re-directed towards synthesis of the specific proteins and nucleic acids of the phage. These phage components are formed separately as can be shown by immuno-

logical and biochemical methods as well as by the electron-microscope.

Stage E.—Phage components are assembled to form the mature particles which accumulate within the cell.

3. *Bacterial Lysis* (lysis-from-within)

When the number of mature phages within the cell reaches a critical value, the bacterium lyses, liberating the viruses which may then attack further cells.

As a result of this cycle, which may occupy twenty to sixty minutes, a single phage may produce up to 200 or more progeny (known as the *burst-size*).

Lysogeny and Temperate Phages.—Infection of a bacterium with phage may result in no perceptible change in the host cell, and in this case the phage is said to be *temperate* (Fig. 32). The initial stages of adsorption of the phage and injection of the DNA are the same as for virulent phage. However, this is not followed by a breakdown of the means of bacterial growth and reproduction, and the bacterium apparently divides normally. Such cells are carrying no detectable phage, but all the progeny of the infected bacterium carry the potentiality of producing further phages and lysing at some later generation. Each cell must contain a component forming part of its genetic constitution which reproduces synchronously with the bacterium and which is capable of forming mature phage. This component is known as *prophage*, the process is known as *lysogenisation* and the host bacterium is said to be *lysogenic*.

Prophage may develop spontaneously into vegetative temperate phage (this was the original phenomenon of sporadic cell lysis observed by Twort), and can be *induced* to the vegetative phase much more efficiently by various non-specific environmental factors such as ultraviolet light. Lysogenic bacteria are immune to infection by phage of the type already carried as prophage. There is thus an analogy between lysogenicity and "latent infection" shown by animal viruses.

Phages are important agents in *transduction* in bacteria, for bacterial genetic material can be carried accidentally from host cells of one genotype to host cells of another genotype by a temperate phage. In other cases (lysogenic conversion) the prophage itself confers the new properties. For instance, non-toxigenic strains of *Corynebacterium diphtheriae* become toxic when lysogenised by certain phages, and similarly *Salmonella* may acquire new somatic antigens. Bacteriophages can thus be regarded as important factors in bacterial ecology.

Isolation of Bacteriophage.—To illustrate the procedure for the demonstration and isolation of bacteriophage, the methods applicable for obtaining phages for typhoid-paratyphoid and dysentery bacilli from faeces and sewage may be described.

A relatively large amount of the material (*e.g.* about 5 g. of faeces or 10 ml. of sewage) is thoroughly disintegrated and emulsified in 50 ml. of broth (pH 7.6) contained in a 200 ml. flask. The broth is incubated under aerobic conditions for twelve to twenty-four hours at 37° C. and then filtered through a Seitz disk. It is preferable, however, to carry out "phage enrichment" by adding to the broth before incubation a few drops of a young broth culture of the organism for which a phage is sought.

To demonstrate the phage in the filtrate obtained by either of these methods, an agar plate is stroked with large loopfuls of a young broth culture of the particular organism, so as to yield fairly broad bands of growth on the medium; after the inoculum has dried on the medium, drops of the filtrate are superimposed on the strokes. The plates are incubated overnight and the presence of phage is denoted by a clear area where the filtrate has been placed on the stroked inocula, the organisms in these areas having been lysed by the phage. (Single colonies of phage-resistant mutant bacteria are sometimes present in the clear area of the plaque.) When a phage has been demonstrated in the filtrate, it can be maintained by adding the original filtrate to broth inoculated with the appropriate organism and preparing a filtrate after incubation. The phage can be propagated serially in the same way by adding each filtrate to a young broth culture of the organism.

The clear areas of lysis may be due to a mixture of phages of different types, and if a pure phage is required its isolation may be effected by the following method. A series of decimal dilutions (in broth) of the phage is prepared, each of which is inoculated with the particular organism; for this purpose a fairly dense suspension from an agar slope culture is made, and sufficient of this is added to the diluted filtrate to yield a slight but distinct turbidity. A few drops of each dilution so inoculated are then spread on an agar plate with a glass spreader, so that most of the surface of the medium is uniformly inoculated. Confluent bacterial growth over the surface of the plate is aimed at. After incubation small discrete, well-separated "plaques" will be observed with certain dilutions of filtrate. At higher concentrations the phage plaques will not be discrete and may be confluent. A plate representing a dilution with good separation of plaques is selected, and with a small inoculating loop single plaques are sub-

inoculated into tubes of broth seeded with the test organism; after incubation these cultures are filtered. To obtain a pure clone it may be necessary to repeat the isolation procedure several times, preparing decimal dilutions, plating, and sub-inoculating discrete plaques.

Phage Typing Technique

The propagation and use of phages for typing bacteria may be illustrated in the case of *Staphylococcus aureus* (pp. 471), where phage typing is of considerable value in the epidemiological study of outbreaks of staphylococcal infection.

Source of the Typing Phages.—Phages may be isolated from lysogenic strains of *Staph. aureus* and selected on the basis of the specificity of their host range (*vide supra*). A number of staphylococcal phages are recognised internationally as a basic typing set, and these have been numbered accordingly (see p. 471). These phages are grown on susceptible host strains of *Staph. aureus* (*propagation strains*), which are numbered accordingly to the phages with the prefix PS, e.g. PS 52. Both the basic typing phages and the propagation strains of *Staph. aureus* may be obtained from the Staphylococcal Reference Laboratory, Colindale, or the National Collection of Type Cultures.

Medium for Propagation and Typing with Phages.—Digest broth containing 0.7 per cent. powdered agar (*i.e.* sufficient to give a rather soft gel) may be used both for propagating and typing. For the latter purpose the digest agar may be under-layered with agar containing 1 per cent. peptone and 0.5 per cent. sodium chloride (peptone agar).

Propagation of Bacteriophages.—Propagation strains of *Staph. aureus* are cultured in nutrient broth from freeze-dried stock preparations. To reduce the chance of contamination and variation occurring in both phage and propagation strain it is best to start the preparation of each batch of phage from freeze-dried stock.

Petri dishes are cast to a depth of 5 mm. with digest agar. The surface of this medium is inoculated with an overnight broth culture of the propagation strain, using a sterile glass spreader to distribute the minimum number of drops which will give confluent growth. When the culture has been absorbed into the agar (usually two hours are needed for this), the phage, reconstituted in broth from freeze-dried material, or in the form of a filtrate, is spread over all save a small segment of the surface of the inoculated medium. This serves as a control area. The plates are then incubated at 37° C. overnight. The concentration of phage particles in the inoculum should be 10-100 times that required to produce con-

fluent lysis of the propagation strain (Routine Test Dilution, *vide infra*).

Following incubation the control area is examined; this must show no evidence of spontaneous lysogenicity of the propagation strain otherwise the plate must be discarded. If clear, the control area is cut out with a sterile knife and the remaining agar frozen by holding at -60° C . for one hour. After freezing, the agar is allowed to thaw at room temperature, when the agar gel disintegrates with extrusion of fluid. The fluid is separated, centrifuged to remove bacterial cells and débris, and titrated by applying 0.02 ml. drops of decimal dilutions in peptone water to the surface of a digest agar plate previously seeded with the propagation strain. Individual plaques of lysis indicate the activity resulting from single phage particles, thus a plaque count gives the number of phage particles in a preparation. When the plaques are numerous they coalesce into confluent lysis. The highest dilution of a phage preparation producing confluent lysis is generally referred to as the Routine Test Dilution (R.T.D.).

If the titre is satisfactory (*i.e.* R.T.D. greater than 1: 1000) the phage preparation is filtered through a Seitz filter to remove remaining bacteria. The sterile filtrate is re-titrated to check on loss of potency during filtration. The identity of the phage is also checked by spotting drops of the undiluted filtrate on plate cultures of a set of indicator strains to confirm that the lytic spectrum (host range) has remained unchanged. Stock, undiluted filtrates of phage are stored at 4° C . and samples are freeze-dried for future propagation.

For typing purposes the phage filtrates are used most often at their R.T.D. or at R.T.D. $\times 1000$. These dilutions are prepared weekly and stored in the refrigerator.

The Typing Technique.—A grid with a number of squares corresponding to the number of phages (usually 20) in the typing set is marked on the bottom of Petri dishes cast with peptone agar and overlaid with digest agar to give a total depth of 2–3 mm. of medium.

Overnight nutrient broth cultures of the strains of *Staph. aureus* to be typed are flooded on to the surface of the plates and excess culture removed. The plates are allowed to dry for two hours at room temperature with their lids partly removed, and when absolutely dry, drops of the typing phages are applied in a constant order with fine capillary pipettes delivering approximately 0.01 ml. Care must be taken not to touch the plate when applying the filtrates, since any of the test strains may be lysogenic and "carry over" may result in "non-specific lysis". The plates are

allowed to dry and are incubated at 35°-37° C. overnight. Next day the plates are examined in a good light and each square of the typing grid examined for lysis which may be reported as follows:

- +++ strong reaction—confluent lysis with or without phage resistant growth.
- ++ moderate reaction—50 or more plaques.
- + weak reaction—up to 50 plaques.

The complete pattern of lysis for each strain is recorded. If no reaction is apparent with any of the phages the strain is reported as non-typable, but may be tested again with stronger preparations of the phages and reactions obtained.

CHAPTER XLIV

PATHOGENIC FUNGI

INFECTIONS produced by the true fungi, or *Eumycetes* (p. 5), are usually designated "mycoses". The *Eumycetes* can be divided into four morphological groups, each of which includes some pathogenic varieties. (1) The *moulds* (filamentous or mycelial fungi) grow as long filaments (hyphae) which branch and interlace to form a meshwork (mycelium), and reproduce by the formation of various kinds of spores. The major part of the mycelium, the *vegetative mycelium*, grows on and penetrates into the substrate, absorbing nutrients for growth; sometimes it forms asexual thallopores. Other hyphae constitute the *aerial mycelium* and protrude from the vegetative mycelium into the air; they form and disseminate into the air various kinds of spores. When grown to a large size on artificial medium, the mycelium is seen as a filamentous mould colony; this may become powdery on its surface due to the abundant formation of spores. The filamentous fungi usually are only locally invasive in the body and spread to fresh hosts by their spores, e.g. the ringworm fungi.

(2) The *yeasts* are unicellular fungi which occur mainly as single spherical or ellipsoidal cells and reproduce by budding. On artificial media they form compact colonies with a creamy, mucoid or pasty consistency (e.g. like those of staphylococcus). They grow diffusely through fluid media and may spread readily through the animal body, e.g. *Cryptococcus neoformans*.

(3) The *yeast-like fungi* grow partly as yeasts and partly as long filamentous cells joined end to end, forming a "pseudomycelium", e.g. *Candida albicans*.

(4) The *dimorphic fungi* grow either as filaments or as yeasts, according to the cultural conditions. Growth usually takes place in the mycelial form, the saprophytic phase, on culture media at 22° C. and in the soil, but in the yeast form, the parasitic phase, on media at 37° C. and in the animal body; e.g. *Blastomyces dermatitidis*.

Systematic Classification

The systematic classification of the fungi is made on different lines. Four classes are distinguished, mainly according to the nature of their sexual spores. (1) The *Phycomycetes* form non-septate hyphae, asexual

"sporangiospores" contained within a swollen spore case, or "sporangium", borne at the ends of aerial hyphae, and sexual spores of the "oospore" or "zygospore" varieties. (2) The *Ascomycetes* form septate hyphae, various kinds of asexual spores including "conidia" which are abstricted successively from the ends of specialised (often aerial) hyphae called "conidiophores", and sexual "ascospores" formed, usually eight together, within a sac or "ascus". (3) The *Basidiomycetes* form septate hyphae and sexual "basidiospores", usually four in number, from the ends of club-shaped structures called "basidia". (4) The *Fungi imperfecti* include the fungi which do not have a sexual stage and thus cannot be placed with certainty in one of the other three classes. Many imperfect fungi form septate hyphae and asexual conidia resembling those of *Ascomycetes*, and their closest affinities lie with this class. A majority of the pathogenic moulds, yeasts, yeast-like fungi and dimorphic fungi belong to the group *Fungi imperfecti*.

Common Saprophytic Moulds

Many species of non-pathogenic moulds occur in the soil and on decomposing organic matter. Aerial dissemination of their spores is widespread and they are commonly found contaminating exposed bacteriological culture media, human foodstuffs and specimens taken from the surfaces of the body. They also occur as secondary invaders, e.g. in the external ear or lung. Their presence in diagnostic cultures must not be taken as denoting an aetiological relationship. Varieties commonly encountered include *Rhizopus*, *Mucor* (e.g. *M. mucedo*), *Aspergillus* (e.g. *A. niger*) and *Penicillium* (e.g. *P. expansum*). *Rhizopus* and *Mucor* are phycomycetes with non-septate hyphae, asexual sporangiospores and

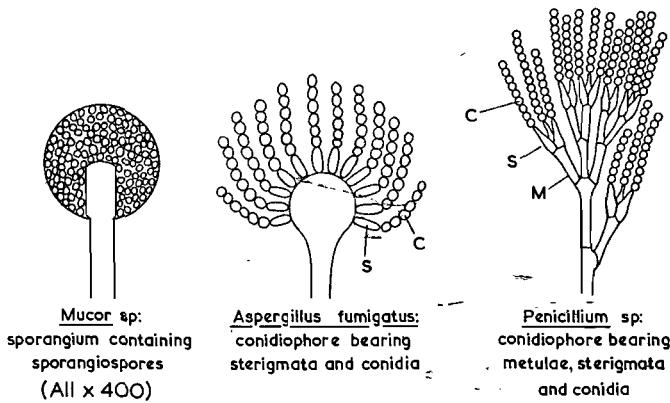


FIG. 33

sexual zygosporcs which are formed by conjugation of two hyphae at their tips. *Aspergillus* and *Penicillium* species are ascomycetes or fungi imperfecti, having septate hyphae, asexual conidia and in some cases sexual ascospores. Their colonies are commonly pigmented yellow, green or black. The conidial chains of *Aspergillus* arise from finger-like "sterigmata" which radiate without branching from the expanded bulbous tip of the conidiophore. Those of *Penicillium* arise brush-like from sterigmata borne on the tips of several terminal branches of the conidiophore (Fig. 33). The important antibiotic substance penicillin is derived from *Penicillium notatum*.

THE DERMATOPHYTES OR RINGWORM FUNGI

Tinea, or ringworm, is caused by three related genera of filamentous *Fungi imperfecti*, namely *Microsporum*, *Trichophyton* and *Epidermophyton*. These are dermatophytic fungi, having the unusual ability of digesting and utilising keratin, and being specialised for parasitising the keratinous structures of the body, i.e. the horny outer layer of the skin, the hairs and the nails. They do not invade the underlying living tissues which form the keratin, or the deeper tissues and organs of the body. The skin lesions are varied and may include inflammation, scaling, hyperkeratosis, vesiculation, pustulation, maceration or ulceration; secondary suppurative infection with pyogenic bacteria may ensue. In ringworm of the body, the spreading edge of the lesion is commonly seen as a red inflammatory ring (e.g. 5–50 mm. diam.) studded with vesicles and pustules, while the recovering central region is scaly and less reddened. Infected hairs become weakened and readily broken off, so that bald patches are produced; in some cases they show a characteristic green fluorescence under ultra-violet irradiation. Infected nails become deformed, discoloured, brittle and broken.

In their host the fungi occur in only two forms: (1) as a *vegetative mycelium* which grows through the keratinous structures, and (2) as chains of *arthrospores* formed by the septation of hyphae into short cylindrical or rounded segments which become widened and thick-walled. The arthrospores appear capable of infecting intact skin on which they are deposited, but more readily infect skin subjected to minor injury by rubbing, scratching or prolonged moistening. They germinate and give rise to hyphae which spread as a mycelium through the whole depth of the horny layer and extend radially into adjoining areas of skin. The mechanism whereby this superficial fungal growth causes

inflammation of the skin is uncertain. In hair-infecting species, the hyphae grow down the walls of the hair follicles which they encounter as they spread through the skin; they pass over into the shaft of the hair and grow in the newly keratinised zone just above the root bulb. After two to three weeks' hair-growth has carried the first infected part above the skin surface, this has become so weakened that it breaks off leaving a short (2 mm.) stump. Hyphae in skin, nail or hair eventually give rise to arthrospores. In hair, the arthrospore formation may be "endothrix", i.e. from hyphae within the hair shaft, or "ectothrix", i.e. from hyphae that have grown out over the surface of the hair. In the latter case, the affected part of the hair comes to bear a thick white coat of spores several layers deep. Some species form small arthrospores, of 2-3 μ diameter, and others form large spores of 4-6 μ (Fig. 34).

Immunologic and Hypersensitive Reactions.—The blood of patients with superficial dermatomycoses can not be shown to contain agglutinins, precipitins or complement-fixing antibodies. Nevertheless, patient's serum and normal human serum may

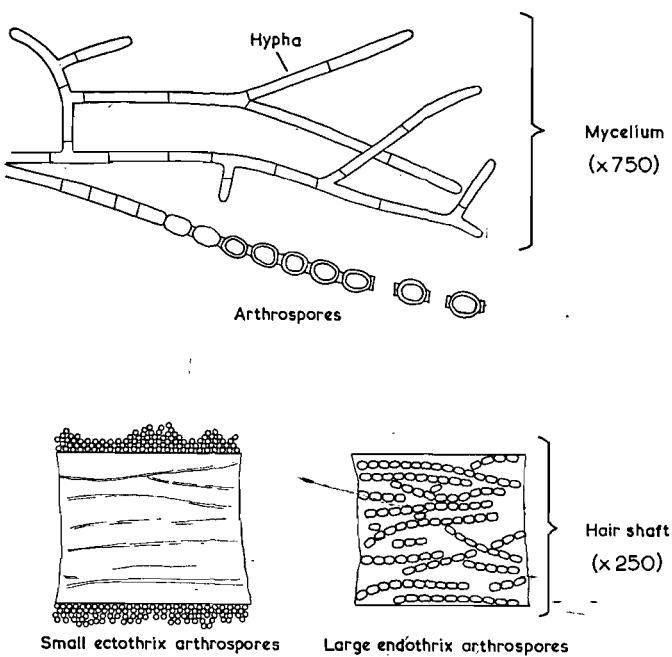


FIG. 34

Forms of ringworm fungi seen *in vivo* in skin, nail or hair.

contain fungistatic substances. Moreover, some kind of immunity mechanism appears operative in producing the eventual spontaneous healing of the affected parts. Many patients, especially those with *Trichophyton* infections, develop a widespread cutaneous hypersensitivity to the antigenic products of the fungus. In the course of the infection, secondary generalised skin eruptions of an allergic nature may occur, *i.e.* "dermatophytids" or "trichophytids", which are apparently due to blood-borne spread of fungal fragments or antigens. The commonest manifestation is a series of vesicles (pomphlox) on the fingers and hands, though other parts of the skin are sometimes also affected. No fungus is found in these allergic lesions. Evidence of hypersensitivity may be elicited by injecting extracts of the organism, *e.g.* trichophyton; this usually evokes an inflammatory reaction after twenty-four to forty-eight hours, like the tuberculin reaction (p. 543). The reactions are not species specific.

