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HANDBOOK OF PRACTICAL BACTERIOLOGY

A Guide to
Bacteriological Laboratory Work

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NINTH EDITION



E. & S. LIVINGSTONE LTD.
EDINBURGH AND LONDON

1953

<i>First Edition</i>	1925
<i>Second Edition</i>	1928
<i>Third Edition</i>	1931
<i>Fourth Edition</i>	1934
<i>Fifth Edition</i>	1938
<i>Sixth Edition</i>	1942
<i>Seventh Edition</i>	1945
<i>Reprinted</i>	1946
<i>Eighth Edition</i>	1948
<i>Reprinted</i>	1949
<i>Reprinted</i>	1950
<i>Ninth Edition</i>	1053

Printed in Great Britain

PREFACE

THE publication of this ninth edition of the *Handbook of Practical Bacteriology* calls for little in the way of prefatory remarks. The text has been revised throughout on the basis of recent advances in bacteriological knowledge and technique, but the general character of the book has been continued from earlier editions and we have endeavoured also so far as possible to avoid any substantial expansion of the text by omitting methods (described in the previous edition) which have now been generally superseded by new procedures or have less application in bacteriological practice. We trust that the contents of the new edition will be acceptable and serviceable to students and laboratory workers, who may use it as a guide to the study and practice of bacteriology.

In the preparation of this edition we owe much to various colleagues who have given us advice and information on special topics, and we have to express our grateful thanks to the following for their generous help in this respect: Drs. J. H. Bowie, J. C. Broom, Joyce D. Cranfield, J. P. Duguid, W. M. Henderson, J. C. Gould, A. F. Maccabe, R. H. A. Swain, A. Wilson Taylor, A. T. Wallace, J. F. Wilkinson, D. R. Wilson, and Helen A. Wright.

We have to thank specially Dr. J. P. Duguid for his valuable assistance in the reading of proofs, and for his careful review of the whole text before publication.

We would also like to record our appreciation of all the helpful co-operation we have received from the publishers in the production of the book.

T. J. M.
J. E. McC.

1953.

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P A R T I

General Biology of Micro-Organisms and Immunity in Relation to Practical Bacteriology

CHAPTER I

THE GENERAL BIOLOGY OF MICRO-ORGANISMS

THE BIOLOGICAL GROUPS REPRESENTED BY THE PATHOGENIC MICROBES

BACTERIOLOGY or Microbiology, as applied to medicine, embraces the study of those micro-organisms which are pathogenic to or commensals of man. The term "pathogenic" implies the power of producing disease; organisms which occur on the skin or in certain parts of the body—*e.g.* mouth, throat, intestine—without exerting any harmful effect are described as "commensals." Various commensal organisms are, however, potential pathogens, and some recognised pathogenic microbes may, under certain conditions, assume a commensal rôle, *e.g.* in the so-called infection carriers.

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, however, show great diversity in their parasitism to different animal species, certain being associated with disease in the human species only, while others are highly virulent towards particular animals though non-pathogenic to man.

The pathogenic and commensal micro-organisms may be classified broadly in the following large biological groups: (a) BACTERIA or SCHIZOMYCETES ("fission fungi"); (b) FUNGI PROPER, which include the moulds and the yeasts; (c) PROTOZOA.

The exact biological relationships of some pathogenic micro-organisms, *e.g.* the Rickettsiae (which include the organism of typhus fever), still remain undetermined, and it is difficult to assign such forms to any one of these groups.

Moreover, certain infective agents are so minute that they can pass through filters which are impervious to the recognised bacteria and have been designated "filterable viruses" or simply "viruses." Some of these are beyond the range of

ordinary microscopic visibility and were at one time designated "ultramicroscopic." Certain viruses, however, have been demonstrated microscopically by special staining methods as minute bodies which are smaller than the bacteria, and many of them have now been observed as organised structures by electron microscopy. The nature of these viruses and the question whether they are organismal entities will be discussed in a later chapter.

The differential characterisation of the biological groups referred to is as follows :—

BACTERIA.—Organisms of microscopical dimensions and generally unicellular, though cellular units may be attached to one another and form chains, filaments or other aggregates ; morphologically simple as observed by ordinary microscopic methods ; multiply usually with great rapidity and by simple fission ; units spheroidal or cylindrical and rod-shaped, comma-shaped, spiral or filamentous ; devoid of chlorophyll ; do not exhibit a nucleus when unstained or stained by the ordinary methods, but nuclear bodies can be demonstrated by special methods ; certain species develop a resting-phase in the form of "spores" ; some of the filamentous types reproduce by means of "conidia" (p. 11) ; in certain species the cells are motile and possess flagella ; some forms are flexuous.

FUNGI: *Mould* forms.—Branching filaments (hyphae) interlacing and forming a meshwork (mycelium) ; more highly organised than the bacteria, often septate and multicellular, and reproduce usually by means of spores developed in "fruiting organs." *Yeast* forms.—Round, oval or elongated units, generally larger than bacteria and multiply by "budding" ; in certain species multiple "endospores" formed ; in some, hyphae occur.

PROTOZOA.—Generally regarded as the lowest forms of animal life ; minute unicellular organisms with the protoplasm well differentiated into nucleus and cytoplasm ; reproduce by fission and spore-formation, and often exhibit a definite life-cycle with both sexual and asexual phases.

The bacteria and the viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and sub-tropical countries.

THE BACTERIA

For practical work some scheme of biological classification is necessary. Various systems have been used, and one of

these, which has been elaborated in recent years, will be outlined later (p. 29). In the first instance, bacteria can be classified broadly as follows :—

HIGHER BACTERIA

Elongated and sometimes sheathed filaments, often showing true branching ; units may be interdependent, e.g. some being specialised for reproduction ; more highly organised than the lower bacteria—e.g. *Actinomyces* (p. 524).

LOWER BACTERIA (or EUBACTERIA)

Simple and typically unicellular structures, never in the form of sheathed filaments ; each unit biologically equivalent and autonomous ; many species motile, and this is usually associated with the possession of flagella.

Main Morphological Forms

1. Cocc—spheroidal in shape—e.g. the streptococci (p. 335).
2. Bacilli—relatively straight rod-shaped organisms—e.g. the typhoid bacillus (p. 434).
3. Vibrios and spirilla—definitely curved non-flexuous rods (vibrios) or spirals (spirilla)—e.g. *Vibrio cholerae* (p. 469).
4. Spirochaetes—flexuous spiral filaments—e.g. *Treponema pallidum* of syphilis (p. 538).

MORPHOLOGICAL STUDY OF THE BACTERIA

UNSTAINED PREPARATIONS OF LIVING ORGANISMS.—The morphology of the bacteria can be studied in the first place by examining them microscopically in the unstained condition, suspended in fluid. In this way their general shape can be observed and motility determined (pp. 10, 70). Certain very slender bacteria, however, such as the spirochaetes, are so feebly refractile that they cannot be seen by the ordinary microscopic methods, and *dark-ground illumination* (p. 71) is necessary for their demonstration.

Electron microscopy (p. 81) is now applied to the morphological study of the bacteria, and has demonstrated many features not hitherto recognised.

For the study of the development of individual organisms and the growth of bacteria in communities or colonies (p. 14), the “ agar-block ” method of Ørskov, or the microscope-

incubator may be used (p. 227). 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 microscopic examination of stained bacteria is usually an essential routine procedure. For this purpose various dyes, *e.g.* methylene blue, basic fuchsin, methyl violet, are employed, sometimes along with a mordant.

“*Negative*” staining is of value for the simple morphological study of bacteria—*i.e.* the organisms are mixed with some substance which, in film preparations, yields a dark or coloured background, while the organisms stand out as clear unstained objects. India ink and nigrosin are examples of substances used for this purpose (p. 88).

Silver impregnation methods (p. 113) are utilised for the staining of spirochaetes and are particularly applicable for demonstrating these organisms in tissues.

“*Impression preparations*” are valuable for the microscopic study of bacteria in their natural relationships in a colony on culture medium. For this purpose whole colonies are transferred to cover-slips and then suitably stained (p. 118).

STAINING REACTIONS.—The staining reactions of the bacteria are of the greatest importance both in their morphological study and for their differentiation and identification. Thus, all the bacteria can be divided into two categories by the so-called Gram’s staining reaction (p. 89)—*i.e.* according to whether they resist decolorisation with aniline oil, alcohol or acetone after staining with a pararosaniline dye—*e.g.* crystal or methyl violet, and subsequent treatment with iodine. Those retaining the dye are designated “Gram-positive”; those decolorised by this method are spoken of as “Gram-negative.”

The mechanism of the Gram-staining reaction is not yet fully understood. Gram-positive organisms are able to retain basic dyes at a higher hydrogen-ion concentration than the Gram-negative species, and it has been suggested that a greater affinity for a basic dye, due to a more acidic character of the protoplasm, may assist such retention. Another suggested explanation for the difference in Gram-reaction is that it depends on a difference in the permeability of the cell wall or the surface of the cell protoplasm. Thus, the dye-complex formed in the cell after staining and treatment with iodine can diffuse freely from the Gram-negative cell under the action of the decoloriser in

which it is soluble, but not from the Gram-positive organism, presumably because of a relative impermeability of its surface. 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 the magnesium salt of ribonucleic acid combined with protein ; thus Gram-positive bacteria become Gram-negative if they are mechanically ruptured or if their magnesium ribonucleate is removed by treatment with bile salt, or separated, by means of the enzyme ribonuclease, from the protein with which it is combined at the surface of the cell protoplasm.

Some bacteria are relatively resistant to simple stains, but when stained by a strong staining solution (applied with heat) resist decolorisation by acid, and are spoken of as "acid-fast"—*e.g.* the tubercle bacillus (p. 397).

Such bacteria have a high lipid content and their acid-fastness has been attributed to this. When lipoids are removed by extraction with suitable solvents, the cells are no longer acid-fast. One of the lipoids extracted from the tubercle bacillus, an unsaponifiable wax, known as "mycolic acid," exhibits the property of acid-fastness in the free state. But the mere presence in the cell of such lipid is not in itself sufficient to explain acid-fastness, since the character is lost by autolysis or mechanical disintegration. Acid-fastness depends on the structural integrity of the cell and possibly on a special disposition of lipid within the cell.

Certain bacteria do not stain uniformly. Thus, the diphtheria bacillus shows a "beaded" or "barred" appearance when stained with methylene blue. The plague bacillus displays "bipolar staining," the ends being more deeply coloured than the centre.

Such uneven staining may depend on various factors : nuclear and cytoplasmic constituents of the cell may stain with different intensity ; the cell may contain granules with specially weak or strong affinity for the stain ; and irregular appearances are sometimes the result of artifact.

INTRACELLULAR GRANULES.—In many species of bacteria, 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.* reserve food material or waste products. They consist of fats, glycogen, starch, or "volutin," the last being possibly a metaphosphate, as indicated by studies of these granules in yeasts. The presence, nature and disposition of intracellular granules is characteristic of certain organisms and may aid in

their identification. Volutin granules are sometimes described as "metachromatic" on account of their peculiar staining reactions, having a strong affinity for basic dyes, and with these being more deeply coloured than the rest of the protoplasm. By special methods they may be stained with one dye while the rest of the cell is stained with another dye of different colour (p. 97). The diphtheria bacillus is one of the organisms exhibiting volutin granules and their demonstration is utilised for its identification.

BACTERIAL PROTOPLASM AND NUCLEAR BODIES.—When unstained bacteria are examined, or bacteria stained by the usual methods, no differentiation into nucleus and cytoplasm is observed. However, by a special method it is possible to demonstrate the presence in bacteria of bodies which correspond to nuclei or chromosomes, though differing morphologically from the organised nuclei of animal or plant cells. After suitable fixation, the bacteria are treated with hydrochloric acid to reduce the affinity of the cytoplasm for stain ; on subsequent staining the nuclear bodies become deeply stained and the cytoplasm but slightly (p. 119). The nuclear bodies are oval or dumb-bell shaped and may be placed 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 granules described above, they are constantly present in all cells and under all conditions of culture. Only a single nuclear body is present in some cells, while in others, as a result of nuclear division preceding cell fission, two, four or even more nuclear bodies may be present.

CELL WALL AND CYTOPLASMIC MEMBRANE.—The bacterial substance is contained within a very thin *cytoplasmic membrane*, and external to this a relatively rigid *cell wall*. It is this latter structure which maintains the characteristic shape of the organism. The chemical composition of the cell wall is not known. By the usual staining methods it remains invisible, but may be revealed by special methods : by staining after treatment with tannic acid, or after treatment with strong salt or alkali solution to cause shrinkage of the protoplasm away from the cell wall. The cell wall is clearly demonstrated by electron microscopy.

BACTERIAL CAPSULES AND EXTRACELLULAR SLIME.—Certain bacteria may exhibit a relatively thick outer capsule and are described as "capsulate." The capsule is a structure quite

distinct from the cell wall and lies outside it. The development of capsules is usually dependent on certain favourable environmental conditions, e.g. the presence of abundant carbohydrate and, in the case of pathogenic bacteria, growth in animal tissues or in a culture medium enriched with unaltered animal protein. In certain species complex carbohydrate substances of polysaccharide nature enter into the composition of such capsules, in other species polypeptides or proteins ; such substances are of great importance in determining specific characters (p. 39). Virulence may also depend on capsule formation (*vide pneumococcus*, p. 356). By ordinary methods of staining the capsule is invisible, though in microscopic preparations from animal tissues or body fluids it often appears as an unstained zone round the organism. Special methods are available for the differential staining of capsules (p. 100). "Negative" staining is also of particular value for demonstrating capsules (p. 88).

Many capsulate organisms and some non-capsulate species produce extracellular slime. This is a colloidal viscid material which is similar in chemical nature to capsular polysaccharides. The slime is situated outside the cells and their capsules. It is amorphous and does not constitute a definite structure like a capsule ; it also readily disperses into solution in liquid culture medium, though in growths on solid medium it remains as a matrix in which the bacteria are embedded, conferring on the growths a viscid or "mucoid" character. Slime can be demonstrated by some of the methods applicable to capsules, e.g. the India ink method (p. 88).

BACTERIAL SPORES.—Some species, mainly those of bacillary morphology, develop a highly resistant resting-phase or spore, by which the individual can survive unfavourable external conditions. The spore is not a reproductive structure. In the vast majority of spore-bearing species only one spore is developed by each vegetative cell. The spore is formed in the protoplasm (hence called "endospore") and includes part of the nuclear material of the cell. As it develops it acquires a dense outer envelope ("spore case" or "spore coat") and becomes resistant to ordinary staining. When mature it takes up a "central," "subterminal" or "terminal" position. The relative size of the spore varies with different species. It may be spherical, oval or elongated. Spores can withstand all injurious chemical and physical influences better than the vegetative forms, and probably owe their resistant properties to their envelopes and their low

content of unbound water, though the total water-content of spores and vegetative forms may be the same. Spores also differ from vegetative cells in lacking enzymic activity. They may be antigenically different from the vegetative phase (p. 38), which indicates some essential difference in chemical constitution.

Under favourable external conditions, *e.g.* as regards the presence of moisture and nutrient materials, the envelope ruptures at one pole or equatorially, and the vegetative organism then emerges. This process is described as the "germination" of the spore. Sometimes the envelope disappears without obvious rupture. By electron microscopy it has been shown that this envelope is a structure quite distinct from the vegetative form which emerges from it.

In unstained preparations the spore is a highly refractile body as compared with the vegetative cell. It is not stained by the ordinary methods but appears as a clear, unstained portion of the bacterial structure. In some cases, however, the spore stains by Gram's method. Special methods are employed for the differential staining of spores (p. 99).

MOTILITY.—The motility of bacteria (observed in a fluid medium) has generally been regarded as due to delicate prolongations of the protoplasm (flagella) which act as locomotory organs; and their propellant action has been described as the result of contractions moving spirally over their surface. They are not seen in unstained preparations and can be demonstrated only by special staining methods (p. 102) or by electron microscopy. Brownian movement must not be confused with true motility. In the latter case the organism definitely changes its position in the microscope field (p. 71). Brownian movement is due to the impact of the molecules of the medium in which the organisms are suspended ("molecular bombardment"), and is an oscillatory movement within a limited radius.

Flagella may be "terminal" (or "polar")—*i.e.* at one or both ends of the bacterium—and single or multiple. They may appear in stained preparations to be distributed all round the organism, and this arrangement is described as "peritrichous"; but possibly such disposition may not be a natural one when the organism is in motion. Thus, under such conditions the flagella may be coiled into a single spiral "tail" which projects from one end.

It has been claimed by Pijper that the motive force of various bacteria resides in the protoplasm and is due to wave-like spiral con-

tractions which are communicated to the cell wall. He suggests that the flagella are the products of movement and derived in this way from mucoid material on the surface of the cell. Electron microscopy, however, has clearly shown that flagella are organised structures as originally supposed, and originate from the cytoplasm.

When there is a single terminal flagellum the term "monotrichous" can be applied; "amphitrichous" indicates the presence of a flagellum at each pole; "lophotrichous" refers to the arrangement of multiple flagella at one or both poles.

Among the spirochaetes motility is generally considered to be a function of the contractility of the cell protoplasm. However, flagella, or flagella-like structures, have been demonstrated in certain spirochaetes, e.g. *Treponema pallidum*, by special staining methods and by electron microscopy (p. 81). The most characteristic movement is a spiral rotation on the long axis with progression in the axial line. Movements of flexion, and sometimes lashing movements, are also observed.

PLEOMORPHISM AND INVOLUTION.—It must be remembered that, especially in artificial culture, bacteria may show considerable variation in shape and size (pleomorphism), and may also exhibit degeneration or "involution" forms which are different morphologically from the normal cell. Such abnormal forms are commonly found when bacteria are growing in the presence of antagonistic substances, or when cultures on artificial media are ageing.

MULTIPLICATION AMONG THE BACTERIA.—Among the lower bacteria, multiplication takes place by simple binary fission. The cell enlarges or elongates, and the protoplasm becomes divided transversely or equatorially. In some species, division of the cell wall immediately follows, and two free and independent units are formed; in others, the cell walls of the new individuals remain continuous (as is well demonstrated by electron microscopy) and the organisms tend to adhere and grow in pairs, chains or clusters (*vide infra*). Division may occur with great rapidity, e.g. every half-hour, so that one individual may soon reproduce several millions of new organisms. Among the spirochaetes transverse fission occurs as in other bacteria.

In the higher bacteria transverse division of the filaments into shorter forms is observed. Certain filaments also form at their free ends a chain of oval "conidia," which are set free, and each of these, besides representing a resting-phase, may develop a new colony.

Some observers have described more complex processes of reproduction among bacteria, including in some species sexual reproduction, and postulate life-cycles comprising different morphological phases. The evidence at present does not warrant acceptance of such views as applicable generally to the pathogenic bacteria.

COCCI

Cocci can be classified and named morphologically as follows :—

Staphylococcus.—The individuals tend to be aggregated in irregular clusters, due to the irregularity of the planes of successive divisions and the adhesion of newly-formed cells.

Streptococcus.—The cocci are arranged and adhere together in chains, successive divisions being always in a similar axis.

Diplococcus.—The cells tend to be arranged uniformly in pairs.

Tetracoccus.—Plates of four (or multiples of four) adherent cocci, division occurring successively in two planes at right angles.

Sarcina.—Packets of eight (or multiples of eight) adherent cocci, division occurring successively in three planes at right angles.

The names *Staphylococcus*, *Streptococcus*, *Diplococcus* and *Sarcina* have been generally used as generic designations.

The different cocci are relatively uniform in size, 1μ ($0\cdot001$ millimetre) being the average diameter. Some species are capsulate. Form varies with species, being spherical, oval, lanceolate or kidney-shaped. The reaction to Gram's stain is an important criterion in their identification.

BACILLI

Morphological features of importance in the study and identification of these organisms are :—

Size—some being relatively large—e.g. the anthrax bacillus (p. 414), others small—e.g. the influenza bacillus (p. 492).

Shape—rectangular—e.g. the anthrax bacillus ; oval (cocco-bacilli)—e.g. the plague bacillus (p. 475).

Arrangement—in pairs—e.g. the diplobacillus of Morax (p. 495) ; in chains—e.g. the anthrax bacillus.

Occurrence of a capsule—e.g. the pneumobacillus.

Motility, flagella and their arrangement—e.g. the typhoid bacillus possesses numerous flagella which are peritrichous in arrangement.

Spores, their shape and position—e.g. the tetanus bacillus is characterised by its spherical terminal spore.

Reaction to Gram's stain—e.g. the diphtheria bacillus is Gram-positive, the typhoid bacillus Gram-negative.

Acid-fastness—e.g. the tubercle bacillus.

Staining of cytoplasm—e.g. “beading,” bipolar staining, volutin granules (*vide supra*).

The bacilli cannot be classified into morphological genera, and physiological characters have to be taken into account in their classification. In the past the term “bacillus” was used as a generic name for all the straight rod-shaped forms, but it must be recognised that such application of the term cannot be justified in the biological sense in view of the great diversity of species which exhibit this morphological form. On the other hand, the long-established use of this name in medical literature would provisionally warrant its continuance for the simple designation of certain bacteria, e.g. “tubercle bacillus,” “diphtheria bacillus,” “typhoid bacillus,” etc.

VIBRIOS AND SPIRILLA

Comma- and S-shaped forms and non-flexuous spirals are characteristic (*vide supra*). Most species are very actively motile (e.g. “darting” motility) and the flagella are terminal. They are mostly Gram-negative.

Two morphological genera have generally been recognised : *Vibrio*—the typically short curved rod-shaped form ; and *Spirillum*—the spiral filamentous type ; though there is no sharp line of demarcation between them.

SPIROCHAETES

Some spirochaetes are relatively large, refractile and easily stained by ordinary methods, e.g. *Borrelia refringens* ; others are delicate, feebly refractile and difficult to stain, e.g. *Treponema pallidum*. Among different species the coils vary considerably in wave-length and amplitude. Movement is mainly rotatory. Flagella-like structures have been demonstrated in some spirochaetes. It has been stated that in certain pathogenic species granules are developed in the protoplasm and extruded from the cell, and these have been regarded as a phase in the life history of the organism.

The pathogenic forms can be classified broadly into the following genera :—

- (1) *Borrelia*—coils are large, and generally three to seven

in number, the wave-length being about $2\text{--}3\mu$. These organisms are usually more refractile and more readily stained by the ordinary dye solutions than the other pathogenic spirochaetes. They are exemplified by the relapsing fever spirochaetes (p. 546) and certain commensal species, such as *Borr. refringens* (p. 545).

(2) *Treponema*—coils are of shorter wave-length (*e.g.* 1μ), generally regular, sometimes relatively rigid, the organism presenting typically a “corkscrew-like” structure. These spirochaetes are usually feebly refractile and difficult to stain by the ordinary dye solutions. *Tr. pallidum* exemplifies this genus (p. 538).

(Some systematists classify all the spirochaetes included in these two genera into one composite genus, designated *Treponema*.)

(3) *Leptospira*—coils are fine, closely wound and numerous, and may be difficult to demonstrate except by dark-ground illumination; one or both extremities of the filament are “hooked” or recurved. Spirochaetes of this group, like the *Treponemata*, are feebly refractile and not readily stained by the ordinary methods. (They differ from the other genera in being able to survive in water.) *Leptospira icterohaemorrhagiae* of infectious jaundice is an example of the genus (p. 549).

MORPHOLOGY OF BACTERIAL GROWTHS ON CULTURE MEDIUM

It is of prime importance in the practical study of the bacteria that these organisms can flourish in the laboratory in artificial food media. Such growth is designated a *culture* and in general is easily visible to the naked eye within a day or two under suitable conditions, as explained later in relation to the physiology of the bacteria. Moreover, on solid media growths of particular species may present a characteristic morphology. When individual bacteria are placed on the surface of a solid medium sufficiently separate from one another, a visible *colony* of growth results from each viable unit. Just as the bacterial cells of a species may possess a characteristic morphology, so also bacterial colonies in artificial culture may show special morphological features, and these are often significant in the identification of the pathogenic and commensal organisms. While such morphological features may be readily visible to the naked eye,

magnification by means of a hand lens or a low-power microscope is frequently necessary for detailed study (p. 212). The morphological types of colonies are exemplified in the descriptions of the various organisms dealt with in Part III.

PHYSIOLOGY OF THE BACTERIA

The physiology and biochemistry of bacteria can be studied by observations made with cultures grown in the laboratory in a nutrient medium.

Bacteria are subject, as regards their growth and vitality, to various external influences—*e.g.* nutrient materials, atmosphere, temperature, moisture and hydrogen-ion concentration of their environment, light, and a great variety of chemical substances.

BACTERIAL NUTRITION.—The growth of bacteria is, of course, dependent on an adequate supply of suitable food material, and this varies with the natural adaptations of different species. It is, moreover, of fundamental importance that in the metabolism of bacteria, as of other living organisms, enzymes play an essential part, and the utilisation of nutrient substances is dependent on the enzymic content of the bacterial cell. The growth of bacteria involves the synthesis of complex materials—proteins, carbohydrates and lipoids—of which the bacterial substance is constituted. Some organisms are able to synthesise these components from the simplest starting materials or “building stones.” Thus, certain non-parasitic forms utilise inorganic nitrogen and the carbon dioxide of the atmosphere, like plants, and are designated *autotrophic*; from ammonia, for example, amino-acids are produced and from these in turn protein is built up, while energy for growth is derived from sunlight or by oxidation of simple inorganic compounds. On the other hand the pathogenic bacteria are *heterotrophic* and require organic material, *e.g.* preformed amino-acids, while energy for growth is obtained mainly by oxidative decomposition of carbohydrates from which they may also derive their supply of carbon. Some of these, however, are able to synthesise amino-acids, *e.g.* tryptophane from ammonium salts if a suitable organic source of carbon is also available. The free-living non-parasitic bacteria are capable of synthesising for themselves all the various metabolites necessary for growth; but as bacteria have become more parasitic in

their evolution, they have increasingly obtained essential metabolites from their hosts and have lost the power of themselves elaborating these compounds. Similarly also, essential components of various enzyme systems necessary for metabolism are lacking in the parasitic and pathogenic bacteria ; and the viruses would seem to represent the extreme of parasitism in which all or practically all enzyme systems have been lost and metabolic activity is completely absent, apart from the tissues of the host from which the necessary metabolites and enzyme systems may, as it were, be borrowed (p. 569).

The transformation of simple substances into the complex constituents of bacteria probably involves the formation in stages of various intermediate substances of increasing chemical complexity, each stage in the synthesis being catalysed by an appropriate enzyme or enzyme system. Any interference with a metabolite representing one of these stages or with its associated enzyme would inhibit growth by preventing the metabolite from being utilised for further synthesis. It is by such interference that many antiseptics, chemotherapeutic substances and antibiotics bring about inhibition of growth and death of the organism (p. 21).

The preformed amino-acids which are essential for the metabolism and growth of the pathogenic organisms are exemplified by tryptophane, leucine, methionine, cystine and histidine. Such substances are obtained naturally from the tissues and body fluids of the animal host, and in artificial culture are contained in the main ingredients of the medium ; thus, commercial peptone, so often used for the preparation of culture media, is a convenient source of amino-acids for bacterial growth.'

Even when all the necessary materials for the synthesis of proteins, carbohydrates and lipoids have been supplied, certain accessory organic compounds are still required for growth; these are analogous to the vitamins required for animal nutrition and have sometimes been designated "*bacterial vitamins.*" In fact, some are identical with the components of the vitamin B complex. Exceedingly minute amounts as in the case of the animal vitamins may suffice. Such compounds, like the essential amino-acids, cannot be synthesised by various heterotrophic bacteria and have to be obtained from the body of the host, or, in artificial culture, from ingredients of the medium, e.g. meat extract, blood or serum. In general, these *accessory growth factors*

function as catalysts or form parts of the molecules of bacterial enzymes and are not building materials for the cell substance. Examples of such compounds are nicotinic acid and pimelic acid (required for the growth of the diphtheria bacillus), pantothenic acid, pyridoxine and thiamin (aneurin or vitamin B₁) (for *Streptococcus pyogenes*), biotin (for *Staphylococcus aureus*), haematin and cozymase (for *Haemophilus influenzae*), uracil and riboflavin (for the tetanus bacillus), folic acid (for *Streptococcus lactis*). The part played by all these compounds in the metabolism of bacteria is not yet fully understood, but, as mentioned above, many of them catalyse essential metabolic reactions or enter into the chemical constitution of essential enzymes, e.g. those involved in the "respiratory" or oxidation-reduction processes of the bacterial cell. Thus, cozymase is an essential catalyst for certain of these reactions; haematin enters into the constitution of the cytochrome system, which also plays a similar part; and riboflavin is a component of the functional prosthetic group of the "respiratory" enzyme, flavoprotein.

The practical applications of the principles of bacterial nutrition outlined above are illustrated in more detail in Chapter V in relation to the preparation of culture media for the parasitic bacteria.

INFLUENCE OF ATMOSPHERE.—Some species grow most abundantly in the presence of *free* oxygen—i.e. when exposed to air—and are described as "aerobes." Others fail to grow in the presence of *free* oxygen, "obligatory anaerobes"—e.g. the tetanus bacillus. The ultimate determining factor, however, is not the presence or absence of free oxygen, but the state of oxidation or reduction of the environment, this condition being usually referred to in terms of the "oxidation-reduction potential" (pp. 219, 220). The majority of pathogens are, however, indifferent as regards atmospheric conditions and will flourish in either the presence or absence of oxygen ("facultative anaerobes"—e.g. the typhoid bacillus). Certain pathogenic bacteria grow best in the presence of a trace only of *free* oxygen and are designated "micro-aerophilic"—e.g. some streptococci. An atmosphere with a certain concentration of carbon dioxide (5–10 per cent.) is essential for the growth of particular species, e.g. *Brucella abortus* when first isolated from the body. It is now recognised that various heterotrophic bacteria assimilate carbon dioxide.

BACTERIAL OXIDATIONS.—As shown above, some bacteria utilise molecular oxygen and they also liberate carbon dioxide

like animal cells. Others effect the oxidations necessary for energy requirements in the complete absence of molecular oxygen. In this connection it should be clearly understood that such oxidation is a coupled oxidation-reduction reaction : hydrogen is given up by one substance (the hydrogen donator) which is thus oxidised, while simultaneously the hydrogen is transferred to another substance (hydrogen acceptor) which is thus reduced. Among the aerobes molecular oxygen serves as a hydrogen acceptor and is reduced ultimately to water, but if the organism can reduce other substances oxidation may proceed under anaerobic conditions by transfer of hydrogen. Various enzymes of the bacterial cell are responsible for the transport of hydrogen, and certain growth factors referred to above are components of these enzymes. The whole subject of bacterial oxidations is of some complexity and its full and detailed consideration is beyond the scope of this book. The oxidation-reduction potential is briefly considered from the practical standpoint in Chapter VI, but for further information on the oxidation-reduction systems of the bacteria, reference should be made to works on bacteriological chemistry.¹

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 the best growth occurs. The optimum temperature of a bacterium is approximately that of its natural habitat, *i.e.* about 37° C. in the case of the micro-organisms which are pathogenic to man. Some organisms have a wide temperature range (*e.g.* 5°–44° C.), others are more restricted (*e.g.* 25°–40° C.). Below the minimum, viability is not necessarily interfered with, but above the maximum, death ensues sooner or later, except in spore-bearing species.

Some species of saprophytic bacteria grow best at remarkably high temperatures (*e.g.* 60°–70° C.). These are designated *thermophilic*.

(b) *on Viability.*—Heat is an important agent in the artificial destruction of micro-organisms, the effect depending on coagulation and denaturation of cell proteins. In general,

¹ Anderson, C. G., *An Introduction to Bacteriological Chemistry*, 2nd Ed., Edinburgh, 1946; Gale, E. F., *The Chemical Activities of Bacteria*, London, 1951.

among the bacteria which are parasites of mammalian animals, non-sporing forms, in the moist state, cannot withstand temperatures above 45° C. for any length of time.

The "thermal death-point" is the lowest temperature *approximately* (above the maximum for growth) which kills a particular organism in a given time, e.g. ten minutes. Thus, the thermal death-point of the pneumococcus is *approximately* 52° C.

Bacteria are more susceptible to moist heat, e.g. in a steam steriliser (p. 129), than dry heat, e.g. in a hot-air oven (p. 126). Spores are much more resistant to heat than vegetative bacteria, and this is probably due to the high content of bound water which cannot take part in the coagulation of the protein (p. 9); but their degree of resistance varies in different species. Further data on this subject are given in later chapters.

Some species die if kept at 0° C., but most survive for long periods at this temperature and even at much lower temperatures.

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 necessary for growth. Drying in air is injurious to many species; spores, however, can resist this influence for long periods—e.g. spores of *Bacillus anthracis* for several years. Certain non-sporing bacteria also may exhibit considerable resistance to natural drying—e.g. the tubercle bacillus (*q.v.*). If cultures of non-sporing bacteria are rapidly dried at low temperature and kept *in vacuo* in the dark they may survive for long periods, even for several years, and this procedure is used as a practical means of preserving cultures (p. 221).