Species; Sources and Modes of Infection.—The various dermatophyte species differ in their adaptation to different regions of the body and to different animal hosts. Among species infecting man, some such as *Trichophyton mentagrophytes*, *T. interdigitale* and *T. sulphureum* can attack most regions: *e.g.* the scalp and hair (tinea capitis), the beard skin and hair (tinea barbae), the skin of the feet (tinea pedis), groin (tinea cruris) and other regions (tinea corporis) and the nails (tinea unguium). Other species are less versatile. *T. rubrum*, a common cause of chronic body, foot and nail ringworm, rarely affects the scalp. *Epidermophyton floccosum*, the commonest cause of tinea cruris, can infect other areas of skin, the feet and the nails, but does not infect the scalp. *Microsporum* species affect the scalp and sometimes other regions of skin, but not the nails. *M. audouini* is the commonest cause of epidemic scalp ringworm in children; it does not attack adults and infection usually disappears spontaneously at puberty.

The dermatophyte species are divided into those which are "anthropophilic", *i.e.* primarily parasites of man and rarely of animals, and those which are "zoophilic", *i.e.* primarily parasites of certain animals and occasionally infecting man from these. The anthropophilic species include *M. audouini*, *T. interdigitale*, *T. rubrum*, *T. sulphureum*, *T. violaceum*, *T. schoenleini* and *E. floccosum*. They tend to produce chronic lesions with slight tissue reaction, *e.g.* hyperkeratotic and non-inflammatory lesions, and sometimes wholly subclinical infections. *T. schoenleini* and *T. violaceum* are exceptional in causing favus. Favus occurs most commonly on the scalp. It arises from hair follicles as minute yellow-red papules; these grow to form cup-shaped yellow

"scutula", or crusts of mycelium, and ultimately destroy the involved hairs. The zoophilic species include *M. canis* (cat and dog), *M. gypseum* (horse and soil), *T. mentagrophytes* (cat, dog, mouse, ox, horse, soil), *T. verrucosum* (ox and horse), *T. quincke-anum* (mouse favus) and *M. equinum* and *T. equinum* (horse). When these fungi infect man (as do the first four), they tend to cause an acute inflammatory lesion (kerion) with vesiculation and maybe suppuration, easy to cure and often healing spontaneously.

Ringworm due to an anthropophilic species is contracted by man from another infected human, as in the epidemic spread of tinea capitis due to *M. audouini* and tinea pedis ("athlete's foot") due to *T. interdigitale* or *T. rubrum*. Infections by zoophilic species are contracted from infected pets, farm animals or vermin, and rarely from infected humans. It is thus important to identify the species causing an infection if an indication is required as to whether the source should be sought among human or animal contacts. Infected cats and dogs should be destroyed since they cannot be effectively treated. The fungus is spread by contact and through the air in the form of arthrospores. These are shed from the affected parts often in scales of skin, in fragments of hair or nail, and in finer debris. The arthrospores are highly resistant to environmental conditions; they may remain alive for years in infected premises and survive on clothing through many successive launderings. They can be killed by brief boiling. Formalin may be used for disinfecting shoes, socks and other articles.

Ringworm in Animals

Ringworm in dogs and cats is usually caused by *M. canis*, but other fungi, e.g. *M. gypseum*, *M. audouini*, *T. mentagrophytes* and *T. quincke-anum*, are sometimes responsible. Although cats frequently fail to show clinical symptoms, bare patches with broken hairs may be seen on the head, face and paws. In dogs the lesions are more obvious, circular bare areas appearing on any part of the body. Ringworm in cattle is especially prevalent in young animals, the commonest pathogen being *T. verrucosum*. The lesions vary from small circumscribed hairless areas to extensive white or yellowish-brown crusts on the head, neck or other parts of the body. Ringworm in the horse may be caused by species of *Microsporum* and *Trichophyton*, the commonest being *M. equinum*, *M. gypseum*, *T. mentagrophytes* and *T. equinum*. Typical lesions are circumscribed bare patches or plaques covered by a soft crust which is easily removed. Ringworm in sheep and goats is rare, but pigs are susceptible to infection with *T. mentagrophytes*. In favus of poultry, the lesions are mostly confined to the comb and wattles as dirty white crusts of fungus tissue. In more generalised infections the

base of the feathers and the skin may be involved. The causal fungus *T. gallinae* (*Achorion gallinae*) is transmissible to man.

Laboratory Diagnosis of Ringworm

The presence of fungus is demonstrated by direct microscopic examination of a specimen of the infected skin, hair or nail. Identification of the genus and species requires isolation of the organism in artificial culture. The specimen must be taken with care. A preliminary cleansing of the lesion with 70 per cent. alcohol reduces bacterial contamination. Scales of skin (e.g. 2-3 mm. diameter) are scraped with a blunt scalpel from the active periphery of the inflamed area, avoiding white macerated material in flexures. The domes of vesicles are snipped off for examination. The scales may be stored between sterile glass slides, and bacterial contaminants are partly eliminated after a few weeks of such storage. In the case of infected nails, scrapings are taken from friable or discoloured parts together with some of the underlying powdery material. Infected hairs must be chosen carefully. It is useless to take *healthy* hairs from the infected regions of skin. The stumps of broken hairs should be plucked with fine forceps, or else lustreless hairs may be taken. If the infecting fungus is *M. audouini*, *M. canis* or *T. schoenleinii*, the infected hairs may be recognised by their fluorescence under ultra-violet irradiation. The head is viewed in a darkened room under filtered ultra-violet radiation from a Wood's lamp. (A suitable lamp is a 125 watts ultra-violet black glass lamp, Type MBW/V, General Electric Co., with a choke for A.C. 230 volts, Type Z 1832, and condenser. By filtration through sodium-barium-silicate glass containing nickel oxide it yields only rays outside the visible spectrum with fungi not causing fluorescence.) Specimens for despatch to the laboratory are best sent in packages of clean folded paper.

Microscopic Examination of Skin, Nail and Hair.—The specimen must be "cleared", i.e. rendered transparent, to allow observation of the fungi within it. This is done by hydrolysing and partly dissolving the keratin with alkali. The fragments of skin, hair or nail are placed in a drop of 10-20 per cent. potassium or sodium hydroxide solution on a glass slide, and a cover-slip is applied. The preparation is left for a while at room temperature, or is warmed very gently to hasten digestion. Thin scales of skin may be cleared within several minutes at room temperature, while a piece of nail may require up to three hours at 37° C., the solution being replenished as necessary. Thick pieces of nail may be incubated with the alkali in a small tube. Excessive digestion

must be avoided. When ready, the cover-slip is squeezed down gently under blotting-paper to give a thin film without disruption of the specimen. The specimen is then examined microscopically in the unstained condition using a 4 mm. objective. Mycelium, chains of arthrospores and free arthrospores may be seen and their recognition permits a diagnosis of fungus infection. Artefacts may be mistaken for fungal elements by inexperienced observers; e.g. oil or fat droplets, air bubbles and spaces between epithelial cells. Thus, cholesterol crystals having the appearance of chains of spores, so-called "mosaic fungus", are often seen between the epithelial cells of skin specimens. These artifacts can be recognised by their failure to stain with lactophenol blue.

Staining is unnecessary for routine diagnostic work, but can be carried out if desired. The alkali is replaced by lactophenol blue stain (p. 138); a drop of stain is placed at one edge of the cover slip and the alkali is withdrawn at the other side by application of blotting-paper. The slide is warmed gently and excess stain is removed by pressing lightly under blotting-paper.

With specimens of hair, the size and arrangement of the arthrospores affords some indication of the variety of fungus. Thus, *Microsporum* species and *T. mentagrophytes* give small-spored ectothrix infections, *T. verrucosum* large-spored ectothrix infections, and *T. sulphureum* and *T. violaceum* large-spored endothrix infections. In favus due to *T. schoenleinii* the infection is endothrix and is further distinguished by the presence of air bubbles in the hair.

Culture.—In artificial culture the dermatophyte fungi produce a variety of spore-forms additional to the arthrospores found *in vivo*. These include small unicellular "microconidia" and large multicellular "macroconidia" (Fig. 35). The genera are distinguished according to the morphology of their macroconidia, and the species according to other microscopic features and the naked-eye morphology of their colonies.

Skin, hair and nail specimens are likely to bear contaminating bacteria; these may be reduced by storage between sterile slides for one or more weeks, or by immersion in 70 per cent. ethyl alcohol for two to three minutes. Since the dermatophytes are aerobic, surface cultures are grown on agar slopes in tubes or vials stoppered with cotton-wool. Fungal growth is favoured by a high sugar concentration and is relatively tolerant of acidity. Sugar-containing media of low pH are therefore selective for fungi and restrain the growth of most bacterial contaminants. Sabouraud's glucose peptone agar at pH 5·4 (p. 236) or malt extract agar at pH 5·4 (p. 237) are recommended. The malt agar may be made more

selective for fungi by addition of 0·036 per cent. potassium tellurite, and Sabouraud's medium may be supplemented with 10 mg. thiamine per l. to enable spore formation in *T. verrucosum*, *T. violaceum* and *T. schoenleini*.

The fragments of skin, hair or nail are planted with a firm straight-pointed wire. Nickel-chrome wire S.W.G. 18 is suitable for this and other manipulations of fungi. At intervals of about 1 cm., three or four fragments are pressed into the surface of the agar so as to be partly submerged. The cultures should be incubated at 25° C. for at least one week, or at room temperature for over two weeks. They are examined every two to three days for the appearance of growth round the edges of the implanted specimen. The fungal colony spreads outwards over the medium and usually extends beyond any contaminating bacterial growth. Material should be taken from near the edge of the colony and transplanted to a fresh slope in order to obtain a pure culture; a single speck of material is planted in the middle of the slope to yield a giant colony suitable for observation of naked-eye morphology.

The texture and pigmentation of the colony are observed on both its upper and lower sides. Most species form large, spreading filamentous colonies and the rhizoid hyphal outgrowth may give the appearance of radial striation (e.g. in *M. canis*). The filamentous structure of the vegetative mycelium is usually soon obscured by a velvety, cottony or powdery covering of aerial mycelium and spores. *T. verrucosum*, *T. violaceum* and *T. schoenleini* are exceptionally slow growing and form compact bacteria-like colonies which are at first smooth and waxy, and later become corrugated and covered with a sparse velvety growth of aerial mycelium. In most species the colonies ultimately develop some degree of pigmentation, commonly cream-pink, buff or yellow-green, though non-pigmented variant strains may be encountered. *T. rubrum* is distinguished by its formation of a red pigment (brown to rose-purple) which diffuses into the medium and may also colour the aerial mycelium. *T. violaceum* forms a deep violet pigment mainly confined to the colony. The colony of *T. sulphureum* changes from white to sulphur yellow as the spores are formed, and its centre eventually becomes folded and crateriform. White variants of this species have been termed *T. tonsurans*. The colony of *T. mentagrophytes* becomes covered with buff-coloured spores and forms a brown-red pigment which diffuses for a short distance into the agar. *Microsporum* colonies become covered with buff-coloured spores, while the underlying medium is tinted yellow-orange by *M. canis* and salmon-pink by

M. audouini. The colony of *E. floccosum* is green-brown and radially furrowed.

Microscopical Examination of Culture.—A "needle-mount" is made as soon as spore formation is sufficiently advanced at the centre of the colony. Some sporing mycelium is removed on the

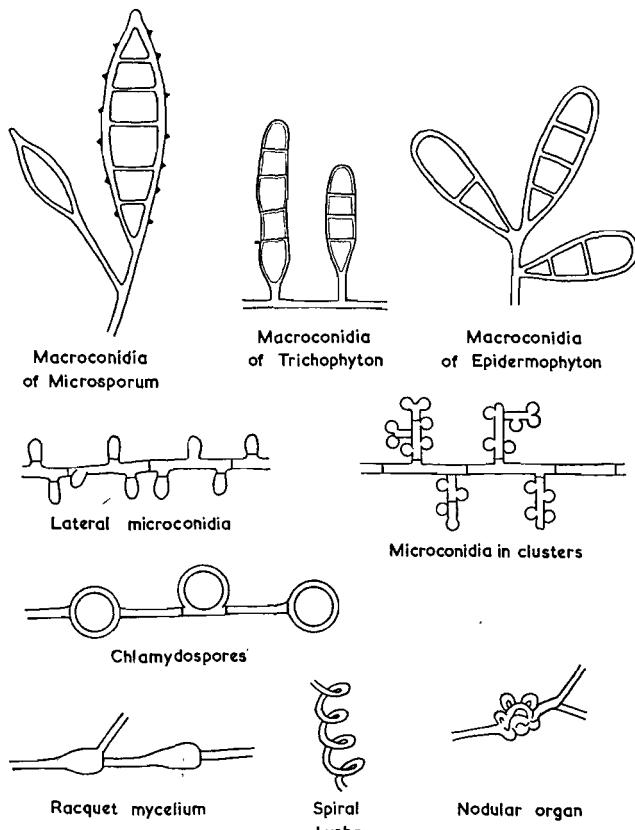


FIG. 35

Types of spores and abnormal mycelial forms found in artificial cultures of ringworm fungi. ($\times 600$.)

point of an inoculating wire and transferred into a drop of lactophenol blue stain (p. 138) on a microscope slide. There it is teased out using two wires or dissecting needles. A cover-slip is applied and excess stain removed after some minutes by pressing down gently under blotting-paper. The needle-mount is then examined microscopically, first with the low-power objective and then with the 4 mm. dry objective.

A search is first made for the presence of *macroconidia*. These are large, elongated bodies which are formed at the tips of certain hyphae and, when mature, are divided by visible transverse septa into several (*e.g.* 2–10) segments or cells. Those of *Microsporum* are largest, $40\text{--}150\ \mu \times 8\text{--}15\ \mu$, and fusiform with pointed ends. Those of *Trichophyton* are smallest, $10\text{--}50\ \mu \times 4\text{--}6\ \mu$, and generally cylindrical, though sometimes curved, with a rounded free end. Those of *Epidermophyton* are $30\text{--}40\ \mu \times 10\ \mu$, and pear-shaped with the narrow end attached to the conidiophore (Fig. 35).

Unfortunately, macroconidia are rare or absent in Sabouraud agar cultures of certain species, *e.g.* *M. audouini* and most *Trichophyton* species. The smaller *microconidia* are usually numerous, though they are rare or absent in *T. verrucosum*, *T. violaceum*, *T. schoenleinii* and *E. floccosum*. Microconidia are single cells of $2\text{--}6\ \mu$ diam., spherical, pear-shaped or elongated, and borne singly from the sides of the hyphae or in grape-like clusters on short stalks. Several species usually show a number of *chlamydospores* (*e.g.* *T. sulphureum*, *T. verrucosum*, *T. violaceum*, *T. schoenleinii* and *E. floccosum*); these are large, roughly spherical cells with very thick walls and are borne singly in the length of the vegetative hypha, bulging to one side or at its end.

Slide Culture by Riddell's Method.—The fungus may be subcultivated on an agar block held between a slide and cover-slip. This enables the arrangement of mycelium and spores to be observed undisturbed at various stages during the course of growth. Fill a Petri dish to the depth of about 2 mm. with Sabouraud's medium. When set, cut the agar into square blocks, 1 cm. \times 1 cm., with a sterile blade. Place an agar square on a sterile slide, inoculate a "needle-tip" of culture into the mid-point of each of its four edges and apply a sterile $\frac{7}{8}$ in. cover-slip to its upper surface. Place the preparation in an open rack within a large sealed jar containing some water with 20 per cent. glycerol at the bottom. The culture is thus well aerated in an atmosphere kept humid to prevent drying. Incubate at room temperature or at 25° C . Each day or two, remove the slide culture from the jar and, without disturbing the cover-slip, examine it microscopically with dry objectives.

A stained preparation is made when the growth is sufficient, *e.g.* 1–3 mm. wide. Remove the cover-slip and discard the agar block without unduly disturbing the rings of growth which adhere to the slide and cover-slip. Apply a drop of 95 per cent. ethyl alcohol to the growth on the slide (or cover-slip); just before this dries, add a drop of lactophenol blue stain and gently apply a cover-slip (or slide). After standing overnight, blot the excess

stain from the edges of the cover-slip and seal the preparation with cellulose lacquer.

OTHER FILAMENTOUS FUNGI CAUSING SUPERFICIAL INFECTIONS

Various other microbes cause superficial infections of man, invading the hair or the horny layer of the skin, though not necessarily digesting the keratin. They include the filamentous fungi causing otomycosis and piedra, the yeast-like organisms *Candida albicans* and *Malassezia furfur* (p. 900), the yeast *Pityrosporum ovale* (p. 897), and the bacteria *Nocardia minutissima* (causing erythrasma) and *Nocardia tenuis* (causing trichinocardiosis axillaris).

Aspergillus Species Causing Otomycosis

Fungal otomycosis is a subacute or chronic infection of the skin of the external auditory meatus and is commonly associated with bacterial infection. *Aspergillus niger* and *A. flavus* are the fungi most frequently present, but species of *Penicillium*, *Mucor* and *Rhizopus* occur in some cases. For diagnosis, the mycelium or conidiophore heads must be demonstrated in films of exudate mounted in 10–20 per cent. sodium hydroxide. The species may be identified by culture on Sabouraud's medium.

Piedra Fungi

Piedra is a trivial infection of hairs by filamentous fungi which grow on the hair shaft forming hard, gritty nodules. The nodules are dark in "black piedra" caused by *Piedraia hortai* and light in "white piedra" caused by *Trichosporon beigeli*. For diagnosis, a fragment of infected hair is mounted in 10–20 per cent. sodium hydroxide and, after softening of the nodules, gently crushed by pressing down the cover-slip. The nodules are seen to consist of tightly packed hyphae which are closely segmented into rectangular or oval cells with thick walls. In *P. hortai* the cells are 4–8 μ wide and ascospores containing 2–8 fusiform ascospores are also present. In *T. beigeli* the cells are 2–4 μ wide and ascospores are absent.

FILAMENTOUS FUNGI CAUSING DEEP INFECTIONS

Broncho-pulmonary Aspergillosis

Various normally saprophytic species of *Aspergillus*, *Penicillium*, *Mucor* and *Rhizopus* are found in infections of the bronchi and lungs. *Aspergillus fumigatus* is the most important of these. Birds, e.g. penguins, are especially susceptible to a primary and frequently fatal pulmonary aspergillosis. The infection may be acute, as in the commonly fatal Brooder pneumonia of chicks, or chronic, with the formation of yellow caseous nodules in the lungs.

A. fumigatus has also been isolated from mastitis and mycotic abortion in cattle; in the latter condition numerous hyphae are present in impression smears of the placenta and foetal stomach contents. In man, the fungal infection appears usually to supervene on a pre-existing pulmonary disease such as bronchiectasis; it occurs most frequently in agricultural workers and other persons who commonly inhale dusts of organic materials heavily contaminated with fungus spores. Diagnosis depends on a microscopical and cultural demonstration of the presence of abundant fungal elements in repeated specimens of sputum. The fungi are common contaminants in the home, hospital and laboratory, so that the occasional finding of a few fungal elements in sputum is of no diagnostic value. Since contaminating spores may germinate and the fungi overgrow other organisms, the examination of postal specimens or specimens stored at room temperature is usually worthless.

Fungi Causing Maduromycosis

Mycetoma or Madura foot of man (p. 665) can be produced not only by actinomycetes such as *Nocardia madurae*, *Noc. pelletieri* and *Streptomyces somaliensis*, but also by a variety of fungi including *Madurella mycetomi*, *M. grisei*, *Allescheria boydii* (*Monosporium apiospermum*), *Phialophora jeanselmei* and *Cephalosporium falciforme*. These fungi are thought to occur as saprophytes in the soil and on vegetable matter, and to enter the tissues of the foot through thorn pricks and other minor injuries. For diagnosis, some pus is obtained from a sinus or aspirated from an unopened abscess. It is spread thinly in a sterile Petri dish and searched for the presence of granules about 0·5–2·0 mm. in diameter. Granules are mounted on a slide in a drop of 10 per cent. sodium hydroxide, gently crushed under a cover-slip and examined without staining. In addition, granules are crushed between two slides and the slide preparations fixed by flaming and stained, one by Gram's method and the other by the modified Ziehl-Neelsen method, as in the diagnosis of actinomycosis (p. 660). The fungal granules consist of a mass of hyphae 2–5 μ wide and thus readily distinguishable from the 1 μ hyphae of actinomycetes. Chlamydospores may be present and the peripheral hyphae may bear "clubs" similar to those in actinomycotic granules. Cultures are grown on Sabouraud's glucose agar for at least three weeks at room temperature or at 22° C.; granules washed in sterile saline are inoculated on a number of slopes. *Monosporium apiospermum* grows rapidly as a white cottony colony in which ovoid conidia, about 6 \times 9 μ , are

borne singly at the ends of conidiophores. *Madurella* colonies grow slowly and are dark grey or black in colour.

Chromoblastomycosis in man is a chronic verrucous and granulomatous infection of the skin and subcutaneous tissue which extends slowly along the lymphatics. It occurs in North and South America, Russia, South Africa and Japan, and is caused by the filamentous fungi *Hormodendrum pedrosoi*, *H. compactum* and *Phialophora verrucosa*. These live saprophytically on wood and vegetation and are commonly introduced through the skin of the legs by an injury with wood. Pus or crusts from the warty lesions are examined in a wet film with 10 per cent. sodium hydroxide. The fungi appear as spherical, dark brown thick-walled cells, about $5-10\ \mu$ in diameter, occurring in small clusters and characteristically dividing by septation. Cultures are grown on Sabouraud's glucose agar for at least three weeks at 22°C . Brown-black or green-black mycelial colonies slowly develop. They contain hyphae $2-3\ \mu$ wide and characteristic conidiophores on aerial hyphae. The conidia are oval single cells, $1.5-3\ \mu \times 3-6\ \mu$, but more nearly spherical in *H. compactum*. *P. verrucosa* forms the conidia at the cup-like ends of flask-shaped conidiophores. *Hormodendrum* species produce these and also two other kinds of conidiophores, one forming the conidia in branching chains from its end and the other bearing the conidia around its sides.

Mucormycosis.—Disease of animals, and rarely man, may be caused by certain species of *Mucor*, *Absidia* and *Rhizopus*, the lesions being either granulomatous or ulcerative. In guinea-pigs, lesions may be produced in the mesenteric lymph nodes similar to those of pseudo-tuberculosis and in cattle and pigs granulomatous lesions may be produced in the lymph nodes, liver, lungs and kidneys. The ulcerative forms of mucormycosis with involvement of the gastric and intestinal mucosa are generally associated with scours and debilitating conditions of younger animals.

PATHOGENIC YEASTS

***Cryptococcus neoformans* (*Torula histolytica*)**

C. neoformans is a true yeast which reproduces by budding and does not give rise to a mycelium or pseudomycelium. It occurs as spherical cells, $5-20\ \mu$ in diameter, Gram-positive and surrounded by a very wide gelatinous capsule. It produces sporadic, commonly fatal infections (cryptococcosis or torulosis) in man, dog, cat, horse, ox and pig. The infections are subacute or chronic, and most commonly affect the meninges and brain, though sometimes the lungs or skin. In cattle, outbreaks of cryptococcal mastitis and metritis occur. Infection is not transmitted from man to man; its source is unknown, but may be endogenous, from animals and birds or from the soil. *Cryptococcus*

strains have been found on the skin and in the gut of healthy humans; these were culturally identical with *C. neoformans* but much less pathogenic for experimental animals (mice). The yeast withstands drying in soil and dust, and it is thought usually to enter the body by inhalation, but maybe sometimes through the skin or intestine.

Laboratory Diagnosis.—The specimens taken for examination may include cerebrospinal fluid (or its centrifuged deposit), sputum or pus from acneform skin lesions. These are observed microscopically, with condenser defocused, in an unstained wet film under a cover-slip and in a second wet film mixed with an equal volume of India ink (p. 122). The presence of budding, thick-walled, nearly spherical cells with capsules is diagnostic.

Cultures are made on Sabouraud's glucose agar at 37° C. and at 22° C., and on blood agar at 37° C., incubating for at least two weeks. Sputum and exudate contaminated with bacteria should be cultivated on the tellurite malt agar (p. 237) which is selective for the yeasts. Cultures are cream to light brown in colour, and become mucoid rapidly at 37° C. and slowly at 22° C. Before becoming mucoid, the colonies may be similar to those of *Staph. albus*. Films of the cultures show the capsulated yeast cells and no mycelium.

The pathogenicity of a strain can be demonstrated by injecting mice intraperitoneally with 0.5 ml. of a saline suspension of an infected exudate or a 1 per cent. (v/v) suspension of a pure culture. The mouse dies within three to four weeks with gelatinous masses of yeast in the abdomen and brain.

Pityrosporum ovale

P. ovale, or the "bottle bacillus", is a small Gram-positive yeast. It is a common commensal of normal skin and occurs on dandruff scales, being regarded by some authors as causative of dandruff and seborrhoeic dermatitis, though there is little evidence to support this belief. It is ovoid or flask-shaped, $1-2 \mu \times 2-4 \mu$, and reproduces by a process intermediate between budding and fission; the bud is separated from the parent cell by a septum formed across its relatively large base. The organism can be grown on nutrient agar smeared with butter fat which supplies oleic acid.

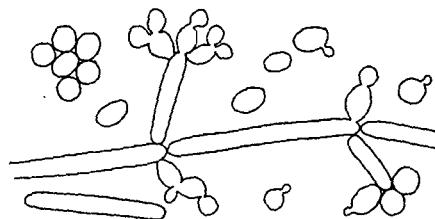
PATHOGENIC YEAST-LIKE FUNGI

Candida albicans (*Monilia albicans*)

C. albicans grows partly as spherical or oval yeast cells, $2-4 \mu$ in diameter, which reproduce by budding, and partly as a pseudo-

mycelium of non-branching filamentous cells which divide by constriction and give rise to yeast cells by budding from these division sites (Fig. 36). Both forms are thin walled, Gram-positive and non-capsulate. Some of the yeast cells form thick walls and become equivalent to chlamydospores.

The organism is a common cause of various acute and subacute infections (candidiasis or moniliasis) in man, animals and birds. Other species of *Candida*, e.g. *C. krusei*, *C. parakrusei*, *C. tropicalis*, *C. pseudotropicalis* and *C. guilliermondii*, occur as commensals or saprophytes, and rarely cause disease. *C. albicans* produces superficial infections of the skin and mucous membranes, and occasionally deep infections such as meningitis, endocarditis and septicaemia. Infection is generally dependent on a weakening of the



Candida albicans: hyphae forming a pseudomycelium
and giving rise to budding yeast-like cells
(blastospores) (x 1000)

FIG. 36

body defence mechanisms, e.g. by diabetes, leukaemia, neonatal debility, senility, local maceration of skin or suppression of the normal bacterial flora of mucosae with antibiotic drugs. Skin infection, inflammatory, exudative and desquamative, is generally localised in damp folds, e.g. axilla, groin, interdigital clefts and the napkin area of infants; it may involve the nails and lead to chronic paronychia and onychia. Oral thrush is especially common in infants, white patches of exudate developing over a raw, inflamed mucous membrane. Vaginal thrush occurs commonly in pregnancy, remitting spontaneously after parturition, and in diabetes. Bronchial and pulmonary candidiasis may occur, but *C. albicans* is more commonly present in the respiratory tract as a secondary invader, e.g. in tuberculosis and bronchiectasis.

Avian moniliasis is an infection of the upper alimentary canal of chickens, turkeys and pigeons, the lesions being mostly confined to the crop and proventriculus. Although mostly of sporadic occurrence, epidemics of "thrush" with an average mortality of over 60 per cent.

have occurred, the species responsible being *Candida albicans* and *C. krusei*. Moniliasis has occurred in pigs, probably as a result of feeding antibiotic-supplemented food, while in cattle thrush-like lesions have been observed in the rumen and intestinal mucosa. A variety of *Candida* species have been associated with mycotic mastitis of cattle and goats.

Source of Infection.—*C. albicans* is a common commensal of man and animals, occurring in the mouth, throat, intestine, vagina and skin; it occurs in the faeces of 20–30 per cent. of healthy persons. Infection is thus commonly endogenous. Infants, however, may be infected from their mothers or from other infants during an epidemic in a maternity hospital. Artificially fed babies are more often affected than breast fed babies. Unless aseptic precautions are taken, infection may be spread among pregnant women by the obstetrician's gloved hand.

Laboratory Diagnosis.—Since *C. albicans* commonly occurs as a commensal in the same situations as it causes infection, the mere demonstration of its presence is not proof of infection. This diagnosis requires demonstration that the organism is abundantly present on several occasions, and also the exclusion of other possible causative agents. Specimens must be examined with minimum delay to avoid multiplication of originally scanty contaminating yeasts.

Sputum and exudate from mucosae should be examined microscopically in Gram-stained smears and in unstained wet films. Skin and nail scrapings are examined in a wet film with 10–20 per cent. sodium hydroxide. Budding yeast cells mixed with long filaments are indicative of a yeast-like fungus.

For culture, exudate may be collected on swabs soaked in Sabouraud broth. The cultures are grown on plates of blood agar at 37° C. and on Sabouraud's glucose agar at 37° C. and at 22° C. Specimens of faeces or sputum should be cultivated on the selective tellurite malt agar (p. 237) or penicillin-streptomycin blood agar (p. 237) to control the growth of bacterial contaminants. In two to three days, large creamy bacteria-like colonies develop on the Sabouraud's medium and smaller grey colonies on blood agar. The culture, which has a characteristic odour, is examined in unstained wet films for budding yeast forms. A pure culture is isolated and tested as follows to enable species identification.

(1) A wet film with India ink is examined to confirm the absence of capsules. (2) A deep-streak culture is grown at 22° C. in plates of corn meal agar (p. 237) to encourage the development of pseudomycelium and chlamydospores. A stiff wire tip bearing the inoculum is cut in strokes through the substance of the agar. As

growth proceeds it is observed *in situ* using a microscope with the low power objective. The pseudomycelium of *C. albicans* forms clusters of budding yeast cells and, at the tips of certain hyphae, large spherical thick-walled chlamydospores. Other *Candida* species do not form chlamydospores. (3) Sugar fermentation reactions are tested by culture at 37° C. for forty-eight hours in broth containing 3 per cent. of sugar. *C. albicans* ferments glucose and maltose to give acid and gas, sucrose to acid only and fails to ferment lactose; *C. tropicalis* forms acid and gas from glucose, maltose and sucrose; *C. pseudotropicalis* from glucose, sucrose and lactose; *C. krusei* and *C. parakrusei* from glucose only; and *C. guilliermondi* ferments none of these sugars. (4) Pathogenicity is demonstrated by injecting a rabbit intravenously with 1 ml. of a 1 per cent. suspension of the culture in saline; the animal dies in four to five days and small abscesses are seen throughout the cortex of its kidneys.

Immunology.—Agglutinating antibodies are found in the blood of many patients. However, they are found also in normal persons, so that their demonstration is of little diagnostic value. The various species of *Candida* are closely related antigenically.

Malassezia furfur.—This is a yeast-like fungus which causes the superficial skin infection known as pityriasis versicolor, a chronic asymptomatic infection producing irregular brownish and desquamating macules. Diagnosis is confirmed by microscopic examination of skin scales mounted in 10–20 per cent. sodium hydroxide; the fungus is seen as clusters of round, budding thick-walled cells, 3–8 μ in diameter, together with short fragments of mycelium. It is doubtful whether *M. furfur* can be grown on artificial culture medium.

DIMORPHIC FUNGI CAUSING DEEP INFECTIONS

Sporotrichum schenckii

This dimorphic fungus is the cause of sporotrichosis in man, horses, cats, dogs, rats and other animals. The infection is chronic, giving firm nodules in the subcutaneous tissue with subsequent inflammation and ulceration of the overlying skin, and spread along the draining lymphatics. Blood-borne dissemination is rare, but when it occurs is often fatal.

Source of Infection.—The fungus is primarily a saprophyte which grows on dead plant material and wood. It can also infect living plants, e.g. berberis, and is found in the soil and in dust on the coats of animals. Man and animals are infected from such contaminated materials through an injury to the skin, e.g. by an

infected thorn or splinter of wood. Infection is most frequent in farm workers, gardeners and manual labourers. Men are sometimes infected by contact with infected animals, and occasionally by an animal bite or sting, but natural spread from man to man has not been recorded.

Laboratory Diagnosis.—The fungus occurs in the yeast form (parasitic phase) when in animal tissues. However, *it can rarely be seen in exudates and tissue sections from human lesions*, so that direct microscopic examination of these is of no positive diagnostic value.

In artificial culture, the fungus grows in the yeast form at 37° C. and in the mycelial form (saprophytic phase) at 22° C. Pus swabbed from an ulcer or, preferably, aspirated from an unruptured abscess, is heavily inoculated on to blood agar and Sabouraud's glucose agar. After two to three days at 37° C. on blood agar, the colonies are moist, wrinkled and cream-coloured, and consist of Gram-positive budding yeast cells which are fusiform, cigar-like or oval in shape, and about 3–5 μ in length. After three to five days at 22° C. on Sabouraud's medium, the colonies are small, white and waxy; later, they become wrinkled, membranous and pigmented cream, brown or black, but never develop a cottony aerial mycelium. These colonies are composed of a mycelium (2–3 μ diameter) bearing pear-shaped conidia (3–6 μ long) which radiate from the tips of hyphae as a cluster or project from their sides as a sheath.

Animal inoculation may be attempted. A suspension of pus or culture is injected intraperitoneally into mice. The animals develop peritonitis and the fusiform and oval yeast cells may be demonstrated both extracellularly and intracellularly in macrophages.

Blastomyces dermatitidis

This dimorphic fungus is the cause of North American blastomycosis, a chronic suppurative and granulomatous infection of man. *Cutaneous blastomycosis* is usually a primary infection of the skin; there is papule and pustule formation followed by ulceration and a slow peripheral spread in the skin, but no systemic dissemination. *Systemic blastomycosis* usually results from a primary infection of the lungs with subsequent blood-borne dissemination to the skin, subcutaneous tissues, bones, kidneys, brain and other organs. Natural infections also occur in dogs and horses.

Source of Infection.—This is unknown. The fungus has not been found as a saprophyte in the soil or elsewhere, and apparently does

not spread from man to man or from animal to man. It presumably enters the body by inoculation through the skin or by inhalation into the lungs.

Laboratory Diagnosis.—Pus or tissue scrapings are examined microscopically in an unstained wet film; this may be mounted with 10 per cent. sodium hydroxide if the specimen requires to be rendered more transparent. When in tissues, the fungus occurs in the yeast form (parasitic phase) and is seen as thick-walled spherical cells which are 8–15 μ in diameter, occur either free or within phagocytes, and on budding bear only single buds.

In artificial culture the fungus grows in the yeast form at 37° C. and in the mycelial form (saprophytic phase) at 22° C. Growth is slow, taking one to three weeks at either temperature. On blood agar at 37° C. it forms wrinkled, creamy or waxy colonies consisting of budding yeast cells as seen in tissues. On Sabouraud's glucose agar at 22° C. the colonies are initially like those of the yeast form, but eventually become covered with a white cottony aerial mycelium which later turns brown. The 22° C. colony comprises a mycelium of broad, thick-walled and closely septate hyphae, later developing many round or oval conidia, 3–5 μ in diameter, and eventually many smooth and thick-walled chlamydospores, 7–18 μ in diameter.

If pus or culture is inoculated intraperitoneally in mice, the budding yeast forms are later seen in the peritoneal exudate.

Paracoccidioides brasiliensis

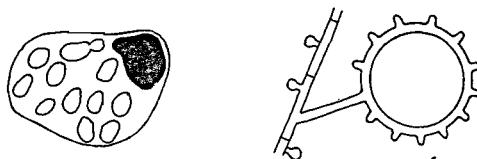
This dimorphic fungus causes South American blastomycosis, a chronic granulomatous infection of the skin, mucosae, lymph nodes and internal organs. The source of infection is unknown. It occurs chiefly in manual workers in rural areas and does not spread from man to man.

Pus or tissue examined in a wet film with sodium hydroxide shows thick-walled yeast cells, 10–60 μ in diameter; some of these bear single buds, as in *B. dermatitidis*, but others bear multiple buds radiating from their surface. After several days on blood agar at 37° C. the fungus forms moist wrinkled creamy colonies of yeast cells. In two to three weeks on Sabouraud's agar at 22° C. it forms white wrinkled cottony colonies which later turn brown; these consist of a septate mycelium bearing a few round or oval conidia. Pus and yeast-form cultures are pathogenic when inoculated intraperitoneally in mice; in five to six weeks, nodules containing budding yeast cells are found in the mesentery.