INFLUENCE OF HYDROGEN-ION CONCENTRATION.—This is an essential factor in bacterial metabolism and growth. The majority of the commensal and pathogenic bacteria grow best at a slightly alkaline reaction (pH 7.2–7.6). Some bacteria, however, flourish in the presence of a certain degree of acidity and are named *acidophilic*, e.g. *Lactobacillus* (p. 467); others are very sensitive to acid but tolerant of alkali, e.g. *Vibrio cholerae* (p. 469).

INFLUENCE OF LIGHT AND OTHER RADIATIONS.—A favourable condition for growth and viability is darkness. Ultra-violet rays are markedly bactericidal, e.g. direct sunlight or radiation from an arc or mercury-vapour lamp. Bacteria are likewise killed by cathode and Röntgen rays and by radium

emanations. They are also susceptible to sonic and super-sonic waves.

ELECTRICAL INFLUENCES.—Bacteria may be killed by the passage of an electrical current through the medium in which they are contained. This is not necessarily due to any direct electrical effect. In the case of an alternating current, the resultant heating effect may be bactericidal *per se*, and, if a direct current is used, electrolytic changes with the liberation of acid, oxygen or chlorine may lead to bactericidal effects of a chemical nature.

OSMOTIC EFFECTS.—Like other cells bacteria are subject to osmotic phenomena. Thus plasmolysis may result in hypertonic salt solutions, while bacterial cells may rupture in hypotonic solutions.

ANTISEPTICS, CHEMOTHERAPEUTIC SUBSTANCES, AND ANTI-BIOTICS.—A great variety of inorganic and organic chemicals are bacteriostatic (inhibit bacterial growth) or bactericidal (kill bacteria), depending on the concentration brought into contact with the particular organism.

Substances possessing marked bacteriostatic or bactericidal properties are usually designated *antiseptics*, and their practical applications are now well known. Various chemical substances exhibit antiseptic action and have been used for that purpose; *acids*—e.g. boric acid, benzoic acid; *alkalis*—e.g. sodium hydroxide; *metallic salts*—e.g. mercuric chloride (corrosive sublimate) and mercuric iodide (biniodide of mercury); *organic metallic compounds*—e.g. sodium ethylmercuri-thiosalicylate (merthiolate); *halogens*—e.g. chlorine (as derived from hypochlorites such as bleaching powder, etc.), iodine; *alcohols* and *ethers*—e.g. ethyl alcohol; *aldehydes*—e.g. formalin; *aromatic hydrocarbons*—e.g. toluene; *phenols*—e.g. phenol (carbolic acid), cresols; *volatile oils and their products*—e.g. thymol; *oxidising agents*—e.g. hydrogen peroxide, potassium permanganate; *reducing agents*—e.g. sulphurous acid; various *organic dyes*—e.g. brilliant green, proflavine; *soaps and other detergents*—e.g. sodium lauryl sulphate. It should be noted that even *neutral salts* such as sodium chloride may exert bacteriostatic or bactericidal properties in certain concentrations.

These various antiseptic chemicals may exhibit considerable differences in their intensity and rapidity of action. Moreover, bacterial species may differ greatly in susceptibility to particular antiseptics; this will be illustrated later in connection with selective methods of cultivation.

Certain chemical substances of low toxicity to the animal body are successfully used for therapeutic purposes in bacterial and other infections, in virtue of their capacity for directly or indirectly controlling the development of the particular micro-organism in the blood or tissues. These substances, however, in the concentrations in which they can be applied in the body may not directly kill the parasitic organism though they may interfere with its metabolism, inhibit growth or reduce virulence, thus allowing the natural defences of the body to play their full part in combating the infection. This is exemplified by the *sulphonamide compounds* (sulphanilamide, sulphapyridine, sulphathiazole, sulphadiazine, sulphamezathine, etc.).

The sulphonamide compounds inhibit growth of bacteria *in vitro* and *in vivo*, and the available evidence suggests that the mode of action consists in an interference with some essential metabolite or with the enzyme associated with that metabolite. Extracts of yeast have been shown to reverse the action of the sulphonamide compounds, *i.e.*, the extracts are antagonistic to them; the substance responsible for this antagonistic action has proved to be *p*-aminobenzoic acid.^{1, 2} This is an essential metabolite and a growth factor for many bacteria, and it has a structural similarity to the sulphonamide compounds. It should be noted that the inhibitory effect is observed only when the ratio sulphonamide/*p*-aminobenzoic acid exceeds a certain value, irrespective of the absolute concentrations of the two substances. This is characteristic of the phenomenon of "competitive inhibition" and suggests that, in virtue of their similar structure, *p*-aminobenzoic acid and sulphonamide "compete" for some enzyme surface in the bacteria. Under normal conditions *p*-aminobenzoic acid is metabolised by enzymic action into some compound necessary for growth (*e.g.* folic acid which is a conjugated form of *p*-aminobenzoic acid). If, however, the relative concentration of sulphonamide is sufficiently high, the enzyme will be unable to act upon sufficient quantity of *p*-aminobenzoic acid for this phase of the metabolism to proceed, with the result that growth of the organism will cease. In other words, the sulphonamide compounds probably act by inhibiting an enzymic reaction, fundamental in the metabolism of the organism.

It seems possible that this theory may explain the action of many antiseptics and chemotherapeutic substances. Studies of certain other growth factors and their chemical analogues have supported the theory.³ For example, it has been possible to "design" substances which will inhibit bacterial growth by substituting $-\text{SO}_3\text{H}$ for $-\text{COOH}$ in a growth-promoting compound; thus, "pantoyltaurine" is derived

¹ Woods, D. D., *Brit. J. Exp. Path.*, 1940, **21**, 74.

² Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 67.

³ McIlwain, H., *Brit. J. Exp. Path.*, 1940 **21**, 136; 1942, **3**, 95.

in this way from pantothenic acid which is necessary for the growth of *Streptococcus pyogenes*, pneumococcus and certain other organisms, and it is found that pantoyltaurine is bacteriostatic to these organisms.

Various *antibiotic* substances derived from plants, fungi and saprophytic bacteria, e.g. penicillin, streptomycin, chloramphenicol, aureomycin, etc., are potent bactericidal or bacteriostatic agents towards pathogenic organisms. It may be noted that, while different species of bacteria may flourish well together, the presence of one organism favouring the growth of another—*symbiosis*—e.g. the enhanced growth of *Haemophilus influenzae* in the presence of staphylococci, the reverse effect may also be observed—*antibiosis*—one species being antagonistic to another. This effect is due to chemical products of the antagonistic organism, and it is to these that the term “antibiotic” is now applied.

The phenomenon of antibiosis as it occurs in nature and in laboratory cultures has long been recognised. *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) was known to have such effect on various bacterial species, and the active principle was designated *pyocyanase*. This product is lytic to many other bacteria, and it has been found to be of lipoid nature, its activity being due to unsaturated fatty acids. This organism, however, yields another antibiotic product, *pyocyanin*, the blue-green pigment of the organism (p. 351), and a further product has been described, *hemipyocyanin*, which is more active towards the pathogenic fungi than towards bacteria.

The failure of non-sporing pathogenic bacteria to survive in soil also drew attention to the antagonism of soil organisms, e.g. sporing aerobic bacilli, actinomycetes and fungi, to the former. Thus, plate cultures of mixtures of soil organisms along with a pathogen have often revealed this effect by the occurrence of zones of lysis or absence of growth of the pathogen round the antibiotic colonies of the soil organisms. A particular species of sporing bacillus derived from soil, *Bacillus brevis*, yields a substance, *gramicidin*, which is highly bactericidal to Gram-positive bacteria such as *Staphylococcus aureus*, various streptococci and the pneumococcus. It is a polypeptide of relatively simple structure. It acts both *in vitro* and *in vivo*, and its toxicity to animals is relatively low.

*Penicillin*¹ is derived from the mould, *Penicillium notatum*, and comprises a group of closely related complex organic acids possessing similar but not identical antibacterial properties. These contain nitrogen and sulphur in their molecules. They have been designated Penicillin F, G, X and K in America and I, II, III and K in this country.

¹ Reference may be made to Fleming, A., *Penicillin: Its Practical Application*, London, 1946.

Penicillin G (or II) is produced in largest amount by the present manufacturing methods. Penicillin is active mainly against Gram-positive bacteria and its potency in inhibiting the growth of these organisms is of a very high order. It is bactericidal and also bacteriolytic, but acts only on organisms in their active phase; in the resting state bacteria are not affected. Its properties were first applied by Fleming in the selective culture of certain Gram-negative organisms, e.g. *Haemophilus influenzae*, when mixed with Gram-positive species in pathological material. Reference to this is made on pp. 494, 498.

Susceptible organisms.

The Gram-positive pyogenic cocci (except enterococcus).
Group of *Clostridia*, e.g. *Cl. welchii*.
Gonococcus and *Meningococcus*.
Diphtheria bacillus, *Bacillus anthracis* and *Actinomyces*.
Pathogenic spirochaetes.

Non-susceptible or relatively non-susceptible organisms.

Coliform bacilli, *Salmonella* and dysentery groups.
Proteus, *Pseudomonas aeruginosa* and *Vibrio cholerae*.
Pasteurella, *Brucella*, and *Haemophilus* groups.
Tubercle bacillus.
Pathogenic fungi, Rickettsiae, Viruses and Protozoa.

The substance is present in solution in the culture fluid when *Penicillium notatum* is grown on the surface of a shallow layer of fluid medium, e.g. in flat bottles, or in medium which is specially aerated, e.g. in large tanks of 12,000 gallons, as in the large-scale manufacture of penicillin; and processes for extracting penicillin in the purest and most concentrated form have now been perfected. It may be pointed out that penicillin is unstable unless preserved in a desiccated form. It is quickly inactivated by acids, alkalis, metallic salts, alcohols and oxidising agents, and is also thermolabile. For therapeutic purposes the sodium, potassium and calcium salts are generally used; these are readily soluble in water or physiological saline and the solution is administered by injection, e.g. intramuscularly. The earlier preparations of penicillin salts were impure and were of a yellow colour due to the pigment of the mould, which was one of the impurities present. Colourless crystalline products containing over 95 per cent. of penicillin G (II) are now available for therapeutic purposes: these represent about 1600 units per mgm. (*vide infra*). The pure sodium salt has a potency of 1660 units per mgm. The therapeutic value of penicillin depends not only on its high quantitative potency (e.g. *in vitro* inhibiting *Staphylococcus aureus* in a dilution of $1:3 \times 10^7$) but also its non-toxicity and the maintenance of its effects even in the presence of blood, serum or pus. (Penicillin K, however, is rapidly inactivated by plasma.) - It is readily absorbed after intramuscular injection, but is rapidly excreted, e.g. in the urine, and this necessitates the injection of large and repeated doses. Alternatively, a preparation

is injected which will delay absorption of penicillin, *e.g.* the procaine salt of penicillin G suspended with aluminium monostearate in peanut oil. For regulating dosage penicillin has been standardised in terms of an arbitrary unit ("Oxford unit") originally defined as that amount which, dissolved in 50 ml. meat extract broth, just inhibits completely the growth of the test strain of *Staphylococcus aureus*. At present the unit may be defined as the activity contained in a certain weight (0.6 microgram) of the pure sodium salt of penicillin G. A total dosage of 2,000,000 or 3,000,000 such units or more may be required for the treatment of severe infections.

It should be noted that a penicillin-destroying ferment (penicillinase) is produced by various bacteria which may also be resistant to penicillin. It is used in certain laboratory tests for inactivating penicillin, and its production is described on p. 316.

The mode of action of penicillin depends on an interference with the early stages of protein synthesis, *e.g.* the assimilation of glutamic acid which is a property of Gram-positive bacteria. When penicillin in certain concentrations acts on growing bacteria the cells become greatly enlarged and fail to divide; they may show protoplasmic protrusions and appear to burst and disintegrate. With high concentrations of penicillin disintegration may occur without previous enlargement. It seems possible that the effect is mainly on the cell wall or outer layer of the cell cytoplasm.

Polymyxin (aerosporin) derived from an aerobic sporing bacillus (*Bacillus polymyxa*) comprises five polypeptide antibiotics ("A"—"E"). It has a special effect on Gram-negative bacteria, and its antibacterial potency is of a very high order. It is active in dilutions up to 1 in 10,000,000, and shows relatively low toxicity to leucocytes and animal tissues. Polymyxin is rapidly bactericidal, and organisms do not apparently acquire resistance to it. Its successful chemotherapeutic use has been claimed in certain human infections due to Gram-negative bacteria. Polymyxin B and E are the most suitable for clinical use.

Streptomycin is one of several antibiotic substances isolated from actinomycetes. It is derived from *Streptomyces griseus*, and constitutes a complex organic substance which on hydrolysis yields a disaccharide (streptobiosamine) and a basic compound (streptidine). The latter has some structural resemblance to inositol, which is a growth factor for certain bacteria. The toxicity of streptomycin is low, and its antibiotic action embraces a wide range of both Gram-positive and Gram-negative organisms. It is active against many pathogenic bacteria, including the tubercle bacillus. Clinical trials have established its therapeutic value, but a serious drawback is the great facility with which some organisms become resistant to it.

Aureomycin, which is derived from *Streptomyces aureofaciens*, is active against both Gram-positive and Gram-negative organisms, and its successful chemotherapeutic use has been claimed in human brucellosis, certain rickettsial infections and other conditions. It is administered orally.

Chloromycetin, derived from *Streptomyces venezuelae*, is another of the numerous antibiotics obtained from actinomycetes, and has proved to be a successful agent in the treatment of rickettsial diseases, e.g. scrub typhus, and also enteric fever. It is also active against certain viruses. It is administered orally. This antibiotic has now been synthesised (chloramphenicol).

Bacitracin, an antibiotic obtained from a strain of the *Bacillus subtilis* group, has recently received some attention. It is neutral, water-soluble, relatively heat-stable, and comparatively non-toxic. *In vitro* it is active mainly against Gram-positive bacteria, but also Meningococcus and Gonococcus. The chemical nature of the active principle has not yet been determined.

Terramycin is obtained from *Streptomyces rimosus* and is active towards a wide range of pathogenic bacteria, Rickettsiae and also certain viruses. After administration by the mouth it reaches a high concentration in the urine and has been specially recommended for the treatment of urinary infections, but is not effective against *Proteus*. Under treatment, organisms may acquire resistance to terramycin, and also to aureomycin.

It must be emphasised that while certain bacterial species may be generally sensitive to an antibiotic, variation in sensitivity may occur among individual strains within these species. In the clinical use of antibiotics it is therefore of importance that the sensitivity of the strain present in the particular case should be determined. Laboratory tests for this purpose have been elaborated and these will be described in a later chapter.

It is noteworthy that antibiotics may exhibit *synergism* or *antagonism* when allowed to act together on micro-organisms.

BIOCHEMICAL REACTIONS OF BACTERIAL CULTURES.—These are the results of the enzymic activities of the organisms, and are similar to many of the chemical decompositions which constitute one of the great functions of bacteria in nature. Among the pathogens, 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, gases (e.g. carbon dioxide and hydrogen). Certain species are also capable of breaking down salts of the organic acids.

Some bacteria possess marked proteolytic properties and digest complex protein substances, such as gelatin, coagulated serum, etc., liberating the various products of protein disintegration—e.g. amino-acids, ammonia compounds, indole, skatole, hydrogen sulphide, etc.

CHROMOGENESIS.—Some bacteria, including certain patho-

gens, produce characteristic pigments, e.g. yellow, green, etc., which in some cases diffuse readily from the bacterial cells into the surrounding medium.

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 (e.g. during seven to eight hours at optimum temperature); the increase in number is in geometric progression and the logarithmic curve of growth is a straight line; this is called the "logarithmic" phase; then follows a "stationary" phase, multiplication gradually ceasing; in this phase (lasting from a few hours to several days) the number of new individuals tends to be equalled by those dying; 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. It should be noted that the initial "lag" phase may be absent when fresh medium is inoculated with organisms which are already in the phase of multiplication. It has been suggested that the "lag" period is due to the fact that the organisms after being "injured" by the products of their previous growth require to undergo "rejuvenation" before they begin to divide and multiply.

BACTERIAL TOXINS.—These 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 have generally been regarded as of protein nature, but only a few have been isolated in chemically pure form. In some cases complex carbohydrates and phosphatides may enter into the composition of bacterial toxins.

They are classified broadly as :—

(1) Extracellular toxins, or *exotoxins*, which diffuse readily from the bacteria into the surrounding medium.

(2) Intracellular toxins, or *endotoxins*, which are retained within the cells until the bacteria die and disintegrate.

The majority of the pathogens produce endotoxin only, but some develop powerful exotoxins—e.g. the diphtheria and tetanus bacilli, certain types of staphylococci and streptococci.

Exotoxins are generally unstable substances, their toxic effect being annulled readily by chemicals, free oxygen, and heat—e.g. in the case of the diphtheria toxin, at 65° C. They tend to be highly specialised in their action on particular tissues. Preparations of these toxins can be obtained by growing the bacteria in fluid culture, which is then filtered through an earthenware or other bacterial filter—the

filtrate contains the toxin (p. 132). After introduction into the body there is usually a short latent period (*e.g.* some hours) before symptoms of poisoning are manifest. By immunising animals with such preparations a specific neutralising antibody (antitoxin) is developed which can be demonstrated in the blood serum of the immune animals (p. 40). Certain exotoxins, *e.g.* tetanus toxin, have now been isolated as pure crystalline proteins (p. 501). It may also be noted that an exotoxin of *Clostridium welchii* has been defined as an enzyme, *viz.* a lecithinase (p. 509).

Most pathogenic organisms do not produce such diffusible toxins, and their culture-filtrates are non-toxic; on the other hand, their dead bodies may be toxic, and it is assumed that their poisons are bound up in the bacterial cell—hence the designation endotoxin.

These toxins are less specialised in their action and produce a more general poisoning effect. They are more stable than exotoxins and can withstand a temperature of 100° C. They can be demonstrated experimentally by injecting dead cultures or bacterial extracts into animals. There is no definite latent period following their introduction as in the case of the exotoxins. Immunisation with endotoxins does not lead to the formation of antitoxin. The endotoxins of certain bacteria, *e.g.* members of the *Salmonella* group, consist of phosphatide-polysaccharide-protein complexes.

Certain toxins, mainly of the extracellular type, are designated according to their particular effects, irrespective of the organisms producing them—*e.g.* *haemolysin* (producing lysis of red blood cells), *leucocidin* (destructive to leucocytes), *necrotoxin* (causing necrosis of tissue).

Some pathogenic bacteria neither produce exotoxin in culture nor are their dead bodies definitely toxic, *e.g.* *Bacillus anthracis*. It is possible that such organisms can produce their toxins only when growing in the tissues.

Many pathogenic bacteria, especially when growing in the tissues, form substances which increase their aggressiveness (*vide infra*), and this type of product has been designated *aggressin*. It is supposed that it acts mainly by interfering with opsonisation and phagocytosis (p. 48), and that by its agency bacteria are able to break down the first defences of the tissues. Various bacterial products, however, may possess aggressin-like properties, and the existence of a specialised substance of this type is doubtful.

In the course of an infective disease the tissues may become

hypersensitive to specific products of the infecting organism : such hypersensitiveness (or allergy) may thus contribute to the toxic manifestations of the disease, since bacterial products which are normally non-toxic or weakly toxic thus become highly toxic (p. 49).

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. Virulence is estimated by the *minimum lethal dose*—*i.e.* the smallest dose of the organism (*e.g.* in the form of 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. The symbol M.L.D. (minimum lethal dose) may be taken to denote the average lethal dose. It must be noted that virulence depends on two factors which may be quite independent of one another : the invasive power or aggressiveness, and the toxigenic property of the organism. Thus, the tetanus bacillus is highly toxigenic but only weakly aggressive ; the anthrax bacillus is markedly aggressive but its toxicity is relatively less pronounced.

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 individuals of the same species, inoculating the animals one from another in succession—*i.e.* "passage." In this way the virulence is increased 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. Other methods of lowering virulence are referred to on p. 37.

VARIATION AND DISSOCIATION.—Bacterial cultures may, under certain conditions, "dissociate" and develop variants which differ in certain respects from the original strain—*e.g.* morphological features, absence of motility, loss of capsules, the characters of colonies on culture medium, fermentative and other biochemical properties, virulence, antigenic characters as indicated by serological reactions, etc. (p. 33). The variant strain may retain the new feature or property as a stable character. Such dissociation is met with in various

groups, but has been specially investigated among the Gram-negative bacilli occurring as commensals or pathogens in the intestine (Chapter XV). For example, a strain of coliform bacillus may at first fail to ferment lactose (p. 430) but develop a lactose-fermenting variant. In colonies on solid media such variants may appear as small papillae. Variation in the physical characters of colonies may also be noted in pure cultures of certain organisms—e.g. the “smooth” (S) and “rough” (R) colonies as seen in the *Salmonella* and other groups. These differences in colony-characters are associated with difference in virulence, antigenic constitution and immunising properties. Thus, the rough (R) variant of the pneumococcus is avirulent and this variation is associated also with absence of capsule-formation and loss of a specific chemical constituent present in the capsule (p. 39). Motile bacteria may develop non-motile variants. The relationship of these variations to antigenic characters is dealt with later in connection with agglutinating antibodies (p. 44).

CLASSIFICATION AND NOMENCLATURE

At present no standard classification is universally accepted and applied. The older methods of classification and nomenclature 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* has been used in the past as a generic name and is still conveniently employed, e.g. “tubercle bacillus,” but clearly the bacillary organisms require subdivision into many separate genera in view of their heterogeneity. A system of classification and nomenclature of the Bacteria (or Schizomycetes) was introduced some years ago by the Society of American Bacteriologists following strictly 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 various type-species quoted, the characters of which are described in later chapters. For full details, the sixth 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 still being applied in medical and bacteriological literature. There is also diversity in the use of particular names. Thus, the generic name *Bacterium* has been applied in recent bacteriological literature in this country to the organisms which have often been described as the “coliform”

bacilli, i.e. *Escherichia*, *Aerobacter* and *Klebsiella*, though in the American classification the name *Bacterium* is now given only to certain non-defined rod-shaped forms whose taxonomic position has not yet been sporing (*vide infra*). The latter seems the more appropriate usage. It is, of course, customary to speak of all the schizomycetes as "bacteria."

In Part III of this book the new names of the American system are in general given priority, but the older conventional designations are also indicated, and in certain instances these are used for convenience in the text, e.g. "tubercle bacillus." Biological names adopted by British writers, differing from those of the American system, are also indicated as alternative designations and in some cases given priority.

Abbreviations of generic names, e.g. "N." "Myco." etc., used throughout the text of the book are those adopted in the following summary.

ORDERS OF BACTERIA (OR SCHIZOMYCETES)

EUBACTERIALES.—Undifferentiated simple forms, without true branching—spherical or rod-shaped, short or long, straight or curved or spiral—some motile, and showing flagella—non-flexuous—some species produce endospores—some pigmented—some store volutin, glycogen or fat—include many pathogenic species.

ACTINOMYCETALES.—Mould-like differentiated forms, rod-shaped or elongated and filamentous—may show true branching and formation of mycelium—endospores not produced—conidia may be formed—non-motile—usually Gram-positive—include a number of important pathogenic species.

SPIROCHAETALES.—Usually slender flexuous spiral forms—endospores absent—include certain important pathogenic species.

It has been suggested that *Rickettsia* (p. 559) and also the viruses (p. 567) should be assigned to additional orders in this classification of the bacteria. Another suggestion is that the viruses might be placed in a biological class along with the bacteria; but it is questionable whether the viruses, in the present state of knowledge, can be named and classified on a strict biological basis.

EUBACTERIALES

FAMILIES :

Pseudomonadaceae.—Straight or spirally curved rod-shaped or elongated forms—usually motile with polar flagella—non-flexuous—Gram-negative.

Achromobacteriaceae.—Gram-negative rod-shaped organisms—motile with peritrichous flagella or non-motile—non-pigmented or producing yellow, orange or brown pigments—non-saccharolytic or feebly saccharolytic.

Micrococcaceae.—Spherical forms—usually non-motile—not arranged in chains—usually Gram-positive—not obligate parasites.

Neisseriaceae.—Spheroidal Gram-negative forms—generally obligate parasites.

Lactobacteriaceae (or Streptobacteriaceae).—Spheroidal or rod-shaped units with tendency to chain formation—Gram-positive—decompose sugars with marked acid production.

Corynebacteriaceae.—Gram-positive rod-shaped organisms showing beaded or barred staining and sometimes metachromatic granules—usually non-motile—aerobic or micro-aerophilic.

Parvobacteriaceae.—Small rod-shaped forms—Gram-negative— aerobic or anaerobic—usually obligate parasites—frequently require body-fluids for growth—not active fermenters of carbohydrates.

Enterobacteriaceae.—Rod-shaped forms—frequently motile with peritrichous flagella—Gram-negative—active fermenters of various sugars with acid, or acid and gas, production.

Bacteriaceae.—Miscellaneous non-sporing rod-shaped forms not included in other families.

Bacillaceae.—Rod-shaped forms—with endospores—usually Gram-positive—aerobic or anaerobic.

GENERA :

Pseudomonadaceae

Pseudomonas.—e.g. *Ps. aeruginosa* (p. 351).

Vibrio.—e.g. *V. cholerae* (p. 469).

Spirillum.—Rigid spiral organisms—usually motile with multiple polar flagella—mostly found in water.

Achromobacteriaceae

Alcaligenes.—e.g. *Alc. faecalis* (p. 457).

Micrococcaceae

Micrococcus.—Cocci occurring in plates or irregular masses—Gram-positive—some produce yellow, orange or red pigment—saprophytes or facultative parasites—e.g. *M. pyogenes* var. *aureus* or *Staph. aureus* (p. 329).

Gaffkya.—e.g. *Gaff. tetragena* (p. 350).

Sarcina.—Cocci arranged in regular packets of eight or multiples of eight (p. 12).

Neisseriaceae

Neisseria.—e.g. *N. meningitidis*, *N. gonorrhoeae* (p. 362).

Veillonella.—e.g. *Veill. parvula* (p. 373).

Lactobacteriaceae (or Streptobacteriaceae)

Diplococcus.—e.g. *D. pneumoniae* (p. 355).

Streptococcus.—e.g. *Strept. pyogenes* (p. 335).

Lactobacillus.—e.g. *Lacto. acidophilus* (p. 467).

Corynebacteriaceae

- Corynebacterium*.—e.g. *C. diphtheriae* (p. 374).
Listeria.—e.g. *List. monocytogenes* (p. 395).
Erysipelothrix.—e.g. *Ery. rhusiopathiae* (p. 531).

Parvobacteriaceae

- Pasteurella*.—e.g. *P. pestis* (p. 475).
Brucella.—e.g. *Br. melitensis* (p. 483).
Malleomyces.—e.g. *Mall. mallei* (p. 422).
Haemophilus.—e.g. *H. influenzae* (p. 492).
Noguchia.—e.g. *Nog. granulosis* (p. 606).
Dialister.—e.g. *Dial. pneumosintes* (p. 535).
Actinobacillus (p. 528).
Bacteroides.—e.g. *Bacteroides fragilis* (p. 534).
Fusobacterium.—e.g. *F. fusiformis* (p. 533).
Moraxella.—e.g. *Morax. lacunatus* (p. 495).

Enterobacteriaceae

- Escherichia*.—e.g. *Esch. coli* (p. 426).
Aerobacter.—e.g. *Aero. aerogenes* (p. 429).
Klebsiella.—e.g. *Kl. pneumoniae* (p. 432).
Proteus (p. 351).
Salmonella.—e.g. *S. typhi* (p. 434) and *S. enteritidis* (p. 446).
Shigella.—e.g. *Sh. dysenteriae* (p. 453).
Serratia.—e.g. *Serr. marcescens* (*Bacillus prodigiosus*, p. 572).

Bacteriaceae

- Bacterium*.—Generic term retained for non-sporing, rod-shaped forms whose taxonomic position has not yet been definitely established.

Bacillaceae

- Bacillus*.—e.g. *B. anthracis* (p. 414).
Clostridium.—e.g. *Cl. tetani* (p. 500).

ACTINOMYCETALES**FAMILIES :**

- Actinomycetaceae**.—Filaments, often branched and forming mycelium—conidia sometimes produced.
Mycobacteriaceae.—Rod-shaped, rarely filamentous—only occasional branching—no conidia.

GENERA :**Actinomycetaceae**

- Actinomyces* (p. 524).
Nocardia.—e.g. *Noc. farcinica* (p. 530).

Mycobacteriaceae

Mycobacterium—e.g. *Myco. tuberculosis* (p. 397).

SPIROCHAETALES**GENERA :**

Borrelia.—e.g. *Borr. recurrentis* (p. 546).

Treponema.—e.g. *Tr. pallidum* (p. 538).

Leptospira.—e.g. *Lept. icterohaemorrhagiae* (p. 549).

SYSTEM OF IDENTIFICATION OF THE BACTERIA

(1) THE MORPHOLOGY, TOGETHER WITH THE STAINING REACTIONS, OF INDIVIDUAL ORGANISMS generally serves as a preliminary criterion, particularly for placing an unknown species in its appropriate biological group. In medical bacteriology the microscopical 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, OR THE MORPHOLOGY OF GROWTHS ON CULTURE MEDIUM—e.g. the appearances of colonies to the naked eye or with certain magnifications (p. 14). This criterion is important in identification, but may also be insufficient to differentiate species—e.g. different species of the *Salmonella* and dysentery groups produce indistinguishable colonies.

(3) PHYSIOLOGICAL, CHARACTERS AND BIOCHEMICAL REACTIONS—e.g. the fermentation of various carbohydrates. Species which cannot 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) SEROLOGICAL REACTIONS.—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 organism (p. 41). Such antisera, for example, may agglutinate or clump the homo-

logous organism in test-tube experiment, and this effect can be observed easily with the naked eye.

It should be noted here that the serum of a person or animal suffering from a bacterial infection may also exhibit specific antibody reactions, and in this way the agglutination reaction can be used for diagnostic purposes—*e.g.* the Widal reaction in enteric fever.

(5) ANIMAL EXPERIMENT.—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.

FUNGI ; PROTOZOA

The general morphology and physiology of these organisms will not be dealt with here. The biological aspects of the groups that are of special importance in practical bacteriology as applied to medicine will be referred to later in the consideration of particular organisms (Chapters XV, XXIII and XXIV).

FILTERABLE VIRUSES

The biology of these viruses still presents many difficult problems. It has been often assumed that they are living organisms, owing mainly to the fact that they are self-reproducing and can be propagated through a series of animals and cultivated in the fertile egg (p. 577). Many of them, though demonstrable as viruses by the experimental method, have not been observed by ordinary microscopic methods. In certain cases, however, exceedingly minute bodies ("elementary bodies") within the limits of ordinary microscopic visibility have been accepted as the actual virus. Recently, electron microscopy has demonstrated many viruses as well-defined structures. So far none of these filterable viruses has been cultivated like the bacteria, *i.e.* on a substrate of inanimate material, though they may flourish *in vitro* in association with living tissue cells, *e.g.* a tissue culture or the embryonated egg.

It should be noted that certain very minute bacteria which are easily recognised by ordinary microscopic methods and are quite readily cultivable may pass the coarser types of filter, *e.g.* the organism of bovine pleuro-pneumonia (p. 535).

The viruses are easily inactivated by various physical and chemical agencies, *e.g.* heat and antiseptics, but on the whole

are more resistant than the non-sporing bacteria. They often possess a high degree of resistance to glycerol.

In general their demonstration and identification depend on the experimental production of a characteristic pathological condition in animals (or man) by means of filtrates after subjecting material from the particular disease (*e.g.* nasal washings, serum) to filtration through a filter capable of arresting the ordinary bacteria (p. 132).

As an alternative to animal experiment, inoculation into certain tissues or cavities in the embryonated hen's egg may sometimes be adopted, characteristic effects being produced. Serological methods may also be used for the identification of particular viruses or different types of a virus (p. 569).

The subject of the viruses is dealt with more fully in Chapter XXII.

CHAPTER II

IMMUNITY IN RELATION TO PRACTICAL BACTERIOLOGY

THE subject of immunity is intimately related to practical bacteriology, and immunological principles underlie certain bacteriological methods.

The term "immunity," in its usual application, signifies the power of the animal body to resist (*a*) infection by parasitic micro-organisms,¹ or (*b*) the injurious effects of their products or toxins.

Immunity may be *acquired*, as in the natural recovery from infection, and is due to the development, during the illness, of *specific* resisting powers against the causal organism or its toxin. Once developed, acquired immunity may persist for long periods, even practically throughout life, as exemplified by the immunity following smallpox. On the other hand it may be of short duration, e.g. after pneumonia.

An acquired immunity may also be developed artificially by inoculating an animal with a micro-organism or toxin in such manner or in such modified form that it is incapable of reproducing the typical disease though still able to bring about an immunity reaction. Such immunity is described as an *active artificial immunity*.

A modified form of micro-organism used for immunisation has generally been designated a *vaccine*, and the inoculation of it is described as *vaccination*, these terms being derived from Jenner's method of inoculating vaccinia virus for preventive immunisation against smallpox (*vide infra*).

The blood serum of an actively immunised animal injected into the body of a non-immune animal renders the latter temporarily immune, and this state is termed *passive immunity*.

The most frequent *methods of producing active artificial immunity* are :—

(1) Introduction into the body (usually by parenteral injection) of living organisms in a state of attenuated virulence.

¹ The terms "micro-organism" and "organism" used in this chapter on immunity apply also to the viruses (p. 34).

Various methods have been used to reduce virulence for purposes of immunisation—*e.g.* (a) cultivating at a temperature above the optimum, as in the case of Pasteur's anthrax vaccine; (b) passage through animals of a different species; thus vaccinia (cow-pox) virus (as used for smallpox vaccination) may be regarded as a form of the smallpox virus attenuated for the human species by its propagation in bovines; (c) continued artificial cultivation, particularly in the presence of an antagonistic substance; thus, the "B.C.G." vaccine used for immunisation against tuberculosis is an avirulent strain of the tubercle bacillus attenuated by prolonged cultivation on a medium containing bile.

(2) Introduction of organisms—usually in the form of cultures—killed by heat or antiseptics—*e.g.* the usual form of *bacterial vaccine*. Repeated and increasing doses are administered. In killing the organisms the lowest effective temperature or the lowest concentration of antiseptic is used to avoid, if possible, alteration in the chemical constituents on which immunisation depends. This system is applied in the prophylaxis of certain infections (*e.g.* antityphoid vaccination), in therapeutic immunisation (*e.g.* treatment of chronic infections with vaccines), and also in the preparation of anti-bacterial sera (*vide infra*) in animals. Viruses inactivated by antiseptics—*e.g.* phenol—are likewise used for immunisation.

Immunity may be produced in some cases by means of bacterial extracts, which illustrates the fact that the immunising stimulus depends on a chemical constituent of the bacterial cell (*i.e.* the "antigen"—*vide infra*), and a further development in artificial immunisation has been the use of purified preparations of the specific antigen extracted from bacterial cultures.

In immunising animals the process may be started with dead organisms, followed by doses of virulent living organisms. In some cases, immunisation is carried out by giving non-lethal doses of unaltered living organisms without previous injection of dead or attenuated organisms, but this method may be difficult to control. A further method is to introduce living organisms and at the same time a specific antiserum which affords the necessary protection against them.

(3) Introduction of exotoxins in repeated and increasing doses, starting with small and harmless doses, and graduating the series, so that, as the immunity progressively develops, each dose is devoid of harmful effect. This is exemplified in the preparation of diphtheria and tetanus antitoxins by immunising animals—*e.g.* horses—with the respective toxins.

To ensure that no injurious effects result from immunisation, toxin which has been rendered non-toxic (*toxoid* or *anatoxin*) by certain chemicals—*e.g.* formalin, etc.—or by heat is generally used. Thus, immunisation with diphtheria toxoid has been extensively applied in the prophylaxis of diphtheria in the human subject (p. 385).

In some cases the treatment of a culture of a toxicogenic sporing bacterium, *e.g.* *Clostridium septicum*, with formalin in certain concentrations may yield a most effective vaccine for immunising animals against the particular infection; the organisms are killed and the toxin is converted to toxoid. This type of vaccine has sometimes been designated *anaculture*.

The injection of an exudate rendered free from bacteria by filtration (the so-called “aggressin,” p. 27) may, in certain cases, be used to immunise animals against the organism responsible for the infection from which the exudate is obtained. Such “aggressins” have been used in the control of particular animal diseases, *e.g.* blackleg (p. 525).

The serum of an actively immunised animal is designated an *immune serum* or *antisera*m, and owes its effect to specific *immune bodies* or *antibodies* which act adversely on the homologous organism or neutralise its toxins.

The special constituent or product of the organism which incites antibody production is termed an *antigen* and is generally of protein nature though other substances such as carbohydrates may enter into the composition of bacterial antigens.

Antibodies may be regarded as modified forms of serum globulin with specific combining affinity for the respective antigens. Thus, the presence of an antigen in the tissues seems to bring about an alteration in the synthesis of serum globulin.

A serum containing antibodies antagonistic to the particular bacterium is spoken of as an *antibacterial serum*; one containing antibodies which neutralise toxin, as an *antitoxic serum*.

It has to be noted that, apart from bacteria and their toxins, other foreign cells, and all soluble complete protein substances foreign to the animal body, may act as antigens and incite specific antibody production—*e.g.* red blood corpuscles, blood serum, animal venoms, etc.

Thus, the red corpuscles of one species injected into an animal of different species incite the development of an antibody which, under certain conditions, can effect lysis of the red cells of the former. This antibody is described as

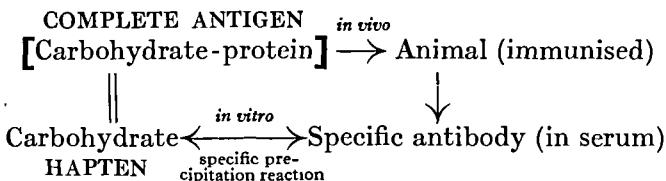
a *haemolysin* and the serum containing it as a *haemolytic antiserum*.

As a general rule, *an antigen, to produce immunity, must be injected parenterally*—*i.e.* by some route other than the alimentary tract (subcutaneously, intravenously, etc.). An antigen, being a protein, if administered by the mouth would generally be so altered chemically by the digestive secretions as to lose its specific immunising properties.

Usually an animal can be immunised only against an antigen which is foreign to its own tissues. Thus, a rabbit can be immunised against ox or sheep red cells, but not against its own cells.

Certain non-protein substances—*e.g.* lipoids, carbohydrates, etc.—though unable to act as antigens *in vivo*,¹ may, when combined with protein, be capable of inciting the formation of antibodies which *in vitro*¹ react specifically with the non-protein substance—*e.g.* in the precipitation reaction (p. 46). To these substances the term *hapten* is applied. The specific characters of the antigen of a bacterium may, in fact, depend on a non-protein constituent—*e.g.* a polysaccharide as in the case of the different types of the pneumococcus (p. 359), and groups of haemolytic streptococci (p. 339).

The action of haptens may be represented schematically as follows :—



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 supposed species, such as the serological types of the pneumococcus.

The specificity of antibodies depends on the specificity of the corresponding antigens. An organism may contain more than one antigenic constituent, and for each of these, on immunisation, a separate antibody is produced. When related bacterial species have

¹ “*In vivo*” denotes “in the living body”; “*in vitro*” (literally, “in glass”) means “in test-tube experiment.”

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.

It must be remembered that, in some instances, the occurrence of antibodies may have no aetiological significance. Thus, 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. 561).

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." Thus, as originally shown by Forsmann, 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 "Forsmann's antibody."

Specific antibodies may also occur naturally: thus, the serum of the guinea-pig contains a haemolytic antibody for ox red corpuscles; specific bactericidal and agglutinating antibodies (*vide infra*) for various bacteria may be demonstrated in the serum of normal animals.

Antitoxic sera are produced by immunising an animal with exotoxins. For example, "diphtheria antitoxin" is the serum of a horse which has been immunised with graded doses of diphtheria toxin or toxoid. When appropriate amounts of toxin and antitoxin are mixed together the mixture is non-toxic. This process of neutralisation is complex and need not be dealt with here. It occurs both *in vivo* and *in vitro*.

The antitoxic principle, like other antibodies, is associated with the globulin of the serum, and in fact may be regarded as a modified form of serum globulin; by a process of precipitating the globulin with half-saturated ammonium sulphate solution, separating the precipitate, dialysing out the ammonium sulphate, and then redissolving the precipitated globulin in a quantity of physiological salt solution much less than the original volume of the serum, an antitoxic serum can be "concentrated" and "refined." Other methods of concentrating and refining antisera have also been used, e.g. precipitation by diluting serum with a large bulk of distilled water or weakly acid buffer solutions (Felton). Recently "refined" antitoxins have been obtained by

treating the serum with proteolytic enzymes (p. 383). By electrophoresis different serum globulins have been defined, with certain of which, " β " and " γ ," antibody properties are associated; γ globulin representing the antibody fraction of certain sera can be separated by precipitation with certain concentrations of alcohol at low temperatures (p. 595).

Endotoxins unlike exotoxins do not incite the production of antitoxins.

Antibacterial sera are generally produced by immunising with the actual bacteria, or bacterial extracts. These sera may exhibit the following properties :—

· *Bacteriolytic* or *bactericidal*—directly lysing or destroying the bacteria.

Opsonic or *bacteriotropic*—rendering the bacteria susceptible to phagocytosis.

Agglutinating—immobilising and clumping the bacteria.

Precipitating—producing a precipitate along with the soluble products of the bacteria, e.g. a bacterial extract.

Complement-fixing—along with the bacterial antigen "fixing" or "absorbing" complement, a normal constituent of serum (*vide infra*).

These effects may be demonstrated *in vitro*. They are all relatively specific and are quantitatively marked—e.g. they may be produced by exceedingly minute amounts of the antigen or antiserum.

The serum of a person or animal infected with a pathogenic bacterium may exhibit also similar properties and constitutes an antibacterial serum. Thus, the serum of a person suffering from typhoid fever exhibits these properties when tested *in vitro* with the typhoid bacillus.

It has to be noted that normal serum may possess bactericidal, opsonic and agglutinating effects. These are relatively weak as compared with the corresponding effects produced by an immune serum.

The *bactericidal action of an immune serum* is due to a specific thermostable¹ antibody (*bacteriolysin* or *bactericidin*) acting along with a normal non-specific constituent of serum (*complement*) which is thermolabile² (at 55° C.). The anti-

¹ "Thermostable" denotes the ability to withstand a certain degree of heat.

² "Thermolabile" indicates that a particular property is lost on exposure to a certain degree of heat.

body 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 :

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

It may be noted here that complement is an exceedingly unstable substance and becomes quickly inactivated when serum is kept under ordinary conditions.

Owing to the original mistaken idea that the antibody acts merely as a combining link between the antigen and complement, the term "amboceptor" has been applied to it.

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.) or preservation for some time, 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.

Bactericidal action by a normal serum, in many cases, is analogous to that produced by an immune serum, and is dependent on a natural antibody (*vide supra*) and complement. This mechanism, which is active mainly on Gram-negative bacteria, has been designated the "α lysin." Normal bactericidal effects towards various Gram-positive bacteria are independent of complement and are due to a thermostable (55° C.) principle "β lysin," the exact nature of which is still undetermined.

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.

Thus :

Red Cells + { Specific antibody } + Complement = Haemolysis.

Red Cells + { Specific antibody } = No effect.

Red Cells + Complement = No effect.

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 annul 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. 257).

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 also play a part in this effect as in the bactericidal action of the α lysin of normal serum.

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

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.

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 vitality of the bacteria. It also clumps dead bacteria in the same way as the living organisms. It is relatively thermostable, e.g. as compared with complement. An electrolyte, e.g. 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 (p. 33).

As in the case of other antibody reactions, normal serum may contain 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*

¹ Wright, *Technique of the Teat and Capillary Glass Tube*, London, 1921; *Studies in Immunity*, 1909.

(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. Further, H agglutination is of a "large-flake" type, i.e. large easily visible clumps, whereas O agglutination is of the "small-flake" or 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 motile 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 interferes with O agglutination. Treatment of the suspension with alcohol, on the other hand, inactivates the H antigen and an alcoholised suspension is therefore a suitable reagent for testing O agglutination (p. 248). 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 20 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. 449). Other species are *monophasic* 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. 449 which should be referred to.

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 either unaltered or lost, the variant then becoming non-flagellate. Among pathogenic bacteria this transformation, S→R, 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 also been shown that the typhoid bacillus and certain related organisms when freshly isolated possesses an additional somatic antigen which is associated with its 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 antigen and can be detected by agglutination tests with an appropriate antiserum. Further reference to this subject is made later (p. 436). It seems likely that various organisms possess analogous "virulence" antigens, and the capsular hapten of the pneumococcus has a somewhat similar rôle (*g.v.*).

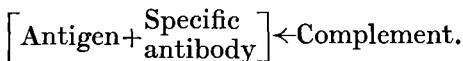
These facts summarise briefly the variations in antigenic composition which have to be allowed for in the practical application of the agglutination phenomenon for diagnostic purposes. Further references to this question are made in later chapters.

The *precipitating action of an antibacterial serum* is analogous in many respects to agglutination and is spoken of as being due to an antibody called *precipitin*. For this test, a suitable extract of the bacterial substance is required ; this is exemplified in the precipitation test used for determining groups among the streptococci (p. 339).

Complement-fixation.—Complement plays an essential part in the bactericidal action of an immune serum, and is absorbed by the bacteria *plus* bactericidal immune body. 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 (p. 43).

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

The various immune properties of an antibacterial serum have been described above in terms of multiple antibodies, each designated according to its effect on the antigen (bacteriolysin, agglutinin, opsonin, precipitin, complement-fixing antibody), but it is still an open question whether these reactions are actually due to separate principles in the serum or are merely different manifestations of the activity of a single antibody.

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. This is well exemplified in the case of the elementary bodies of vaccinia (p. 590).

ANAPHYLAXIS AND HYPERSENSITIVENESS

Under certain conditions the parenteral injection of foreign protein leads to a specific sensitising effect so that the subsequent injection of the same substance may produce toxic and even fatal results.

Thus, if a guinea-pig is injected subcutaneously with horse

serum (even an extremely small dose—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 may develop a sudden illness or *anaphylactic shock* in which the chief manifestation is spasmodyc contraction of unstriped muscle, particularly that of the small bronchi. The serum may be quite non-toxic *per se* when given to an unsensitised animal even in large doses.

Substances that lead to anaphylaxis (*anaphylactogens*) are those which act as antigens in relation to immunity, and the phenomenon of anaphylaxis is regarded as due to the interaction of antigen and antibody (*precipitin*) in the tissues. Passive hypersensitivity can also be conferred by injecting the serum of a sensitised animal into a normal animal.

The results of anaphylaxis depend on the mode of injection of the substance into the sensitised animal and the quantity introduced. Anaphylactic shock is more liable to occur and is more marked when the injection is intravenous or intraspinal, 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 small fractions may not lead to an anaphylactic shock and the animal is “desensitised” in this way. The state of hypersensitivity, once developed, may persist for long periods. If, after the sensitising injection but before sensitivity has developed, a second dose of the substance is given, the animal is protected for a time against a subsequent injection—i.e. *anti-anaphylaxis* has been developed.

Haptens (p. 39) may produce anaphylactic shock in animals specifically sensitised with the complete antigen of which the hapten is a constituent.

The nature of these phenomena has not been completely elucidated. Various explanations have been advanced on the basis of experimental data. It would appear that the interaction of antigen and precipitating antibody in certain relative proportions in the tissues leads to the liberation of histamine or similar substances which are responsible for the toxic manifestations, and anaphylactic shock presents many similarities to histamine poisoning. For further details, one of the larger works should be consulted. Serum anaphylaxis is, of course, of practical importance in medicine in relation to serum therapy—for example, when it is necessary to give a second dose of a therapeutic serum after an interval, and especially by intravenous injection; the risk

of anaphylactic shock can, in such cases, be obviated by desensitisation with very small doses of serum (*vide supra*). The human subject, however, is not so liable to anaphylaxis as certain animals.

Such hypersensitivity can be tested for by injecting intracutaneously 0·1 ml. of the serum. A positive result is denoted by the occurrence within thirty minutes of an urticarial reaction at the site of inoculation, which may develop a vesicle and may be surrounded by a wide erythematous zone. Hypersensitivity can also be demonstrated by instillation of serum into the conjunctival sac, or by applying it to a small scarified area of skin.

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 have been regarded as due to a natural hypersensitivity to the horse serum. It may, however, 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 *intravenous* injection of foreign serum, especially in large amount, may, in certain persons, produce immediate symptoms of shock. In the administration of therapeutic antisera such severe reactions can be avoided by a preliminary test for hypersensitivity (*vide supra*). If this is detected, the required dose should be subdivided into fractions separately administered, starting with a small quantity.

Specific hypersensitivity to the products of the infecting organism (*allergy of infection*) is a feature of various diseases in man and animals, and can be demonstrated by the general and local reaction manifested on injection of preparations from cultures of the particular organism—*e.g.* the tuberculin reaction (p. 406). In such cases, the sensitiveness can also be elicited by simple cutaneous or intracutaneous tests. This form of hypersensitivity differs from anaphylaxis in certain respects. Thus, it is doubtful whether such allergy is dependent on a serum antibody, and it has not been generally possible to transfer the sensitiveness to a normal individual by injection of serum.

Atopy.—In certain persons, probably as a result of genetic

factors, hypersensitiveness may occur towards a considerable variety of substances of 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, gastro-intestinal disturbance, etc. This form of sensitiveness has been designated *atopy*, and is responsible for such conditions as hay fever, asthma, etc. Substances to which such sensitiveness can be attributed (*atopens*) are : plant pollens (as in hay fever), dandruff of animals (*e.g.* horse), proteins of various articles of food (*e.g.* shell fish), bacteria, moulds, etc. Atopy can be tested for by cutaneous reactions with preparations of the particular *atopen*, as in testing infection-allergy. While atopy cannot be transferred in the same way as anaphylaxis, it has been found that when the serum of an atopic person is injected into the skin of a non-sensitive person, and after an interval the *atopen* is injected at the same site, an urticarial wheal results (Prausnitz-Küstner reaction). The serum therefore contains some active substance (designated *reagin*). Whether this principle is an antibody still remains doubtful.

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 and after 24 hours the same filtrate was injected intravenously, an intense reaction occurred at the site of the intradermal injection, *viz.* an area of haemorrhagic inflammation with subsequent necrosis. After a longer period had elapsed, *e.g.* 32 hours, intravenous injection might have no such effect. The reaction is not specific : thus, after the intradermal injection of typhoid bacillus filtrate, a coliform bacillus filtrate injected intravenously may excite the reaction. The precise significance and the underlying biological mechanism of this phenomenon are still somewhat obscure.

PART II

Bacteriological Technique

MICROSCOPY; STAINING METHODS; CULTIVATION OF MICRO-ORGANISMS; ANIMAL INOCULATION AND AUTOPSY; IMMUNOLOGICAL AND SEROLOGICAL METHODS; BACTERIOLOGICAL EXAMINATION OF AIR, WATER, MILK, ETC.; TESTING OF ANTISEPTICS, ANTIBIOTICS, ETC.; COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

CHAPTER III

THE USE OF THE MICROSCOPE IN BACTERIOLOGY

MICROSCOPY is of primary importance in bacteriology, and a suitable microscope and a knowledge of its use are essential to those engaged in bacteriological work. To obtain satisfactory results, the microscope and its illuminant must be used under the best possible conditions, and to do this the worker must know the various components of the instrument and their uses.

The component parts of the microscope are as follows :—

The *eye-piece* fits into a graduated *draw-tube*, which in turn slides in the *body-tube*. The lower end of the tube is furnished with a *revolving nose-piece* ("triple" or "quadruple") into which are screwed the *objectives*. The tube and objectives are moved up and down by means of a rack and pinion termed the *coarse adjustment*, while the fine movements necessary for accurate focussing are performed with the *fine adjustment*. The tube and adjustments are supported by an *upright* which is connected to the *foot* by means of a *hinged joint*. Attached to the upright near the joint is a platform called the *stage*, on which is placed the microscopic preparation to be examined. In the centre of the stage is an aperture through which the object can be illuminated from below. A *mechanical stage* may be fitted either to the stand itself or to the fixed stage, so that the preparation can be moved about horizontally, thus ensuring steady and controlled movement. Below the stage is the *substage*, which is raised or lowered by a rack and pinion or, in older models, by a spiral screw. The substage is fitted with a *substage condenser*, attached to which is the *iris diaphragm*. Fitted to the end of the *tail-piece* is a *mirror*, mounted on a gimbal fitting. The plane side of the mirror is employed when a condenser is used ; the concave side is used only in the absence of a condenser, and its focal length is such that light comes to a focus on the object examined.

The working of the fine adjustment varies according to the make of the microscope, for different manufacturers have their own particular type of mechanism. The older forms were actuated by means of a

milled head mounted on a pillar behind the coarse adjustment, which turned a screw with a fine thread. Present models have a milled head parallel to the coarse adjustment. The movements of the milled head raise or lower the tube in the same direction as the coarse adjustment. The milled head is graduated in $\frac{1}{50}$ ths, and one division corresponds usually to a movement of the tube of 0.002 mm. The manner of securing a fine movement is by a system of levers, cams or cogwheels, according to the make of microscope.

The draw-tube should be adjusted to the *tube length* (p. 56) 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 sub-stage, 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 $\frac{4}{3}$ -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 $\frac{1}{4}$ -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 :—

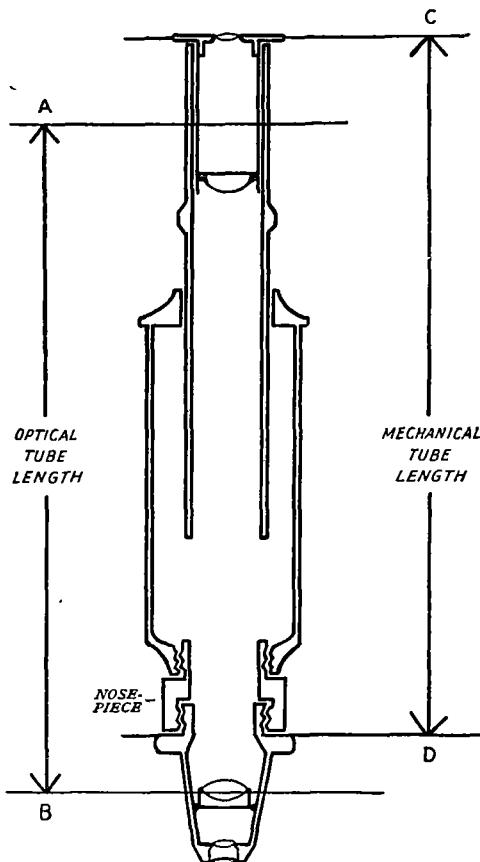
- (1) The focal length of the objective.
- (2) The magnifying power of the eye-piece.
- (3) The distance between the lens system of the objective and the image produced—the "*optical tube length*."

In actual practice, however, when calculating the magnification, the "*mechanical tube length*" is used; the diagram on p. 57 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 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 :—

$$\begin{aligned} \text{magnification of objective} &= \frac{\text{size of image}}{\text{size of object}} \\ &= \frac{\text{distance of image from objective}}{\text{distance of object from objective}} \end{aligned}$$

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. ($\frac{1}{6}$ -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 conjunction 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. ($\frac{1}{6}$ -in.) is 40/0.65, and the 2-mm. ($\frac{1}{12}$ -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 $\frac{1}{12}$ -in. objective has, in reality, a shorter focal length than that by which it is designated, and gives a magnification of 95–100 diameters, 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.

The $\frac{1}{12}$ -in. oil-immersion lens works very close to the cover-slip, and the intervening space between objective and cover-

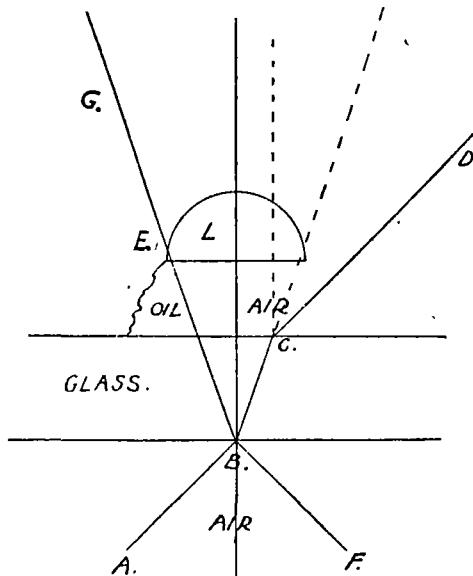


Diagram showing the paths of rays through (1) a dry lens (on right), and (2) an oil-immersion lens (on left) (after Spitta).

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.

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,¹ the rays of light do not undergo refraction and pass into the objective (see diagram—FBEG).

The high power ($\frac{1}{2}$ -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. 61).

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

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

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

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).

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.

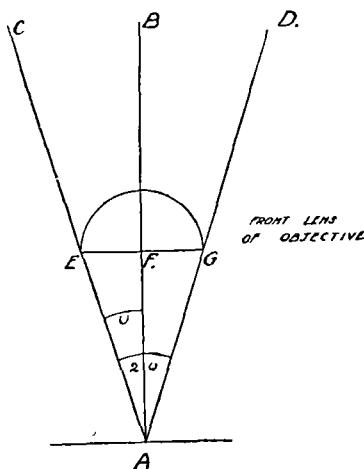


Diagram to illustrate numerical aperture.

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.

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 wave-length 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. (0.25μ).

Resolution, however, must not be confused with visibility, because it is possible to see "elementary bodies" (of virus diseases) as small as 0.074μ with ordinary white light, and even smaller, 0.067μ , 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. 117), 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 0.25μ 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 wave-length than visible light, greater resolution (2-3 times) can be obtained. With the introduction of the electron microscope (p. 81), in which the electrons have an equivalent wave-length 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 over-run 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 over-run 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 over-run 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., *J. Clin. Path.*, 1951, 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. 402. 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 camel-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

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

"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. 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 $\frac{1}{2}$ -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 sub-stage, 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. 65 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. ($\frac{1}{8}$ -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 has 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. 206) 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. 204). 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 ($\frac{1}{8}$ -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. 10).

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 movement 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. \times 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 illumin-

ated 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.

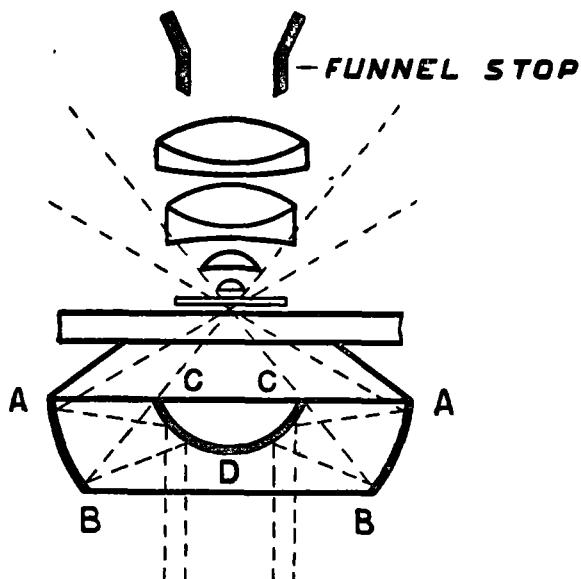


Diagram showing the paths of rays through the condenser and a $\frac{1}{2}$ -in. oil-immersion lens fitted with a funnel stop. AB and CDC are reflecting surfaces. The surface at CC is opaque. (*After E. Leitz.*)

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. 65). 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 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 adapter*, with a small iris diaphragm, may be used. The front part of the oil-immersion objective is removed and screwed to the adapter, 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 adapter. 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 $\frac{1}{7}$ -in. N.A. 0·95. The latter lens (referred to on p. 56) 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 adapter, 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 ($\frac{2}{3}$ -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 0·8, 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 $\frac{1}{6}$ -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 adapter with iris diaphragm (p. 73) is often of value in reducing the N.A. of the $\frac{1}{6}$ -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 $\frac{1}{12}$ -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.

$$\begin{aligned} 100 \text{ eye-piece divisions} &= 11 \text{ stage divisions} = 11 \text{ mm.} \\ 1 \text{ eye-piece division} &= .0011 \text{ mm.} = 1.1\mu. \\ 1 \text{ eye-piece division, therefore, with the} \\ \text{given objective, eye-piece and tube} \\ \text{length} &= 1.1\mu. \end{aligned}$$

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 wave-length 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 wave-lengths) 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 wave-length 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. For the blue filter 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. 65) 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 gm.
Phenol	.	.	.	3.0 gm.
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.

¹ Richards, O. W., Kline, E. K., and Leach, R. E., *Amer. Rev. Tubercl.*, 1941, **44**, 255; Bogen, E., *ibid.*, 1941, **44**, 267; Oscarrson, P. N., *Acta Med. Scand.*, 1941, **108**, 240; Richards, O. W., and Miller, D. K., *Amer. J. Clin. Path.*, 1941, ii. (Technical section), 1; Lampert, H., *Lancet*, 1944, **2**, 818.

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 MICROSCOPY¹

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.

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

It is necessary to have :—

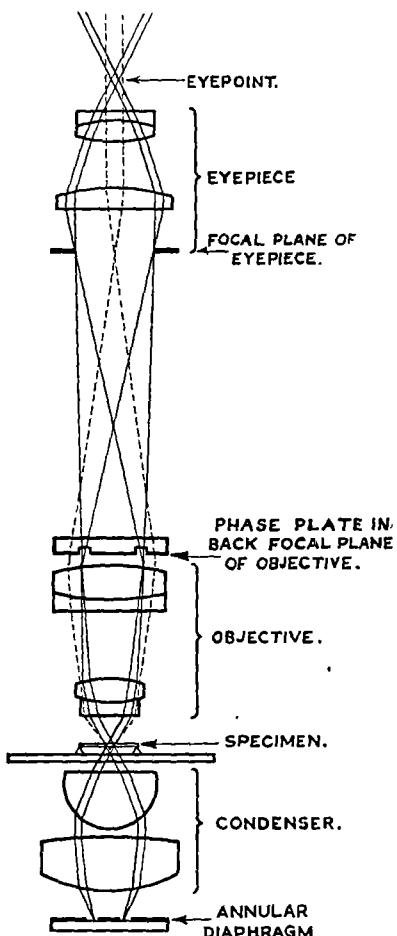
- (1) A special condenser below which is a rotating metal disk carrying a series of annular diaphragms. These are disks of glass rendered opaque but with a narrow ring of clear glass. Each objective requires a different size of annulus according to its numerical aperture; thus, for the 16-mm. objective 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.
- (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. 80).

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 eyepiece and through it the back focal plane of the objective is observed. The annulus and phase ring should coincide. If not exactly coincident

¹ *Phase Microscopy: Principles and Applications*, by Bennett, Jupnik, Osterberg and Richards, New York and London, 1951; Martin, L. C., *J. Quek. Micr. Club*, 1951, Ser. 4, 3, 237; Taylor, E. W., *J. Roy. Micro. Soc.*, 1946, 66, 1.

the centring screws under the condenser are adjusted to achieve this. The eye-piece is now re-inserted and the specimen examined.

DIAGRAM ILLUSTRATING THE PATHS OF LIGHT RAYS IN PHASE CONTRAST MICROSCOPY¹



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

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.

¹ Reproduced by permission of American Optical Company.

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 wave-length 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 principles of its construction are comparatively easy to understand. The resolution, and hence the degree of magnification of a microscope, depends on the wave-length of light used (p. 62). It has been shown that electrons have an equivalent wave-length inversely proportional to their speed, and the particular wave-length used in the electron microscope is $1/100,000$ that of ordinary light so that high resolution and great magnification are possible. The electron micro-

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

scope 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 ($1m\mu$).

The instrument is costly, elaborate and technically difficult to maintain. It is mentioned here to draw attention to a recent development in the study of microbial structure which has already yielded important results and which will in the future help to elucidate many problems in microbiology.

CHAPTER IV

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. Their protoplasm reacts to stains in a manner somewhat similar to the nuclei of tissue cells, and therefore the various basic dyes are the commonest stains employed. The action of these stains may be intensified by the use of mordants such as phenol or weak alkalis, by the application of heat, or by prolonging the time of staining.

Staining is also of special importance in the identification of bacteria, in virtue of the fact that, among these organisms, there are pronounced differences in their reactions to particular staining methods, such as those involving the use of decolorising agents after treatment with certain dye solutions.

The general principles of bacterial staining have been dealt with 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. 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.

Slides.—These may be treated in a manner similar to cover-slips. A quicker and quite satisfactory method for ordinary routine use 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. 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.¹

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. 206) 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 either by passing the 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

¹ Difficulties of supply may necessitate slides being re-used. They should be boiled for ten minutes in $\frac{1}{2}$ -1 per cent. solution of "Kin-Ray" (p. 126), and then *both* sides cleaned with "Bon ami" or similar soap. It is essential to remove the film of bacteria in this way, otherwise when the slide is used again, the organisms will re-stain and cause erroneous conclusions. We have seen serious errors in diagnosis following the re-use of slides not properly cleaned. It is for this reason that we have recommended above that used slides should be discarded, and slides should only be re-used when difficulties of supply make this *absolutely necessary*.

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

The method of staining varies with the nature of the preparation (film or section).

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

TISSUE SECTIONS

The sections being embedded in paraffin (p. 122), it is necessary to remove the paraffin so that a watery stain may penetrate. The paraffin is first removed with xylol,¹ the xylol removed with alcohol,² 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 xylol¹ and finally mounted in Canada balsam under a cover-slip. The Canada balsam (which is a

¹ Benzol may be substituted for xylol.

² 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.

resin) is dissolved in xylol in order to render it of suitable consistence.

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. 90), 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.

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

¹ J. Path. Bact., 1939, 49, 592; 1941, 53, 441.

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 " ¹	10 grams

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.
Solution of caustic potash in water (1 : 10,000)	100 ml.

(This caustic potash solution is made by adding 1 ml. of a 1 per cent. solution to 99 ml. of water.)