Histoplasma capsulatum

This dimorphic fungus is the cause of histoplasmosis, a granulomatous disease which occurs in man either as a primary benign, usually asymptomatic infection of the lungs, or, rarely, as a progressive, usually fatal systemic infection involving the reticulo-endothelial tissues; in the latter case the primary infection is often extra-pulmonary, e.g. in the skin, upper respiratory tract, mouth or intestine. The disease is endemic in the Mississippi valley, but sporadic cases occur throughout the world. Natural infections also occur in cows, horses, dogs, cats, rodents and other animals.

Source of Infection.—This fungus is found in the soil of endemic areas, but whether it grows in the soil as a saprophyte or enters the soil from small infected animals, is uncertain. Infection seems



Histoplasma capsulatum: left, yeast-like cells in macrophage; right, mycelium from culture bearing microconidia and a tuberculate chlamydospore. (x1000)

FIG. 37

mainly to occur by inhalation of infected soil dust. It does not spread from man to man.

Laboratory Diagnosis.—The fungus occurs as the yeast form when in the tissues. Characteristically it is an *intracellular parasite of reticulo-endothelial macrophages*. Smears of sputum, blood, sternal bone marrow, lymph node pulp and mucosal lesion scrapings are stained by Giemsa's stain. Numerous capsule oval yeast cells, 1–5 μ in diameter, are seen within the macrophages and a very few extracellularly (Fig. 37, left).

Sputum, gastric washings and other clinical specimens are cultivated on blood agar at 37° C. (or glucose blood agar containing penicillin and streptomycin to control bacterial contaminants, p. 237) and Sabouraud's glucose agar at 22° C. On blood agar at 37° C., the colonies are moist, wrinkled and creamy, and consist of budding yeast cells. On Sabouraud's medium at 22° C., they are slow growing, cottony in appearance due to development of an aerial mycelium, white at first and later turning brownish;

they consist of septate mycelium bearing small lateral conidia (2–3 μ in diameter) and eventually form large round "tuberculate" chlamydospores, 7–15 μ . in diameter; the latter are characterised by a covering of small rounded tubercles projecting from the outer surface of the thick spore wall and they are diagnostic of *H. capsulatum* (Fig. 37, right).

The fungus is pathogenic to laboratory animals. Mice injected intraperitoneally with infected material or culture die after varying periods of time.

Immune and Hypersensitive Reactions.—Within a few weeks of first becoming infected, the patients develop complement-fixing antibodies in their blood and become hypersensitive to *H. capsulatum* antigens. Hypersensitivity is demonstrated by injecting intradermally 0·1 ml. of a 1 in 100 dilution of a standardised preparation "histoplasmin"; this gives rise in twenty-four to forty-eight hours to an area of induration more than 0·5 cm. in diameter. The significance of a positive histoplasmin reaction is analogous to that of a positive tuberculin reaction, indicating present or past infection. Partial cross-reactions may be given with *Blastomyces* and *Coccidioides* antigens, which should therefore be tested simultaneously for quantitative comparison.

Histoplasma farciminosum is the causal agent of epizootic lymphangitis of horses and other equidae. This is a chronic infection of the subcutaneous lymphatics with abscess formation, discharges from which contain numerous yeast-like organisms surrounded by a thick cell-wall or capsule.

Coccidioides immitis

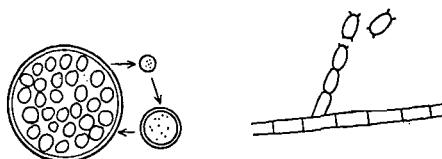
This dimorphic fungus is the cause of coccidioidomycosis, a granulomatous disease affecting man either as a primary benign, often asymptomatic infection of the lungs or, occasionally, as a progressive infection which disseminates to the lungs, skin, bones, meninges and other organs, and usually proves fatal. Coccidioidomycosis is endemic in the arid south-west of the United States of America, and also occurs endemically or sporadically in various other parts of the world. Natural infections occur in rodents, dogs, cows and sheep in endemic areas.

Source of Infection.—The fungus occurs in the soil in the endemic localities, perhaps being a soil saprophyte or perhaps being derived from infected animals. Infection probably occurs in most cases by infected soil dust being inhaled into the lungs or introduced through injured skin. It does not appear to spread from man to man or from animals to man. *The arthrospheres*

formed in artificial cultures readily become airborne and are highly infective, so that infection is liable to occur in laboratory workers unless special precautions are taken.

Laboratory Diagnosis.—The fungus occurs in the tissues as non-budding, spherical thick-walled sporangia. These are 20–70 μ in diameter and contain numerous small spherical endospores (2–5 μ diam.). The spores are liberated by rupture of the sporangium and subsequently themselves develop into sporangia (Fig. 38, left). Sputum and other infective materials from patients are examined in wet films with 10 per cent. sodium hydroxide in order to demonstrate these forms.

When infected materials are cultivated on blood agar at 37° C. and on Sabouraud's glucose agar at 22° C., the growth is mycelial



Coccidioides immitis: left, mature spherule containing endospores and immature spherules as found in tissues; right, mycelium segmenting to form arthrospores in culture. (x500)

FIG. 38

in both cases. The colonies are whitish, at first moist and membranous, but later becoming cottony with the development of an abundant aerial mycelium. The hyphae segment into arthrospores (about $3 \times 4 \mu$) which become very thick-walled and are highly infective (Fig. 38, right).

Cultures and infected exudates are pathogenic for mice and cause demonstrable infection one to two weeks after intraperitoneal injection.

Immune and Hypersensitive Reactions.—Precipitins and complement-fixing antibodies appear in the patient's blood in severe primary infections. Hypersensitivity to *C. immitis* antigens appears during the first or second week of severe or mild illness. Intradermal injection of 0.1 ml. of a 1 in 100 dilution of standardised "coccidioidin" gives rise to an area of erythema and induration of at least 0.5 cm. in diameter. Patients with disseminated coccidioidomycosis usually fail to give a reaction. A positive coccidioidin reaction has a significance analogous to that of a positive tuberculin reaction.

Rhinosporidium seeberi

Rhinosporidiosis is a chronic polyp-forming infection of the submucous tissue of the nose, eyes, ears and larynx, and occasionally of the genitalia and skin. It is commonest in India and occurs sporadically in man, horses and cows. The source of infection is unknown, though persons swimming in rivers and stagnant water are frequently infected. The causative organism, *Rhinosporidium seeberi*, has not been cultured, but its appearance in tissues suggests that it is a fungus. For microscopic diagnosis, material is taken from a polyp and squeezed in water between a slide and cover-slip. Free thick-walled spherical spores about $5-7\ \mu$ in diameter are seen and various stages of their growth into spherical sporangia of $200-300\ \mu$ diameter. The sporangia contain thousands of spores and eventually release these by bursting at a pore.

CHAPTER XLV

PROTOZOA

MALARIA PLASMODIA; BABESIAE; TRYPANOSOMES; LEISHMANIAE; INTESTINAL PROTOZOA

THE MALARIA PLASMODIA

MALARIA is a protozoal disease of man in which the causative organism—*Plasmodium*—invades the red cells of the blood. Analogous diseases occur in certain animals and birds, e.g. “monkey-malaria”, “bird-malaria”.

Four well-defined species of the malaria plasmodium are recognised:

Plasmodium vivax—Benign tertian malaria.

Plasmodium malariae—Quartan malaria.

Plasmodium falciparum (*Laverania falciparum*)—Malignant malaria.

Plasmodium ovale—Some cases of malaria in Africa.

These organisms belong to the order Haemosporidia of the Sporozoa. The plasmodium is transmitted by female anopheline mosquitoes, and goes through the sexual cycle of its life cycle in the body of the insect. Only the asexual cycle is completed in the human subject.

Some features of the organism can be observed in unstained preparations, but most of the important characters are seen in preparations stained with one of the Romanowsky stains, e.g. Leishman's.

Outline of the Life History of the Malaria Plasmodium.—It is introduced into the body by the bite of the mosquito (*vide infra*) as a minute spindle-shaped motile cell or sporozoite, containing nuclear material in the form of a chromatin granule. The sporozoite rapidly leaves the general circulation and invades tissue cells to commence the *pre-erythrocytic* phase of the asexual cycle. In these cells the sporozoites develop into large schizonts which divide to form merozoites and these are liberated when the host's cell bursts. Some merozoites initiate the *exo-erythrocytic* phase by parasitising other tissue cells; others invade red cells to start the *erythrocytic* phase and enter the circulation (Fig. 39). In the

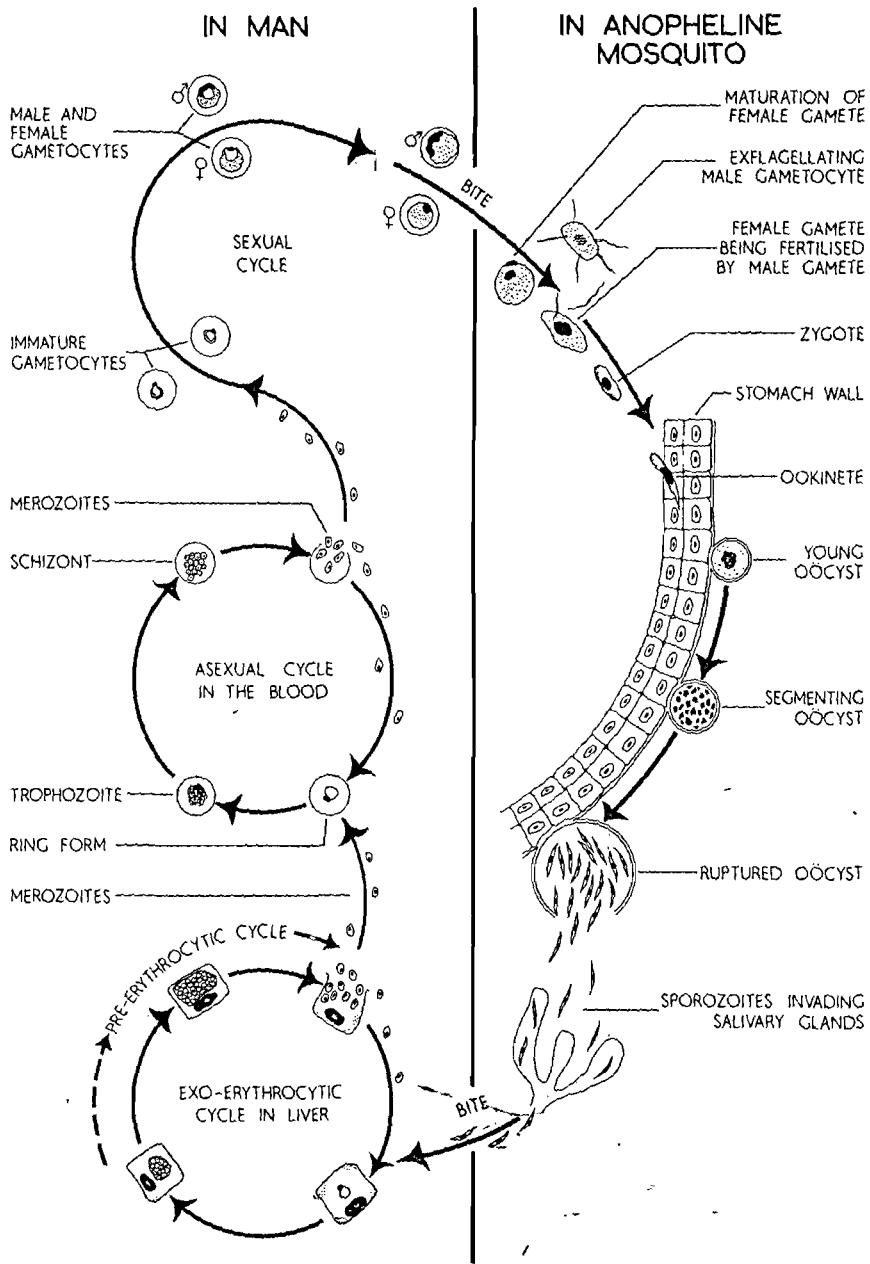


FIG. 39

Reproductive cycles of the malarial parasite *Plasmodium vivax*.¹

¹ Modified from Blacklock and Southwell *A Guide to Human Parasitology*, 6th Edition (1958). London, Lewis.

red cells the merozoites grow at the expense of the cells to form a trophozoite and altered blood pigment accumulates as brownish granules. It may display amoeboid movement and protrude pseudopodia. More than one trophozoite may attack a single corpuscle. The corpuscle may show considerable alteration in size as the trophozoite develops. The trophozoite tends to assume a characteristic ring-form due to the formation of a vacuole-like structure, with the chromatin granule at one side, so that the whole organism resembles a signet-ring. When full-grown it is more or less rounded, and may appear to occupy the greater part of the corpuscle. These mature trophozoites usually contain a considerable amount of blood pigment.

When fully developed, the trophozoite becomes a schizont, and schizogony takes place. The pigment accumulates towards the centre, the chromatin breaks up into smaller particles, the protoplasm subdivides and forms a number of small round or oval merozoites (about 2μ in diameter) each containing a fragment of the original chromatin. The residual protoplasm and pigment remain in the centre of the group of merozoites. The number of merozoites resulting from schizogony varies with the different species. Finally the individual merozoites are liberated as free structures in the blood. The merozoite attacks another red cell and thus the asexual cycle is repeated. The pigment is taken up by leucocytes.

In *Plasmodium vivax* infections exo-erythrocytic schizogony carries on in parallel with erythrocytic development and frequently persists for long periods after the parasite has left the peripheral blood. This may lead to relapse as erythrocytic invasion is always liable to occur. In *Plasmodium falciparum* infections exo-erythrocytic schizogony does not persist along with erythrocytic development so that once the erythrocytic parasite has been eradicated relapse does not occur.

The length of time the organism takes to complete the asexual cycle varies with the species:

<i>Plasmodium vivax</i>	Two days.
<i>Plasmodium malariae</i>	Three days.
<i>Plasmodium falciparum</i>	One or two days.

The fever develops at the stage of schizogony; thus in *Plasmodium vivax* infections the febrile paroxysm occurs every second day (tertian malaria), in *Plasmodium malariae* infections every third day (quartan malaria).¹

¹ The terms "tertian" and "quartan" are derived from the occurrence of the successive attacks on the third and fourth days respectively, counting the day of the previous attack as the first.

In malignant malaria the parasite leaves the peripheral blood before it becomes a mature trophozoite, and schizogony occurs, as a rule, only in the blood of internal organs.

Differentiation of the Malaria Plasmodia

	P. vivax (Benign tertian)	P. malariae (Quartan)	P. falciparum (Malignant)
Asexual life-cycle	48 hours	72 hours	24 to 48 hours
Trophozoites in fresh unstained preparations of blood . . .	Not refractile, hyaline, not easily observed; usually one organism only in red cell; active movement	Refractile, "frosted-glass" appearance; more easily observed; less amoeboid and active	Small, about $\frac{1}{6}$ or $\frac{1}{8}$ of diameter of red cell; often more than one organism in cell; active at first
Pigment in trophozoites . . .	Fine, yellowish-brown, and evenly distributed	Coarse, brownish-black	Scanty, fine
Ring forms (stained preparations) . . .	Large, irregular, not well defined, about $\frac{1}{3}$ diameter of red cell; usually single chromatin granule	Thick round rings, about $\frac{1}{6}$ diameter of red cell; often in the form of equatorial bands	Small, multiple, thin; often 2 chromatin granules; often situated at the edge of red cell; about $\frac{1}{5}$ or $\frac{1}{6}$ diameter of red cell
Red cells (stained preparations) . . .	Swollen, pale, showing deeply stained rose to purple points, "Schüffner's dots"	Not altered	Shrivelled, deeper colour, but may be swollen and pale; may have a few cleft-like irregular purple dots, "Maurer's dots",
Schizonts (stained preparations) . . .	Large, mulberry-like; about same size as red cell; 12-25 oval merozoites	Small, "daisy-head" - like; smaller than red cell; 6-16 round merozoites	Small; segmentation irregular; 8-32 (usually 12) very small merozoites; rarely seen in peripheral blood
Gametocytes . . .	Rounded; macro-gametocyte about one and a half times the size of red cell	Rounded; about the same size as red cell or smaller	Elongated, crescentic or sausage-shaped

While some of the organisms develop into schizonts, others become gametocytes. In the case of *Plasmodium vivax* and

Plasmodium malariae, these are rounded and about the same size as a mature trophozoite. The gametocytes of *Plasmodium falciparum* are sausage-shaped or crescentic, with the envelope of the corpuscle stretched across the poles of the crescent. Male and female gametocytes are distinguished, and designated respectively micro- and macro-gametocytes, the former generally smaller than the latter. The female form of *Plasmodium falciparum* is narrower than the male. The micro-gametocyte, as compared with the macro-gametocyte, contains a nuclear structure which is relatively large, diffuse and often disposed across the body in the form of a spindle. The protoplasm stains faintly and the pigment is diffuse. In the macro-gametocyte the protoplasm stains dark blue, the nucleus is small, compact and often peripheral in situation; but in the female gametocyte of *Plasmodium falciparum* the nucleus is situated centrally with the pigment accumulated round it.

These gametocytes remain unchanged in the blood until it is withdrawn from the body, e.g. by the mosquito or when a drop is exposed to air and transferred to a warm stage for microscopic observation. In the stomach of the mosquito the following changes occur. The gametocytes of the crescent type become rounded. The female gametocytes undergo "maturation" by the formation of one or two "polar bodies" which contain part of the original nuclear chromatin, and are protruded and detached. The mature macro-gametocyte constitutes the macro-gamete. From the male cell, four to eight flagella-like structures are quickly protruded ("exflagellation"); these are long, slender processes with somewhat enlarged free ends, each containing a chromatin granule derived from the parent nucleus, and are the micro-gametes; they are ultimately detached, and move with a kind of lashing motility. A micro- and macro-gamete unite and form the zygote which develops movement (oökinete), elongates slightly and penetrates the stomach wall, embedding itself under the outer layer or between muscle fibres; it becomes spheroidal, forms an encysting membrane (oöcyst) and increases in size until it projects into the body cavity; division into rounded sporoblasts (or sporoblastoids) occurs, and these divide again into the spindle-shaped sporozoites, thousands of which are formed from the original oöcyst. The cyst ultimately ruptures and the sporozoites are set free in the body cavity and settle in the salivary gland, from which they are injected with the salivary secretion when the insect bites. This phase in the life history is that of sporogony as contrasted with the asexual schizogony in the human subject; it takes seven to ten days (under favourable temperature conditions).

Plasmodium ovale resembles *Plasmodium malariae*, but the

erythrocytes are often markedly enlarged, oval in shape, show an irregular outline and exhibit to a marked degree the stippling (Schüffner's dots) as seen in the benign tertian form of malaria. The schizont comprises six to twelve merozoites. The illness is tertian in periodicity. The infection has been transmitted experimentally by the bite of *Anopheles maculipennis*.

Certain other supposed sub-species of *Plasmodium* have also been described.