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 complete. The preparation is used in a manner similar to Löffler's methylene blue; it is employed in McFadyean's reaction (p. 414).

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

Similar staining results are obtained with

Borax Methylene Blue (Masson)

Methylene blue	10 grams
Borax	25 grams
Water	500 ml.

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

CARBOL THIONIN

Stock Solution :

Thionin	1 gram
Phenol, 1 : 40 watery solution	100 ml.

For Use :

Stock solution	1 part
Distilled water.	3 parts

(Filter before use.)

This stain is useful for demonstrating in tissues organisms such as the typhoid and glanders bacilli.

- (1) Stain sections, five to ten minutes.
- (2) Wash well with water.
- (3) Differentiate in a bowl of water to which a few drops of acetic acid have been added.
- (4) Wash well with water.
- (5) Blot, dehydrate with absolute alcohol, clear in xylol and mount in Canada balsam.

DILUTE CARBOL FUCHSIN

Made by diluting Ziehl-Neelsen's stain (p. 94) 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.

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 (p. 99).

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 aniline—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.)

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

Iodine	1 gram
Potassium iodide	2 grams
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 gram
1 per cent. acetic acid	2 ml.
Distilled water	1 litre

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

² 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. 147) 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.

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 gram
Pyronine	0·15 gram
Distilled water	to 100 ml.

(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.

¹ Kopeloff, N., and Beerman, P., *Proc. Soc. Exp. Biology*, 1922–1923, 20, 71.

- (2) Flood slide with stain made up as follows :

Stain :

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 grams
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.
 - (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.)

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) Gram's Iodine ¹ :						
Iodine	1 gram
Potassium iodide	2 grams
Distilled water	300 ml.
(3) Aniline-xylol :						
Aniline	2 parts
Xylol.	1 part
(4) Dilute Carbol fuchsin :						
Ziehl-Neelsen's carbol fuchsin (p. 94)	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.

Sections.—

After removing the paraffin with xylol or benzol, treating with alcohol and washing with water, counter-stain first with carmalum ² 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.

¹ See footnote on p. 90.

² 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.

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. 96) 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, and is thought by some workers to make scanty organisms more easily noticed. This counter-stain is required for the method described on p. 402, 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 gram
Absolute alcohol	10 ml.
Solution of phenol (1 : 20)	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 grams
Phenol (cryst.)	25 grams
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 THE DIPHTHERIA BACILLUS

The diphtheria bacillus gives its characteristic staining reactions best in a young culture (eighteen to twenty-four hours) on a blood or serum medium (p. 374).

NEISSEER'S METHOD (Modified)

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

<i>Solution A :</i>	Methylene blue	1 gram
	Alcohol (95 per cent.)	50 ml.
	Glacial acetic acid	50 ml.
	Distilled water	1 litre

<i>Solution B :</i>	Crystal-violet	1 gram
	Alcohol (95 per cent.)	10 ml.
	Distilled water	300 ml.

Counter-Stain :

<i>(a)</i>	Chrysoidin	1 gram
	Distilled water	300 ml.
	(Dissolve by gentle heat, and filter)	
<i>or (b)</i>	Bismarck brown	1 gram

Distilled water 500 ml.

(1) Mix together two parts of solution A and one part of solution B. Stain films in the mixture for a few seconds.

(2) Counter-stain with chrysoidin or Bismarck brown for thirty seconds (or longer if necessary).

(3) Wash rapidly in water, blot and dry.

The volutin granules of the diphtheria bacillus appear bluish black, while the protoplasm is stained yellowish brown.

A further modification of this method gives excellent results :—

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

(2) Wash off with dilute iodine solution (iodine solution of Kopeloff and Beerman's modification of Gram's method, p. 92, diluted 1 in 10 with water) and leave 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. 90).

(5) Wash in water and dry.

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

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 :

Toluidin blue	0·15 gram
Malachite green	0·2 "
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 grams
Potassium iodide	3 "
Distilled water	300 ml.

Note.—The iodine solution used in Jensen's modification of Gram's method (p. 90) 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.

¹ J. Amer. Med. Assoc., 1924, 83, 121.

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.

STAINING OF SPORES

If spore-bearing organisms are stained by ordinary dyes, the body of the bacillus is deeply coloured, whereas the spore is unstained and appears as a clear area in the organism. It has been supposed that the envelope prevents the stain from penetrating the protoplasm of the spore. Once the spore is stained, it tends to retain the dye after treatment with decolorising agents, and in this respect behaves similarly to the tubercle bacillus (*q.v.*).

The following is a simple and satisfactory method for staining 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 for three to five minutes, heating the preparation until steam rises.

(2) Wash in water.

(3) Treat with 5 per cent. sodium sulphite solution for thirty seconds.

(4) Wash with water.

(5) Counter-stain with 1 per cent. aqueous methylene blue for one minute.

(6) Wash in water, blot and dry.

It is advantageous after (2) to treat with 30 per cent. ferric chloride for a minute or two and then proceed to (3) without washing in water.

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.

Fleming's nigrosin method as described on p. 88 is also a simple and effective means of demonstrating spores, which stand out as clear unstained spaces on a dark background.

FLEMING'S METHOD FOR DEMONSTRATING SPORES BY CARBOL FUCHSIN AND NIGROSIN

(1) Make films on a slide in the usual way and dry.

(2) Stain for five minutes with hot carbol fuchsin.

(3) Wash off excess of stain.

- (4) Decolorise by either of the following methods :
- Cover with, or immerse in, 1 per cent. nigrosin for five to ten minutes.
 - Cover with 5 per cent. sodium sulphite for five to thirty seconds.

(The length of time for decolorisation varies with the organism, and a large organism like the anthrax bacillus may take five times as long to decolorise as a smaller one like *Cl. sporogenes*.)

- (5) Wash with water and dry.

(6) Place a small drop of 10 per cent. nigrosin at one end of the film and spread this in an even film over the stained specimen with the edge of another slide.

The thickness of the film depends largely on the rate of spread—the faster the spread is made the thicker is the film—and the proper thickness can be rapidly learned in a few trials.

The spores appear as clear-cut, bright red objects in the unstained bacillary body, which is clearly defined by being surrounded by a perfectly homogeneous background of nigrosin.

The preliminary decolorisation with nigrosin, sodium sulphite or other decolorising agent need only be partial, and the final decolorisation is effected by the 10 per cent. nigrosin before it dries on the film.

STAINING OF CAPSULES

Several methods are described for general routine work.

DEMONSTRATION OF CAPSULES BY INDIA INK¹

(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 BACTERIAL 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,

¹ Butt, E. M., Bonyng, C. W., and Joyce, R. L., 1936, *J. Inf. Dis.*, 58, 5.

as described above ; otherwise capsules may best be observed in very thin wet films of India ink.¹ 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 the highly refractile outline of the bacterium is seen. 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.

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 EOSIN²

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.

¹ Rowland, S., *J. Hyg.*, 1914, **13**, Plague suppl., 3, 418.

² Howie, J. W., and Kirkpatrick, J., *J. Path. Bact.*, 1934, **39**, 165.

In the case of Gram-positive cocci and bacilli, 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."

HISSE'S METHOD

This method can be used for the demonstration of capsules when the organisms are present in blood or serous fluids. It is not applicable to the ordinary cultures.

Solutions required.—

- | | |
|-------------------------------------------------------------------|----------|
| (1) Saturated alcoholic solution of gentian-violet ¹ . | 1 part |
| Distilled water | 19 parts |
| (2) Copper sulphate solution 20 per cent. in distilled water. | |

Films should be thin and fixed by heat in the usual manner.

(1) Pour on the stain and gently heat until steam rises. Allow the stain to act for fifteen to twenty seconds.

(2) Wash off the stain with the 20 per cent. copper sulphate solution without washing in water, dry the film between blotting-paper, and mount, if necessary, in balsam.

In order to avoid an excessive deposit of copper sulphate crystals on the film, successive amounts of copper sulphate solution are poured on the slide until it is quite cool.

The bacteria are stained deep violet, while the capsules are pale violet in colour.

STAINING OF FLAGELLA

Thin films are made from agar cultures. A small amount of the culture is emulsified in water, the quantity of culture being only as much as will cause the faintest turbidity of the water. A film is made from a drop of the emulsion on a clean slide and allowed to dry in the air. Strict attention should be paid to the cleanliness of the slides, which are treated with the bichromate-nitric-acid solution described on p. 83, well washed, and carefully dried. They are then passed through the Bunsen flame and allowed to cool before spreading the films.

In Fleming's method, the organisms are grown on cellophane which avoids the transference of any culture medium to the slide (*vide infra*).

¹ Basic fuchsin may be substituted for gentian-violet.

KIRKPATRICK'S METHOD¹*Solutions required.*—(1) *Fixing solution*:

Absolute alcohol	60 ml.
Chloroform	30 ml.
Formalin	10 ml.

(2) *Mordant*:

Ferric chloride, 5 per cent. solution	1 part
Tannic acid, 20 per cent. solution (dissolved by heat and allowed to cool)	3 parts

Before use, dilute the mordant with an equal quantity of water.

(3) *Silver Solutions*:

- (a) *Silver stock solution*.—Add 200 ml. distilled water to 10 grams of silver sulphate² in a screw-capped bottle, and incubate for twenty-four hours at 37° C., shaking occasionally. This solution keeps well.
- (b) *Silver staining solution*.—Rinse a clean 100 ml. conical flask with distilled water. Place 40 ml. of the filtered silver stock solution in the flask, and add quickly 0·6 ml. ethylamine "33 per cent. W/V"^{2,3}. A precipitate forms which is immediately dissolved. From a clean drawn-out capillary pipette add filtered silver stock solution until the solution remains permanently opalescent. Now add 10 ml. distilled water.

Method

Use agar cultures thirty-six to forty-eight hours old. Heat a clean 3×½-in. test-tube and allow to cool. Suspend a loopful of the agar culture in 1 ml. sterile distilled water in the tube, rotating the loop very gently. Add distilled water until the suspension is faintly opalescent.

(1) Place a loopful of the suspension on a clean slide and draw the excess to one side by means of the wire loop, so that a thicker part of the film (which acts as a control spot in the staining process) is formed. Allow the film to dry in the air.

(2) Treat the film with the fixing solution for one to three minutes.

(3) Rinse in alcohol and wash thoroughly in water.

(4) Treat with the mordant for three to five minutes.

(5) Wash well with water and dry the under-surface of the slide.

(6) Filter on the silver staining solution and heat gently until the thick control spot becomes dark brown in colour, and a metallic scum appears on the edges of the fluid—about fifteen seconds. Cease

¹ Method described in 10th edition of Muir and Ritchie's *Manual of Bacteriology*, 1937.

² British Drug Houses Ltd.

³ W/V=Weight-Volume—i.e. 33 per cent. by weight of ethylamine.

heating and allow the solution to act for a further fifteen to thirty seconds.

- (7) Wash off the staining solution in running water (do not pour off).
- (8) Dry the film and mount in balsam.

If the staining is successful the organisms are stained black, while the flagella are clearly defined and are light brown or grey in colour. A granular appearance in the flagella may be due to excess of ethylamine, over-heating or over-treatment in the mordant. Too little ethylamine or excessive heating may cause a crystalline deposit. If the directions are carefully followed the method gives good results.

FLEMING'S METHOD FOR THE DEMONSTRATION OF FLAGELLA

A cellophane disk sterilised in distilled water in the autoclave is placed on the surface of an agar plate. This is dried off in the incubator for a short time. The surface of the cellophane is then inoculated with the organism, *S. typhi*, *Esch. coli*, *Proteus*, etc., which is grown for a period not exceeding twenty-four hours. By means of forceps the cellophane disk is removed, together with the whole of the culture, and transferred to another Petri dish, after which about 20 ml. of sterile distilled water is added (sufficient to cover the disk). This is incubated for a few hours to allow the bacteria to float off into the water. Then 1 ml. of formalin is very gently added to fix the bacteria, and the plate is left overnight undisturbed to allow fixation to take place, with the minimum disturbance and separation of flagella. The suspension is poured into a screw-capped bottle and preserved. This suspension keeps perfectly well for a long time.

For use remove sufficient from the upper part of the bottle and add to distilled water.

Technique.—

Slides.—It is usually stated that slides must be specially cleaned for staining flagella, but ordinary cleansing is quite satisfactory with this method.

Making films.—Place a drop of a thin suspension on the slide and spread over a large part of the slide with a wire or pipette. This is best allowed to dry at room temperature.

Mordant.—Stock solutions of 20 per cent. tannic acid and 5 per cent. tartar emetic (potassium antimony tartrate) can be kept. (20 per cent. tannic acid is convenient as this can also be used for Kirkpatrick's method.)

To three parts of 20 per cent. tannic acid add two parts of 5 per cent. tartar emetic and five parts of water, (A heavy precipitate forms which dissolves on boiling.) Heat to boiling point in a test-tube and flood the slide with it. After two minutes wash well with tap water.

Silver solution.—Dilute a saturated solution of silver sulphate with an equal volume of distilled water and add ethylamine until the precipitate is just re-dissolved. This solution keeps indefinitely.

Heat, but not quite to the boiling point, and flood the slide. Allow to act without further heating for about one minute, then wash off rapidly with tap water. The slide may then be blotted and dried in the usual way.

To make permanent preparations immerse the silvered slide for half to one hour in weak gold chloride solution.

The features of this method are :

No culture medium is present in the suspension except the minute amount on the cellophane disk.

The bacteria are fixed with the minimum disturbance so that their flagella are preserved intact, while the formalin appears to assist the staining.

The silver ethylamine solution keeps for a long time and is always ready for use.

This method, which is essentially an improved and simpler modification of Zettnow's method, is very suitable for class purposes.

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

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 N/100 NaOH.

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.

The Romanowsky stains, except Jenner's stain, are usually diluted for staining purposes with distilled water, when a precipitate is formed which is removed by subsequent washing.

JENNER'S STAIN

This can be purchased ready for use, but may be made by dissolving 0.5 gram of powdered stain in 100 ml. pure methyl alcohol. This stain was devised for the cytological examination of blood films, but is not so suitable for parasites.

Pour the stain on the dried but *unfixed* film and allow to act for one to three minutes. Wash the film with distilled water until pink. Blot the slide and allow to dry in the air.

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 bottle—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 :—

Disodium hydrogen phosphate (anhydrous)	5.447 grams
Potassium dihydrogen phosphate	4.752 grams

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

Disodium hydrogen phosphate (anhydrous)	4.539 grams
Potassium dihydrogen phosphate	5.940 grams

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. monosodium phosphate.

Shute¹ maintains that 15 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 2-3 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

¹ Shute, P. G., *Trans. Roy. Soc. Trop. Med. Hyg.*, 1950, 43, 364.

of Giemsa's stain following Leishman's stain has been recommended as follows¹ :—

Fix thin blood film with Leishman's stain for 15–60 seconds. Dilute with twice the volume of buffer solution at pH 7·0 and stain for 15 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 30 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. 86).

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.

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,² which gives consistent and reliable results.

¹ Dinscombe, G., *Brit. Med. J.*, 1945, 1, 298.

² Lillie, R. D., *U.S. Publ. Health Reps.*, 1943, 58, 449.

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 mgm. azure B eosinate, 100 mgm. azure A eosinate, 400 mgm. methylene blue eosinate, and 200 mgm. 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 (A.R.). The proportion of stains given above should yield a satisfactory staining picture. The diluting fluid is buffer solution pH 7.0 (p. 107).

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. 107), 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 con-

taining 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. 556) and can also be applied in the staining of delicate spirochaetes. Fix the preparation for thirty to sixty seconds by osmic acid vapour over the following solution : osmic 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 grams
Disodium hydrogen phosphate (anhydrous)	5·0 grams
(If crystalline salt is used, 12·6 grams.)	

Dissolve in 50 ml. distilled water, bring to the boil and evaporate almost to dryness in a water-bath, then add potassium dihydrogen phosphate (anhydrous) 6·25 grams (crystalline salt 8·0 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 the polychroming of the methylene blue as outlined above, and *Solution A* can be made as follows :—

Methylene blue	0·8 gram
Azur I	0·5 gram
Disodium hydrogen phosphate (anhydrous)	5·0 grams
(Crystalline salt, 12·6 grams.)	
Potassium dihydrogen phosphate (anhydrous)	6·25 grams
(Crystalline salt, 8·0 grams.)	
Distilled water	500 ml.

¹ *Trans. Roy. Soc. Trop. Med. Hyg.*, 1941, 35, 35.

² This stain in tablet form can be obtained from G. T. Gurr, London.

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	1·8 grams
Disodium hydrogen phosphate (anhydrous) . .	5·0 grams
(Crystalline salt, 12·6 grams.)	
Potassium dihydrogen phosphate (anhydrous) . .	6·25 grams
(Crystalline salt, 8·0 grams.)	
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.

**SIMEONS' METHOD FOR STAINING THICK BLOOD FILMS
FOR MALARIA PARASITES¹**

Solution 1 (eosin solution).—

Eosin pure	1·0 gram
Distilled water	1 litre

¹ Indian Med. Gaz., 1942, 77, 725.

Solution 2 (Stévenel's blue).—

(a) Medicinal methylene blue	1·0 gram ✓
Distilled water	75 ml.
(Dissolve completely.)	
(b) Potassium permanganate	1·5 grams
Distilled water	75 ml.
(Dissolve completely.)	

Mix (a) and (b) in a flask. A massive precipitate forms at once. Keep the flask in a water-bath at boiling point for half an hour during which the precipitate re-dissolves.

Filter. The stain is now ready for use: it requires no further dilution.

Procedure for Staining Thick Films.—

- (1) Dehaemoglobinise by immersion in tap water in beaker No. 1, one minute.
- (2) Immerse in eosin solution, twenty seconds.
- (3) Rinse by dipping into tap water in beaker No. 2, four seconds.
- (4) Immerse in Stévenel's blue, thirty seconds.
- (5) Rinse by dipping into tap water in beaker No. 3, four seconds.
- (6) Immerse again in eosin solution, ten seconds.
- (7) Rinse by dipping into tap water in beaker No. 2, four seconds.

STAINING OF SPIROCHAETES**FONTANA'S METHOD***Solutions required.—*

(a) <i>Fixative</i> :						
Acetic acid						1 ml.
Formalin						2 ml.
Distilled water						100 ml.
(b) <i>Mordant</i> :						
Phenol						1 gram
Tannic acid						5 grams
Distilled water						100 ml.
(c) <i>Ammoniated silver nitrate</i> :						

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.

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

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. 122). 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 a mixture of

Alcohol	1 part
Saturated aqueous solution of mercuric chloride	2 parts

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 gram
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 Method.¹

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.

¹ *Parasitology*, 1942, 34, 109.

STAINING OF VIRUS INCLUSION AND ELEMENTARY BODIES, AND RICKETTSIAE

INCLUSION BODIES

For intranuclear and cytoplasmic inclusions Giemsa's stain, p. 108, 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. 122) or Zenker's fluid (p. 121), and cut paraffin sections in the usual way. Stain for 12 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 3 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.

Lépine's Stain.¹

Solution 1.

A. Basic fuchsin	1 gram
Alcohol (50 per cent.)	200 ml.
B. Watery solution of safranin (1 in 500)	

Mix equal parts A and B.

Solution 2. Permanganate Blue. (*vide* Stévenel's blue, p. 113)

Dissolve in one flask 1 gram ordinary medicinal methylene blue in 75 ml. distilled water and dissolve in another flask 1.5 grams potassium permanganate in 75 ml. distilled water.

Mix and put the flask in the water-bath (boiling).

A heavy precipitate forms—this precipitate dissolves little by little in the water-bath and the liquid becomes dark blue at first, then a violet blue.

Leave in the water-bath at least half an hour, then filter through ordinary filter-paper

¹ Modification by R. M. McKinnon, Stack Medical Research Laboratories, Khartoum.

This stain remains stable for about two months at an average temperature of 32°–40° C.

Method of Staining.

Pass sections through xylol and alcohol to water.

Stain for ten minutes in solution 1.

Wash off stain with tap water.

Stain thirty seconds to one minute with solution 2.

Differentiate with absolute alcohol until a magenta colour is obtained.
(This step requires some practice to get the correct degree of differentiation.)

Pass direct to xylol. Mount in balsam.

ELEMENTARY BODIES

Giemsa's Stain.

This has already been described on p. 108, 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.

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. 68, 73), 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.

IMPRESSION PREPARATIONS

These have been used in the morphological study of the pleuropneumonia 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 Bouin's fluid (p. 122) may be used, or Flemming's solution.³ For staining, methylene blue or dilute carbol fuchsin may be employed, but Giemsa's stain, applied by the slow method (p. 110), 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.

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

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

³ 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.

ROBINOW'S METHOD FOR DEMONSTRATING CHROMATINIC MATERIAL IN BACTERIA¹

Robinow has shown that chromatinic structures can be differentiated from the protoplasm if osmic-acid fixed cells are first treated with N/1 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 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 2-3 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 (p. 115). Wash in water and store in 70 per cent. alcohol.

Staining.

Transfer films from 70 per cent. alcohol to N/1HCl at 60° C. for 10 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 stain² per ml. of phosphate buffer (p. 107). Stain for 30 minutes at 37° C., rinse and mount in water, and examine at once. This method shows the chromatinic structures quite clearly.

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 10 minutes each in xylol, and mount in Canada balsam.

A quick method of showing chromatinic structures is as follows: dip air-dried impression preparations made as above for 5 seconds into boiling N/5 HCl.

To demonstrate the cell wall, make impression preparations fixed in Bouin's fluid, as described on p. 122. Mordant for 20-30 minutes with 5-10 per cent. tannic acid and stain with 0.02 per cent. crystal violet in water for 5-10 seconds. Mount in water.

To examine the nucleus in resting spores, e.g. *B. mycoides*, *B. mesentericus*, make a film, which need not be fixed, from the culture, mount in a drop of N/1 HCl containing 0.05 per cent. acid fuchsin and examine after an interval of 5-10 minutes.

¹ Robinow, C. F., *J. Hyg.*, 1944, **43**, 413; "The Bacterial Cell," by Dubos, R. J., 1949, Addendum.

² G. T. Gurr's R66 Giemsa stain.

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.

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 grams
Potassium bichromate	2·5 grams
Sodium sulphate	1 gram
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 grams
Distilled water	800 ml.
Sodium chloride	5 grams
Trichloracetic acid	20 grams
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 1-12 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.

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 5μ 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 V

CULTIVATION OF MICRO-ORGANISMS (INCLUDING METHODS OF STERILISATION)

ONLY in exceptional cases can the identity of a bacterium be established by its morphological characters (p. 38). 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. 210). 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.* glass ware, 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.

In the following pages the original methods are described, as in previous editions of the book, but the use of screw-capped bottles and bottled media is specially dealt with for

the guidance of laboratory workers to whom media in this form are issued, as apart from students who may only be interested in the principles underlying the preparation and use of the simpler culture media.

PREPARATION OF GLASS-WARE

Washing and Cleansing.—New glass-ware requires special attention because of the resistant spores which may be present in the straw and other packing material. Thorough mechanical cleansing with soap and hot water (and the aid of a brush) is not sufficient ; neither are the spores always killed in the hot-air oven (*vide-infra*). It is advisable to boil new glass for fifteen minutes in one of the proprietary sodium metasilicate detergents such as "Kin-Ray."¹ Soap powders should not be used. Glass containers with discarded cultures are usually placed in 3 per cent. lysol immediately after use, but those containing tubercle bacilli or spore-bearing pathogenic organisms, such as *B. anthracis* or *Cl. tetani*, must be autoclaved (p. 129). The discarded cultures and their containers are then boiled in a $\frac{1}{2}$ -1 per cent. "Kin-Ray" solution (depending on the state of the glass and hardness of the water) for fifteen minutes in a covered boiler. The glass-ware is cleansed with a test-tube brush (or other suitable brush) and well rinsed in running water. If the tap water is very "hard" and contains a considerable amount of calcium salts, rinsing in distilled water is necessary. The glass-ware is then allowed to drain and dry. After treatment as above the glass-ware has a shine and sparkle not obtained with soap preparations.

Neutralisation of Glass-Ware.—New glass-ware, especially the cheaper varieties, gives off free alkali, and this may be sufficient to interfere with the growth of certain organisms. Where such slight changes of reaction are of importance, the glass-ware should be placed in 1 per cent. commercial hydrochloric acid for several hours, thereafter well washed in tap water and finally in distilled water.

Screw-capped bottles (described later) are subjected to a special cleansing process by the makers whereby surface alkali is removed, and the treatment described above is not necessary. The bottles may be used without further treatment, as received from the manufacturers, but used bottles are cleansed as above.

METHODS OF STERILISATION

Various methods are employed for the destruction of bacteria, according to the nature of the article to be sterilised ; and each method has its own particular use, with well-defined limitations. The usual methods of sterilising are either

¹ Made by the Reddish Chemical Co. Ltd., Globe Works, Reddish, Stockport.

(a) destruction of the organisms by some form of heat or by chemical antiseptics, or (b) mechanical removal by filtration.

Chemical methods of sterilisation are not employed in the preparation of culture media, as the presence of the chemical which destroys the contaminating organisms will either kill or prevent the growth of the bacteria artificially introduced into the medium.

For the sterilisation of most culture media and apparatus, heat is applied in some appropriate form, depending on the nature of the object to be sterilised.

STERILISATION BY HEAT

Articles may be sterilised by heat in two forms—dry heat (Bunsen flame or hot air) and moist heat (steam or hot water). Sterilisation by hot air requires a much higher temperature, or a much longer time at the same temperature, than in the case of moist heat.

To ensure complete sterilisation, all forms of bacterial life must be destroyed, *and the time of sterilisation is that necessary to kill the most resistant forms.* Spores are extremely resistant to all methods of destruction, whether by means of heat or chemicals, and whereas an organism such as *B. anthracis* in the vegetative phase is killed by moist heat at 100° C. in a few seconds, the spores may resist boiling for five minutes. Spores of some saprophytic bacteria may survive boiling for periods up to 1½ hours. The time for effective sterilisation, therefore, by any method is that which ensures complete destruction of all spores.

STERILISATION BY DRY HEAT

(1) *Red Heat.*—Inoculating wires, points of forceps and searing spatulas are sterilised by this method, the heat from an ordinary Bunsen burner being utilised.

(2) *Hot-Air Oven.*—In its original form this consists of a chamber having double walls between which hot air passes from a Bunsen burner. Most ovens nowadays are heated electrically and there is incorporated an automatic device which keeps the temperature constant at any predetermined level. A temperature of 160° C. for one hour is necessary for complete destruction of bacterial spores. (It is obvious that such a temperature cannot be utilised for the sterilisation of culture media.) This is the best method for sterilising dry

glass-ware, such as test-tubes, Petri dishes, flasks, throat swabs, graduated and capillary pipettes, and certain instruments, such as forceps and scissors and *all-glass* syringes. Before sterilisation, test-tubes or flasks should be plugged with cotton-wool stoppers; other types of glass-ware, e.g. pipettes, may be wrapped in kraft paper (p. 140).

Although screw-capped bottles themselves will withstand the temperature of the hot-air oven, the rubber liners or washers in the screw-caps will not. Bottles already capped, therefore, cannot be sterilised by this method, but should be autoclaved (p. 129).

It should be noted that from certain brands of cotton wool, volatile substances are given off during sterilisation; these condense on the tube and may interfere later with the growth of certain bacteria, e.g. pneumococcus.

Certain precautions have to be observed when sterilising glass-ware: (a) the glass should be perfectly dry; (b) the oven must be cold when the apparatus is inserted, then heated to the requisite temperature and kept at that temperature for the full time necessary for sterilisation; (c) the oven should be allowed to cool before the articles are removed, as sudden or uneven cooling is apt to cause cracking of the glass.

The advantage of this method is that all the articles are kept dry.

(3) *Flaming*, by passing the article through the Bunsen flame without allowing it to become red hot. This method is useful for sterilising scalpels, needles, the mouths of culture tubes, cotton-wool stoppers, glass slides and cover-slips. Needles and scalpels may also be sterilised by dipping them in methylated spirit and then burning off the spirit, the process being repeated several times.

STERILISATION BY MOIST HEAT

(1) *Boiling in a Water-Bath*.—A suitable form of steriliser 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. Five minutes at 100° C. is sufficient to kill all non-sporing and many sporing organisms; the spores of certain species, however, may resist longer exposures, for one hour or more. This method of sterilisation has only a limited use in bacteriology, and is employed for tubes, instruments (forceps, scissors, etc.), syringes (metal and glass types), pipettes, measuring cylinders, etc. The addition of a small amount

of sodium carbonate obviates rusting of steel instruments, but with stainless steel this is not necessary.

If the water supply is "hard," distilled water should be used, otherwise the instruments on removal become covered with a film of calcium salts.

The interior of a test-tube may be sterilised quickly for ordinary purposes by boiling water in it.

(2) *Steam at 100° C.*—This method is much used in bacteriology. A Koch or Arnold steam steriliser (sometimes called "steamer") heated by steam, gas or electricity with an automatic regulator is employed, and is particularly useful for the sterilisation of culture media. In its simplest form this is a vertical metal cylinder with a removable conical lid (having a small opening at the top for the escaping steam) and containing water which is heated by a gas burner under the bottom of the cylinder; but various modifications of this form of steriliser have been used. A perforated diaphragm situated above the water holds the articles to be sterilised. Its advantages are: (a) the apparatus need not be costly; (b) both the container and the medium are sterilised; (c) as the medium is in an atmosphere of steam, there is no loss from evaporation; (d) the apparatus requires little or no attention.

Sterilisation is effected in two ways:—

(a) One exposure for $1\frac{1}{2}$ hours. This usually ensures complete sterilisation, and can be used for such media as broth or nutrient agar (p. 149). It cannot be used for nutrient gelatin (p. 153), as this medium, after prolonged heating, fails to solidify on cooling.

(b) Exposure at 100° C. for twenty minutes on each of three successive days. The usually accepted principle of this "intermittent" method of sterilisation is that one exposure is sufficient to kill the vegetative forms of bacteria; between the heatings, the spores, being in a favourable medium, become vegetative forms which are destroyed during the subsequent heating. This method, which is sometimes referred to as "Tyndallisation," is employed in sterilising media containing sugars (p. 166) which may be decomposed by higher temperatures or by prolonged heating. It can also be used for the sterilisation of gelatin media.

If tubes or flasks are used it is advisable to cover the cotton-wool stoppers with two layers of parchment paper or kraft paper to avoid drenching.

(3) *Steam at High Pressure in the Autoclave.*—The principle on which the autoclave is used is that water boils when its vapour pressure is equal to the pressure of the surrounding atmosphere. If, therefore, the pressure be increased inside a closed vessel, the temperature at which the water boils will rise above 100° C., the exact temperature depending on the pressure employed. The usual form of laboratory autoclave consists of a vertical cylinder of gun-metal which is supported by a sheet-iron case. (The larger forms of autoclave have the cylinder arranged horizontally.) The lid 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.* 8½ in. for a vertical autoclave of 19 in. internal height) and this is heated by a Bunsén gas ring below the cylinder. The bottles, tubes, etc., to be sterilised are placed on a perforated diaphragm situated above the water level. The apparatus is furnished with a steam-tap and pressure-gauge, and also a safety-valve, which can be set to blow off at any desired pressure. The pressure originally employed was 15 lbs. per square inch above atmospheric pressure, making a total of 30 lbs. per square inch absolute pressure. Under this pressure water boils at 120° C., and fifteen minutes' exposure to this temperature kills (with few exceptions) all forms of organisms, including spores. This pressure is still used for sterilising surgical dressings, empty glass containers, pathogenic cultures containing spores, etc. When sterilising broth and agar, the high temperature resulting from 15 lbs. of steam pressure may have a detrimental effect on the nutritive properties of the medium. An exposure of thirty minutes at 5 lbs. pressure is preferable. Autoclaving should not be used for sugar media or gelatin, as the former are decomposed and the latter may not solidify on cooling.

Directions for Using the Autoclave.—See that there is sufficient water in the cylinder. Insert material to be sterilised and light the gas. Place the lid in position, see that the tap is *open*, adjust the safety-valve to the required pressure¹ and screw down the lid. As the temperature inside the autoclave rises, air is forced out of the tap and eventually steam issues. *Make sure that all air has been expelled from the cylinder*, as indicated by steam issuing freely, and then close the tap. The pressure now rises until it reaches the desired

¹ In some varieties of autoclave the adjustment of the safety-valve has to be determined previously by trial.

level, when the safety-valve opens and the excess steam escapes. Allow the sterilisation to continue for the requisite time, fifteen or thirty minutes as the case may be, *from this point*. When sterilisation is complete, turn off the gas and allow the autoclave to cool until the pressure-gauge indicates that the inside is at atmospheric pressure. Now open the tap very slowly to allow air to pass into the autoclave, and remove the lid. If the pressure is suddenly released without cooling, the liquid media, being at a temperature above 100° C. and suddenly exposed to ordinary atmospheric pressure, will boil violently and be expelled from their containers with almost explosive force. In order to avoid drenching by the steam, cotton-wool stoppers should be covered with parchment paper or kraft paper.

Autoclaves heated by other means, *e.g.* steam or electricity, are operated in a similar manner.