Diagnosis of Malaria

THIN BLOOD FILMS.—Two or three films are made on microscope slides or $\frac{3}{4}$ -in. square No. 1 cover-slips, which have been carefully cleansed (p. 107) and polished with a smooth cloth. Slides are generally preferred for the purpose as they are more easily handled than cover-slips, though with the necessary skill better blood films can be made on cover-slips.

Requisites.—Straight Hagedorn needle, slides or cover-slips, spirit lamp or Bunsen, gauze, industrial alcohol.

Films on Slides.—The blood is obtained by puncturing the lobe of the ear, or the finger close to the base of the nail. The needle is sterilised by flaming or by some other efficient process and the

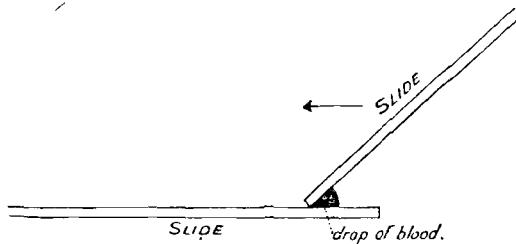


FIG. 40

area to be punctured is cleansed with 70 per cent. alcohol or 90 per cent. isopropyl alcohol. Touch the exuding drop of blood with the surface of a slide close to one end; place the narrow edge of a second slide, kept at an angle of 45° , on the drop of blood and allow the blood to spread out across the slide in this angle before drawing out the film; now spread the blood uniformly on the slide in the form of a thin film (diagram).

Films on Cover-slips.—Touch the drop of blood (obtained as above) with the surface of a cover-slip held by the edges between the thumb and first finger of one hand, and place a second cover-slip over the first so that the drop spreads out between them. Then at once take the second slip by the edges between the thumb

and forefinger of the other hand, and slide the two apart without exerting pressure.

The films are allowed to dry, and are then fixed and stained with Leishman's stain. They are mounted and then examined, first with a dry $\frac{1}{8}$ -in. lens, and later with the oil-immersion objective. In searching for crescents it is advisable to use the former. The $\frac{1}{7}$ -in. oil-immersion lens is particularly useful for this purpose.

It is essential that the film should be well stained, otherwise it is useless searching for the parasite. A valuable guide is the staining of the leucocytes in the film—if this is satisfactory, malaria parasites should be detectable.

THICK BLOOD FILMS.—As a routine measure, and especially when the organisms are likely to be scanty, thick films should be prepared and examined. A large drop of blood is deposited on a slide, and spread with the head of a pin in the form of a thick film about $\frac{3}{4}$ in. in diameter, the density being such that the hands of a watch can just be seen through the film; it is thoroughly dried, *e.g.* in the incubator. The unfixed film can be stained by Field's stain (p. 134) or for thirty minutes in Leishman's stain diluted one part in twenty with distilled water buffered at pH 7.2. Dilute Giemsa's stain may also be used. Alternatively the haemoglobin is removed by treating with acid-alcohol (alcohol 50 ml., hydrochloric acid 10 drops) or a mixture of 4 parts of 2.5 per cent. glacial acetic acid in distilled water and 1 part of 2 per cent. crystalline tartaric acid in distilled water, and then washing in water. The film can now be stained by Leishman's or Giemsa's stains.

FRESH PREPARATIONS OF BLOOD FOR MICROSCOPIC EXAMINATION.—A drop of blood is deposited on a slide, covered with a cover-slip, and the edges of the glass are smeared with vaseline to prevent drying of the film. A warm-stage apparatus should be used during the microscopic examination.

Only stained films are examined as a rule in routine work. They should, if possible, be taken during the pyrexia and no anti-malarial drugs should have been administered beforehand. The organisms can be recognised by their various characteristic appearances, and it is possible to determine the species or type present (Table, p. 910). It must be remembered, however, that the young trophozoites of the three types may be almost indistinguishable from one another, and if only young forms are present in the film, it may be difficult to determine the species. To inexperienced workers, artificial appearances may sometimes simulate malaria parasites, and a blood platelet overlying a red corpuscle may be mistaken for a young form of the plasmodium.

In certain cases prolonged search may be required. It is advisable, in searching thin films for scanty malaria organisms, to examine particularly the edges of the film. They may be more numerous there than in the centre. The thick-film method greatly facilitates the detection of the parasite. The absence of parasites during an apyrexial interval by no means excludes malaria and the repeated examination of films may be required before the diagnosis can be established.

Malaria organisms can sometimes be detected in *films from bone marrow aspirated by sternal puncture*, and in some cases this method may be used for diagnostic purposes.

BABESIAE (or PIROPLASMS)

These protozoal organisms (classified with the Sporozoa) produce disease in various domestic animals, but are not known to infect the human subject.

They invade red blood corpuscles like the malaria plasmodia, and multiple infection of these cells is characteristic. The individual organisms are generally pyriform bodies about $2\text{ }\mu$ to $4\text{ }\mu$ in length, containing a well-defined chromatin structure. The central part of the organism often stains less deeply than the periphery, and ring-forms like those of the malaria parasite may be observed. Some species show small rod-shaped forms. Multiplication occurs by binary fission, and pairs of individuals partially attached to one another may be seen inside the red cells. For microscopic demonstration, blood films are stained by a Romanowsky stain, e.g. Leishman's or Giemsa's. These organisms are usually transmitted by ticks, of which the eggs may become infected. For detailed information regarding the biology and life-cycle of these organisms, one of the works on protozoology should be consulted.

Babesia bigemina is the causative organism of Red Water Fever of cattle in North and South America, Africa and Australia. It is transmitted by ticks (e.g. *Margaropus annulatus*). It occurs in characteristic pairs in the red cells. The individuals are pear-shaped with the pointed ends in apposition.

Babesia canis is the organism of Biliary Fever, or tick fever, of dogs. In the blood the babesiae are seen both in the red cells and plasma. The intracellular forms are pear-shaped or irregular, and several may be found in one corpuscle. The free forms are rounded.

Organisms resembling *Babesia bigemina* occur in Red Water Fever of cattle in Europe (*Babesia bovis*), Biliary Fever of horses (*Babesia equi*) and haemoglobinuria of sheep (*Babesia ovis*).

Babesia (or *Theileria*) *parva* of East African Coast Fever in cattle appears as a small rod-shaped body inside red cells. Ring-forms may also be observed. *Babesia* (or *Theileria*) *mutans* is a somewhat similar organism producing a relatively mild infection of cattle in South Africa.

An organism designated *Anaplasma*, originally thought to be related to *Babesia*, has been described in Gall Sickness of cattle in South Africa. As seen in blood films stained by Giemsa's stain, it appears as a minute rounded body situated near the margin of the red cell (*Anaplasma marginale*), in size about one-tenth of the diameter of the cell. It is described as possessing a central granule. Such structures may possibly be related to *Bartonella*.

TRYPANOSOMES

Protozoa of the class Mastigophora. Infection with these organisms is designated by the general term Trypanosomiasis.

Three species which are pathogenic to man have been described: *Trypanosoma gambiense* and *Trypanosoma rhodesiense* of African Sleeping Sickness, and *Trypanosoma cruzi* of Brazilian trypanosomiasis. It is doubtful whether the first two are separate species. The last-named is sometimes classified in a separate genus, *Schizotrypanum*.

Trypanosoma gambiense

Biological Characters.—This organism is an elongated, sinuous, fusiform structure, 12–40 μ long by 1.5–3 μ broad, with a longitudinal undulating membrane, and a flagellum projecting from one end. It is motile and, in moving, the flagellum is anterior. In stained preparations two nuclear structures are noted, the larger or trophonucleus situated about the middle of the organism, and the smaller micro- or kineto-nucleus (or kinetoplast) at the posterior end. The latter stains deeply and is surrounded by an unstained halo. Two constituent structures have been distinguished in the micronucleus: a granule (blepharoplast) from which the axoneme arises (*vide infra*) and the "parabasal" body. Chromatin-like granules are seen in the protoplasm independently of the nuclei. From the blepharoplast arises a filament, the axoneme, which forms the free edge of the undulating membrane and is continued into the flagellum, forming its central core. This structure stains like chromatin. Morphological variation (polymorphism) is noted among individual organisms, some being relatively long and slender with long "free" flagella, other shorter

and broader and with a short flagellum or lacking a free flagellum (Fig. 41). Intermediate forms are also observed.

Multiplication is by longitudinal fission.

Occurrence.—The infection is transmitted by the bite of *Glossina palpalis* (*Tsetse fly*) and in certain areas by *Glossina tachinoides*, *Glossina morsitans* and *Glossina pallidipes* (in Uganda). Certain wild animals are reservoirs of infection, e.g. the antelope, but the disease is essentially transmitted from person to person. Shortly after blood from an infected person is ingested by the

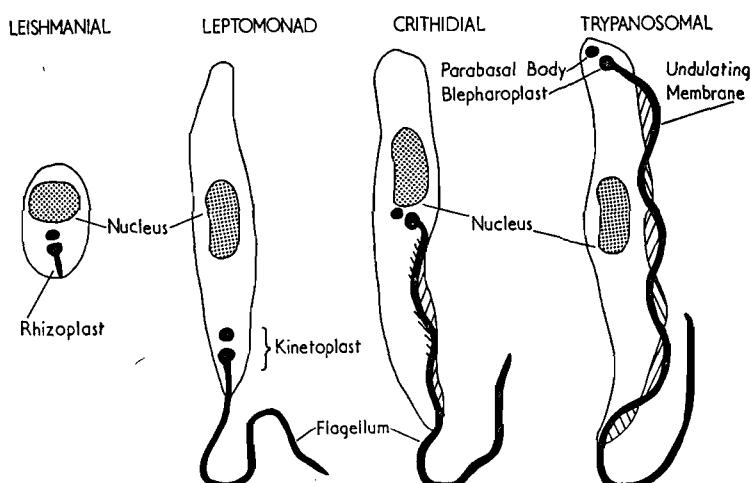


FIG. 41
Forms of Trypanosomidae.

insect, transmission is possible in a mechanical fashion. A later stage of infectivity occurs after about twenty days; the trypanosomes have multiplied in the intestine and passed to the proventriculus, the salivary gland and proboscis. Multiplying in the insect, the organism may show considerable change of form. Thus the undulating membrane may be lost, the kinetonucleus becomes situated just in front of the macronucleus, and a free flagellum may or may not be present (crithidial form). From these crithidia in the salivary gland the so-called "metacyclic" forms develop, i.e. resembling the short and broad trypanosomes seen in the blood (*vide supra*). The fly does not become infective until the metacyclic forms are present in the salivary gland.

Two or three weeks after infection a febrile condition develops,

and trypanosomes are present in the blood, though not as a rule sufficiently numerous to be readily demonstrable. The superficial lymph glands, e.g. posterior cervical, become enlarged, and trypanosomes can be demonstrated by puncture and aspiration with a syringe. In the advanced stages of the disease, when the characteristic lethargy has developed, the parasites can be detected in the cerebro-spinal fluid.

Experimental Inoculation.—Monkeys injected with infective material develop a disease which is more or less similar to human trypanosomiasis.

Guinea-pigs can be infected, and trypanosomes appear in considerable numbers in the blood, but the infection is either unassociated with any obvious pathological condition, or the resulting disease is very chronic in its course.

Cultivation of Trypanosomes.—p. 921.

Trypanosoma rhodesiense

Associated often with a more acute form of Sleeping Sickness than that produced by *Tryp. gambiense*.

Glossina morsitans is the insect vector.

Morphologically it resembles *Tryp. gambiense*, but, *in an inoculated animal*, a certain number of the trypanosomes (about 5 per cent.) show the trophonucleus situated posteriorly near the kinetonucleus ("posterior nucleated" forms).

This organism is also stated to be more virulent to laboratory animals. The question whether or not it represents a separate species is still unsettled, and it has been pointed out that *Tryp. gambiense* may also exhibit posterior nucleated forms in inoculated animals, though this is infrequent.

It has been supposed that this organism may be identical with *Tryp. brucei*, the organism of Nagana, but it has been shown by inoculation of man that *Tryp. brucei* is not pathogenic to the human subject.

Trypanosoma (or Schizotrypanum) cruzi

The cause of human trypanosomiasis in Brazil (Chagas' disease).

Its first development after infection occurs in the endothelial and tissue cells of internal organs, in the muscles and in the heart wall. It is non-flagellate at first, and resembles *Leishmania* (*vide infra*). It may, however, appear in the blood as a typical flagellate trypanosome with a very prominent kinetonucleus.

Certain other vertebrate hosts harbour the organism, e.g. the armadillo and opossum.

It is transmitted by Reduviid bugs, e.g. *Panstrongylus megistus* (syn. *Conorhinus megistus*). These bugs are readily infected experimentally, unlike *Glossina* flies with African trypanosomes, and may be used in laboratory diagnosis of *Tryp. cruzi* infections by allowing them to feed on suspect cases, and recovering the trypanosome from their droppings (xenodiagnosis).

Various laboratory animals are susceptible to experimental inoculation, e.g. guinea-pigs, white rats, monkeys.

Diagnosis of Trypanosome Infection

In the first place the peripheral blood should be examined. As trypanosomes may be scanty, especially in *Tryp. gambiense* infection, "thick films" are prepared and stained by Leishman's stain as in malaria diagnosis (*q.v.*). Fresh preparations of the blood may also be examined microscopically.

A method of concentrating trypanosomes in the blood has been applied as follows: 5–10 ml. of blood are withdrawn from a vein into 20 ml. of 1 per cent. sodium citrate solution, and the mixture is centrifuged for about ten minutes; the plasma and the leucocyte layer on the surface of the blood sediment are withdrawn and re-centrifuged; this is repeated two or three times; the deposit is examined, in the form of fresh preparations and stained films, after each centrifuging.

If superficial lymph glands are enlarged, puncture and aspiration with a syringe may be carried out, and the "juice" examined. The syringe should be perfectly dry if this procedure is to be successful.

Blood, gland "juice" or an emulsion of an excised gland injected into a guinea-pig may yield a positive diagnosis where other methods fail. The blood of the animal is examined, in fresh preparations of stained films, at intervals after the inoculation.

In the lethargic state, 10 ml. of cerebro-spinal fluid are withdrawn, and centrifuged for fifteen to twenty minutes; the deposit is then examined either in the form of a fresh preparation under a cover-slip ringed with vaseline, or in stained films.

Trypanosomiasis of Animals

Trypanosome infections occur in a variety of animals. Different species of trypanosomes are recognised. These have the general

characters of the genus as described in the case of *Tryp. gambiense*, but show variations in certain of their characters.

Tryp. brucei is the organism of Nagana or Tsetse Fly Disease occurring in horses, other equidae, dogs and cattle in Africa; it is transmitted by *Glossina morsitans* and certain other species of *Glossina*. It is sluggish in movement and corresponds in its morphology with *Tryp. rhodesiense* (*vide supra*), showing posterior nucleated forms.

Tryp. vivax, so called in virtue of its active movement, and *Tryp. congolense* produce infections in domesticated animals in Africa and are transmitted by tsetse flies. *Tryp. vivax* possesses a "free" flagellum. *Tryp. congolense* is a smaller organism (9–18 μ long) and has no free flagellum.

Tryp. equinum is the organism of Mal de Caderas, a South American disease of horses. A feature of the organism is the inconspicuous kinetonucleus which stains feebly.

Tryp. evansi is the organism of Surra affecting domesticated animals (including camels) in various parts of the world. It is actively motile, with a free flagellum and a blunt posterior end. It is conveyed mechanically by *Stomoxys* and Tabanid flies.

Tryp. equiperdum occurs in horses, producing the disease known as Dourine; the infection is transmitted by coitus. The organism shows a free flagellum and is very similar to *Tryp. evansi*.

Tryp. lewisi is an exceedingly common blood parasite of rats, and is world-wide in distribution. It is an actively motile, narrow trypanosome, with a pointed posterior end and anteriorly a free flagellum. The kinetonucleus is rod-shaped and stains deeply. It produces little disturbance in the health of the host. Infection is transmitted by rat fleas.

Tryp. theileri occurs in cattle in South Africa. It is unusually large (25–70 μ in length). The flagellum is free. It is possibly transmitted by a Tabanid fly. The pathogenicity of this organism is doubtful.

For further details of the pathogenic trypanosomes one of the larger works on bacteriology or protozoology should be consulted.

LEISHMANIAE

These are pathogenic protozoa with certain biological relationships to the trypanosomes.

Leishmania donovani—of kala-azar, occurring predominantly in children in countries in North Africa, and in both children and adults in parts of East Africa, the Indian sub-continent and the Far East.

Leishmania tropica—of Tropical Sore or Delhi Boil. This is generally regarded as a separate species.

Leishmania braziliensis—of Espundia (or Uta), occurring in South America. This disease resembles, in some respects, Tropical Sore, but affects specially the nasal and pharyngeal mucous membranes.

Leishmania donovani

In morphology it is a round or oval organism about $2\text{--}5 \mu$ in its longest diameter. Sometimes in films from the blood in the spleen and bone marrow, torpedo-shaped forms are seen.

Stained with a Romanowsky stain, two nuclear structures are observed, one large and rounded (macronucleus), and the other small, deeply staining, and rod-shaped (kinetoplast). As in the trypanosomes, this latter structure consists of a parabasal body and a prolongation corresponding to the axoneme (rhizoplast). The protoplasm may be vacuolated (*vide supra*). The organism multiplies by binary fission.

Leishmania donovani is typically intracellular in the tissues, situated in the endothelial cells of the spleen, liver, bone marrow and lymphatic glands. It may also be found, though infrequently, in large mononuclear cells in the peripheral blood. One endothelial cell may contain a considerable number of organisms.

Cultures can be obtained from the spleen on N.N.N. medium incubated at $20^{\circ}\text{--}24^{\circ}$ C. In culture the organisms increase in size and elongate; the kinetoplast becomes situated at one end, and from it a flagellum arises. No undulating membrane develops. Thus, *Leishmania* in culture assumes the biological characters of a *Leptomonas*.

Monkeys and dogs can be infected experimentally. The Chinese hamster (*Cricetus griseus*) is susceptible to inoculation and has been used for experimental studies.

Transmission of the disease is by insect agency. The evidence points to the sand-fly, *Phlebotomus argentipes*, as the vector of infection in India.

When the insect ingests infected blood the organisms become transformed to leptomonads in the gut of the vector and undergo multiplication, ultimately migrating to the buccal cavity from which they are inoculated when the insect bites another person.

In certain parts of the world human leishmaniasis is associated with the same infection among dogs which may act as reservoirs of the disease.

Diagnosis of Kala-azar

*Gland Puncture.*¹—Gland " juice " is aspirated from the inguinal or femoral lymphatic glands; films are made and stained with Leishman's stain as in malaria diagnosis. This method gives excellent results in a large proportion of cases and is without risk to the patient.