Steam-heated Autoclaves.—Where steam is available autoclaves should be heated from the main steam supply. Such autoclaves are of the horizontal pattern, made of copper or gun-metal tinned inside, with a swing door fastened either with swing bolts and nuts, or by a "capstan head" which, on being turned, manipulates radial bolts. A convenient size is 20 in. diameter and 30 in. long (internally) fitted with a perforated shelf of stainless steel. The steam pipe entering the autoclave should have a baffle plate to distribute the steam and prevent it from impinging directly on the material to be autoclaved. A special reducing valve, which permits any pressure of steam from 0-15 lbs., is an advantage. It is important to have a thermometer, either of the ordinary mercury or recording type, fixed to the autoclave when steam is obtained direct from a steam main. When the steam is turned on the pressure-gauge rapidly registers the desired pressure, whereas the thermometer may show that the necessary temperature has not yet been reached. The duration of sterilising should be reckoned from the time when the proper temperature is attained.

Attention should be paid to the steam trap of the autoclave. If a bottle or tube containing agar breaks during sterilisation and the agar gains access to the trap and solidifies there, the autoclave may become half-filled with condensed water, which is unable to escape.

Autoclaving in "Free Steam."—A useful method, particularly when the autoclave is connected to a main steam supply, is to sterilise by "free steam." The culture medium is placed in the autoclave, which is

then tightly closed and the steam supply turned on. When all the air has been expelled from the autoclave, steam issues freely from the escape-tap. Adjust the steam supply so that an adequate amount of steam is emitted from the autoclave, and allow this to continue during the period of sterilisation. Although the interior of the autoclave is open to the air, the steam pressure inside is about 1-2 lbs. (above atmospheric pressure), which maintains the temperature just over 100° C., while the latent heat of the steam quickly warms up the contents of the autoclave and maintains them at 100° C. or over during the time of sterilisation, which can be less than with the ordinary steam steriliser.

Alternatively, the contents of the autoclave can be thoroughly heated up by half-an-hour's exposure to "free steam," the escape-tap is then closed and the contents raised to 5 lbs. pressure for fifteen minutes. This is a very useful method for culture media such as broth and agar.

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. 128). 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 should be 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.

METHODS OF STERILISATION EMPLOYED FOR SPECIAL PURPOSES

STERILISATION 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 (p. 252).

(3) Metallic salts or organic compounds of metals—*e.g.* mercuric chloride (or perchloride of mercury) sometimes used as a disinfectant in a 1 : 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) Glycerol.—This is used as a 50 per cent. solution for the preservation of certain of the viruses, which retain their virulence in it for many months. In addition the glycerol kills off contaminating organisms, so that in time the virus-material contains no living bacteria of the ordinary type. This method is used in the preparation of vaccinia virus for vaccination against smallpox (p. 591). Glycerol is also used as a preservative in agglutinating and other sera (p. 252).

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. 572.

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* (p. 572).

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.)

CHAMBERLAND FILTERS

These are made of unglazed porcelain and are produced in various grades of porosity. The finer grades will pass only certain viruses of 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. 171). 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 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 $10m\mu$ 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.

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., *J. Path. Bact.*, 1931, **34**, 505, and *Proc. Roy. Soc., B.*, 1933, **112**, 384.

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.

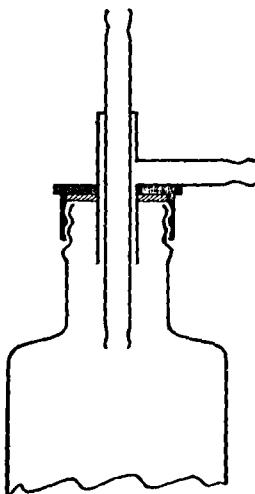
A disadvantage of the filtering flask is that the filtered fluid has to be transferred later to another container, and where it is desired to store filtered fluids, *e.g.* serum or culture media, contamination may occur in the process. It has also been our experience that rubber bungs are not resilient 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, p. 136). 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. 148), 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.

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. 224) 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. 167,

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. 167) 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. 167 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. 136. 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.

Collodion Filters.—Larger works or original papers should be consulted regarding these.¹

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

¹ See footnote 2 on p. 134.

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.

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 $\frac{1}{4}$ -, $\frac{1}{2}$ - 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 2½ 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.

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

¹ Medical Research Council War Memorandum, No. 15. "The Sterilisation, Use, and Care of Syringes," 1945, H.M. Stationery Office.

to British Standards Specification, No. 1263 (1945).¹ Syringes of 5 ml. capacity and upwards should have excentric 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 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

¹ Obtainable from British Standards Institution, 28 Victoria Street, London, S.W. 1.

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. 126). 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.

Careful attention should be paid to the process of sterilisation. The hot-air oven must have a reliable thermostatic control and be provided with a thermometer, the bulb of which is near the syringes. The temperature should be checked from time to time and control tests with material containing *B. subtilis* spores should be made.

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

¹ See reference in footnote on p. 140 for details.

film envelopes¹ 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 $160^{\circ}\text{C}.$ ($\pm 2^{\circ}\text{ C.}$) 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.

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.

FOOD SUPPLY AND ENERGY REQUIREMENTS OF BACTERIA

The general subject of bacterial nutrition has been dealt with in Chapter I, but those aspects which are directly referable to the practical cultivation of bacteria in the laboratory will be considered here.

The food requirements of bacteria vary with the natural environment and the particular rôle they have in nature. Some organisms are able to grow under a wide range of conditions, whereas other more highly parasitic bacteria, such as the gonococcus, are restricted in their requirements, with regard not only to food but also to temperature and other factors. It is usually impossible to reproduce exactly the natural conditions under which pathogenic micro-organisms flourish. On the other hand it must be realised that a considerable degree of adaptability exists among them, and for the great majority of pathogenic bacteria suitable artificial media have been devised.

¹ See "The Use of Transparent Film Envelopes for a Syringe Service," McCartney, J. E., *Lancet*, 1951, 1, 509.

Bacteria require nitrogenous food material, and in the case of the pathogenic organisms this generally consists of protein derivatives. In the usual culture media nitrogen is conveniently supplied in the form of "peptone," which is a commercial product obtained by peptic or papain digestion of some protein material, *e.g.* meat. Peptone is a crude product and consists of a mixture of polypeptides, dipeptides and amino-acids. It is soluble and does not coagulate on heating; and can therefore be incorporated in media which later have to undergo sterilisation by heat. Moreover, it is well adapted to the synthetic metabolism of bacteria, especially if it contains a sufficiency of amino-acids. Alternatively, instead of adding the digested protein in the form of commercial peptone, natural proteins are split up during the preparation of the medium by the action of pepsin, papain or trypsin. This is the principle of the so-called "digest media," of which Hartley's broth (p. 151) is an example.

Carbon as a constituent of bacterial protoplasm is derived mainly by the decomposition of carbohydrates. This process is essential in the case of many organisms. Carbohydrates are supplied generally in the form of sugars (p. 164), and if fermented they are usually valuable in promoting growth—*e.g.* the diphtheria bacillus grows much better if a small quantity of glucose is added.

Various accessory growth factors are important in the cultivation of pathogenic bacteria, and these can be supplied in meat extracts and particularly *fresh* body fluids, such as blood, serum and ascitic fluid, which at the same time may yield other nutritive substances. It must be recognised that these growth factors are often susceptible to heat and adsorption, and that excessive heating and filtration of media must be avoided if they are to be retained.

The value of meat extract incorporated in culture media is recognised although its precise properties have not yet been fully determined. In composition it is practically devoid of protein, and consists of mineral salts, a small amount of sugar and nitrogenous muscle extractives. It probably supplies certain amino-acids and growth factors.

Mineral salts are essential to growth, particularly the chlorides, phosphates and sulphates among the acid radicles, and calcium, potassium and sodium among the bases.

There are certain pathogens with particular requirements which have to be fulfilled before growth takes place. Some highly parasitic organisms require the presence of blood or

serum—e.g. the gonococcus. The haemophilic group of bacteria—e.g. *H. influenzae*—do not grow on ordinary media, but multiply if blood is present; two growth-promoting factors present in blood, designated “X” and “V,” appear to be essential. The “X” factor is thermostable, resists autoclaving at 120° C. and consists of haematin (p. 492). The “V” factor is more easily destroyed by heat; it is now known to be coenzyme I (cozymase) (p. 492). Egg media are especially suitable for growth of the tubercle bacillus. The organism of Johne’s disease can be grown if an extract of other acid-fast bacteria is present, and it would appear that some substance is synthesised by these organisms which is necessary for the growth of the former.

The filterable viruses are unable to multiply on the usual artificial media, but many of them have been cultivated in association with embryonic cells, as in the developing chick.

Certain atmospheric conditions also are essential for growth of various bacteria; these are dealt with on pp. 218-219.

The 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. 21), 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 mgm. per 100 ml. before the blood-culture bottles are made up (p. 224). 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.

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 tubed 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. 151).

BOTTLED CULTURE MEDIA

In the past, culture media have been stored in bulk in flasks with cotton-wool or rubber stoppers, or in some form of "milk" or "whisky" bottle. In recent years screw-capped bottles which enable the medium to be stored over long periods have come into general use.¹ This system consists essentially in placing the medium in a container, which is then hermetically sealed and thereafter sterilised. The result is, that the container being completely closed, the contents remain sterile indefinitely, in exactly the same way as canned foods. There is no necessity to store in a cold room, while transport is much more easily accomplished. 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. It should be noted that though the use of screw-capped bottles was introduced to facilitate the distribution, storage and transport of culture media in a large laboratory organisation, it can equally well be applied in smaller laboratories, and where comparatively small quantities of culture media are made and used. The methods of using these bottles are particularly recommended, as considerable saving in material and labour will result.

The original methods of employing flasks and test-tubes as containers of culture media are given throughout this chapter for the information of the student, and the practical application of the above-mentioned system is described for the laboratory worker.

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 $\frac{1}{2}$ -, $\frac{1}{4}$ - 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

¹ McCartney, J. E., "Screw-capped Bottles in the Preparation and Storage of Culture Media," *Lancet*, 1933, 2, 433.

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. 224).

Bottle.	Capacity in ml.	Cap.	Washer.
1 gallon, narrow mouth .	4600	Special, to fit	" Compo " cork and " resistol "
80 oz. Winchester series .	2400	KN31	
¹ 40 oz. " " .	1190	Ditto	
¹ 20 oz. " " .	600	Ditto	
10 oz. " " .	290	KN134	
5 oz. round . . .	140	KN133 sprayed white	
3 oz. " . . .	85	KN133 ,	
2 oz. medical flat . . .	60	KN132 ,	
² 1 oz. round (H 53) . . .	28	KN133 ,	
$\frac{1}{2}$ oz. " . . .	15	KN132 ,	
$\frac{1}{4}$ oz. " (bijou) . . .	6	KN132 ,	
1 oz. Universal container .	28	KN 86	aluminium lacquered

As " medical flat " bottles with the cap on may crack when sterilised in the autoclave, their general use in laboratory work is not recommended. The round bottles stand autoclaving repeatedly.

These bottles are made by the United Glass Bottle Manufacturers, Ltd., 8 Leicester Street, London, W.C.2. 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, 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

¹ These bottles are also used for intravenous infusion solutions, e.g. saline, glucose-saline, etc.

² See Fig. on p. 204.

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.

These bottles can be autoclaved with the caps tightly screwed on, either empty or containing media, with little risk of breakage. 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.

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 should never 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. 320.

8-oz. pot, p. 320.

$\frac{1}{2}$ -lb. jar } same size }
1-lb. jar } of cap. } p. 324.

$\frac{1}{2}$ -gallon wide-mouth jar } for the preparation of culture media.
1-gallon " " " "

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.

It should be noted here that the various "digest" media to be described are of great value for obtaining very luxuriant growths of organisms, but the cultures may die out rapidly. For maintaining stock cultures the use of media prepared from ordinary meat extract is advisable.

BROTH ("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. 159), 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. Commercial brands of peptone may contain copper salts derived from the vessels used in their manufacture, and it is essential that any peptone used should be copper-free.

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

¹ For ordinary purposes "commercial" peptone is satisfactory, but for special purposes sugar and indole must be absent.

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 20 minutes at 68° C., stirring at intervals. Shake well and steam in the steam steriliser for 30 minutes, filter through paper and adjust the reaction to pH 7.8-8.0 (p. 159). Again steam for 30 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 10 minutes at 10 lbs. pressure. This broth can be used for ordinary purposes, when the glucose may be omitted.

Lab.-Lemco.—A meat extract known as "Lab.-Lemco" may be used as a substitute for the extract of fresh meat in the proportion of 10 grams to a litre of water. The addition of salt (0.5 per cent.) and peptone (1 per cent.) converts it into broth. The reaction must be standardised as in the case of the ordinary infusion broth.

Infusion broth and nutrient agar made from it are identified by means of a YELLOW bead in the container (p. 201).

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.

A useful form of "digest" medium for general use is :

HARTLEY'S BROTH

Ox heart or lean beef (free from fat and minced)	1500 grams
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.
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 grams
Distilled water	1500 ml.
Absolute alcohol	500 ml.

Industrial methylated spirit may be used instead of absolute alcohol (see note on p. 85).

Shake the mixture thoroughly in a large stoppered bottle and allow to stand for three days at room temperature, the shaking being repeated occa-

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. 159) and distribute in 250 ml. amounts in 10-oz. screw-capped bottles, or in smaller amounts, *e.g.* in blood-culture bottles (p. 224) 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. 201).

HORSE FLESH DIGEST MEDIUM

This medium is specially suitable for cultivating haemolytic streptococci when an abundant growth is required.

Mix 2 lbs. 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. peptone (de Fresne's or other 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.

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. 210).

sionally. Strain through muslin, and filter through Chardin paper. 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.

It can, however, be rendered solid by the addition of gelatin, 15 per cent., or agar-agar, 2 per cent.

NUTRIENT GELATIN

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 10 minutes. Cool quickly to 45° C. and add the beaten white of an egg (see Nutrient Agar, p. 154) slowly to the gelatin. Steam for 30 minutes, stirring occasionally. Filter through paper pulp. 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 10 minutes in free steam and then at 10 lbs. 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. 208).

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. 147). When the medium is made it is distributed 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, comes into the market in the form of dried strands prepared from a seaweed found in the Japanese and Chinese seas.¹ It is also obtained powdered, which is preferable.

Agar powder or fibre, as bought, contains many solid impurities, and when nutrient agar is made it must be filtered to produce a clear and transparent medium. Owing to the large amount of minute colloidal particles present, filtration through filter-paper is slow, and, in consequence, before filtration the nutrient agar is "cleared" with egg albumin.

The proportion of agar generally used to solidify broth is 2 per cent., whether powder or fibre. After the agar has been added, the broth is placed in the steam steriliser for one hour to effect solution. The mixture is then cooled to 55° C., and the switched whites of two eggs, or 10 grams of egg albumin dissolved in 50 ml. water, are added per litre.² The medium is now steamed for two hours, or preferably autoclaved for one hour at a pressure of 5 lbs. per square inch. The agar solution (standardised to optimum pH, *vide infra*) is then filtered through Chardin filter-paper in the steamer, distributed into bottles or tubes, and finally sterilised either by autoclaving at 5 lbs. pressure for half an hour, or by steaming for half an hour each day on three successive days.

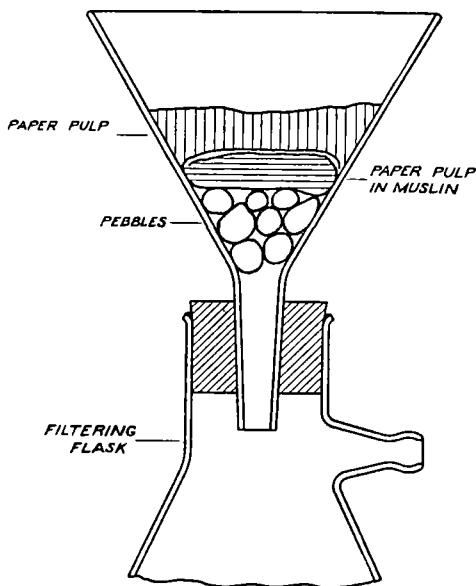
When dealing with large quantities of medium, it will be found quicker and easier to filter nutrient agar through paper pulp³ instead of through filter-paper. For amounts up to five litres we can recommend the following method. Into a 10-in. glass funnel (fitted to a filter flask as shown in the figure) place about a dozen round clean pebbles to form a support for the paper pulp. The paper pulp is thoroughly shredded and soaked in warm water. Take a large sheet of muslin 24 in. square, and place it over the funnel with the ends hanging over the side. Place moist paper pulp on top of the muslin until the layer is 2 in. deep, and pack it firmly (with the bottom of a 100 ml. cylindrical measure), especially round the edge. Turn the ends of the muslin over the surface of the paper pulp, so as to form a sort of bag. Now superimpose on this a 1-in. layer of moist paper pulp and pack firmly. The funnel and its contents are placed in the

¹ Agar is also prepared now from seaweeds in New Zealand and California.

² As an alternative to eggs, 40 ml. of ox serum per litre of medium may be used.

³ A suitable paper pulp is "White Heather" brand, or T. B. Ford's filter pulp. Both these are sold in slabs.

steamer or autoclave at the same time as the broth and agar powder when the latter is to be dissolved. It is important to note that no egg or egg albumin is required. The funnel is taken while hot from the steriliser, connected to the water-pump or other suction apparatus (p. 135), and melted agar poured into it. Gentle suction is applied and the water in the pulp first runs from the lower end of the funnel and after a few seconds agar begins to appear. Suction is stopped, the water and dilute agar discarded, and the filtration now proceeded with. As the filtration continues the amount of suction should be slightly increased. The agar passes through very rapidly, and with the



apparatus figured five litres can be filtered in thirty to forty minutes. It is not advisable to filter any greater quantity without changing the upper layer of paper pulp. For larger quantities of medium, a one- or two-gallon cylindrical stone jar with a false bottom or a specially made tinned copper container is used, and amounts of agar up to fifty litres an hour can be dealt with.

This agar-broth medium is properly described as Nutrient Agar, but is generally referred to in the cultivation of bacteria as "Agar."

When fluid, it is perfectly clear, but when solidified is faintly opalescent. Its advantage is that it is solid at 37° C. which is the optimum temperature for most pathogenic

organisms. *The medium must be heated to 98° C. to melt it, but when melted it may be cooled down to 42° C. before solidifying.* This property is utilised in the preparation of serum-agar and blood-agar (p. 178). The agar may thus be cooled below the coagulating point of the serum proteins—60° C.—and yet remain fluid so that the serum or blood is present unaltered in the medium.

Agar differs from gelatin inasmuch as it is a carbohydrate and is not generally liquefied by bacteria.

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. 201).

Both broth and agar media may be enriched or modified by the addition of various substances.

GLUCOSE BROTH.—Broth *plus* 0·25 per cent. of glucose. Since glucose acts as a reducing agent, this medium may be used for the growth of anaerobes.

GLYCEROL BROTH.—Broth to which glycerol is added in the proportion of 5–8 per cent. This medium is sometimes used for the growth of the tubercle bacillus.

Similarly, **GLUCOSE AGAR** is made by the addition of 0·25 per cent. of glucose, and **GLYCEROL AGAR** by adding 5–8 per cent. of glycerol to ordinary agar. The former is used chiefly for deep stab cultures of anaerobes (p. 218).

STANDARDISATION OF MEDIA

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 organisms have a particular reaction at which the growth is optimal.

In order, therefore, to secure the best growth, particularly of the highly parasitic organisms, it is necessary that the adjustment of the reaction should be made as accurately as possible.

Two methods have been used—the one in which the acidity or alkalinity is expressed in terms of the absolute acidity which depends on the hydrogen-ion concentration ; the other, in terms of the neutral point of an indicator (phenolphthalein),

and referred to as Eyre's method. The former method is the more satisfactory and is now almost exclusively used.

STANDARDISATION ACCORDING TO HYDROGEN-ION CONCENTRATION.—The true acidity of any fluid depends on the number of dissociated hydrogen ions present, and the reaction of the medium is dependent on, and measured by, the hydrogen-ion concentration. The greater the concentration, the more acid the medium.

Even a typically neutral liquid such as pure water undergoes dissociation (though extremely slight) into hydrogen and hydroxyl ions. It can readily be shown (*a*) that the hydrogen and hydroxyl ions are equal in number and exactly balance and neutralise each other; (*b*) that the greater part of the water is undissociated (un-ionised); (*c*) that an equilibrium exists between the ions and un-ionised water; and (*d*) that, at equilibrium, the product of the concentrations of the hydrogen and hydroxyl ions is a constant, which is termed the ionisation constant (or dissociation constant). From conductivity measurements it has been found that the concentration of the hydrogen ions (and therefore of the hydroxyl ions) in pure water is 10^{-7} . This means that in one litre of pure neutral water there is $1/10^7$ gram of hydrogen ions and an equivalent weight of hydroxyl ions. If an acid, *e.g.* hydrochloric acid, is now added, it dissociates liberating hydrogen ions, the amount of which depends on the amount of acid added and the degree to which it dissociates. The hydrogen-ion concentration is accordingly increased, while to maintain equilibrium, the number of hydroxyl ions is proportionately reduced. When an alkali, *e.g.* sodium hydroxide, is dissolved in water, it undergoes ionisation with the production of hydroxyl ions, the amount of these being proportional to the amount of alkali and its degree of ionisation, and a corresponding decrease in hydrogen ions occurs. However alkaline the solution may be, there will still be some hydrogen ions left, in such proportion that the ionisation constant remains unchanged. It will be seen, therefore, 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 the hydrogen ions.

It is, however, inconvenient to express acidity, for example, as 2×10^{-6} , or alkalinity as 1.5×10^{-9} grams of hydrogen ions per litre, and to simplify the matter the pH or hydrogen exponent scale was introduced.

The pH value of a liquid is defined as the logarithm of the reciprocal of the hydrogen-ion concentration. Thus :

$$\text{pH} = \log \frac{1}{\text{concentration of H-ions}} \propto \frac{1}{\text{acidity}}$$

For neutral water where the concentration of hydrogen ions is 10^{-7} grams per litre the pH is $\log \frac{1}{10^{-7}} = 7$. In the two examples quoted above, for the acid the $\text{pH} = \log \frac{1}{2 \times 10^{-6}} = 6 - \log 2 = 5.699$,

while for the alkali the $\text{pH} = \log \frac{1}{1.5 \times 10^{-9}} = 8.824$.

Since this is a logarithmic scale a change of one unit in pH is equivalent to a tenfold change of hydrogen-ion concentration, that is a tenfold change of acidity ; thus, a liquid of pH 5 is ten times more acid than one of pH 6, while a liquid of pH 9 is ten times more alkaline than one of pH 8. It will also be seen that as the pH depends on the *reciprocal* of the hydrogen-ion concentration, the lower the pH number the greater will be the acidity. As the neutral value is pH 7, a pH value of less than this number indicates an acid solution, and greater than this an alkaline solution.¹

The pH value of blood plasma is about 7.5, that is, it is slightly alkaline. This reaction is about the optimum for the growth of most pathogenic organisms.

Though it is not practicable to use the hydrogen electrode for general laboratory purposes, a pH meter utilising a "glass electrode" is applicable. Small portable models worked from dry batteries are now available and in laboratories where numerous routine determinations of pH are required, a pH meter is a necessary piece of standard laboratory equipment.

Media can, however, be adjusted to any desired pH by a simple and satisfactory colorimetric method. This depends on the fact that when a fluid contains buffer salts such as phosphates, and an indicator, the addition of alkali or acid does not cause an abrupt change in the colour of the indicator. For example, phenol sulphone-phthalein (phenol red) is yellow in acid solution and purplish pink in alkaline solution. If an alkali be added gradually to an acid phosphate solution containing phenol red, the change in colour will commence at

¹ N/10 HCl has an approximate pH value of 1
N/100 HCl " " " " 2.

pH 6·8, and the colour will become more purplish pink, until the final change is reached at pH 8·4; thus the "range" is pH 6·8–pH 8·4, and as it covers the optimum reaction for culture media—namely, pH 7·2 to 7·6—this particular indicator is used.

Other dyes have their own definite range of pH in which colour change occurs, and there is now available a complete series of indicators which exhibit colour changes between pH 1 and pH 11 as follows:—

<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.
Cresolphthalein	8·2–9·8	colourless to red.
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 procedure for adjusting culture media to a definite pH is comparatively simple. Solutions of fixed and known hydrogen-ion concentrations (buffer solutions) are prepared, and to each solution a definite amount of indicator (for this purpose phenol red) is added. The resultant tint is the standard to which the medium must be brought by titration with alkali, and so the amount of alkali to be added per litre may easily be calculated.

Apparatus required and Method of Titration :

1. A set of tubes of standard bore containing buffer solutions of known pH to which the indicator has been added. These solutions are made up by mixing N/15 Na_2HPO_4 and N/15 KH_2PO_4 in certain proportions. The tubes with the standard solutions, which have a range of pH 6·6–8·0 at intervals of pH 0·2, can be purchased.¹ Details of their preparation may be found in larger works.

2. A special comparator rack.

¹ These can be obtained from British Drug Houses, Ltd.

3. "Cordite" tubes, which have a uniform thickness of wall and bore, and are identical with the tubes containing the standard solutions.

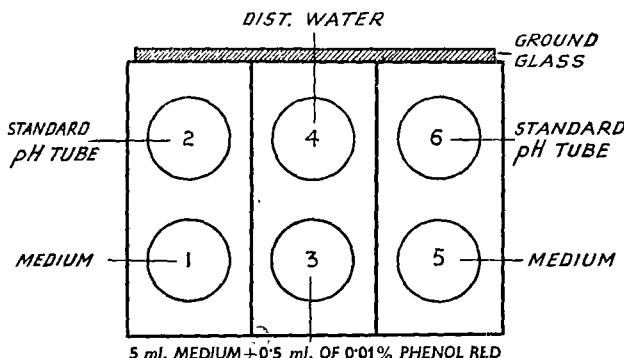
4. A solution of phenol red, 0·01 per cent.,¹ in distilled water.

5. N/20 NaOH made up as follows :—

500 ml. N/10 NaOH
91 ml. 0·01 per cent. phenol red
distilled water to 1 litre.

6. A burette, preferably a microburette, measuring to 0·01 ml. (An improvised one may be made from a 1-ml.

PLAN OF COMPARATOR RACK



graduated pipette, a short piece of rubber tubing, a glass tube drawn out to a fine point and a pinchcock.)

Tube 3 contains 5 ml. of the medium+0·5 ml. of 0·01 per cent. solution of phenol red.

Tubes 1 and 5 contain the medium only.

Tube 4 contains distilled water only.

Tubes 2 and 6 are the standard tubes for comparison. By this arrangement of the tubes the colours of 1 and 2 and of 5 and 6 are superimposed when examined in the rack.

¹ First prepare a stock 0·02 per cent. solution as follows. Weigh out 0·1 gram phenol red, add to this 10 ml. (accurately) of N/10 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. N/10 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.

It has been found easier to bring the solution to a tint midway between two standard colours than to make the tint match a given standard. Thus, suppose a reaction of pH 7.5 is required, then standard tubes pH 7.4 and pH 7.6 are placed in positions 2 and 6 of the rack.

The N/20 NaOH solution is run into tube 3 until the tint produced is midway between the tints of tubes 2 and 6, and the amount noted. The average of two readings is taken and the calculation is as follows.

Let the number of ml. of N/20 NaOH = y .

5 ml. medium require y ml. N/20 NaOH

to adjust reaction to pH 7.5.

1 litre medium requires $200y$ ml. N/20 NaOH.

1 litre medium requires $10y$ ml. N/1 NaOH.

Example: Suppose $y=1.15$, then 11.5 ml. N/1 NaOH are required per litre of the medium to adjust the reaction to pH 7.5.

It will readily be seen that the tint due to the mixture of the medium and the indicator in tube 3 is compensated for by the medium in tubes 1 and 5.

The indicator is incorporated in the standard alkali solution, so that when the medium in tube 3 is titrated, the actual concentration of the dye always remains constant.

It is preferable when making media in bulk to have the reaction slightly alkaline, and to adjust it for use by the addition of acid. The addition of alkali to an acid medium causes a precipitation of phosphates and the medium has to be filtered again before use. If, however, the medium be made slightly alkaline, and acid then added to obtain the correct reaction, no precipitate occurs and the medium is perfectly clear. In this case the titration with the standard pH tubes is carried out in exactly the same manner, except that, instead of sodium hydroxide solution, N/20 HCl containing the indicator 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.

Other indicators used for bacteriological work are bromo-cresol purple, which changes colour from yellow to violet over the range pH 5.2-6.8, and bromo-thymol blue, which has a colour range from yellow to blue between pH 6.0-7.6 (p. 159).

*Lovibond Comparator.*¹—This instrument is very convenient for estimating the pH of culture media. The indicator is added to a tube of medium and the colour is compared with that of a series of standard coloured glasses corresponding to various pH values. In matching colours the natural tint of the medium is compensated for by viewing the colour of the glass with a tube of the medium placed behind it.

DETERMINATION OF pH VALUES OF BACTERIAL CULTURES

A knowledge of the pH of bacterial cultures and of the pH changes which they undergo during cultivation is often of importance and is sometimes of practical value (e.g. in the differentiation of *Streptococcus agalactiae* from *Streptococcus pyogenes*). Accurate determination of pH values can be obtained by use of the glass or hydrogen electrode, but, for general purposes, the colorimetric methods already described (p. 159) are suitable. However, in the case of bacterial cultures often only small quantities of the fluid are available and it becomes necessary to use the capilliator for the pH determinations. The "B.D.H. Capilliator Outfit"² is the best method and is available with indicators and cards to cover a range from pH 1.2-pH 11.0 (p. 159).

The technique is as follows. The pH is first approximately determined by the use of a universal indicator. Such an indicator is a mixture of indicators which operate over a wide range of pH (e.g. the B.D.H. Universal Indicator—range pH 3-pH 11; or better, an indicator such as the B.D.H. "Four-Eleven," which is also available in capilliator sets, and has a range pH 4-pH 11). A list of the colours which may be obtained and the corresponding pH values are supplied with the indicator. A small quantity of the bacterial culture is withdrawn with a sterile capillary pipette and transferred to a white tile and the appropriate amount of indicator added. From the resulting colour of the mixture the approximate value of the pH is obtained; for example, pH 4. The tile is appropriately sterilised after use with a 3 per cent. lysol solution.

¹ Obtainable from British Drug Houses, Ltd.

² For full details see catalogue, British Drug Houses, Ltd.

An alternative "Universal Indicator" contained in test-papers, as supplied by Messrs. Johnson & Sons, Hendon, London, N.W. 4, can be recommended. A drop of culture fluid is withdrawn by means of a wire loop from the culture and placed on the paper. The resulting colour is compared with the chart supplied with the papers, and the correct indicator can then be chosen for the final determination with the capilliator as described below. 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 operates over the desired range ; for the example given above bromo-cresol green (pH range 3·6-5·2) would be chosen. The capilliator consists of a series of capillary tubes filled with buffer solutions containing an 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 determination of the pH is carried out by mixing together equal quantities of the indicator and culture, and then matching against the colour standards. A capillary tube, identical in size with those in the capilliator, is fitted with a teat and is used for withdrawing the indicator, which is pipetted on to a tile or small watch glass. The same "pipette" is used for withdrawing an equal amount of culture and the two fluids are mixed on the tile or watch glass, sucked back into the capillary tube, and the resulting colour matched against the standards and the pH value thus obtained.

Errors due to the colour of the medium itself can be corrected by using a compensating cell. Care should be taken when working with pathogenic cultures, and the used capillary tubes should be dropped into lysol solution.

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 lbs. pressure for thirty minutes. The peptone water is later distributed into tubes or small bottles as required.

Plain peptone water *without indicator* is distinguished by a WHITE bead in the container, while peptone water *with indicator* is identified by a BROWN bead (p. 201).

Peptone water is used to test the formation of indole (p. 427), and also for the enrichment of the *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 quercitin (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 (erythrite ; 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 (sorbitate), 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

¹ Made by adding N/1 sodium hydroxide to a 0·5 per cent. solution of acid fuchsin until the colour just becomes yellow.

footnote on p. 160, 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 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. 202), 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. 199).

The following alternative method of making sugar media is recommended.

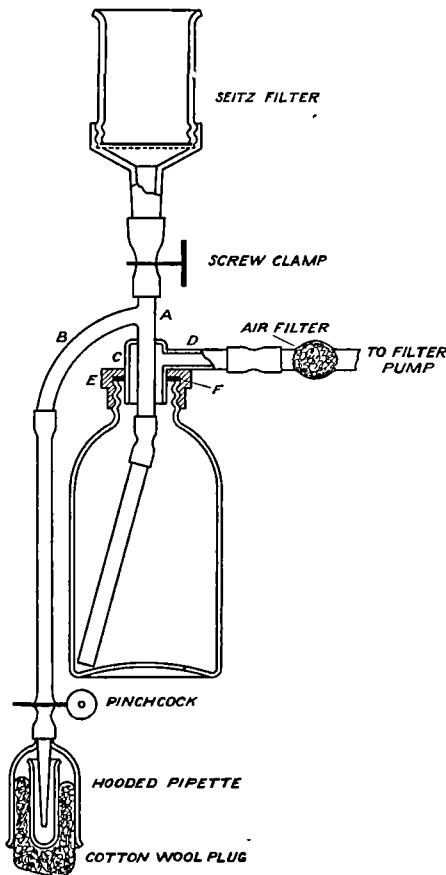
Peptone water with an indicator is tubed, the Durham fermentation tube inserted, and the test-tubes are stoppered with coloured cotton wool. It is preferable to use small screw-capped bottles (p. 148). They are then sterilised in the steam steriliser, or in the autoclave for half an hour at 5 lbs. pressure. 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 (p. 167).

This method of obtaining small quantities of sterile fluid from bulk can be applied to serum as well as to sugars (p. 171).

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

¹ The colour denotes the particular sugar used.

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 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 convenient for placing a test-tube (or bottle) under it to receive the sugar solution. The coloured 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. 138).

It is recommended that sugar media as above be distributed in 3 ml. amounts into small screw-capped bottles, $\frac{1}{2}$ 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. 200).

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 $2\frac{1}{2}$ per cent. of an alcoholic solution.¹ The medium is distributed in 5 ml. amounts 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.

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.

MEDIA CONSISTING ALMOST ENTIRELY OF SERUM

LÖFFLER'S BLOOD SERUM.—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° C. and maintained for six hours, when the serum coagulates to a yellowish-white solid. The tubes are thereafter sterilised at 85° C. (in the top of the steam steriliser) for twenty minutes on each of three successive days. If sterile serum is used, only two hours' inspissation at 85° C. is necessary. Further

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 N/1 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.

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 lbs. 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° C. and maintained for two hours. The culture medium should be allowed to cool before being handled.

In these containers Löffler's serum is most useful for diphtheria diagnosis in school-clinics, small hospitals, etc., where cultures are made only from time to time. The medium can be stored for long periods, and the small amount of water of condensation present keeps the surface constantly moist. A very profuse growth occurs after incubation for a few hours. The caps should be only loosely screwed on during incubation. The use of these bottles is most economical.

Löffler's serum 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. 97).

Collection of Blood.—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. With care, contamination can be avoided.

Defibrinated blood is collected in a similar way. The stoppered bottle, however, contains glass beads. The bottle is only half filled, and immediately a sufficient quantity of blood has been collected the stopper is replaced and the bottle continuously shaken for about five minutes. The blood so treated does not clot on standing.

Another useful method is the following.

Horse blood is obtained from the slaughter-house, the horse being

bled directly into jars containing 10 ml. of a 10 per cent. solution of neutral potassium oxalate per litre of blood. The red corpuscles are allowed to settle overnight in the cold and the plasma is siphoned off into a Winchester quart bottle; 22.5 ml. of a 4 per cent. solution of calcium chloride per litre of plasma are added, and the bottle is shaken immediately on a machine until the fibrin has separated. (The plasma coagulates more quickly and fibrin separates more easily if it is warmed to room or body temperature before the calcium chloride is added.) The serum is now filtered through a Seitz bacterial filter (14 cm. diameter disk) into a large sterile screw-capped bottle of 1-5 litres capacity, fitted with siphon delivery tube and hooded pipette, as described on p. 167. The serum is stored in the refrigerator and used as required.

Sterile specimens can be obtained by inserting a cannula or wide-bore needle 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. 148), 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.

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 or bottles are at the correct angle for forming slopes. The temperature used is generally 75°-80° 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.

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. 165), 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. 166.

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. 168).

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. 166. The caps are then painted with cellulose paint, according to the sugar used (p. 200). Alternatively, small quantities of the rarer sugars in 10 per cent. aqueous solutions may be sterilised (p. 168) and stored in $\frac{1}{4}$ -oz. screw-capped bottles fitted with a perforated cap and rubber washer similar to the blood-culture bottles described on p. 224. When the sugar solution is required it is withdrawn from the bottle by perforating the rubber with the needle of a sterile syringe in exactly the same manner as the rubber cap of a vaccine bottle. Contamination will not occur if the procedure is carried out carefully and under a hood (p. 209).

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. 375). 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 saucepan 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. Animal serum is ordinarily used, and can be obtained in the laboratory by bleeding a rabbit (*vide infra*), allowing the blood to coagulate in a sterile measuring cylinder stoppered with cotton wool, or in an agar-lined tube,¹ and removing the serum after it has been fully separated. All the necessary precautions must be taken to avoid contamination. Serum can be stored in sealed tubes after heating for one hour at 56° C. on three successive days, but fresh serum yields much better results than heated serum in the culture of certain pathogens—*e.g.* gonococcus.

Horse serum kept in a sterile bottle, as described on p. 166, is recommended where large quantities of serum-agar are used.

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. 205).

¹ The agar-lined tube prevents the clot from adhering to the wall of the tube and ensures its free contraction, thus giving a large yield of serum. 1·5 per cent. agar in normal saline is used and it need not be filtered. It is stored in 100 ml. amounts in bottles and melted as required. 10 ml. of the melted agar are added to a stoppered sterile 8×1 in. boiling tube, which is tilted and rotated until the agar flows all over the interior surface and sets in the form of a thin layer.

As serum-agar is indistinguishable from ordinary agar, the tube should be marked “+S” or plugged with coloured 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. 204). 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 grams
Sodium chloride	5 grams
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 lbs. 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 lbs. 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 $\frac{1}{4}$ -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.

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.

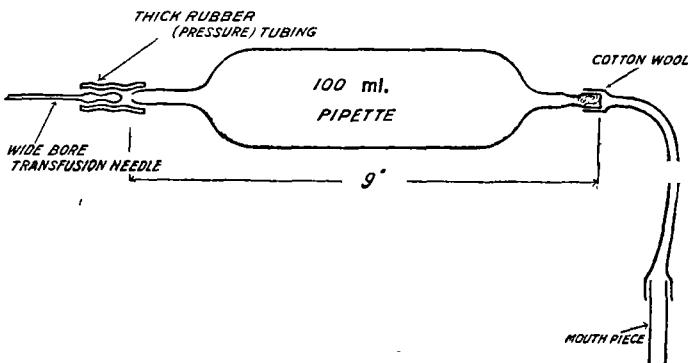
Either human or animal blood is suitable.

Human blood may be obtained easily by means of vein puncture (*vide* blood culture, p. 223).

Defibrinated rabbit blood obtained from the ear vein or by cardiac puncture can be recommended for general use.

¹ Made as described in the footnote on p. 160, except that the phenol red is ten times as strong. See also p. 165.

20 to 30 ml. of blood may be obtained easily from the ear vein of a large rabbit without 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 (*vide infra*). 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 pledge 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. This is a very useful method for obtaining small quantities of sterile rabbit blood.

For cardiac puncture the procedure is as follows.

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 short length (1½ 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. 246). 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 kilo. of body-weight can be obtained. The blood is then transferred to a sterile 500-ml.

flask or bottle containing glass beads. Agitation should be kept up for at least five minutes, to ensure that all the fibrin is separated.

The blood is added to melted 2 per cent. 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. Since the surface layer only is utilised for growth, blood is not required in the lower part of the dish. A fairly thick layer of medium is required to prevent excessive drying during incubation. If this were entirely 10 per cent. blood-agar, the medium would be almost opaque when viewed by transmitted light through the dish, and methaemoglobin formation by organisms would be difficult to see. Moreover, haemolysis would not be easy to determine in young cultures if a thick blood-agar layer were present. The method of the double layer of agar and blood-agar is not only more economical, but it yields a bright, light-transmitting medium on which methaemoglobin formation and haemolysis can easily be observed.

If only one plate is required, two 5 ml. amounts of agar are melted. The contents of one are poured into the Petri dish as the first layer. The contents of the other are cooled to 55° C. and 0·5 ml. blood added. After mixing, this is poured on the surface of the first layer in the dish and allowed to cool.

It is advisable, however, to prepare several plates at one time as follows. Two 100-ml. screw-capped bottles of agar (*p. 154*), 10 ml. defibrinated horse blood in a $\frac{1}{2}$ -oz. screw-capped bottle (*p. 148*), 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 content 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. Two 50 ml. amounts of agar and 5 ml. of blood will make seven plates.

For special purposes, amounts of blood up to 50 per cent. may be added.

Messrs. Burroughs Wellcome Ltd. supply sterile oxalated horse blood suitable for making blood-agar. This may conveniently be used

in laboratories where there is difficulty in obtaining directly sterile animal or human blood.

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.

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 (p. 202), and solidified in the sloped position in the serum inspissator at 80° C. The tubes are then sterilised at 85° C. (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 may take some weeks to grow, the tubes are sealed after inoculation by pushing down the cotton-wool stopper below the top of the tube, and pouring in a little melted paraffin wax.

We strongly advocate, however, that all media for growth of the tubercle bacillus be distributed into $\frac{1}{2}$ - or 1-oz. screw-capped bottles as described on p. 148.

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° C. Sterilise by heating in the inspissator at 80° 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.*

Potassium dihydrogen phosphate, KH ₂ PO ₄ (Analar)	0·4	per cent.
Magnesium sulphate (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 5 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. 148) and screw the caps tightly on. Lay the bottles horizontally in the inspissator and heat at 80° C. for half an hour. Allow them to remain in the inspissator overnight, and heat again the following day at 75° 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. 404) in ten to twelve days. It can be strongly recommended, particularly for the human type.

PETRAGNANI'S MEDIUM (for the 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.

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 ("Universal container," pp. 148, 319).

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 lbs. 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.

OTHER MEDIA FOR SPECIAL PURPOSES

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. 154) and filter. (Large quantities should be filtered through paper pulp in the same way as agar—p. 154.) 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 media 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. 201). 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.

MACCONKEY'S BILE-SALT FLUID MEDIUM**(1) Single Strength.**

Sodium taurocholate (commercial)	.	.	.	5 grams
Peptone (any good make)	.	.	.	20 grams
Sodium chloride	.	.	.	5 grams
Lactose	.	.	.	10 grams
Distilled water	.	.	.	1 litre

Dissolve the taurocholate, peptone and sodium chloride, steam for 2 hours and add the lactose during the last 15 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., 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 45 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 2-3 months and the double strength within 6-8 weeks.

GLUCOSE-PHOSPHATE MEDIUM
(for Methyl-red and Voges-Proskauer tests)

Peptone	.	.	.	5 grams
Dipotassium hydrogen phosphate (anhydrous)	.	.	.	5 grams
Distilled water	.	.	.	1 litre

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 $6 \times \frac{1}{2}$ in. test-tubes or $\frac{1}{2}$ -oz. bottles and autoclave at 10 lbs. for 10 minutes.

CITRATE MEDIUM
(for differentiation of coliform bacilli)

Sodium chloride	.	.	.	5.0 grams
Magnesium sulphate	.	.	.	0.2 gram
Ammonium dihydrogen phosphate ($\text{NH}_4\text{H}_2\text{PO}_4$)	.	.	.	1.0 gram
Dipotassium hydrogen phosphate (anhydrous)	.	.	.	1.0 gram
Distilled water	.	.	.	1 litre

Dissolve, add 2 grams of citric acid and bring back the reaction to pH 6.8 with N/1 NaOH solution when the mixture forms a clear

colourless solution. Tube or bottle in 5 ml. quantities, and autoclave at 15 lbs. for 10 minutes. (See alternative formula, p. 429).

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 grams
Lab.-Lemco	5.0 grams
Peptone (Difco proteose or Evans)	5.0 grams
Lactose	10.0 grams
Sodium citrate	8.5 grams
Sodium thiosulphate	8.5 grams
Ferric citrate	1.0 gram
Sodium desoxycholate	5.0 grams
Neutral red (as indicator)	

Tap water to 1 litre.

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 lbs. 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 grams
Sodium thiosulphate (Analar, $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$)	17 grams
Ferric citrate (scales)	2 grams
Distilled water	100 ml.

Dissolve by heat or by standing at room temperature for two days.

Solution B.

Sodium desoxycholate	10 grams
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

¹ Wilson, W. J., *J. Hygiene*, 1938, 38, 507, and personal communication.

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.

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 lbs. 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 M/1 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 M/2 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 grams
Peptone	5 grams
Lactose	4 grams
Disodium hydrogen phosphate (Na_2HPO_4)	9.5 grams
Sodium dihydrogen phosphate (NaH_2PO_4)	0.5 gram
Distilled water	1 litre

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.

McLEOD'S MEDIUM (for the diphtheria bacillus)²

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 lbs. 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.

¹ Knox, R., Gell, P. G.-H., and Pollock, M. R., *J. Path. Bact.*, 1942, **44**, 469.

² See Anderson, J., Happold, F., McLeod, J. W., and Thomson, J., *J. Path. Bact.*, 1931, **34**, 667.

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 N/10 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'S¹ MODIFICATION OF NEILL'S MEDIUM (for the diphtheria bacillus)

1. Lab.-Lemco	10 grams
Peptone (Difco proteose, or Evans)	10 grams
Sodium chloride	5 grams
Agar	20 grams
Water	1 litre

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 saponin in a final dilution of 1 in 200 is more simple and convenient.²

Prepare a 10 per cent. solution of saponin (white) in distilled water and sterilise in the autoclave at 10 lbs. 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.³) in a final dilution of 1 in 500 can be used. A 1 per cent. solu-

¹ *Lancet*, 1941, 1, 175.

² Young, M. Y., *J. Path. Bact.*, 1942, 54, 263.

³ Cyanamid Products, Ltd., Shootersway, Berkhamsted, Herts.

tion 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	0·7 gram
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. 388.

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 ISOLATION OF THE DIPHTHERIA BACILLUS

(*Vide* p. 390 for method of use, etc.)

Mix together

Sterile Hartley's broth pH 7·7-7·8 (p. 151)	100 ml.
Potassium tellurite, 3·5 per cent. aqueous solution	1·0 ml.
Copper sulphate (Analar) 10 per cent. aqueous solution	0·25 ml.
Laked horse blood	5·0 ml.

Lake the blood with saponin as described on p. 186 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.

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.

DUBOS' 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 in-

¹ See *Lancet*, 1948, 2, 862.

² See Dubos, R. J., and Davis, B. D., *J. Expt. Med.*, 1946, 83, 409.

gredient, "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 (p. 309).

Basic Medium.

Potassium dihydrogen phosphate (KH_2PO_4)	1.0 gram
Disodium hydrogen phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	6.25 grams
Sodium citrate	1.25 grams
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.6 gram
Asparagine	2.0 grams

Dissolve one at a time in distilled water, then add :—

Tween 80, ¹ 10 per cent. solution	5 ml.
Casein hydrolysate, ² 20 per cent. solution	10 ml.
Distilled water to	1 litre

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 lbs. pressure for 10 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 5N 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.

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., Lindsey Street, London, E.C. 1.

**MODIFIED KIRSCHNER MEDIUM FOR THE GROWTH OF
THE TUBERCLE BACILLUS**

Na ₂ HPO ₄ , 12H ₂ O	19 grams
KH ₂ PO ₄	2.5 grams
Magnesium sulphate	0.6 gram
Sodium citrate	2.5 grams
Asparagine	5 grams
Glycerol ¹	20 ml.
Phenol red (0.4 per cent.)	3 ml.
Distilled water	1 litre

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 lbs. pressure for 10 minutes.

Before use add 1 ml. of sterile horse serum containing 100 units penicillin per ml. to each 9 ml. of medium. The penicillin is added to reduce contamination to a minimum.

**BORDET-GENGOU MEDIUM
(for *Haemophilus pertussis*)**

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 lbs. 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 weeks after preparation.

**TODD-HEWITT BROTH (MODIFIED)
(for use in typing streptococci—p. 344)**

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

¹ All the salts are dissolved before the glycerol is added.

85° C. for 30 minutes. Filter through Chardin paper and add Eupeptone 2 per cent. Adjust the reaction to pH 7.0 with 10N NaOH (about 3 ml. per litre required), then add :

Sodium bicarbonate	0·2 per cent.
Glucose	0·2 "
Sodium chloride	0·2 "
Sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$)	0·1 "

Boil the mixture for 15 minutes, filter through Chardin paper, bottle and autoclave at 10 lbs. pressure for 10 minutes.

The final pH is 7.8. The medium does not require any further adjustment, but the pH should be checked.

FILDES' PEPTIC-BLOOD-BROTH (for the isolation of *Cl. 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 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.

DIEUDONNÉ'S MEDIUM—Blood-alkali-agar (for the isolation of the cholera vibrio)

Mix equal parts of defibrinated ox blood and N/1 sodium hydroxide and heat for one and a half hours in the steam steriliser. At this stage 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) Sodium carbonate (anhydrous) | 10 per cent. solution. |
| (2) Sodium sulphite | 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 20 minutes.

A precipitate forms, but rapidly sediments, and the clear supernatant material is used for making plates. Colonies of the cholera vibrio appear in 12 hours, and after 24 hours these are relatively large with red centres, while coliform bacilli generally produce small slightly pink colonies.

LIVER INFUSION AGAR¹

(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. 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.

SELECTIVE MEDIUM FOR ISOLATION OF STAPHYLOCOCCUS AUREUS FROM HEAVILY CONTAMINATED MATERIAL²

Lab.-Lemco	10 grams
Peptone, "Bacto" or Evans	10 grams
Dipotassium hydrogen phosphate (anhydrous)	5 grams
Lithium chloride	5 grams
Mannitol	10 grams
Distilled water	1 litre

¹ Huddleson, I. F., *Brucelloses in Man and Animals*, 1939, New York, p. 13.

² Ludlam, G. B., *Month. Bull. Min. Hlth. and P.H.L.S.*, 1949, 8, 15.

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 20 minutes. Before use add sterile 1 in 400 potassium tellurite solution to the melted medium to give a final concentration of 1 in 20,000.

On this medium *Staph. aureus* produces after 48 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 aerobic sporing bacilli. It is specially useful for isolating *Staph. aureus* from air, dust, clothes and faeces.

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 N/1NaOH, 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 20-minutes, add 1.0 ml. of pure HCl and filter. Bring the reaction of the filtrate to pH 8.2, steam for 30 minutes and adjust reaction to pH 7.7-7.8. Use perforated screw caps (p. 224) 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.

THIOLYCOLLATE MEDIA (for anaerobes)

It has been shown by Brewer¹ that the addition of sodium thioglycollate (0.1 per cent.) maintains the anaerobic condition which

¹ Brewer, J. H., *J. Amer. Med. Assoc.*, 1940, 115, 598.

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	1 per cent.
Peptone (thio)	1 "
Sodium chloride	0·5 "
Sodium thioglycollate	0·1 "
Agar	0·05 "
Glucose	0 to 1 per cent.
Methylene blue	0·0002 (1 in 500,000)

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.

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 (Bacto or Evans)	1 gram
Sodium chloride	5 grams
Monopotassium phosphate	2 grams
Phenol red	0·012 gram
Agar	20 grams
Distilled water	1 litre

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. 167.

¹ Christensen, W. B., *J. Bact.*, 1946, 52, 461.

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 N/5 NaOH and 4 grams urea to 50 ml. of M/5 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 glass-ware must be scrupulously clean but not necessarily sterile.

Method.

Emulsify sufficient of a 24-hours culture of the organism to be tested, in 0·5 ml. of the substrate in a $3 \times \frac{3}{8}$ in. tube. The fluid should be distinctly opalescent. Place the tube in a water bath at 37° C. for exactly 3 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 3 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 4–5 minutes after nesslerisation.

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 lbs. pressure for fifteen minutes, and filter.

¹ Elek, S. D., *J. Path. Bact.*, 1948, **60**, 183.

Whey Agar.—Made from whey broth by the addition of 1·5 per cent. agar.

SABOURAUD'S MEDIUM
(for the pathogenic fungi)

Peptone ("granulée de Chassaing")	1 per cent.
Maltose ("brute de Chanut")	4 "
Agar	2·3 "

Make up as in the case of ordinary nutrient agar but standardise to pH 5·0-5·5. For the special peptone, "mycological peptone" (Oxoid)¹ can be used; and for the special maltose, ordinary commercial malt extract or pure glucose can be substituted.²

SMITH-NOGUCHI MEDIUM
(for spirochaetes)

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° C.) or by filtration may render it unsuitable.

Technique.—Special long narrow tubes (8×½ 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. 175). 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 8×½ 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

¹ Supplied by Oxo Ltd. (Medical Dept.), London, E.C. 4.

² See Carlier, G. I. M., *Brit. J. Dermat. Syph.*, 1948, **60**, 61.

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

Noguchi's Medium.—In a tube place 8 ml. saline or Ringer's solution¹ at 55° C. Add 1 ml. melted nutrient agar. Allow 20 drops of blood from the ear vein of a rabbit to fall into the tube (p. 175). The tube is not shaken and the contents are allowed to become semi-solid without mixing. Test for sterility by incubation at 37° C. for twenty-four hours. This medium is applicable when the organisms have been accustomed for some time to artificial cultivation.

Schüffner's Medium (modified).—To 1500 ml. tap water add 1.5 grams Difco neopeptone or Witte's peptone, and boil; then add 300 ml. Ringer's solution and 150 ml. Sorensen's solution (prepared by mixing 108 ml. of M/15 Na₂HPO₄ and 42 ml. M/15 KH₂PO₄); boil until phosphates have precipitated, filter and cool. The reaction of the medium should be between pH 6.8 and pH 7.2. Place 3 ml. quantities in clean, new, stoppered test-tubes or screw-capped bottles and autoclave for twenty minutes; then to each add 0.3 ml. fresh guinea-pig serum which has been sterilised by filtration through an L₅ Chamberland candle. Heat at 56° C. for half an hour and test for sterility by incubation overnight.

Fletcher's Medium (modified).—Bleed several rabbits and separate the serum from each with aseptic precautions. As individual animals vary considerably in the suitability of their serum for cultivation of leptospirae, it has been recommended that pooled serum should be used. It is preferable, however, that separate batches of the medium should be made from each serum, samples being tested for their growth-promoting qualities and the batch giving the best results then selected for the routine cultivation of the organism. All the necessary precautions should be taken to ensure sterility of the serum. Prepare a solution consisting of 0.2 per cent. peptone (good quality) and 0.1 per cent. sodium chloride in distilled water (adjusted to pH 7.2), add in measured quantities to sterile screw-capped bottles and sterilise by steaming. After cooling, add 10 to 20 per cent. of serum. Incubate the bottles at 37° C. to test for sterility. To obtain satisfactory growths use large inocula introduced with a sterile pipette.

This medium may be converted to a semi-solid form, which is also suitable for cultivating leptospirae, by incorporating in the peptone solution 0.1 per cent. agar, the peptone-agar being melted by heat and cooled to 55° C. before addition of the serum. This semi-solid medium is more satisfactory than the fluid one for maintaining stock cultures.

¹ Sodium chloride, 9 grams; calcium chloride, 0.25 gram; and potassium chloride, 0.42 gram per litre.

Stuart's Medium¹*Basic Medium, less Phosphate.*

	<i>Concentration.</i>	<i>Volume in ml.</i>	<i>Final Concentration.</i>
Asparagine (<i>d</i> -rot.) . .	M/10	2	M/1000
Ammonium chloride . .	M/10	10	M/200
Magnesium chloride . .	M/10	4	M/500
Sodium chloride . .	M/10	66	M/30
Glycerol (Analar.) —	—	1	0·5 per cent.
Phenol red in distilled water	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 30 minutes to remove carbon dioxide. Add 16 ml. of Sorensen'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.

Distribute the medium in 2-3 ml. amounts in carefully washed sterile screw-capped bottles, add 5-10 per cent. of sterile rabbit serum, and place the bottles in a water bath at 60° C. for one hour.

Note.—All glass-ware must be perfectly clean and free from any trace of soap or other detergent, as this is lethal to the spirochaete. It is advisable to soak the bottles in the buffer solution for 24 hours and then rinse with water preparatory to sterilisation. Filtered rabbit serum should be used. (The difficulty, referred to on p. 196, may be encountered of certain individual samples of serum being unsuitable.) Heavy inocula should be used, e.g. 0·5 ml. of a well-grown culture. Incubation is carried out at 30° C.

Korthof's Medium (modified)²*Peptone-salt Solution.*

Peptone (any good make)	0·8 gram
Sodium chloride	1·4 grams
Sodium bicarbonate	0·02 gram
Potassium chloride	0·04 gram
Calcium chloride	0·04 gram
Monopotassium phosphate	0·24 gram
Disodium phosphate (Na ₂ HPO ₄ , 2H ₂ O)	0·88 gram
Redistilled water to	1 litre

¹ Stuart, R. D., *J. Path. Bact.*, 1946, **58**, 343.

² Broom, J. C., Personal Communication.

Boil (on a water bath) for 20 minutes. Filter through Chardin-type paper, bottle in 100 ml. amounts and sterilise in the autoclave. The pH should be approximately 7.2.

Serum.—Take blood from the ear veins of rabbits which from previous experience are known to be suitable. Allow the blood to clot, pipette off the serum, sterilise by filtration through a Seitz disk, and inactivate by heating at 56° C. for 30 minutes.

"Haemoglobin Solution."—After the serum has been removed from the clot (see previous paragraph), 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 10 ml. amounts in hard glass tubes. Test for sterility at 30° C. for 4 days.

We have found Korthof's medium excellent for the cultivation of leptospirae.

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. normal 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. 175). 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 grams
Glucose	20 grams
Slightly alkaline broth	1 litre

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 DRBOHĽAV'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 solution¹ and shake the mixture thoroughly. 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.

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

¹ Sodium chlóride, 9 grams; potassium chloride, 0·42 gram; calcium chloride, 0·24 gram; sodium bicarbonate, 0·1-0·3 gram; water, 1 litre.

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

Solutions, etc.

Distilled water	White
Normal saline (0·85 per cent.)	Dark blue
Glucose in saline	Blue and green

CHAPTER VI

CULTIVATION OF MICRO-ORGANISMS (Continued)

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. 148), 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. nutrient agar, MacConkey's medium—or when melted and cooled to 55° C. the addition of 10 ml. defibrinated horse blood or serum will make sufficient blood-agar or serum-agar for seven plate cultures in 4-in. Petri dishes (p. 205).

USE OF SOLID MEDIA

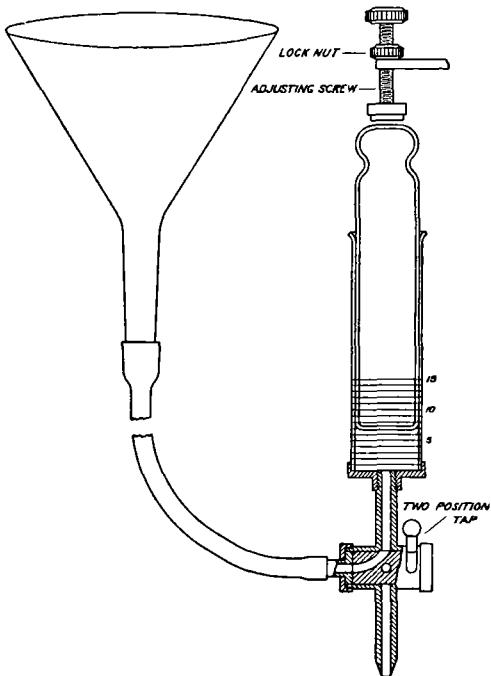
For immediate use the medium is allowed to solidify in sterile stoppered test-tubes either by cooling after having been melted by heat, as in the case of agar or gelatin (p. 155), or by coagulation in an inspissator, as in the case of solidified serum or egg media (p. 177). The tubes are plugged with cotton wool, and sterilised in the hot-air oven before the addition of the medium.

Alternatively, 1-oz., $\frac{1}{2}$ -oz. or $\frac{1}{4}$ -oz. screw-capped bottles (p. 148) 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 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 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

¹ Supplied by R. B. Turner & Co., London.

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.

Other types of automatic filling devices, some motor driven, are available for distributing culture media in small containers.

When tubing agar or broth with the filler, the medium is run into clean but not sterilised test-tubes. These are then plugged with cotton wool and sterilised in the steamer or in the autoclave as indicated under the description of the various media.

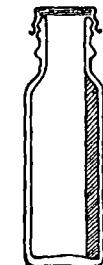
Alternatively, the agar or broth is distributed into small bottles, the caps are screwed tightly on and the containers suitably sterilised.

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. 206), 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 $\frac{1}{2}$ -oz. screw-capped bottles¹ can conveniently be substituted for test-tubes. The aluminium cap should have



*Reproduced by permission of
the "Lancet"
Ltd.*

¹ These bottles are referred to on p. 148.

a black rubber washer 3 mm. thick. 5 ml. amounts of the medium are added to the 1-oz. bottles and the caps are 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.

Instead of 1-oz. bottles it is more economical to use $\frac{1}{2}$ -oz. (bijou) bottles. Only 2.5 ml. of medium (broth, agar or other solid media) are required. An extensive experience of these small bottles shows them to be very convenient for laboratory use, affording economies in media, storage and conveyance.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures (p. 210), 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 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.

When it is necessary to dry the surface more quickly this can be done by passing the dish containing the medium quickly several times over a Bunsen flame.

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, 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. 224); 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 4½ 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.

Graduated pipettes are employed when measured quantities of material are used for inoculation (see water examination).

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, thus forming two pipettes. 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 $15 \times 2\frac{1}{2}$ in., which is then stoppered with cotton wool and sterilised by dry heat (p. 126). Before use, the tip of the capillary portion is broken off and a rubber teat fitted to the other end. These pipettes are necessary for inoculating cooked-meat medium (*q.v.*) and certain other media, and are very useful in many bacteriological manipulations.

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.

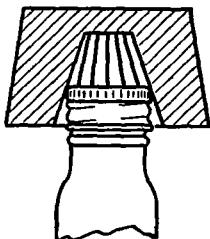
TECHNIQUE OF INOCULATING SCREW-CAPPED BOTTLES

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.

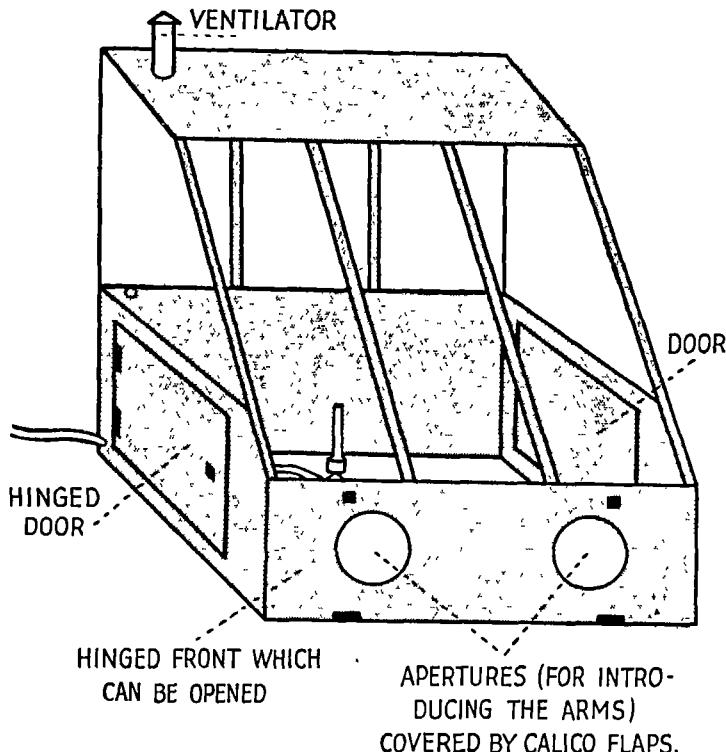
The bottles are held exactly as test-tubes, and the cap is held in the same way as the cotton-wool stopper. The bottles are then unscrewed from the cap, the



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.

A more simple inoculating box which is movable can easily be constructed as shown in the figure, p. 209. 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.

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. 439). 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.

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. 514), 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. 205). 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, Dieudonne'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.

OTHER METHODS.—pp. 373, 405, 528, and 636.

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. 204) 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. 17). 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 (pp. 215-218).

Oxygen by itself is not inimical to the growth of the anaerobes, and it has been suggested that when molecular oxygen is present hydrogen peroxide is formed, which prevents their multiplication, and that, unlike certain other peroxide-forming bacteria, they do not produce catalase which would decompose the peroxide. The production of anaerobic conditions by incorporating in culture medium a source of catalase is exemplified in the *Smith-Noguchi method* for cultivating spirochaetes (p. 195). The cultures are sealed from the air by a vaseline plug superimposed on the medium; and any peroxide that may be formed is at once destroyed by the catalase present in a piece of fresh sterile rabbit kidney.

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. 206). 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. 192), 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. (Noguchi tubes), 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. 192) 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 "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 pp. 516–7 and other organisms such as anaerobic streptococci and *Bacteroides* (p. 534). 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. 224). (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.

REMOVAL OF OXYGEN BY MEANS OF PYROGALLIC ACID AND CAUSTIC SODA

When pyrogallic acid and caustic soda are mixed together, the mixture rapidly absorbs oxygen and becomes dark brown in colour. Many types of apparatus have been devised for the cultivation of anaerobes on plates, in which this method is used for absorbing oxygen. They are, however, inconvenient, and are not specially recommended.