Bone-marrow Aspiration (from the sternum or tibia).—Films are prepared from the marrow and stained as above.

Spleen Puncture.—Fluid is aspirated from the enlarged spleen with a fine hypodermic needle attached to a dry syringe and films are prepared as above. The procedure is not without risk in inexperienced hands and liver puncture is preferred.

Culture.—Aspirated spleen or gland " juice " is cultured on N.N.N. medium.

Blood Films (Thick).—These are prepared as in malaria diagnosis. This method is sometimes successful, especially in India, but is too unreliable for routine diagnosis.

Any one of the above methods may be negative while others are positive and, for tests of cure, as well as diagnosis, it is therefore advisable to use more than one method.

Experimental inoculation—in monkeys or hamsters—with the material obtained by spleen, gland, liver or bone-marrow puncture, though of value for experimental work cannot be regarded as a practical method for routine purposes.

Leucopenia is invariable in uncomplicated kala-azar and there is a relative lymphocytosis.

The *aldehyde reaction* has also been used in the diagnosis of the disease. For this purpose about 5 ml. of blood are withdrawn and allowed to clot. The serum is separated and to 1 ml. are added 2 drops of commercial formalin. A positive reaction is indicated by an immediate opacity, followed within thirty minutes by the development of a firm white gelatinous coagulum (like boiled egg albumin); mere jellification is not accepted as a positive result. This method is commonly used in India, but it has been found unreliable in the diagnosis of kala-azar in other countries.

The *complement-fixation test* is sometimes of definite value; the antigen can be prepared from cultures of the organism, but a similar reaction is apparently obtained with an " antigen " prepared from cultures of an acid-fast bacillus (Sen Gupta).²

¹ Kirk, R., & Sati, M. H. (1939-1940), *Trans. roy. Soc. trop. Med. Hyg.*, 33, 501.

² Sen Gupta (1945), *Ind. med. Gaz.*, 80, 896.

Leishmania tropica

This organism is similar to *Leishmania donovani* and shows the same intracellular distribution. Besides the characteristic oval forms, elongated organisms may be noted. In culture on N.N.N. medium leptomonas forms develop as in the case of *Leishmania donovani*. Monkeys and dogs can be infected experimentally. Transmission is probably by *Phlebotomus papatasii* and *Phlebotomus sergenti*.

Diagnosis.—Films are made from the scrapings from the sore (preferably at the margin) after carefully cleansing the surface and removing the surface discharge. They are stained by Leishman's stain.

THE INTESTINAL PROTOZOA**Entamoeba histolytica**

This is the causative organism of amoebic dysentery and extra intestinal amoebiasis, an infection which occurs mainly in tropical and subtropical countries. The incubation period is measurable in weeks rather than days, in contrast to bacillary dysentery. The onset of symptoms is more gradual and the character and frequency of the stools differ from those of bacillary dysentery.

Biological Characters.—There are two "types" or races of amoeba—the small (average size 9–15 μ) and the large (average size 18–30 μ). The vegetative forms are rounded, elongated or irregular amoebae. The cytoplasm consists of a clear hyaline ectoplasm, and a granular, often vacuolated endoplasm, but this differentiation is not always readily observed. In their most active condition the amoebae show flowing movements of their protoplasm and rapidly protrude and retract pseudopodia, which may be composed at first mostly of ectoplasm. These movements lead to changes in shape and also to active progression, often likened to the motion of a snail. The nucleus is round or oval, and in the unstained condition is not easily distinguished. It is situated in the endoplasm, usually excentric in position. It is poor in chromatin, and the nuclear membrane is thin. The chromatin granules are small, and are collected in a ring just inside the nuclear membrane. The nucleus shows a small central karyosome. The amoebae ingest red corpuscles, leucocytes and tissue cells, which are observed in the endoplasm, but ingested bacteria are less frequently found. The ingested erythrocytes appear smaller than normal. The vegetative forms after leaving

the body tend to become rounded and immobile, and soon die and disintegrate. Multiplication is by mitotic binary fission.

Under conditions unfavourable to the amoebae, e.g. when the disease is becoming arrested, encystment occurs. Cysts are spherical, with a thin, hyaline, refractile cyst wall, which gives them a distinct double contour. The contents are finely granular. The average diameter is 6–11 μ for the small race and 11–18 μ for the large. The cysts usually contain multiple nuclei, *not more than four*, a glycogen mass, and also thick rod-shaped or oval structures which stain deeply with haematoxylin and are called "chromatoid bodies" or "chromidial bars" (see Fig. 42).

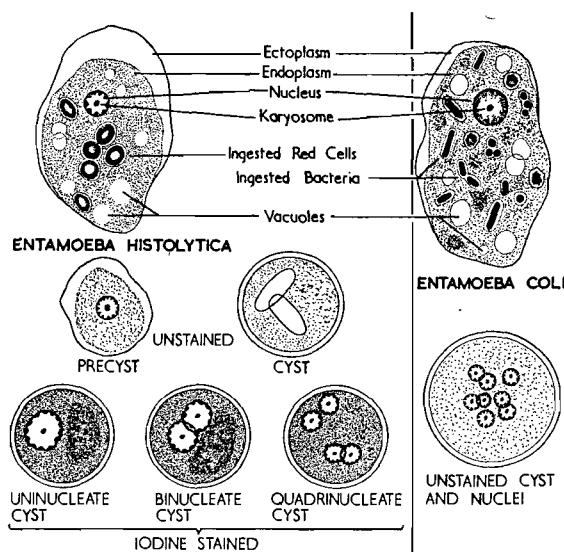


FIG. 42

Stages in the development of *Entamoeba histolytica* and *Entamoeba coli*.

The cysts are developed by division of the vegetative form into smaller and rounded "precystic" forms.

The newly formed cyst has only one nucleus, which later divides into two, with further division to four. The glycogen mass is best seen in young cysts, staining brown with iodine, but is apparently used up as the cyst matures. In unstained preparations the chromatoid bodies appear as refractile structures.

Methods of microscopic demonstration and staining are referred to under dysentery diagnosis.

Occurrence.—In the early stage of amoebic dysentery the vegetative forms are present in considerable numbers in the large intestine and in the stools. They penetrate the mucosa of the large bowel and disintegrate the tissue by their pseudopodia and possibly also by means of a liquefying ferment. The submucosa is invaded, and, occasionally, small veins are penetrated from which the amoebae may be carried to the liver. In the bowel, oval or irregular ulcers are developed with undermined edges, which may sometimes lead to perforation of the bowel wall. There is little inflammatory reaction (*cf.* bacillary dysentery) unless a secondary infection occurs.

The so-called "tropical abscess" results from invasion of the liver, probably through the portal circulation. The abscess contains a slimy chocolate-coloured "pus" consisting of necrotic tissue and altered blood, with relatively few leucocytes or pus cells. The amoebae are found mainly in the wall of the abscess, and may not be present in the pus when first evacuated.

Cysts may be detected in the stools, often in large numbers in chronic cases. After apparent recovery the patient may remain a carrier, and the encysted forms are passed in the faeces. The cyst represents a resting phase with increased powers of resistance and can survive outside the body for some time. This is the form in which the organism is transmitted from person to person, but cysts are infective only when in the quadrinucleate state.

Healthy carriers with no previous history of dysentery are common as shown by cysts of *Ent. histolytica* in their stools. Frequently, however, these strains can be shown to be avirulent, while strains from persons with amoebiasis are always virulent. The relationship between virulent and avirulent strains is unknown.

Cultivation.—This organism can be cultivated artificially by the method of Boeck and Drbohlav or a modification of their method (p. 241).

Laboratory Diagnosis of Amoebic Dysentery

Collection of specimens of stools.—The stool should be examined as soon as possible after being passed and while still warm. They should be unmixed with urine. The specimen for examination may be collected in a faeces specimen tube provided with a cork carrying a metal spoon or scoop which fits into the tube, and by means of which faecal matter may be collected. A satisfactory alternative is the Universal container. The chances of finding motile amoebae are greatly enhanced when special care is taken

to select for microscopic examination a portion of the blood-stained mucus which is so characteristic of the faeces in the acute phase of the infection.

Microscopic Examination.—A microscope slide is gently warmed over the Bunsen flame, and on the middle of one half of the slide a large drop of normal saline solution is placed and, on the other, a drop of Lugol's iodine (p. 103). A loopful of the mucous discharge or the stool is emulsified in the saline drop and another loopful in the iodine solution. (A preparation in 1 per cent. watery eosin also assists in the detection of protozoa.) If specks of blood are observed in the specimen, these should be examined. The preparations are covered with No. 1 cover-slips, and examined first with the low-power objective and then with the $\frac{1}{8}$ -in. and, if necessary, the oil-immersion lens. It is advantageous to use a "warm-stage" attached to the microscope in examining fresh preparations for amoebae (p. 95). Phase-contrast microscopy is specially applicable for demonstrating cytological features of the protozoa.

Vegetative amoebae can usually be recognised without difficulty. In the saline preparation, *Entamoeba histolytica* may often be identified by its active characteristic, amoeboid movement and the inclusion in the cytoplasm of numerous red corpuscles. On the other hand, immobile vegetative amoebae without ingested corpuscles present considerable difficulty in their identification. Large phagocytic cells (macrophages) may be found in dysenteric stools, and may be mistaken for immobile amoebae by inexperienced workers. They often show vacuolation, and may even contain red corpuscles. They are practically immobile, and the nucleus, unless degenerate, occupying one-fourth or one-fifth of the whole cell, is definitely larger than that of an amoeba, and is not of the ring-like or "vesicular" type. This distinction is seen in the iodine preparation. In a heat-fixed film these macrophage cells and their nuclei can be stained with methylene blue, while amoebae cannot thus be demonstrated.

The differences in the morphology of protozoal cysts as seen in iodine-stained preparations makes it possible to diagnose amoebic dysentery in the absence of motile amoebae. It must, however, be emphasised that considerable experience is necessary before the laboratory worker can distinguish pathogenic from non-pathogenic cysts, and that it is often a difficult matter to make this diagnosis with certainty.

Where pathogenic amoebae cannot be detected, the microscopic examination often yields information of diagnostic importance. In a case of bacillary infection there is usually an abundant

and characteristic cellular exudate. The cells present are mostly polymorph leucocytes with a varying number of red cells, and in the early stages, numerous epithelial cells. In addition to these, macrophages are frequently a characteristic feature of the exudate. The leucocytes, as a rule, show marked degeneration.

In amoebic dysentery there are few leucocytes, unless the case is also complicated by a bacterial infection. Charcot-Leyden crystals are frequently seen microscopically in amoebic dysentery and are absent in bacillary dysentery.

The microscopic examination is therefore an important step in diagnosis: the finding of the characteristic *Ent. histolytica* establishes a diagnosis of amoebic dysentery, while an abundant cell exudate and the absence of amoebae would indicate bacillary dysentery.

This preliminary determination enables a report to be made at once as to the nature of the dysentery, so that treatment can be initiated without delay.

Where no amoebae can be found, and if a diagnosis of bacillary dysentery cannot be established, it is essential that further microscopic examinations be carried out before amoebic infection is excluded.

*Demonstration of Amoebic Cysts by the Flotation Method.*¹—A dense, but finely divided, watery suspension of faeces is prepared in a mortar and then strained through previously wetted wire gauze. The suspension is centrifuged for five minutes at 2000 to 3000 r.p.m. in a conical tube. The contents of the tube except the lowest inch of deposit are discarded and to the residue zinc sulphate solution of sp. gr. 1.25 is added in fractions, thorough mixing being effected by stirring with a glass rod. The mixture is finally centrifuged for three minutes. At this stage the cysts float to the surface and are concentrated there. A loopful of the surface scum is removed and mixed on a slide with a loopful of Lugol's iodine, a cover-slip is superimposed and the preparation is examined microscopically.

Stained preparations are of assistance in the identification of intestinal amoebae. Films are made on cover-slips from the stool and are fixed "wet" by floating the cover-slips (film downwards) in a fixing solution consisting of 2 parts saturated perchloride of mercury in saline, with 1 part absolute alcohol. They are then stained with iron-haematoxylin (p. 137).

An alternative method is that of Dobell, in which the preparation is mordanted with ammonium molybdate and then stained with an aqueous solution of haematoxylin (p. 138).

¹ Faust, E. C., et al (1939). *J. Parasit.*, 25, 241.

Non-pathogenic amoebae

Entamoeba coli.—A non-pathogenic intestinal amoeba which, in diagnosis, must be carefully differentiated from *Ent. histolytica*.

The vegetative forms closely resemble those of *Ent. histolytica*, but the cytoplasm is not so distinctly differentiated into endo- and ecto-plasm. The pseudopodia are small and blunt and not so refractile as those of *Ent. histolytica*. The nucleus is usually central in position, easily distinguishable, rich in chromatin which is sometimes arranged in quadrant form, and has a thick, refractile, nuclear membrane. The karyosome is well marked. Amoeboid movement is sluggish. It has been generally agreed that no ingested red cells are seen in the cytoplasm (when this organism is noted in a case of dysentery). It has been pointed out, however, that *in vitro* this organism can ingest red cells as readily as *Ent. histolytica*. Bacteria are ingested often in large numbers. The cysts are larger (15–30 μ) than those of *Ent. histolytica*, the cyst wall is thick, and there may be more than four nuclei, e.g. frequently eight. No bar-shaped chromatoid bodies are observed in the fully developed cysts which occur in the faeces.

Endolimax nana.—A frequent non-pathogenic intestinal amoeba. The vegetative form is 10 μ in diameter or less. In unstained preparations the nucleus is not distinct, but when stained by haematoxylin it is easily demonstrated, and shows a large, irregular, excentric karyosome.

The cysts are oval, and about the same size as the vegetative form. They contain one, two or four small nuclei, but no chromatoid bodies.

Iodamoeba bütschlii and *Dientamoeba fragilis* are also included among the intestinal amoebae of man, but need not be described here. Their characters may be ascertained by reference to works on protozoology.¹

Entamoeba dispar.—This designation has been given to an entamoeba which closely resembles small forms of *Ent. histolytica*. Thus, the encysted forms may be quadrinucleate. It is regarded as non-pathogenic. There is some doubt whether it constitutes a species distinct from *Ent. histolytica* or is a non-virulent form of this organism.

Entamoeba gingivalis.—This organism occurs in considerable numbers in pathological conditions of the mouth, e.g. pyorrhoea, gingivitis, dental caries, but has no definite aetiological relationship

¹ See *Protozoology* (1926). London, Wenyon; *Handbook of Medical Protozoology* (1949). London, Hoare.

to these conditions. It is about 10–20 μ in diameter and resembles *Ent. histolytica* in many respects, showing active amoeboid movement and differentiation of the cytoplasm into ecto- and endoplasm; the nucleus is indistinct in unstained preparations; the organism possesses the property of ingesting free cells, e.g. leucocytes.

Balantidium coli

Balantidium coli is a ciliated protozoon and is the only member of the class *Ciliophora* pathogenic to man. It can cause ulceration of the intestinal wall with symptoms of colitis, diarrhoea or dysentery (balantidial dysentery). The vegetative form is larger than other human intestinal protozoa measuring 60 by 40 μ . The body is uniformly covered with short cilia and at the anterior end is a groove leading to a mouth opening. There is a nucleus and the cytoplasm contains vacuoles. Encystation occurs with the formation of a thick-walled cyst and the cysts may remain alive for weeks in moist faeces. When swallowed the cyst germinates in the intestine, setting free vegetative ciliates.

Balantidiosis is common among pigs who are the natural host and probably form the source of infection for man. The disease may occur in temperate climes, particularly among persons who come into contact with pigs.

Intestinal Flagellates

These organisms are often associated with dysentery and diarrhoea, particularly in the tropics, but their pathogenicity is doubtful, and they may occur as commensals.

Trichomonas hominis.—Is pear-shaped, 9–15 μ long, and shows a nucleus and cytostome. It possesses three to five flagella projecting from the broad end, and also another flagellum forming the border of an undulating membrane and with the free part projecting from the pointed posterior end.

An organism which is biologically similar to *Trich. hominis* may occur in the vagina, and has been named *Trich. vaginalis*. It may be found in cases of vaginitis, and there is a considerable body of evidence, mostly clinical, in support of its pathogenicity. For recognition of *Trich. vaginalis* and its characteristic jerky movements, "wet" preparations of vaginal secretion should be examined first with low magnification and then with the $\frac{1}{8}$ -in. lens of a phase-contrast or dark ground microscope. Dried films stained by Leishman's stain may also be used for diagnostic examination. Its morphological features are similar to those of *Trich. hominis*.

Chilomastix mesnili.—Resembles *Trich. hominis*, but has no undulating membrane and only three flagella. It has an elongated slit-like cytostome. Cysts can easily be recognised; they are oval, about $8\ \mu$ in their long diameter, and contain one nucleus.

Giardia (or *Lamblia*) *intestinalis*.—Inhabits the duodenum and jejunum.

Main characters:—somewhat flattened in shape; flat surface pear-shaped; bilaterally symmetrical; $10\text{--}18\ \mu$ in its long diameter; a large sucking disk, on one surface; two nuclei with karyosomes; two long median parallel axostyles which represent skeletal structures, with blepharoplasts at each end; eight flagella in pairs—two arising from the anterior blepharoplasts (the broad end is spoken of as anterior), two arising near the anterior blepharoplasts but following the axostyles to the posterior edge of the sucker before diverging, two arising at the posterior edge of the sucker and rooted in the axostyles, and two arising from the posterior blepharoplasts.

The cysts are characteristic: oval in shape, about $10\text{--}15\ \mu$ long, with two or four nuclei (the cyst containing two organisms formed by subdivision); the parallel axostyles are observable.

Giardia intestinalis has been found in the faeces of young children suffering from a subacute diarrhoea with bulky offensive stools. Infection occurs particularly in day and residential nurseries and there may be many symptomless carriers. The giardia can be eliminated from the bowels of cases and carriers by treatment with meprazine ($0.1\text{--}0.3$ g. daily for six days).

For further information regarding other intestinal protozoa, reference should be made to works on protozoology.

Toxoplasma

This organism, an unclassified protozoon has been found in cases of encephalitis of young subjects, often associated with chorio-retinitis and various malformations. There may be a resemblance to haemolytic disease of the newborn. It is also associated with lymphadenopathy, resembling glandular fever, and it occurs in exanthematous blood infection with pneumonitis of adults. Sub-clinical or inapparent infection is common, especially in adults, as judged by the presence of antibodies. In congenital toxoplasmosis acute involvement of every organ may occur; in adult acquired disease, acute infection is rare. The infection in the newborn appears to be derived from the mother *in utero*, the maternal infection being an inapparent one. In other cases trans-

mission may occur by droplet infection or contact with excreta or infected tissues.