For media in ordinary test-tubes, BUCHNER'S METHOD is applicable. The test-tube containing the medium is placed in a Buchner's tube, a stout-walled tube, $8\frac{1}{2} \times 1$ in., with the lower end constricted so that the test-tube placed therein does not reach to the bottom of the tube. The tube is furnished with a well-fitting rubber bung. Some solid pyrogallic acid is placed in the bottom of the tube, strong caustic soda solution is added, the inoculated tube is quickly introduced and the rubber bung immediately inserted. The oxygen is rapidly absorbed and fairly satisfactory anaerobic conditions are obtained.

McINTOSH AND FILDES' JAR

This apparatus is easy to manipulate, and the degree of anaerobiosis is readily observed by means of a methylene-blue indicator inside the 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. This consists of a mixture in a test-tube of equal volumes of (a) N/10 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.

(The lid is clamped down and the jar connected to a hydrogen supply (p. 217). The current is turned on so that the palladinised asbestos may be heated. The combination of oxygen and hydrogen takes place quietly in the jar. Water is formed, and more hydrogen enters to take the place of the 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.)

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.

The original McIntosh and Fildes' jar, described in 1916, consisted of a metal tin with a "press on" lid, in the centre of which was soldered a small brass gas-tight tap, and to the under surface was fixed a folded strip of brass holding a flat envelope-shaped piece of fine brass wire gauze containing palladinised asbestos. To produce anaerobic conditions the envelope was heated, the lid replaced, hydrogen introduced, and the lid sealed with plasticine. Its disadvantage was that a visible indicator of anaerobiosis within the jar could not be employed. To overcome this McIntosh and Fildes substituted the glass jar described above. This apparatus is costly, however, and Hudson¹ has introduced a modification of the original tin whereby an external indicator tube shows whether the anaerobiosis is effective. A 3-in. length of compo-metal gas tubing (about $\frac{3}{16}$ in. external diameter) is bent at right angles for an inch of its length, and this end is inserted into a hole punched in the upper part of the tin, and soldered in position to make a gas-tight joint. To the portion of tubing outside the tin is attached a 2-in. length of pressure rubber tubing. A piece of 6-mm. glass tubing is sealed at one end, and the indicator solution added (about 0.5-1.0 ml.). A constriction is made above the liquid (to avoid spilling) and the open end is attached to the pressure tubing. The indicator solution consists of 1 per cent. glucose in Hartley's broth (pH 8.5-9.0), coloured deeply with methylene blue to which is added as preservative 0.01 per cent. merthiolate or 0.01 per cent. phenyl mercuric nitrate. When in use, the indicator should become decolorised after about two to three hours' incubation at 37° C. The indicator tube is left in place and used repeatedly. When the colour change becomes too small a tube of fresh indicator solution can easily be fitted. This is a cheap and effective method of anaerobic cultivation.

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

¹ Hudson, R. E. B., *Brit. J. Exp. Path.*, 1941, 22, 305.

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 lbs. 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.

A considerable saving in both time and hydrogen is effected if the bulk of the air is removed from the jar, by evacuation with a water-pump or other suitable means, before admitting hydrogen.

Anaerobiosis in a closed jar may be secured by generating hydrogen from chromium and sulphuric acid.¹ This is a convenient improvisation if other apparatus is not available. A desiccator with a stopcock is used. The cultures are placed in the desiccator along with a dish containing chromium metal powder, to which is added 15 per cent. sulphuric acid. The stopcock is left open while the vigorous evolution of hydrogen continues, and then closed. The desiccator is placed in the incubator.

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 method² is to use tin containers, size 8×10 in., with press-on lids, and capacity of about 3½ litres.³ (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, together with an open tube, 8×1 in., containing 8 ml. (excess) of 25 per cent. 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. 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

¹ Rosenthal, L., *J. Bact.*, 1937, **34**, 317.

² Gladstone, G. P., and Fildes, P., *Brit. J. Exp. Path.*, 1940, **21**, 161.

³ Obtainable from A. Gallenkamp & Co., Ltd., London.

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.

OXIDATION-REDUCTION POTENTIALS

Sour milk, bacterial cultures, etc., decolorise methylene blue owing to the development of reducing conditions during bacterial growth. In order to follow up qualitative observations of these reducing effects a quantitative measure for evaluating oxidation-reduction conditions is required, and oxidation-reduction potentials enable this to be done. The principle of the method depends on 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. It should be borne in mind that measurements of the electrode potential of a system, *e.g.* of a bacterial culture, indicate the oxidation-reduction *intensity* of the system itself, and not its *capacity* to oxidise or reduce some other component or system.

The electrode potential of a bacterial culture may be measured accurately by electrical methods, but 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 ranges 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 poising,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* it was found that the initial E_h of the medium, approx. $+300$ mv., fell gradually and reached -200 mv. after about forty-eight hours' incubation, and the potential remained at this low level for some considerable time. With haemolytic streptococci, on the other hand, the potential fell from $+300$ mv. to -150 mv. in twelve hours, but then rose fairly rapidly, probably owing to the formation of hydrogen peroxide. In a glucose-broth culture of *Esch. coli*, in which gas formation occurred, the potential fell 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 culture media unless the E_h is below a certain value (e.g. -0.436 volt). 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 (p. 192). It must not be assumed that there is a strict line of differentiation between aerobes and anaerobes. Every grade of behaviour may be observed from the aerobic organisms, such as *M. lysodeikticus* which proliferates actively only when the oxygen supply is abundant, to the strict anaerobes which appear to require absolute exclusion of air at least in the initial stages of growth. Some organisms, such as *Esch. coli*, which are well supplied with enzyme systems of many kinds, are able to multiply over a very wide range of cultural conditions. Bound up with this question is that of accessory growth substances which must be supplied to some organisms whilst others are able to synthesise their own, but this need not be discussed further here.

Oxidation-reduction potentials and oxidation-reduction indicators are employed in the testing of sewage and sewage effluents, in connection with cheese-making and the keeping qualities of beer, in the determination of vitamin C, etc. The metabolic activities 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 quality and keeping powers 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

¹ Corresponds to the buffering effect in pH estimation.

a rapid decolorisation, whilst with good quality milk there is a long lag period and reduction is slow (p. 296).

For full details of this important subject the following monograph should be consulted : *Oxidation-Reduction Potentials in Bacteriology and Biochemistry*, by L. F. Hewitt, 6th edition, 1950, E. & S. Livingstone Ltd., Edinburgh.

PRESERVATION OF CULTURES (AND SERA) BY DRYING IN VACUO

When cultures are dried rapidly *in vacuo* they may retain their viability for several years, and this is a convenient means of keeping stock strains of many organisms. The principle of the so-called "lyophile" method of drying is that the material is frozen solid and dried from the frozen state ("freeze-drying"). In the more elaborate methods the culture (or serum) to be dried is frozen with solid carbon dioxide, but with smaller quantities and simpler apparatus, the speed of the evaporation due to exhaustion by the vacuum pump is relied upon to cause freezing of the material. With small quantities of liquid, however, the container (ampoule or tube) absorbs so much heat that it is difficult if not impossible to freeze the material by its own rapidity of evaporation. In Rayner's method this is overcome by placing the culture (or serum) on thin waterproof cellophane.

*Technique*¹.—Cultures should be grown overnight in digest broth to which a few drops of horse blood have been added. A 4-in. Petri dish is fitted at the bottom with two disks of white filter-paper. On the surface are placed small pieces of waterproof cellophane 2 thousandths of an inch thick and approximately $\frac{3}{8}$ in. square. About twenty-four can be placed on the filter-paper without touching or overlapping. The dish and its contents are then sterilised in the hot-air oven, the lid of the dish being kept slightly open by means of a piece of wooden swab stick cut to fit the inside of the lid. By means of a capillary pipette a single drop of the blood-broth culture is dropped in the centre of each cellophane square. (The end of the pipette must not touch the cellophane.) The dish with the lid slightly open is placed over calcium chloride in a 10-in. desiccator which is exhausted by means of a Hyvac or similar pump. The cultures quickly freeze and dry within twenty minutes, although they are usually left overnight in the exhausted desiccator.

For preservation, each cellophane square with the dried culture is sealed *in vacuo* in a glass container. The pieces of cellophane are picked up with sterile forceps and dropped into sterile thin chemical test-tubes, $5 \times \frac{1}{2}$ in., and the cotton-wool plugs replaced. The tubes are then constricted in the middle by a blowpipe flame to about $\frac{1}{16}$ in. diameter. The tubes are next placed in the desiccator, which is again exhausted and left overnight. To seal the tubes the cotton-wool plug is set alight and pushed down the tube. A soft rubber bung, with

¹ Quoted from Rayner, A. G., *J. Path. Bact.*, 1943, 55, 373.

about 2 in. of 5-mm. glass tubing through it, is fitted into the tube. The glass tubing is connected to a Hyvac pump, and when a vacuum is obtained the tube is sealed at the constriction in the blowpipe flame. After sealing, each tube of dried culture is tested for vacuum (to ensure that there are no leaks) by means of a high-frequency vacuum tester connected to the A.C. mains supply. If a satisfactory vacuum is present a bluish-violet fluorescence is seen in the tube. It is possible to dry cultures of the gonococcus, meningococcus and *H. pertussis* successfully in this way.

For larger quantities of material, e.g. agglutinating sera and guinea-pig complement-serum, disks of waterproof cellophane $2\frac{1}{2}$ in. in diameter are placed over upturned lids of approximately 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 the desiccator, which is exhausted by means of the 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 $6 \times \frac{5}{8}$ in. test-tubes. These are constricted, again desiccated, and sealed with a vacuum as described above. The cellophane disks can be used again.

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 anti-coagulant.

Requisites :—

(1) A 10-ml. "all-glass" syringe (with a firmly fitting needle) sterilised in the hot-air oven as described on p. 141.

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.

(2) Gauze or cotton wool, bandage, antiseptic (*e.g.* 2 per cent. iodine in 70 per cent. alcohol), methylated spirit, collodion, dissecting forceps, Bunsen burner or spirit lamp.

(3) 50 ml. sterile digest broth (*e.g.* Hartley's, p. 151) 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. This is particularly 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.

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. 192) is applicable for cultivating anaerobes and microaerophilic organisms from blood.

"Liquoid," a proprietary name for sodium polyanethol 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 trypsinii 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 mgm. 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. 148). 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

¹ Made by the Viscose Development Co., Ltd., Woldham Road, Bromley, Kent.

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 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 mgm. per cent. *p*-aminobenzoic acid;
- (4) broth + 0·5 per cent. sodium taurosholate (yellow cap and bead).

The size of the viskap is No. 2 semi-opaque cut $1\frac{3}{8}$ 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 lbs. 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. 218).

"Clot" Culture.—When blood samples from suspected enteric fever have been submitted for the Widal test (p. 243) 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.

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. 210). 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.

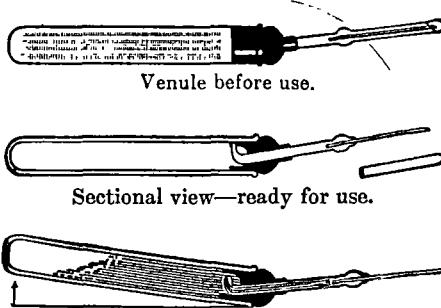
Where an infection with the enteric group is suspected, it is convenient to plate directly from the blood culture on MacConkey's medium to obtain the characteristic colonies on this medium.

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 3 weeks before being discarded as negative.

A convenient instrument for drawing aseptically a blood sample from a vein is the so-called *Behring Venule*.¹ 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



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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.

THE AGAR-BLOCK METHOD OF ØRSKOV FOR STUDYING THE MORPHOLOGY OF GROWING BACTERIAL CULTURES

This method has been applied by Ørskov² and others for the morphological study of *Actinomyces* and it allows the maintenance of living cultures under continuous observation.

¹ These instruments can be obtained from Bayer Products Ltd., London.

² Ørskov, J., *Investigations into the Morphology of the Ray Fungi*, 1923, Copenhagen.

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.¹ 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 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 anti-septics 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 1:1000 perchloride of mercury solution. If the hands become contaminated they should be sterilised in a basin of dilute lysol or perchloride

¹ Phase contrast microscopy is recommended for this (p. 79).

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 pledge 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.

CHAPTER VII

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 bacteriological experiments are the guinea-pig, rabbit, mouse and white rat, 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. 16–18 gauge, for inoculating thick suspensions or emulsions of tissue. The needles should be made from stainless steel.

Syringes may be sterilised by taking them apart, placing in cold water, and boiling for five to fifteen minutes, but sterilisation in the hot-air oven is preferable. A full account of the sterilisation of syringes and their care is given on p. 140 *et seq.*

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

¹ For a specification of all-glass syringes see p. 141.

to pour the culture into a small (2-in.) Petri dish, or a 50-ml. conical test-glass. 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.—These should be cut into small pieces in a sterile porcelain mortar by means of scissors sterilised by boiling. Some clean coarse sand, 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. Recently the Laboratory Animals Bureau, Medical Research Council Laboratories, Holly Hill, Hampstead, London, N.W. 3, 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.

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 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.

Intracutaneous inoculation.—This method is used chiefly in testing cultures of the diphtheria bacillus for virulence (p. 379). The hair is removed from the flanks of the animal by shaving or 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 25 or 26 S.W.G. (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.

The normal rectal temperature of the guinea-pig is $100\cdot8 \pm 1\cdot2^{\circ}$ 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 lepiseptica*) 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.

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. 232. 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.

In removing samples of blood from the rabbit a similar procedure is adopted. The ear is shaved, sterilised with alcohol and painted with sterile vaseline. The vein is dilated by the heat of an electric bulb or, if larger amounts of blood are required, by gently rubbing with xylol or benzol; an incision is made into it by means of a large triangular needle or a sharp scalpel. The blood is then allowed to drop into a test-tube. When sufficient blood has been removed a small piece of cotton wool is pressed firmly over the incision in the vein. If xylol has been used the ear is washed with alcohol and lightly smeared with vaseline (p. 175).

Cardiac puncture.—p. 175.

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 $\frac{1}{16}$ 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.

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.

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 warm water. 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 depilated (p. 232) under slight ether anaesthesia. The animal is completely anaesthetised for the inoculation and the depilated area sterilised with tincture of iodine. A fine-bore needle attached to a 1-ml. syringe (as used for intracutaneous inoculation—p. 233) is employed and easily penetrates the skull. The site of injection is just posterior and lateral to the vertex and the point of the needle is carried through the skull for $\frac{1}{8}$ in. to $\frac{1}{4}$ in. Approximately 0·05 ml. of fluid can be injected with safety.

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 ten minutes. 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.

Separate barrows should be used for food-stuffs 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

Guinea-pigs should be fed twice daily.

Morning—

Oats, 1 part	Bran, 3 parts	Water (for stock in runs).	} 1 oz. per animal.
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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, 1947, Baillière, Tindall & Cox, London; and *Notes on Communicable Diseases of Laboratory Animals*, by H. J. Parish, 1950, E. and S. Livingstone, Edinburgh.

Afternoon—

Hay	2 oz. per animal
Cabbage, kale or other green food supplemented by roots	2 oz. , ,
Water as above.	

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. 480).—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.

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 with 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. typhi-murium*, are often responsible, and epizootics are liable to occur in overcrowded stock.

Virus diseases, such as guinea-pig paralysis, may be met with.

Rabbits

Feeding, per animal:—

Morning—

Oats, 1 part									2½ oz.
Bran, 3 parts									
Water.									

Afternoon—

Hay	4 oz.
Cabbage, kale or other green food supplemented by roots	3 oz.
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. 480).—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 cuniculicida* (p. 480) 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. typhi-murium*.

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. Oöcysts 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

Feed only once daily, preferably in the afternoon. Give mash of boiled meal, mixed with fresh oats and bran and made into balls the size of a walnut. An adult rat requires 2 oz. of this daily. Also give fresh cabbage in the mixture three times weekly and some biscuit and cod-liver oil once a week. Rat food should be given immediately it is made and not allowed to remain over till the next day.

It is more convenient to use "rat feeding cubes"; they are sold commercially¹ and consist of a balanced mixture of ground cereals, meat, fish and milk-proteins, with yeast and cod liver oil. Three cubes per day are provided for each adult animal.

¹ Obtainable from North-Eastern Agricultural Co-operative Society Ltd., Bannermill Place, Aberdeen.

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.

Mice

Feeding.—Feed only *once* daily, preferably in the afternoon, with stale dry bread soaked in water and squeezed and made up in pieces the size of a walnut, or alternatively with a mixture of oats 1 part, and bran 3 parts, moistened and made up into small balls as above; allow one per animal. A little cod-liver oil should be placed in the mash once weekly. A pinch of canary seed and millet seed should be allowed each animal weekly.

Alternatively, one "rat feeding cube" (*vide supra*) per day for each adult mouse provides a completely balanced diet. Drinking water is essential and should be provided from a special glass container.

Cages.—Stock cages for breeding, etc., are made of wood with a zinc bottom. The lid is of perforated zinc with wooden edges. The size is 24×12×12 in.

For individual mice in a one or two days experiment, e.g. pneumococcus inoculation, a 1-lb. or 2-lb. screw-capped preserving jar, with a piece of perforated zinc in place of the glass portion of the lid, is most convenient.

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. An increase of canary and millet seed should be given to breeding animals.

Common Diseases

Intestinal infections due to organisms of the *Salmonella* group (*S. enteritidis* and *S. typhi-murium*), and termed "Mouse typhoid," may produce severe epizootics. Existing stock should be destroyed, boxes disinfected and fresh stock obtained.

Infectious ectromelia (p. 618).—A virus disease occurring in either an acute or chronic form. In the acute disease there is necrosis in the 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.

Epizootic infection by *Actinomyces muris* (p. 532) may sometimes occur in mouse stocks.

Ringworm is met with, and also favus (p. 631).

Worms may occasionally cause ill-health or death.

Tumours are not uncommon, particularly mammary carcinoma.

CHAPTER VIII

IMMUNOLOGICAL AND SEROLOGICAL METHODS AS APPLIED TO BACTERIOLOGY¹

THE WIDAL REACTION ; AND OTHER AGGLUTINATION TESTS

THE nature of the *Widal agglutination reaction* and its applications in the diagnosis of enteric fever are referred to on pp. 34, 41, 441.

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 (p. 247) 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.

WIDAL AGGLUTINATION TEST

The *blood specimen* is taken by vein puncture (p. 223), so as to obtain a satisfactory amount of serum for the complete test as described here, and referred to on p. 441. At least 5 ml. of blood should be obtained, and the blood immediately transferred from the syringe to a stoppered sterile tube or

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.

screw-capped bottle and allowed to clot. When the serum has separated, it is pipetted off into a sterile tube.

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, *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. 248). 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. 561), 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-nl. 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.

Steriliser with boiling distilled water for pipettes, etc. (p. 127) ; sterile 0·85 per cent. saline ; test-tubes 3×½ in. ; agglutination tubes 3×⅓ 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 (p. 207).

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" (p. 45), e.g. *S. paratyphi B*, must represent the specific phase.

It is now a general practice to use standard suspensions such as those described later (p. 248) ; 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 5, Brown's opacity standards (p. 274). 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)	CONTROL- NO SERUM
1 in 15	1 in 30	1 in 60	1 in 120	1 in 240	1 in 480		

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 ; 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)	CONTROL- SUSPENSION -NO SERUM
1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	1 in 960		

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. 207) 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. 45) it is usually sufficient to incubate at 37° C. for two hours and then leave for half an hour at room temperature. "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. 45) 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.

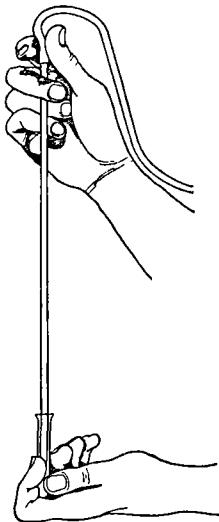
Further references to the Widal reaction are made on pp. 248, 441.

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 forefinger. *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.

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

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.

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. 207). 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. 244) 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	1	2	3	4	5
	Drops				
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,¹ 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 H antigen (p. 45). 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 to a twenty-four-hours' broth culture or by suspending a young agar culture in saline containing 0·1 per cent. formalin.

¹ Standard suspensions for the Widal reaction can be obtained from the Standards Laboratory for Serological Reagents, Central Public Health Laboratory, Colindale Avenue, London, N.W.9.

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.

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. 448, 485, 561). 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.

AGGLUTINATION TESTS USED FOR THE SEROLOGICAL IDENTIFICATION OF CERTAIN ORGANISMS BY MEANS OF SPECIFIC ANTISERA

These tests are carried out by a similar technique. 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 in 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. 448.

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 (p. 497), 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. 212), 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. 344. 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 pledget 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 grams 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. 45, 450).

The rabbits are injected intravenously (p. 234) at intervals of five to seven days with a suspension in saline of a twenty-four hours' 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. 274), 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. 249) 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. 234) 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. 175), 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. 246), 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. 221 for the preservation of complement. The potency of antisera is retained over a considerably longer period when dried than when stored in fluid form.

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

¹ More powerful agglutinating sera may, of course, be obtained; 1 in 1600 is merely the *minimum* titre which should be aimed at.

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. 449, 490). 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 minutes)—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 hours 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. 245), 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 anti-serum 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.

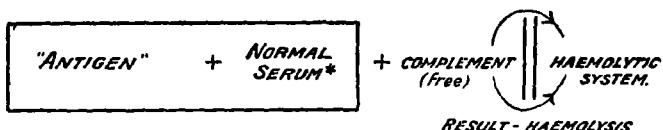
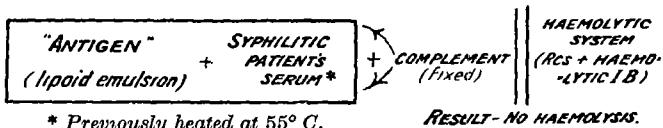
WASSERMANN SYPHILIS REACTION

This reaction depends on the "fixation" of complement by an emulsion of certain lipoid substances (phosphatides, such as lecithin) 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 emulsion *plus* syphilitic serum fixes complement in the same way as a bacterial or other antigen *plus* its homologous anti-serum (p. 46). Possibly the lipoid plays the part of a hapten (p. 39) 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. 43). 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 :



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. Many workers use constant amounts of antigen and of patient's serum with varying amounts of complement. In some cases, constant amounts of antigen and of complement with varying amounts of patient's serum are employed—*e.g.* the standard method recommended by the Royal Medico-psychological Association for mental hospital practice.¹

Examples of two different forms of technique are described : (1) in which the various quantities of the reagents are measured with graduated pipettes and added directly to the tubes in which the test is carried out ; (2) in which the reagents are all used in a standard volume, variation in actual amount, *e.g.* of complement, being effected by varying the dilution in the standard volume ; thus only one standard volume requires to be pipetted instead of varying quantities, and, moreover, the method can be applied to small amounts, but it has the disadvantage that it can become very inaccurate in the hands of other than skilled workers.

The preparation of the reagents is essentially the same in the different methods.

METHOD I.

ANTIGEN

(1) 20 grams of *sheep heart-muscle*, carefully freed from fat and fibrous tissue, are *finely* ground with clean sand in a mortar and extracted for four days at room temperature

¹ See Mann, S. A., and Partner, F., *Memorandum on the Wassermann Reaction in Mental Hospital Practice*, London County Council, 1931 (obtainable from King & Son, London).

with 100 ml. of absolute alcohol. In this way, lecithin and similar substances are extracted from the tissue. The extract is filtered and pure cholesterol is dissolved in it to the point of saturation. The cholesterol acts by intensifying the "antigenic" properties of the tissue extract.

For the test, a suspension is prepared by adding 1 part of the alcoholic extract to 12 parts of normal saline solution. In preparing the emulsion the *maximum turbidity* should be obtained by running the extract slowly on to the salt solution in a cylindrical measure (or test-tube) and then mixing slowly by rotation of the cylinder held in a slanting position.

While a 1 to 12 suspension is usually found to be suitable with an antigen prepared as above described, new batches of antigen should be tested in varying dilutions with known positive and negative sera to determine the optimal dilution for routine use.

(2) The following *alternative antigen* is also recommended : *human heart-muscle* from the left ventricle is obtained at a *post-mortem* examination (if possible a case of accidental death); it is freed from fat, minced finely, and ground for a minute in a mortar with absolute alcohol (1 gram of heart to 9 ml. of alcohol) and clean sand. The mixture is shaken in a shaking machine for $1\frac{1}{2}$ hours and then filtered. It is more easily and quickly prepared by using one of the commercial "blenders" or homogenisers. The minced heart muscle and alcohol are placed in the glass container in convenient amounts and the machine allowed to run for 1-2 minutes. After all the heart muscle and alcohol have been treated the mixture is placed in the incubator for 24 hours and shaken at intervals during the working day. It is then filtered through paper previously washed with ether and dried, placed in the cold room overnight when a heavy precipitate comes down. Filtration as above is repeated. To 1.5 volumes of this extract is added 1 volume of 1 per cent. cholesterol in absolute alcohol. For use, 1 volume of this mixture is placed in a beaker and 29 volumes of saline are measured into a cylinder, the contents of which are then poured rapidly into the beaker. In the actual test, as shown on p. 260, this antigen suspension can be used in place of that described above.

PATIENT'S SERUM

A specimen of blood is obtained by vein puncture (p. 223) as for blood culture. The blood is then placed in a

sterile stoppered test-tube or screw-capped bottle (p. 319) 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 half an hour. 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.

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. The methods of preservation are detailed on pp. 221, 266.

HAEMOLYTIC SYSTEM

With guinea-pig complement, a haemolytic system consisting of ox or sheep red corpuscles sensitised with the appropriate haemolytic antibody is used.

Defibrinated blood is obtained at the abattoir (p. 170). 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 3 per cent. suspension, and five to seven minimum haemolytic doses (M.H.D.) of the haemolytic antiserum are added.

Haemolytic Antiserum.—The method of preparing a haemolytic antiserum and of estimating its M.H.D. may be summarised as follows. A rabbit is injected intravenously at seven to ten days' intervals with increasing amounts of washed red cells—*e.g.* 0.5 ml., 1.0 ml., 1.5 ml. of the sediment after washing and centrifuging, and suspended in normal saline to make up the volume to 2 ml. Alternatively, the animal is injected intraperitoneally with 5 ml., 10 ml. and 15 ml. of the washed red cells at similar intervals. Ten days after the last injection a small quantity of blood is withdrawn from an ear vein; the serum is separated, and its M.H.D. estimated by testing the haemolytic effect of varying amounts (*e.g.* from 0.001 ml. to 0.005 ml.) on 1 ml. of a 3 per cent. suspension of red cells along with an excess of guinea-pig complement (*e.g.* 0.05–0.075 ml.) (methods given later). As guinea-pig serum contains natural haemolysins for ox and sheep corpuscles, the complement-containing serum must previously be freed from this antibody by mixing equal volumes of the serum and the appropriate red corpuscles (washed), keeping the mixture at 0° C. for one hour and then separating the serum by centrifuging. If the M.H.D. is 0.002 ml. or less, the animal is bled from the neck vessels or by cardiac puncture (p. 175). The blood serum is separated, heated for one hour at 55° C., and stored in sealed glass tubes or phenolised and bottled (p. 252). Alternatively, it may be preserved in the dry state (p. 221). Should the M.H.D. exceed 0.002 ml., a further injection of blood may increase the haemolytic potency of the serum.

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.

A haemolytic system of ox cells sensitised with a rabbit *v.* ox immune serum is usually free from marked or rapid haemagglutination.

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

¹ Sawyer, H. P., and Bourke, A. R., *J. Lab. and Clin. Med.*, 1946, 31, 714.

37° C. water-bath for fifteen minutes, dilute to 15 ml. with 0·85 per cent. salt solution, then centrifuge at high speed for five minutes (3000 revolutions per minute). 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 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 minutes. 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.

THE TEST

Apparatus required :

Small test-tubes, $3 \times \frac{1}{2}$ in.; a rack for the tubes; 1-ml. pipette graduated to the tip in 1/10ths and 1/100ths; 0·1-ml. pipette graduated in 1/100ths and 1/500ths (p. 244).

Specimens of complement from different guinea-pigs vary quantitatively in their haemolytic effect, and even if pooled preserved complement-serum is used its degree of activity varies with the specimen of red cells used. It is therefore necessary, first to estimate the M.H.D. of the complement to be used. The M.H.D. is the smallest amount required to produce complete lysis of the given quantity of red cells in the presence of excess of immune body. This usually lies between 0·004 ml. and 0·01 ml. of undiluted guinea-pig serum for 0·5 ml. of the haemolytic system.

To measure amounts less than 0·01 ml. make a 1 in 10 dilution of the serum, and of the diluted serum take ten times the actual amount required, using the 0·1-ml. pipette graduated in 1/100ths and 1/500ths.

The following exemplifies the estimation of the M.H.D. of complement :—

Tube	1	2	3	4	5
Haemolytic system .	0·5 ml.	0·5 ml.	0·5 ml.	0·5 ml.	0·5 ml.
Complement (diluted 1 in 10) .	0·02 ml.	0·04 ml.	0·06 ml.	0·08 ml.	0·1 ml.
Haemolysis . . .	Trace	Marked	Just complete	Complete	Complete

The M.H.D. is therefore 0·06 ml. of a 1 in 10 dilution of complement—i.e. 0·006 ml. undiluted complement.

Tubes 1 and 5 can be omitted from the series unless the complement is unusually active or weak.

The actual Wassermann test is shown in the Table below.

Full Test

Tube . . .	Test proper				Serum Control		Antigen Control	
	1	2	3	4	1	2	1	2
Normal saline .	—	—	—	—	0·5 ml.	0·5 ml.	—	—
Antigen suspension .	0·5 ml.	0·5 ml.	0·5 ml.	0·5 ml.	—	—	0·5 ml.	0·5 ml.
Patient's serum	0·05 ml.	0·05 ml.	0·05 ml.	0·05 ml.	0·05 ml.	0·05 ml.	—	—
Complement .	2 M.H.D.	4 M.H.D.	8 M.H.D.	12 M.H.D.	2 M.H.D.	4 M.H.D.	2 M.H.D.	4 M.H.D.

Complement-dose Control :—

Tube	1	2	3	4
Saline	0·5 ml.	0·5 ml.	0·5 ml.	0·5 ml.
Complement (diluted 1 in 10) .	0·04 ml.	0·06 ml.	0·08 ml.	0·1 ml.

Abridged Test

Where large numbers of Wassermann tests have to be carried out, and there is need for economy in labour and materials, the test may be abridged as follows : tube 4 of the "test" series is omitted and 6 M.H.D. of complement are substituted for 8 M.H.D.

in tube 3 ; tube 2 of the "serum control" may also be omitted, though this may sometimes introduce a difficulty if a serum is anti-complementary ; tube 4 of the complement-dose control can be omitted unless the complement is unusually weak.

The mixtures are incubated at 37° C. for one and a half hours, and then to each tube 0.5 ml. of the haemolytic system is added. The tubes are again incubated for one hour, when the results are noted. Absence of haemolysis in the test series signifies that fixation of complement has occurred, so that not even 1 M.H.D. is left free. The serum and antigen controls determine any possible anti-complementary action of either of these reagents, and the complement dose control indicates any possible deterioration in the haemolytic value of the complement by dilution and incubation. By carrying out the test in this quantitative way the number of doses of complement fixed by antigen *plus* serum can be estimated —*i.e.* the strength of the reaction.

Known negative and positive sera must always be included in each set of Wassermann tests as controls.

It must be remembered that specimens of complement from different guinea-pigs vary in fixability, and it is for this reason that the pooled serum of several animals is used. With a weakly fixable complement it may even be impossible to elicit positive reactions, while with a strongly fixable complement non-specific effects are rendered more marked. The controls with known negative and positive sera are indispensable. The positive control sera should include a weakly reacting one.

Any anti-complementary effect of the serum or antigen must be allowed for in estimating the fixation by serum *plus* antigen, and the results with known positive and negative sera are to be regarded as standards for comparison with the results given by the sera tested.

The following criteria of positive and negative reactions may be adopted : a serum which permits of complete lysis with the same minimum amount of complement as the negative control serum is, of course, negative ; a serum which requires at least twice as much complement as the negative control serum to yield complete lysis is to be regarded as positive. Sera which show intermediate effects may be classified as "doubtful." If the serum in question exhibits any degree of anti-complementary effect, allowance must be made for this.

The practical details and interpretation of the results can be learned only by actual acquaintance with the test.

**EXAMPLES OF THE RESULTS OF WASSERMANN TESTS
CARRIED OUT BY THE METHOD DESCRIBED**

	<i>Test proper</i>				<i>Serum Control</i>		<i>Antigen Control</i>	
	1	2	3	4	1	2	1	2
Tube . . .								
Amount of complement in M.H.D.	2	4	8	12	2	4	2	4
Amount of <i>undiluted</i> complement in ml.	0.012	0.024	0.048	0.072	0.012	0.024	0.012	0.024
Negative serum	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis
Positive serum	No lysis	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	—	—
" Weak positive " serum	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	—	—
" Marked positive " serum	No lysis	No lysis	No lysis	No lysis	Complete lysis	Complete lysis	—	—

Complement dose :—

Undiluted complement in ml.	0.004	0.006	0.008
	Marked lysis	Just complete lysis	Complete lysis

In this experiment the M.H.D. of undiluted complement is 0.006 ml. both in the initial titration and in the complement-dose control.