The organism occurs in the form of oval or crescentic bodies, about $6\text{--}7 \mu \times 2\text{--}4 \mu$, which are found in endothelial and large mononuclear cells, but also in the free state. With a Romanowsky stain, e.g. Giemsa, it shows a reddish nuclear structure and blue cytoplasm. It can be transmitted experimentally to various animals, e.g. guinea-pigs, mice and other laboratory rodents. The origin of human infection has not been defined but similar organisms cause natural infections in many animals throughout the world. Strains isolated from human infections display immunological homogeneity.

For diagnosis during life, body fluids may be inoculated into laboratory rodents. The inoculation of material into young mice by the intracerebral and intraperitoneal routes at the same time is recommended. A complement-fixation test using a toxoplasma-containing material as antigen is available for diagnosis. A neutralisation test¹ using the chorio-allantoic membrane of the chick embryo has also been found satisfactory. Another laboratory test which has been used in diagnosis is the cytoplasm-modifying antibody reaction of Sabin and Feldman. It is based on the fact that the organism when acted on *in vitro* by a specific antibody fails to take up, and stain with, methylene blue. The organisms used in the test are obtained from peritoneal exudate of experimentally inoculated mice. For details of the method and interpretation of the results reference should be made to Sabin and Feldman,² and Beverley and Beattie.³ When using antibody tests it is most important to show evidence of a rising titre.

¹ Macdonald, A. (1949), *Lancet*, i, 950.

² Sabin, A. B., & Feldman, H. A. (1948), *Science*, 108, 660; (1949), *Pediatrics*, 4, 660.

³ Beverley, J. K. A., & Beattie, C. P. (1952), *J. clin. Path.*, 5, 350; (1958), *Lancet*, i, 379.

CHAPTER XLVI

PROPHYLACTIC IMMUNISATION

IN recent years there has developed an increasing interest in the role of immunisation in the control of communicable diseases. This fresh interest in immunisation probably derives from a number of contributory factors, *e.g.* the dramatic success of national programmes for the control of diphtheria by immunisation; the publicity given to large-scale campaigns for the control of tuberculosis and poliomyelitis by prophylactic vaccines; the wider knowledge made available through international bodies like W.H.O. of the prevalence and economic importance of communicable diseases in developing countries; the need for protection against the introduction of quarantinable diseases like smallpox and yellow fever; and the growing realisation that treatment with anti-microbial drugs has definite limitations and disadvantages.

The Rationale of Immunisation

The objective of immunisation is to produce, without harm to the recipient, a degree of resistance as great as, or greater than, that which follows a clinical attack of the natural infection. With this objective in mind, those communicable or infectious diseases amenable to control by vaccination may be considered in four main groups; toxic, acute bacterial, chronic bacterial, viral and rickettsial infections. In the first group, *e.g.* diphtheria and tetanus, the brunt of the infection is due to a specific poison or toxin which can be purified artificially, rendered harmless by treatment with formalin (=toxoid) and used as a very effective antigen or prophylactic, particularly if it is adsorbed on to a mineral carrier, *e.g.* aluminium hydroxide or aluminium phosphate.¹ The potency of toxoid antigens can be measured and standardised with great accuracy, and the amount of antitoxin that is produced in the inoculated person gives a reliable indication of the degree of resistance to infection in that individual.

¹ These alum salts are tissue irritants and therefore should be used in the lowest concentration required for an adjuvant action.

Among the acute bacterial infections there are two categories as far as immunisation procedures are concerned: (a) pyogenic infections (staphylococcal, streptococcal and pneumococcal) against which vaccines are largely ineffective, since there are many different antigenic types within the species, e.g. pneumococcus type 1, 2, 3, etc., so that an infection (or immunisation) with one type does not protect against infection with other types; (b) infections like whooping-cough, cholera, plague and anthrax, where there is one antigenic type of organism, so that a vaccine prepared with the infecting organism might be expected to give a reasonable degree of protection. However, these organisms contain many different antigenic components, of which probably only one or two are particularly concerned with the virulence of the organism. It is important to try to identify and certainly to preserve these so-called "protective antigens" in vaccine preparations.

Another difficulty is that in infections like whooping-cough and cholera, the infection affects predominantly the epithelial surfaces so that antibodies produced as a result of vaccination may not gain easy access to the site where the pathogen is producing the infection. For this and other reasons it was essential to test vaccines against whooping-cough and cholera in properly controlled field trials, so that objective assessment of their value could be obtained.

So far the assumption has been implicit that the production of a specific protecting antibody is the main requirement for effective immunisation, although it should be noted that immunity may persist long after such antibodies cease to be demonstrable, as in whooping-cough. When the chronic bacterial infections are considered (e.g. typhoid, brucellosis, tuberculosis), it must be concluded from knowledge of the natural behaviour of these infections that the specific humoral antibodies which can at present be identified play little part in overcoming the infection. Thus, antibodies to the specific antigens of the typhoid and brucella bacteria are demonstrable in the blood of the patient within a week of onset of the clinical illness, but the fever may go on for many weeks before clinical recovery. In addition, relapses in these *continued fevers* are not uncommon despite the presence of high concentrations of specific antibodies. In contrast to the acute bacterial infections, the infecting organisms in chronic infections are for the most part intracellular parasites, and it seems likely that what is called *cellular immunity* may be more important in overcoming the infection than the presence of humoral antibodies. It is probably for this reason that in tuberculosis and

brucellosis a living attenuated vaccine is needed to produce immunity.

In the viral infections it is known that humoral antibodies are important, but, again, cellular immunity also seems to be important in some diseases. Thus, children with agammaglobulinaemia can recover from infections like measles, chickenpox and mumps with an apparently good immunity without detectable humoral antibody, whereas they rapidly succumb to acute bacterial or toxic infections. Such children can also be successfully vaccinated with smallpox vaccine (and with B.C.G.). These findings indicate that specific humoral antibodies are not essential for recovery from some virus diseases. On the other hand, immunity to certain infections seems to be equated with the presence of antibody; human gammaglobulin can be used effectively to control measles and infectious hepatitis, and killed viral vaccines which probably act mainly in virtue of the production of humoral antibody can protect against diseases like influenza and poliomyelitis.

Immune Response and Duration of Immunity

The newborn baby may contain in its blood antibodies to the agents of certain toxic, bacterial and viral infections according as the corresponding antibodies are present in the mother's blood. This passive immunity gives protection to the infant at a time when it is poorly equipped to produce specific antibodies, but it interferes to a varying extent with the infant's capacity to respond to the stimulus of toxoids or vaccines in the early months of life. For example, killed poliomyelitis vaccines elicit little or no antibody response in most children under 6 months of age because of the presence of maternal antibody. The capacity of the infant's tissues to produce specific antibody to injected antigens is, in any case, poorly developed in the first few months of life although some response is obtained to powerful antigens such as alum-adsorbed toxoids. It should be noted that the newborn infant will respond to living vaccines, *e.g.* B.C.G. and smallpox vaccines.

When a good specific antibody response is being sought to a toxoid or killed antigen, the usual procedure is to give two or three doses of the antigen at intervals of several weeks. The first dose of antigen evokes a poor antibody response after a latent period of approximately two weeks, but after the second dose the amount of antibody produced is multiplied tenfold and after a third dose may be increased a hundredfold. The first or "priming" dose of

toxoid will be more effective the larger it is; or if it is released slowly as from a mineral carrier; or if it is mixed with certain bacterial vaccines, e.g. tetanus toxoid plus typhoid vaccine, diphtheria toxoid plus pertussis vaccine. The second and subsequent doses are effective in much smaller amounts than the first, and without the help of adjuvants. With toxoids the response is much better if the two doses are spaced out at an interval of four to eight weeks, and, provided the priming dose is adequate, the response to the second dose will still be maximal even if given six to twelve months after the first. It is not known whether this phenomenon is applicable also to killed bacterial or viral vaccines. Where there is reason to believe that a community has acquired a basic immunity from the widespread occurrence of clinical or inapparent infection, as in influenza, one dose of antigen will act as the secondary stimulus or *booster*.

As regards the duration of immunity after the basic course, this can be measured precisely in the case of toxic infections according to the level of specific antitoxin in the blood, or, less precisely, in diphtheria by the Schick test. Recent studies have shown that an adequate concentration of diphtheria antitoxin may persist in the blood of children for at least four years after primary immunisation in early infancy. After a primary course of three doses of tetanus toxoid, a satisfactory antitoxin titre may be present for as long as ten years.

The duration of immunity after injections of killed bacterial vaccines cannot be equated with the presence of demonstrable antibody; for example, after a course of three doses of pertussis vaccine given to children (average age one year) there was no change in the degree of protection in successive six months during a follow-up period of two-and-a-half years, although antibodies were no longer demonstrable in a considerable proportion of the children within a year after immunisation. Again, in the chronic bacterial infections, there is no correlation between antibody titres and clinical protection, as was shown by the controlled studies of typhoid vaccines in Yugoslavia. In the viral infections, although antibody titres are accepted as a measure of the degree of protection, there are certain anomalous findings: for example, high titres of neutralising antibody have been found in the early stages of fatal infections in smallpox.

For these bacterial and viral infections, therefore, the only reliable measure of the duration of immunity after vaccination is a careful assessment of the attack rate over a period of time, preferably in comparable groups of vaccinated and non-vaccinated children.

Controlled Field and Laboratory Studies of Prophylactic Vaccines

Evaluation of prophylactic vaccines and toxoids by means of carefully designed field trials has in the past fifteen years resulted in a revolutionary change in the accuracy with which the degree of protection afforded to the inoculated can be assessed. Public health programmes for the use of vaccines so tested can now be planned with the assurance that a known degree of effectiveness will be obtained. Vaccines submitted to controlled field trials so far include those against whooping-cough, tuberculosis, typhoid fever, influenza and poliomyelitis. It will be noted that although B.C.G. and typhoid vaccines had been available and in use for many years prior to the time the new methods of field testing were generally applied, they had ultimately to be submitted to test before a true assessment of their value could be made. It is only by statistically acceptable studies which give unbiased information that controversy is settled and confident use can be made of the vaccines on a large scale.

Well-planned studies, though costly, save money, time and misplaced effort in the long run and can give information not otherwise obtainable on dosage, combined antigens, duration of immunity and the like. They do not of course answer at once all the questions regarding the use of the vaccines; instead they may, and do, pose fresh questions which are still unanswered.

Combined field and laboratory studies of vaccines aim at providing confidence in the efficacy of future vaccination programmes. Sufficient confidence can only rarely be attained if the studies do not observe two basic principles, which may be called (1) the principle of comparability and (2) the principle of reproducibility.

(1) *The Principle of Comparability* ensures confidence that an observed degree of protection apparently conferred upon a population group by vaccination was due to the vaccination and not to other chance influences. It requires comparison of the incidence of the disease in two or more groups, and precautions to ensure that these groups can be regarded as identical in all respects except for the factor of vaccination.

The most convincing evidence will be obtained if one of the groups in a field trial ("the control group") remains unvaccinated, or is vaccinated with an unrelated vaccine. If it is not feasible to have a control group, comparisons may be made between groups treated with vaccines that are prepared in different ways, or some-

times between groups treated with the same vaccine but following a different dosage schedule.

(2) *The Principle of Reproducibility* ensures confidence in obtaining, in future vaccination programmes, the same degree of protection as observed in the field trial. It requires precautions to ensure that the vaccine which was proved to be of value in the field trial can be prepared again and can, if possible, be validly tested in a laboratory assay.

A field trial can only show whether or not the actual preparation of vaccine used in the trial was successful. What is then needed is confidence in the ability to reproduce this particular preparation or of preparing an equally or more efficient preparation. The preparation method must therefore be unambiguously described and the laboratory studies must include assays of different vaccine preparations in laboratory animals, in order to develop an assay method yielding results that parallel those obtained in the field, thus permitting the assessment of the protective value of future vaccine preparations by laboratory assay alone.

If the essential protective antigen of a micro-organism could be isolated, identified and quantitatively assessed with chemical exactitude and if the mechanism of the production of immunity by the host were understood, there would clearly be no need to do more than to measure the amount of protective antigen in the first successful vaccines tested. It could then be ensured that all subsequent vaccines contained similar or greater amounts of the essential constituent.

Such measurement can be made with some approach to accuracy with toxoids such as the diphtheria or tetanus prophylactics, but with most bacterial and virus vaccines, killed or living, this is not possible, and the principle of reproducibility must therefore be carefully observed. It is then necessary to compare a series of vaccines in field studies and at the same time to submit them to as many laboratory studies as possible in the hope that variations of protective power in the field will occur and will be reflected in one or more laboratory tests. The laboratory test which gave results most closely corresponding to the protective value may then be adopted as the test for future batches of vaccine. Such a complete investigation, however, demands that field and laboratory studies be made of several batches of vaccine.

Examples of Controlled Trials of Prophylactic Vaccines.— Some examples are given of well-conducted field trials that have given valuable information on the place of prophylactic vaccination against certain infectious diseases in defined communities,

(a) *Whooping-cough.*—In whooping-cough, following the earlier studies of Kendrick and Eldering in Grand Rapids, Michigan, a continuing series of controlled field and laboratory trials of pertussis vaccines have been carried out in the United Kingdom over the past decade. Altogether some 50,000 children in the age range 6 months to 2 years were inoculated and 25 different vaccines were tested. The following is an extract from the final report:¹

"The results of the trials clearly showed that it was possible by vaccination to produce a high degree of protection against the disease, as shown by the substantial reduction in the attack rate amongst home contacts, and, in those cases where vaccination failed to give complete protection, to reduce the severity

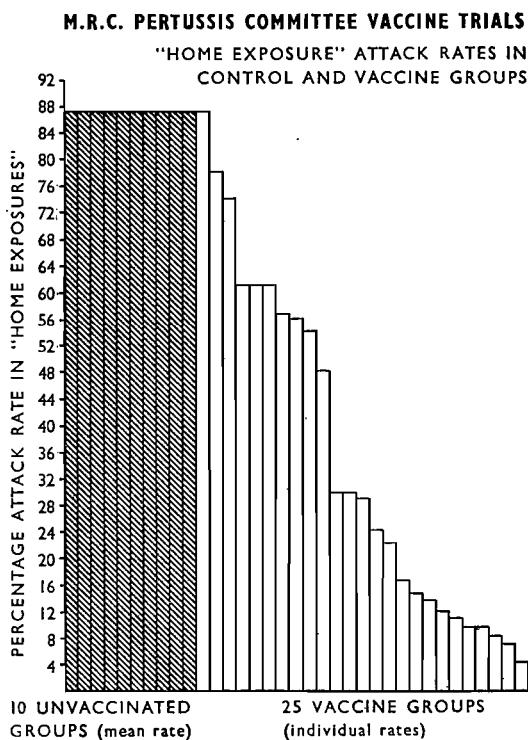
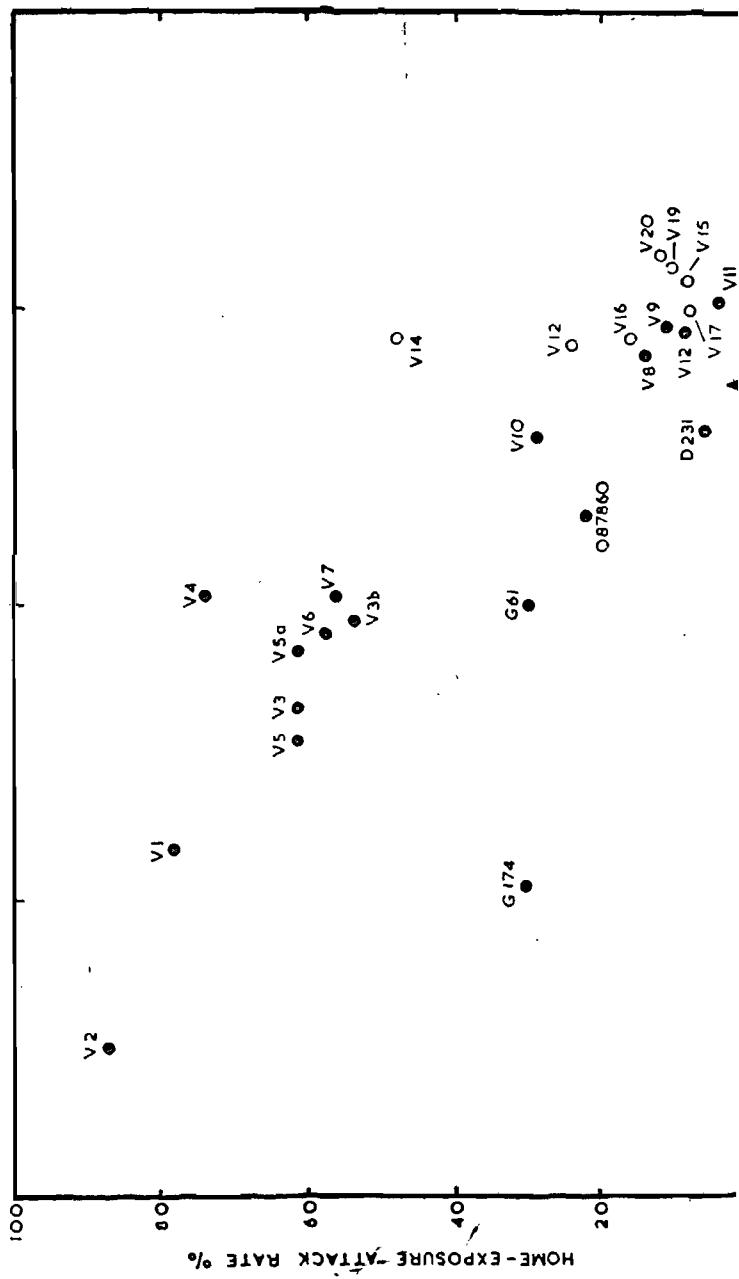


FIG. 43

(*A Symposium on Immunization in Childhood.*
Edinburgh, Livingstone, 1960.)

¹ Report, *Brit. med. J.* (1959), 1, 994.



and duration of the disease. The results also showed that the different vaccines employed varied a great deal in their protective action; the poorest gave an attack rate in home contacts of 87 per cent., and the most effective an attack rate of 4 per cent."

In regard to laboratory assays of potency, three methods of evaluation were used: agglutinin response in the inoculated children, agglutinin response in mice, and the mouse-brain protection test. The first two of these tests correlated well with attack rates in inoculated children with most of the vaccines tested, but there was a lack of correlation with a purified antigenic fraction of *B. pertussis* (the Pillementer fraction) which gave good protection clinically but a poor agglutinin response in mice. It was therefore considered that the mouse-brain protection test was the most satisfactory in assessing prophylactic potency. The results are shown graphically in Figs. 43 and 44.

(b) *Tuberculosis*.—Despite epidemiological evidence, particularly from Scandinavian countries, of the protective effect of B.C.G. vaccination, there were until recently very few well-controlled trials from which a critical assessment of the vaccine could be made. Now, controlled field trials in different age-groups and communities, have shown that B.C.G. and vole bacillus vaccines can give a high degree of protection (around 80 per cent.) to vaccinated infants and adolescents, and that this protection may persist for five to ten years after vaccination. The British data¹ for school leavers in three industrial areas carefully followed up for over seven years are shown in Fig. 45.

Despite these satisfactory results, other trials carried out in the southern states of the United States have shown only a small degree of protection to the vaccinated compared with the control groups. This latter finding may be related to a high incidence of low-degree sensitivity to tuberculin. Some countries follow the original Calmette procedure of giving large repeated doses of B.C.G. vaccine by mouth, but apart from the recent trial in Algeria there seem to be no controlled studies to assess the protective value of the oral route of vaccination.

(c) *Poliomyelitis*. The relatively low incidence of paralytic poliomyelitis, even in well-developed countries, has meant that a critical evaluation of polio vaccine would require very large numbers of children. Such a study was carried out in the United States of America where over half a million school-children aged 6-8 years were involved in a strictly controlled trial in 1954. The overall attack rate of paralytic poliomyelitis in the vaccinated and

¹ Report, *Brit. med. J.* (1959), 2, 379.

placebo groups was in the ratio of 1:3.5. This ratio was much greater for the grave bulbo-spinal (1:18) than for spinal poliomyelitis (1:2.5) and was progressively greater in the 6, 7 and 8 year old children (see table p. 941). Subsequent trials carried out in the United Kingdom and Sweden have supported the American

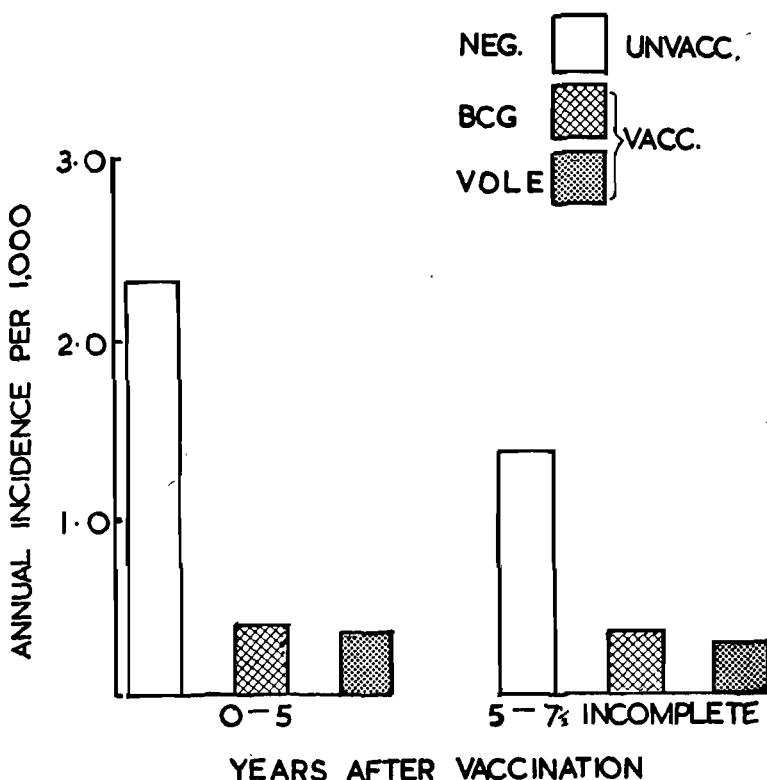


FIG. 45

The incidence of tuberculosis in the unvaccinated and vaccinated groups in the British Medical Research Council's vaccines trial.

conclusion that killed poliovirus vaccine can give a high degree of protection.

(d) *Typhoid*.—Although it has been assumed for many years that anti-typhoid vaccination has made a major contribution to the control of typhoid, particularly for the armed forces in times of war, certain anomalous findings among vaccinated troops during and after the war raised doubts and called for a fresh critical

Paralytic Poliomyelitis Cases by Age and Vaccination Status

Age (1 May 1954)	Vaccinated	Placebo	Per cent. Effectiveness	S.L.
6 years: Number . . Rate per 100,000 .	16 37	21 49	24	N.S.
7 years: Number . . Rate per 100,000 .	12 16	48 64	75	0.001
8 years: Number . . Rate per 100,000 .	4 7	29 53	87	0.001
9 years: Number . . Rate per 100,000 .	1 4	9 37	89	0.01
All ages (6-10 and over): Number . . Rate per 100,000 .	33 16	110 55	71	0.001

S.L.=level of statistical significance.

N.S.=not significant at level of 0.05.

assessment of typhoid vaccination. A controlled trial of two vaccines (heat-killed phenol-preserved, and alcohol-killed alcohol-preserved) with a control group given a Flexner dysentery vaccine, was carried out in an endemic typhoid area in Yugoslavia, involving over 35,000 inoculated persons in the age range 5-40 years. The results, over a two-year follow-up period (1954-55), showed a significant degree of protection in those receiving the phenolised vaccine, whereas there was insignificant protection with the alcoholised vaccine.¹ It is interesting that in the following three years provisional data showed that those who received two doses of vaccine in 1954 and a booster in 1955 still showed a significantly lower attack rate than the control group.

¹ *Bull. World Hlth Org.* (1957), **16**, 897.

Typhoid Cases in Vaccinated Groups

Vaccine	1954		1955		1956	1957	1958	Total cases
	No. of vacc.	No. of cases	No. of vacc.	No. of cases	No. of cases	No. of cases	No. of cases	
Alcohol	12,017	17	8,913	6	1	0	6	30
Phenol	11,503	7	8,595	2	1	1	2	13
Control	11,988	23	9,002	8	4	6	9	50
Total .	35,508	47	26,510	16	6	7	17	93

Hazards of Immunisation

It is axiomatic that prophylactic agents, before they are issued for use, should have been shown to be safe as well as effective, and T.S.A. regulations require certain tests for sterility and toxicity to be carried out on these prophylactics. Nonetheless, there are certain incidental hazards associated with the injection of immunising agents, and the first of these relates to the syringe and needle to be used for the injection. The best way to ensure that a syringe and needle are sterile is to heat the assembled outfit in a hot air oven at a temperature of 160° C. for one hour. The next best is the use of high pressure steam in an autoclave or pressure cooker, which, if properly used, can again ensure absolute sterility, *i.e.* the destruction of both sporing and non-sporing micro-organisms. Although boiling does not kill the more resistant sporing organisms, this procedure is accepted as reasonably safe for the re-sterilisation of syringes that have already been properly sterilised in a hot air oven or autoclave. In other words, where many injections are being given, as at a baby clinic, repeated boiling of the syringe and needle for ten minutes is accepted as adequate protection against the risk of transference of infection. A sterile syringe and needle must be used for each injection to avoid the risk of transferring the agent of homologous serum hepatitis, for a syringe, if used for repeated injections with a fresh sterile needle each time, may carry over minimal amounts of tissue fluid. The skin should, of course, be cleansed and preferably treated with a quick acting antiseptic such as 2 per cent. iodine in 70 per cent. alcohol immediately before the injection. For smallpox vaccination, the skin should simply be cleansed with soap or methylated ether and then allowed to dry. It is unwise to give an injection to a child who is

obviously suffering from skin sepsis; and smallpox vaccination should be avoided in a child with eczema, because of the risk of generalised vaccinia.

In regard to reactions, there is as a rule little or no local systemic reaction following the injection of plain toxoids, but when alum is added to the toxoid there is usually some local reaction because of the irritant effect of the alum. For this reason the injection should be given deep subcutaneously or intramuscularly, so that any fibrous nodule at the site of injection is not easily felt. With killed vaccines, the amount of local or systemic reaction is usually greater than with toxoids and with both T.A.B. and pertussis vaccines there may be local swelling associated with some febrile reaction within the first twenty-four to seventy-two hours after inoculation. After injections of pertussis vaccine, cases of encephalopathy, manifested by convulsions and coma and followed sometimes by mental deterioration, have been reported. It is impossible to estimate the risk of this hazard, but it occurs in probably less than one in 1 million injections and has been noted particularly in the U.S.A. In view of this rare hazard, it is advisable to avoid giving pertussis vaccine to children with a history of repeated convulsions and to children who are convalescent from some other illness.

There is very little risk of reaction following the use of poliomyelitis and influenza virus vaccines. With the former, a few instances of allergic reactions in patients who were already hypersensitive to penicillin have been recorded, but as manufacturers are giving up the use of penicillin in the production of poliovirus vaccine, this hazard is not likely to occur in future. Both local and systemic reactions to flu virus vaccine may occur in 1-3 per cent. of persons; here and also in the use of yellow fever vaccine, enquiry should be made about the patient's sensitivity to egg since these vaccines are prepared from chick embryo tissue.

With smallpox vaccine there is the recognised, if small, risk of post-vaccinal encephalitis when primary vaccination is carried out on a school child or adult. It is perhaps not so widely known that the risk of post-vaccinal encephalitis seems to be rather greater in children under 1 year of age (15.8 per million) than in children between 1 and 4 years of age (2.1 per million). Again, generalised vaccinia is more likely to occur in infants (51 per million) than in 1 to 4 year olds (23 per million). But these hazards are admittedly very small and should not necessarily influence the doctor in deciding on the most convenient time for giving particular inoculations.

A complication that has attracted considerable attention in recent years is the so-called "provocation" poliomyelitis, which may occur as a paralysis in the inoculated limb in children within a month of receiving some prophylactic inoculation. In a recent M.R.C. investigation,¹ the overall incidence of provocation poliomyelitis was 1 per 37,000 inoculations. The risk, however, was greatest with a mixed diphtheria-pertussis vaccine containing alum, where the rate was 1 in 15,000 injections. Significant, but smaller risks, were observed with the diphtheria prophylactics A.P.T. and P.T.A.P., and with the mixed diphtheria-pertussis vaccine without alum. No significant risk was found after plain pertussis vaccine, T.A.F. or formol toxoid, or smallpox vaccine. Because of the hazard associated with an alum-containing prophylactic or even with the plain diphtheria pertussis mixture, the Ministry of Health have advised against giving prophylactic agents in one or other of these forms. However, studies both in this country and in Canada indicate that the hazard associated with combined diphtheria-pertussis or with the triple diphtheria-tetanus-pertussis vaccine is so small as to warrant the continued use of combined prophylactics because of their administrative and other advantages.²

IMMUNISATION SCHEDULES

The aim of immunisation programmes is the control of infection in the community rather than individual protection. A lower level of immunity than is necessary for solid individual protection can effectively reduce the incidence of communicable diseases if a high proportion of the susceptible community is immunised. Thus, in diphtheria, there is a rapid reduction in both morbidity and mortality when 60-70 per cent. of the pre-school and school children are effectively immunised. Smallpox is controlled when approximately 80 per cent. of the whole community has been successfully vaccinated. Tetanus is an exception to this general rule in that protection of a proportion of the population does not reduce the risk to the non-immunised individual.

For countries with well-developed systems for the collection of morbidity and mortality data relating to communicable diseases

¹ Report, *Lancet* (1956), ii, 1223.

² See *A Symposium on Immunization in Childhood*. Edinburgh, Livingstone (1960).

plus good public health and general medical services, it should be relatively easy to decide what vaccinations should be carried out and how best the programme can be effected. Nonetheless, delays and lack of co-ordination in the application of knowledge frequently occur even when a good organisation is available. There is also the risk that the incidence and importance of a disease may be under-estimated or miscalculated if notification is poor, or mortality rates are falling, *e.g.* in tuberculosis and whooping-cough, or deaths are attributed to secondary causes, *e.g.* bronchopneumonia following measles or whooping-cough. In countries with limited medical services it is essential that strenuous efforts be made to provide satisfactory vital statistics and, by sample surveys or other means, to obtain a reasonably accurate assessment of the main causes of morbidity and mortality.

In the United Kingdom most of the immunisation work is done either at child welfare clinics or by the family doctor or paediatrician. While there are obvious advantages in having the immunisations carried out by the family doctor (and the available evidence indicates that the proportion of immunisations done by practitioners is steadily increasing) it is essential that there should be a well-organised system for ensuring that the child receives its injection at the appropriate age and time intervals and that there is an efficient system of recording the immunisations.

An immunisation campaign carried out without provision for its continuation as a routine procedure will not give satisfactory results—except where complete eradication is achieved. Therefore, in planning immunisation schedules, provision must be made to ensure receptivity by the public and, particularly, to secure the co-operation of parents who have to bring their children to the doctor or clinic for repeated inoculations. These measures are essential for the successful execution of the programme.

Knowledge about the duration of immunity following the primary course of immunisation and after booster (or recall) injections is not yet sufficiently precise in a number of communicable diseases. The number and timetable of booster doses must, therefore, be left rather elastic. The need to use efficient prophylactics and, wherever possible, ones that can be standardised, cannot be over-emphasised since the continuance of immunisation programmes with the co-operation and confidence of the public depends on the successful results of these procedures.

The two schedules set out below are suggested models (*a*) for areas with well-developed medical services, and (*b*) for developing areas with inadequate medical services.

(a) *Suggested Schedule of Immunisation in Areas with Adequate Public Health Medical Services*

Age	Prophylactic Vaccine	Visit
2-6 months	Diphtheria-pertussis-tetanus triple vaccine: 3 doses with 1 month's interval between each dose.	1st, 2nd and 3rd
6-7 months	Smallpox vaccination.	4th
7-10 months	Poliomyelitis vaccine (inactivated): 2 doses with 1 month's interval.	5th and 6th
15-18 months	Booster dose of triple vaccine; simultaneously third dose of poliomyelitis vaccine.	7th
2-4 years	Fourth dose of poliomyelitis vaccine.	8th
5-6 years	Booster dose of diphtheria-tetanus vaccine; simultaneously, smallpox revaccination.	9th
10-14 years	B.C.G. vaccination; booster dose of diphtheria-tetanus vaccine if Schick test positive; no injection of diphtheria prophylactic in Schick pseudo-reactors.	10th

Poliomyelitis.—The use of live attenuated poliovirus vaccine is not included in these schedules because of the still limited knowledge about its efficacy when given orally to infants. This procedure may become the method of choice in countries where there is a high incidence of clinical disease in early infancy. In these countries immunisation with inactivated vaccine may have to be started earlier than suggested in the schedule, but in such circumstances the antibody response will be negligible in a considerable proportion of infants because of the presence of maternal antibody.

It is suggested that a fourth dose of poliovirus vaccine be given within one to two years after the third dose of the primary course since (a) antibody titres may have fallen to low levels by that time

(b) *Suggested Schedule of Immunisation in Areas with Inadequate Medical Services*

Age	Prophylactic Vaccine	Visit
0-4 weeks	(1) B.C.G. vaccination.	1st
3-9 months	(2) Smallpox vaccination. (3) Diphtheria-pertussis-tetanus (triple vaccine with alum): 2 doses at an interval of one month. The first injection could be given at the time of smallpox vaccination. Smallpox vaccination is verified at the second visit. Failures of smallpox vaccination are re-vaccinated.	2nd and 3rd
School entry or soon thereafter.	(4) Diphtheria/tetanus booster (plain or with alum). (5) T.A.B. vaccination: 2 doses at an interval of one month. (6) Smallpox re-vaccination: at the time of second T.A.B. injection.	4th and 5th
10-14 years	(7) B.C.G. re-vaccination (in tuberculin negative reactors). (8) Smallpox re-vaccination. (9) T.A.B. booster.	6th and 7th

and (b) clinical disease has occurred in children after 3 doses. If the polio antigens, particularly types I and II, are improved, it may be possible to postpone the fourth dose until school entry.

It is envisaged that schedule (b) will be used in countries with a low incidence of clinical poliomyelitis. In such areas poliomyelitis vaccine should not be employed routinely, but should be available to those at special risk of clinical disease.

Quadruple Vaccine (D.P.T. and polio) is not recommended at present.

Tetanus.—An individual who has been effectively immunised with a primary course of tetanus toxoid followed by 1 or 2 booster doses should be given a further dose of tetanus toxoid if exposed to the risk of tetanus. If the injury is extensive, a dose of tetanus antitoxin should also be given. If tetanus antitoxin is given to a

non-immunised individual, active immunisation with tetanus toxoid should be started two to four weeks later.

Tuberculosis.—B.C.G. vaccination. High prevalence areas: first vaccination within first four weeks of life or, where the modified vaccination schedule is used, B.C.G. vaccine may be given at the same time as the second dose of triple vaccine at 4 to 8 months of age. Routine pre-vaccination tuberculin testing may have to be done at this age, dependent upon the prevailing infection risk during the first year of life. Re-vaccination at school entry and at school leaving age, after tuberculin test.

Low prevalence areas: first vaccination—in school or before leaving school after tuberculin test. Re-vaccination may be performed on military recruits, students, and on the occasion of routine examination of other occupational groups.

APPENDIX

ABBREVIATIONS AND CONVERSION FACTORS

Mass

g.	=gram
kg.	=kilogram (1 kg.=1000 g.)
mg.	=milligram (1 mg.=0.001 g.)
μ g.	=microgram (1 μ g.=0.001 mg.)
lb.	=pound weight avoirdupois (1 lb.=453.6 g.)

Length

m.	=metre
cm.	=centimetre (1 cm.=0.01 m.)
mm.	=millimetre (1 mm.=0.001 m.)
μ	=micron (1 μ =0.001 mm.)
$m\mu$	=millimicron (1 $m\mu$ =0.001 μ =10 Ångström units, Å.)
in.	=inch (1 in.=2.54 cm.)
ft.	=foot (1 ft.=12 in.)

Area

sq. in.	=square inch (1 sq. in.=6.45 sq. cm.)
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Volume

- l. = litre (1 l. = 1.76 pints)
ml. = millilitre (1 ml. = 0.001 l.)
 μ l. = microlitre (1 μ l. = 0.001 ml.)
oz. = fluid ounce (1 oz. = 28.41 ml.)
cu. ft. = cubic foot (1 cu. ft. = 28.3 l.)

Temperature

X° C.=X degrees Centigrade
Conversion of X° Centigrade to X° Fahrenheit:
X° F.=1.8X° C.+32

Time

- hr. = hour
min. = minute
sec. = second

Other Abbreviations

- N = normal (e.g. 2 N HCl)
M = molar (e.g. 0.1 M Na₂CO₃)
r.p.m. = revolutions per minute
mv. = millivolt
p.p.m. = parts per million
L.D.50 = average lethal dose
M.L.D. = minimum lethal dose
M.H.D. = minimum haemolytic dose
per cent. The percentage concentration of solutions is stated as g. of solute per 100 ml. of solution, i.e. as per cent. (w/v). Unless otherwise indicated the solvent is water. Per cent. (v/v)=ml. of substance per 100 ml. of mixture, as in gas mixtures.

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