THE WASSERMANN REACTION WITH CEREBRO-SPINAL FLUID

In testing spinal fluid the reaction is carried out as in the case of serum, but with the following modifications :—

(1) The antigen is prepared by emulsifying 1 part of the cholesterolised sheep heart extract with 12 parts of spinal fluid ; 0.5 ml. of this mixture replaces antigen *plus* serum in the usual test.

The alternative antigen (2) is prepared by mixing 1 part of the cholesterolised extract with 29 parts of the fluid.

(2) In place of saline *plus* serum in the usual serum controls, 0·5 ml. of spinal fluid is tested.

(3) The cerebro-spinal fluid is not usually heated to 55° C. before the test as in the case of serum.

METHOD II. (*standard volume method*)

HARRISON'S TECHNIQUE MODIFIED BY WYLER¹

It is not possible to include all the minutiae of detail described by Wyler, and workers who use this method should consult his monograph. The main essentials of reagents and technique, however, are given below.

In this test a standard volume of each reagent is used, and 0·11 ml. is the amount selected. An ordinary capillary pipette is graduated with mercury² as follows: 0·11 ml. of mercury is accurately measured into a watch glass; the mercury is then gently sucked into the capillary pipette (held almost horizontally) and a mark made at the upper level with glass-writing ink. The pipette is used with the rubber tube and mouth-piece described on p. 246, or with a rubber teat. Instead of adding different amounts of the same dilution of complement, as in the method previously described, the same amount of different dilutions is taken. For example, suppose the M.H.D. was the standard volume of a 1 in 60 dilution of complement, then 3 M.H.D. would be present in the standard volume of a 1 in 20 dilution, while for 5 M.H.D. a 1 in 12 dilution would be used.

Antigen—

An alcoholic extract of *human heart-muscle* is prepared as described on p. 256 (2). For use, place in a wide tube 0·3 ml. antigen and 0·2 ml. 1 per cent. solution of cholesterol in absolute alcohol. Add very rapidly 7 ml. of saline. This 1 in 15 dilution is the antigen suspension referred to in the description of the test proper.

Patient's serum—

This is inactivated and a 1 in 5 dilution in saline is used.

Complement—

Guinea-pig serum (p. 257).

Haemolytic system—

Sheep red corpuscles, 3 per cent., are sensitised with 6 M.H.D. immune body. The cell suspension may be standardised by means of

¹ For full details of this method see "The Wassermann Test," by Wyler, E. J., *Medical Research Council Special Report Series*, No. 129, 1929; *Ministry of Health Reports on Public Health*, No. 67, 1932; *J. Path. Bact.*, 1934, **39**, 521.

² Measurements can also be made with the graduated pipettes referred to on p. 259.

a haemoglobinometer, preferably of the photoelectric type. Either Burroughs Wellcome haemolytic serum, or rabbit *v.* sheep antiserum, can be used.

Titration of complement—

Prepare 2 ml. of a 1 in 10 dilution of the guinea-pig serum in saline. Set out eight tubes. Add to each respectively, starting from the left, 1 volume of each of the following dilutions of complement prepared from the 1 in 10 dilution : 1 in 30, 1 in 40, 1 in 50, 1 in 60, 1 in 70, 1 in 80, 1 in 90, 1 in 100. To each tube add 2 volumes of saline and 1 volume of sensitised red cells. Prepare a similar series of tubes with the complement dilutions, but instead of adding 2 volumes of saline add 1 volume of antigen suspension and 1 volume of saline. Then add 1 volume of sensitised red cells and shake thoroughly. Incubate both sets of tubes in the water-bath for half an hour at 37° C. and read the result. In the first set of tubes (*i.e.* without antigen) the highest dilution showing complete haemolysis is the minimum haemolytic dose. Dilutions of complement equivalent to 2, 3 and 5 M.H.D. are now prepared from the undiluted guinea-pig serum. The second set of tubes (with antigen) indicates whether the antigen suspension has any anti-complementary action.

Test proper—

Four tubes are required for each test, two for serum controls and two for the antigen-serum mixture. The contents of each tube are as follows :—

Tube 1 (serum control)	1 volume saline. 1 volume patient's serum diluted 1 in 5 in saline. 1 volume complement diluted to 3 M.H.D.
Tube 2 (serum control)	Same as tube 1, but the comple- ment present is 2 M.H.D.
Tube 3	1 volume patient's serum diluted 1 in 5 in saline. 1 volume complement 5 M.H.D. 1 volume antigen suspension.
Tube 4	Same as tube 3, except that com- plement is 3 M.H.D.

The reagents are added in the following order :—

- (1) 1 volume of dilution of patient's serum into all tubes.
- (2) 1 volume of saline into tubes 1 and 2.
- (3) 1 volume complement dilution 2 M.H.D. into tube 2.
- (4) 1 volume complement dilution 3 M.H.D. into tubes 1 and 4.
- (5) 1 volume complement dilution 5 M.H.D. into tube 3.
- (6) 1 volume antigen suspension into tubes 3 and 4.

The racks are shaken after each set of ingredients is added to the tubes.

The tubes are incubated first at room temperature for thirty minutes and then in the water-bath at 37° C. for thirty minutes, after which 1 volume of sensitised red cells is added, and the tubes are well shaken and replaced in the water-bath at 37° C. After one or two minutes they are taken out, quickly shaken and replaced. The results are read as soon as the No. 1 tubes and some of the No. 4 tubes, including the No. 4 tube of the negative control, show complete haemolysis—usually in about five minutes.

In addition to the test proper, described above, in which known positive and negative sera are used as controls, an antigen control and a corpuscle control are also carried out at the same time.

Antigen control—

To one tube are added 1 volume saline, 1 volume 2 M.H.D. complement and 1 volume antigen. This is placed in the rack with the test proper, 1 volume of red cells being added later along with the rest of the tubes.

On incubation there should be complete haemolysis within thirty minutes, confirming that the antigen does not absorb more than one dose of complement.

Corpuscle control—

This consists of 1 volume of sensitised red cells with 3 volumes of saline. The red cells are added to the saline at the same time as to the other tubes. The tube is centrifuged when the tests have been read. The supernatant fluid must be colourless, showing that no deterioration of the cells has occurred since the complement was titrated.

Method of Recording the Results of the Test

Symbols—

- ++ = Complete inhibition of lysis with 3 M.H.D. complement and complete or almost complete inhibition with 5 M.H.D. complement.
- + = Complete or almost complete inhibition of lysis with 3 M.H.D. complement and partial inhibition of lysis with 5 M.H.D. complement.
- ± = Partial inhibition of lysis with 3 M.H.D. complement ; slight inhibition or complete lysis with 5 M.H.D. complement.
- = Complete lysis with 3 M.H.D. complement.

Interpretation of symbols—

- ++ = Strong positive } Diagnostic.
- + = Positive }
- ± = Positive in *known* cases of syphilis only. In unknown cases it is regarded as *doubtful*, and as an indication for further investigation.
- = Negative, but the patient is not necessarily non-syphilitic,

Test with cerebro-spinal fluid—

The fluid does not require to be heated.

Into a series of tubes place varying quantities of the cerebro-spinal fluid as follows :—

2 volumes undiluted fluid.

1 volume undiluted fluid.

1 volume 1 in 2·5 dilution.

1 volume 1 in 5 dilution.

To each tube are added 1 volume antigen and 3 M.H.D. complement. As a control, another tube with 2 volumes of undiluted fluid and 3 M.H.D. complement, but without antigen, is used. The remainder of the procedure corresponds to the serum test, and the strength of the reaction is judged by the number of tubes, excluding the control, in which a positive reaction occurs. A strongly reacting fluid gives a positive result with the 1 in 5 dilution, while a weakly reacting specimen yields fixation of complement only in the tube containing 2 volumes of undiluted fluid.

Partial or even complete inhibition of lysis in the tube containing 2 volumes of undiluted cerebro-spinal fluid cannot be accepted as positive for diagnostic purposes unless there is also definite inhibition of lysis in the tube containing 1 volume of undiluted fluid.

Instead of using a constant volume pipette it is convenient, particularly where large numbers of tests are carried out, to use dropping pipettes.

For this technique Wyler's paper (third reference, footnote, p. 263) should be consulted.

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. 221 ; 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

¹ Richardson, G. M., *Lancet*, 1941, 2, 696 ; and personal communication, 1944.

Two stock solutions, which keep indefinitely, are used :—

(A) Boric acid (H_3BO_3) 0·93 gram, borax ($Na_2B_4O_7 \cdot 10H_2O$) 2·29 grams, and sorbitol ($C_6H_{14}O_6 \cdot 1H_2O$) 11·47 grams 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 gram and sodium azide (NaN_3) 0·81 gram 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° to 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.¹ The serum is kept in sterile screw-capped bottles at approximately 4° C. The full haemolytic activity of the serum and the fixability of the complement in the Wassermann reaction are maintained for about six months. It should be noted in using this preserved complement that it represents a 1 in 2 dilution of the original serum.

THE FLOCCULATION TEST FOR SYPHILIS

Since the discovery by Michaelis (1907) that precipitation or flocculation occurs when a syphilitic serum is allowed to interact with organ extracts, various tests depending on this phenomenon have been described and recommended for the routine serum diagnosis of syphilis as substitutes for the Wassermann reaction—e.g. the Sachs-Georgi reaction, the Kahn reaction, the Meinicke test, etc. Most of these have been reviewed in the League of Nations report on the sero-diagnosis of syphilis.² The differences in technique depend

¹ Sonnenschein, C., *Ztschr. f. Immun.*, 1930, 67, 512.

² League of Nations Publications, 1928, No. C.H.726; and 1931, No. C.H.968.

mainly on the manner in which the organ extract is prepared and the method by which the resulting flocculation, precipitation, or clarification (Meinicke test) is observed.

The method of the Bacteriology Department, Edinburgh University, may be recommended as a simple and reliable technique. The results are practically parallel to those of the complement-fixation test, and the flocculation reaction may be substituted for it ; the serum, however, should be tested within two days after withdrawal of the blood specimen. In treated cases of syphilis the flocculation reaction may be definitely positive when the Wassermann reaction is very weak or negative ; sometimes, however, the reverse result is obtained. It is to be noted that the flocculating property of syphilitic serum may be lost if specimens of blood are kept for several days.

The "Antigen" is similar to that used for the Wassermann reaction.

20 grams of sheep heart-muscle are ground with sand in a mortar and extracted for four days at room temperature with 100 ml. of absolute alcohol. The extract is filtered and 0.25 gram cholesterol is added and dissolved by keeping the extract in a water-bath at 55° C. for half an hour. It is then allowed to stand at room temperature for a day and again filtered.

The optimum dilution of this antigen for the test is usually 1 in 18, but may vary for different preparations and must therefore be ascertained for each new preparation. Likewise the "sensitivity" must be compared with a known standard so that, if necessary, the antigen can be corrected or standardised.¹

The reaction is carried out by a method similar to that of an agglutination test (p. 245).

The activity of the serum is at a maximum after heating at 54°–56° C. For the test the patient's serum is therefore heated at 55° C. for half an hour and a series of dilutions is prepared in small test-tubes ($3 \times \frac{1}{2}$ in.) as follows (physiological saline solution being used as the diluent) :—

(1)	(2)	(3)	(4)	(5)	(6)
1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64

0.4 ml. of each concentration is a convenient quantity for the test, and to each tube one-half of this volume (0.2 ml.) of the antigen suspension is added. If the optimum dilution of the

¹ For details of the method of standardising this antigen, reference should be made to *Indian J. Med. Research*, 1929-30, 17, 477 : "The Standardisation of Antigen used in the Syphilis Flocculation Reaction," by K. V. Krishnan.

antigen is 1 in 18, then a 1 in 6 suspension is used ; it is prepared by mixing rapidly equal quantities of the undiluted antigen and saline solution, allowing the mixture to stand for ten minutes and then adding sufficient saline to give the required dilution (*i.e.* 1 in 6). The mixtures of serum and antigen are transferred to narrow agglutination tubes, incubated at 37° C. for four hours and allowed to stand overnight at room temperature, when readings are made of the results. A final reading should be made after thirty-six hours. In reading the results, the tubes should be held in front of a shaded lamp so that they are brightly illuminated without the glare of the lamp reaching the eyes. A control tube containing antigen suspension and saline but no serum should be included. With strongly reacting sera, flocculation may occur even in tube 6. With weak sera definite flocculation may occur only in tube 1. "Zone phenomena" are sometimes observed, *e.g.* with sera after three days or more from the time of withdrawal of the blood, flocculation being less with lower dilutions of serum than with higher dilutions. Such zone effects are specially noticeable with sera which give strongly positive Wassermann reactions.

The reaction can be hastened by shaking the mixtures of serum and antigen for five to ten minutes before incubation. This can easily be done after the addition of the antigen to the serum dilutions by placing the small test-tubes vertically in a shaking machine (such as that used for the Kahn test, *vide infra*).

The test can, if necessary, be abridged by omitting tubes 5 and 6.

The transfer of the mixtures to narrow tubes, as described above, facilitates the reading of results, but flocculation can be observed in the wider tubes (if necessary with the aid of a hand-lens), and transfer to agglutination tubes can be omitted, with saving of time, labour and glass-ware.

THE KAHN FLOCCULATION TEST

Apparatus required—

- (1) Small test-tubes, $3 \times \frac{1}{2}$ in., as used in the Wassermann test ; these tubes should be of perfectly clear glass and thoroughly clean.
- (2) Flat-bottom glass cylinders, $1\frac{1}{2} \times \frac{1}{2}$ 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.
,, <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 minutes. (After shaking, incubation in a water-bath at 37° C. for fifteen minutes or an incubator at 37° C. for twenty minutes 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, St. Peter's Hospital, Vallance Road, London, E.1. For full details of the test and the preparation of the antigen see *The Kahn Test*, by R. L. Kahn, 1928, 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.
,, <i>saline</i>	.	.	0.15 ml.	0.15 ml.	0.15 ml.

Shake tubes as above.

Incubate as above.

Then add *saline* . . . 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 second.

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	++++	+++	+++	+++	Strongly positive
,, D	+++	+++	++++	++	Positive
,, E	+++	++	++	++	Positive
,, F	+	++	++	++	Weakly positive
,, G	+	+	++	+	Doubtful
,, H	—	±	++	±	Negative
,, 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 floccula-

¹ See Khairat, O., *Brit. Med. J.*, 1952, 1, 582.

² Kahn, R. L., *J. Lab. and Clin. Med.*, 1941, 26, 139; and *Arch. Dermat. and Syph.*, 1940, 41, 817; Beveridge, W. J. M., *Edin. Med. J.*, 1943, 50, 344.

tion test are non-specific or definitely significant of syphilitic infection. From studies of "false positive" reactions he concluded that a positive result may in some cases be related to biological changes apart from syphilitic 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. 270).

The various types of comparative result are illustrated in the following Table :—

<i>At 37° C.</i>	<i>At room tempera-ture.</i>	<i>At 2° C.</i>	<i>Classification of result</i>
++ . . . 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.

THE COMPLEMENT-FIXATION TEST IN TUBERCULOSIS

This reaction has sometimes been utilised in the diagnosis of certain cases of tuberculosis and also as an index of the activity of the disease, but is now infrequently carried out. Details have been given in previous editions, to which reference can be made.

THE COMPLEMENT-FIXATION TEST IN GONORRHOEA

The general technique of the test is very similar to that of the Wassermann test described on p. 254 *et seq.*

Antigen.—Various types of antigen prepared from cultures of the gonococcus have been used. The antigen recommended is that devised by Price,¹ who claims that in the first week of the disease 27 per cent. positive results are obtained, rising to 46, 70, 80 and 100 per cent. after two, three, four and five weeks respectively. A suitable antigen for the test can be obtained from Burroughs Wellcome & Co., along with technical details of its use.

Haemolytic System.—3 per cent. sheep cells *plus* 5 M.H.D. haemolytic antiserum.

Complement.—Prepared and titrated as for the Wassermann test (p. 257). Along with this titration, antigen and serum control tests are also made.

Test Proper.—This is similar in technique to Wyler's method for the Wassermann test, a constant-volume method being used. One volume of serum and one volume of diluted antigen are tested with 3 and 5 M.H.D. of complement respectively, together with a serum control containing 2 M.H.D. complement. (One volume of saline is also added to each tube.) The mixtures are well shaken and placed in a 37° C. water-bath for one hour. Thereafter, one volume of the haemolytic system is added, the tubes are well shaken and again placed in the water-bath. Results are read when the normal serum controls are completely haemolysed.

No haemolysis with 5 M.H.D. complement is denominated a ++ result ; haemolysis with 5 M.H.D., but no haemolysis with 3 M.H.D. + ; complete haemolysis with 3 M.H.D. negative.

For full details Price's Monograph should be consulted.

The application of *complement-fixation tests in the diagnosis of various other infections* is dealt with in the appropriate chapters of Part III.

¹ Price, I. N. O., Monograph published by the London County Council, No. 2995, 1933 (obtainable from King & Son, London); *J. Path. Bact.*, 1932, 35, 635.

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 (p. 37). 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 (p. 45). 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, but the most convenient is :—

Comparison with Standard Opacity Tubes (Brown).¹—This consists in comparing the opacity of the suspension

¹ Indian J. Med. Research, 1919, 7, 238-250.

with that of a series of ten standard tubes containing different dilutions of suspended barium sulphate. In making comparisons the bacterial suspension should, of course, be placed in a tube similar 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 Timothy.¹

Showing the Relation of Opacity to the Numerical Equivalent of various Bacteria estimated by means of the Haemacytometer Method

Opacity Tube No.	Staphylococcus aureus	Streptococcus (haemolytic)	Pneumococcus	Gonococcus	Esch. coli	S. typhi	S. para-typhi B	N. catarrhalis (on ord. agar)	H. influenzae
10	3789	3043	7053	3578	3787	4577	4171	3611	11396
9	3410	2739	6348	3220	3408	4119	3754	3250	10256
8	3031	2434	5642	2862	3030	3662	3337	2889	9117
7	2652	2130	4937	2505	2651	3204	2920	2528	7977
6	2273	1826	4232	2147	2272	2746	2503	2167	6838
5	1895	1522	3527	1789	1894	2289	2086	1806	5698
4	1516	1217	2821	1431	1515	1831	1668	1444	4558
3	1137	913	2116	1073	1136	1373	1251	1083	3419
2	758	609	1411	716	757	915	834	722	2279
1	379	304	705	358	379	458	417	361	1140

The figures represent millions per ml.

Standard opacity tubes with the corresponding tables are supplied by Burroughs Wellcome & Co.

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*.)

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

¹ Indian J. Med. Research, 1924, 11, 1253.

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"¹ 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 (p. 224), 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.

¹ A very suitable container is the "Clinbritic" vaccine bottle which is fitted with a skirted type of rubber cap and a screw-on superimposed plastic cap. This bottle is obtainable from Britton, Malcolm & Waymark Ltd., 38 Southwark Bridge Road, London, S.E.1.

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 (1952).

LABORATORY CENTRIFUGES

The use of the centrifuge is to separate cells, organisms, or other particles from a fluid suspension. On standing, the cells, etc., will slowly sediment by gravity, but this sedimentation can be greatly accelerated by means of the centrifuge. The "relative centrifugal force," which indicates the degree to which the normal force of gravity is increased, depends, amongst other factors, on the radial distance of the centrifuged material from the central axis and on the square of the number of revolutions per minute. It should be appreciated also that the shape and size of the particles, the viscosity and surface tension of the fluid, and the difference in specific gravity between the particles and fluid, play an important part in the process. For example, when defibrinated blood is being washed (p. 257), the first sedimentation of the corpuscles from the viscous serum of high specific gravity takes much longer than when the corpuscles are suspended in saline.

Types of Centrifuge.—For general laboratory work they are of three types : (1) Hand-driven ; (2) Water-driven ; (3) Electric.

Hand-driven centrifuges have two or four 15-ml. conical metal "buckets" carrying the glass centrifuge tubes (*vide infra*), and are chiefly used by medical practitioners or in small laboratories where electric power or water is not available. The speed attained rarely exceeds 2000 r.p.m., while the motion is jerky and the column supporting the buckets tends to vibrate. Moreover, the buckets come to rest quickly when the drive ceases, thereby tending to disturb the sediment.

Water-driven centrifuges utilise a water turbine to the spindle of which the "head" is connected. At least 30 lbs. per sq. in. pressure is required to work the machine satisfactorily. These machines are now rarely used.

Electric centrifuges may be obtained for various quantities of fluid up to 1 litre. The machine consists of a motor with a vertical spindle to which the "head" is attached. An assortment of heads may be used according to the amounts and number of the specimens. The portable type of electric centrifuge mounted on rubber cushions on a stand with rubber castors is very useful. Speeds up to 4000 r.p.m. are usually obtained. For greater speeds more powerful and strongly designed machines, which are therefore more costly, are required. With these centrifuges the head is usually mounted on a firm stand and is belt-driven from a separate motor.

Larger centrifuges (often incorporating a refrigerating unit) with capacities up to 13 litres are used in the preparation of human plasma and blood derivatives, but their description and use are beyond the scope of this book. Similarly, the special centrifuges used in connection with virus studies (p. 570) are not described here. The "Angle" centrifuge is considered later.

Speeds up to 3000 r.p.m. are sufficient for ordinary laboratory use. The speed is controlled by means of a rheostat, and it is essential that the centrifuge should be started only when the full resistance in the rheostat is in circuit. The head will then commence to revolve very slowly, and the speed is increased gradually until the desired revolutions are obtained. Electric centrifuges should be fitted with some form of "no-volt release" so that if the current is interrupted (e.g. by a blown fuse or from a main switch) and the machine stops, it will not start again when the current is restored; otherwise the motor will start violently, with damage to the machine and its contents.

The *metal buckets* which hold the glass centrifuge tube are of various capacities. The buckets for conical glass tubes should be cylindrical and not conical, as the conical tubes, which are usually hand-made, are not all of the same slope and therefore may not fit accurately into a conical bucket, but touch only at one place so that, on centrifuging, the tube is broken and the contents are lost.

Centrifuge Tubes.—The thickness of the wall of the tube, varies according to the capacity, but tubes of the same size should have a uniform thickness of wall so that when balanced they contain approximately the same amount of fluid.

The tubes are usually plugged with cotton wool and sterilised in the hot-air oven. When used in the centrifuge, however, the cotton-wool plugs may be drawn to the bottom of the tube unless the upper portion of the plug is folded over the mouth of the tube and secured with a rubber band. Even when the cotton-wool plug is secured in this way, cotton fibres become detached owing to the speed of the centrifuge and are seen microscopically in the deposit.

The most convenient method is the following. Instead of a cotton-wool stopper, a screw-cap without a washer is placed over the mouth of the tube, the size being that of a loose fit. (For the ordinary 15-ml. conical tubes the KN182 screw-cap of a $\frac{1}{4}$ -oz. "bijou" bottle is convenient.) Each centrifuge tube with cap in position is wrapped individually in kraft paper and sterilised in the hot-air oven. During centrifuging the cap is kept on the tube and the contents do not become contaminated. Moreover, the mouth of the centrifuge tube is kept sterile and the supernatant fluid can be poured off without risk of contamination.

Method of Using the Centrifuge.—It is essential that each centrifuge tube and bucket should be balanced accurately by a similar tube and bucket diametrically opposite on the circumference of the head. Various types of balances are catalogued for this purpose, but usually they are made only for one size of tube. Where several heads and different sizes of bucket are used, the simplest and most convenient type is the commercial "2-lb. butter balance" of the Beranger type having two flat pans, both being of porcelain. The larger buckets stand upright on the flat pans, while for the 15-ml. buckets a simple holder may be made from a wooden container such as is used for transmitting test-tubes by post. The top is cut off, leaving the holder $1\frac{1}{2}$ in. high, which is then nailed to a piece of thin wood (plywood) $\frac{3}{16}$ in. thick and $1\frac{1}{2}$ in. square to serve as a base. Two of these are required, and they can be balanced accurately with fine shot or small pieces of sheet lead.

When, for example, a sample of urine has to be centrifuged, a sterile centrifuge tube is filled to within $\frac{1}{2}$ in. of the top, the metal cap replaced, and the tube is put in a holder on the balance. A similar centrifuge tube is filled with water and placed in a holder and the cap is laid on the balance pan beside it; by means of a rubber teat and capillary pipette, water is added or removed from the second tube until both tubes are accurately balanced. The metal cap is placed over the second tube and both tubes are inserted opposite each other in the centrifuge head. The lid of the centrifuge is secured and the centrifuge slowly started. When the head is rotating freely, the speed is gradually increased, with the rheostat or by increasing the water pressure, according to the motive power, until the desired speed is reached. After the prescribed time the current is switched off or the water power shut off, and the head comes slowly to rest. When all movement has ceased, the tubes are lifted from the buckets. Tubes of the larger sizes should be balanced in their metal buckets, which are then fitted into the centrifuge head.

It is essential that the hinged or detachable lid, which is fitted to most centrifuges, should be closed during centrifuging, otherwise a decrease in the speed of revolution due to "windage" will ensue.

The makers' instructions for oiling and overhaul of the machines should regularly be carried out.

Angle Centrifuge.—In this type of instrument the tubes, instead of being allowed to rotate in a horizontal plane, are fixed at an angle

(from 20° to 45°) on the rotating head. The centrifuge is driven by a "universal" motor (suitable for both direct and alternating current and different frequencies). The usual speed is 3000–4000 r.p.m. The advantage of the angular position is that particulate matter is rapidly separated and concentrated, with saving of time and current. Thus, particles have only to traverse a short distance before deposition on the side of the tube. The tubes are encased in a spun metal "bowl," which in its rotation offers very slight resistance to air and so obtains greater speeds. The centrifuge has rubber feet and stands on the laboratory bench without being bolted or fixed. It is very convenient for ordinary routine purposes and can be used also for separating virus bodies.

With high-speed angle centrifuges, glass tubes are apt to become broken, and centrifuge tubes made of cellulose acetate are often used. They are, however, not easy to sterilise and are not recommended for ordinary routine use.

COLD STORAGE

It is essential to have some form of cold storage in the laboratory for the preservation of blood, serum, culture media, vaccines, etc.

Mechanical refrigeration is now universally used, and refrigerators are available in a large number of sizes from 1½ 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.

CHAPTER IX

BACTERIOLOGICAL EXAMINATION OF AIR, WATER, AND MILK; TESTING OF ANTISEPTICS;

TESTING OF SENSITIVITY OF BACTERIA TO SULPHON-AMIDES AND ANTIBIOTICS; TITRATION OF PENICILLIN ACTIVITY IN BLOOD, ETC.; TESTING OF SURGICAL CAT-GUT FOR STERILITY; COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

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. 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 up

¹ *J. Hygiene*, 1941, 41, 197.

to 10 cubic feet are tested. This method has proved valuable in the qualitative and quantitative study of air bacteria. It may be added that 5 per cent. blood-agar containing 1 : 1,000,000 crystal violet which allows streptococci to grow while inhibiting most air and dust organisms is useful for demonstrating the former as indices of contamination of air from the respiratory passages.

BACTERIOLOGICAL EXAMINATION OF WATER

From the hygienic standpoint, the bacteriological examination of water resolves itself into the determination of the presence or absence of any serious excretal contamination.

Under certain conditions, the coliform bacilli represent the most reliable indicators of such pollution.

As this group of organisms may be derived from the intestine of various animals, even water supplies far removed from human contamination contain them in small numbers. In water grossly polluted with excretal matter, e.g. sewage, they are present in large numbers. The test for their presence as an index of impurity must therefore be carried out on a quantitative basis, so that their approximate number in the water can be estimated.

As explained in Chapter XV, this group includes a large number of different types. Some of these ("typical" or "faecal", e.g. *Esch. coli*) are more prevalent in the intestine than others ("atypical," e.g. *Aerobacter aerogenes*), and obviously the former are of more significance as indicators of recent faecal contamination. Therefore, in carrying out the test for coliform bacilli in water, it is essential to determine whether the strains present are typical or atypical (pp. 288, 426).

The presence of streptococci and sporing anaerobic bacilli, e.g. *Clostridium welchii*, is of additional significance in determining water purity. The occurrence of streptococci is strong evidence of faecal pollution, but their absence does not exclude such impurity. The intestinal sporing anaerobes, being highly resistant, do not by themselves indicate a recent or serious pollution.

The enumeration of the total viable bacteria in a water specimen is a useful supplementary test in determining its purity, but this test is of little value by itself.

The demonstration of pathogenic bacteria, e.g. the typhoid bacillus, would obviously constitute the most direct proof of a dangerous impurity, but pathogens, if present, are usually so

scanty that the technical difficulty of their isolation makes this test impracticable for ordinary purposes.

The routine tests generally used in the bacteriological examination of water are :—

- (1) Enumeration of the viable bacteria.
- (2) The quantitative test for coliform bacilli.
- (3) The differential test for " typical " coliform bacilli.

Collection of specimens.¹—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. 140). 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 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.

¹ See Report No. 71, Ministry of Health, 1939, on the Bacteriological Examination of Water Supplies.

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.

Enumeration of viable bacteria.—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, add a smaller quantity, e.g. 0.1 ml., and in dealing with specimens of uncertain purity it is advisable to make a series of plate cultures with varying quantities of the water. Thus, serial dilutions may be made from the sample, e.g. 1 in 10, 1 in 100, as in examination of milk (*q.v.*) and 1 ml. quantities of these plated.

Make *duplicate* plates from each volume tested and incubate one at 37° C. for two days, 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 has been suggested that an incubation of 24 hours at 37° C. gives plate counts which more closely represent the number of parasitic organisms present, since after 2 days certain saprophytic bacteria capable of growing slowly at 37° C. may have developed in the plates.

Count the colonies that develop in the medium. To aid counting, divide the plate into sections by ruling on the glass

with a grease pencil. Count the colonies in each section (using a hand-lens if necessary to detect small colonies) and add the numbers. 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) Ltd. and known as a "colony illuminator" (EDM205). It consists of a box illuminated from the inside by two fluorescent tubes arranged to give uniform illumination of a standard-size Petri dish which rests inverted in a circular space in the black plastic top, supported on a lower black plastic square base, and surrounded by a translucent opal plastic sleeve through which the light diffuses evenly. Five inches above the Petri dish a lens of 5 in. diameter and magnification $2\times$ is fixed. Behind this is fitted a black plastic 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 it is an advantage to divide the plate into sections by ruling lightly on the under surface with a grease pencil (as already mentioned). If the plates prepared from the undiluted water show between 30 and 300 colonies these should be counted. If there are more than 300 colonies and the sample has been diluted, then the plates giving counts between 30 and 300 should be selected and the others discarded. If all plates show more than 300 colonies, then the result should be reported as more than 300 multiplied by the reciprocal of the highest dilution used; e.g. if the sample was diluted 1 in 100, the result would be given as "more than 30,000." Alternatively, provided no more than 500 colonies are present, a count of more than 300 colonies may be made as accurately as possible and the result given as an approximate one. Since only a proportion of the bacteria originally present in the water are capable of developing under the conditions of the test, the total colony count represents the number of organisms per ml. of the sample, which have grown at the specified temperature (*i.e.* 37° C. or 22° C.) in the specified time (*i.e.* 2 days or 3 days). The result is expressed briefly as the plate count per ml. at 37° C. and 22° C.

The number of viable bacteria growing at 20°-22° C. in 3 days in the average municipal water supply, *i.e.* after filtration, should not usually exceed 100 per ml., while the number growing at 37° C. should not usually exceed 10 per ml. The bacterial count from unfiltered water varies greatly, depending on the nature of the supply and the prevailing climatic conditions. A count of 1000 or more per ml. may arouse suspicion regarding the purity of a water supply, but

does not necessarily signify dangerous pollution ; thus, an increase of organic matter in the summer season may lead to bacterial multiplication and an unusually high bacterial count. On the whole, the results of such enumeration are of relatively little value by themselves in estimating the hygienic purity of a water, though, when regular observations of this nature are made on the same water supply, a high count on a particular occasion may draw attention to some condition of the supply requiring further investigation.

In uncontaminated water, since the organisms present are mainly saprophytes, the number of colonies developing at 20°–22° C. is usually much greater than at 37° C., the ratio being higher than 10 : 1. A contaminated water may yield a ratio less than this, e.g. 10 : 3. These ratios, however, are too variable to be of much significance.

Test for Coliform Bacilli.—The object of this test is to ascertain the number of coliform bacilli in a given volume of water or the smallest amount of water which contains such organism. The estimation 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 inverted tubes to show the formation of gas ; acid and gas formation (a "positive" result) indicates the growth of coliform bacilli (pp. 166, 427). In this way it may be 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. 291). This is the method recommended for routine use.

Media required :—

- (1) 2 per cent. peptone water containing 0·5 per cent. sodium taurocholate, 1 per cent. lactose, and neutral red, Andrade's solution or brom cresol purple as indicator (p. 181—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 an inverted 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.

The bottles are incubated for forty-eight hours and those showing acid and gas formation ("positive reaction") are noted. This indicates the growth of coliform bacilli, though further testing is required to determine whether these are typical. The examination up to the present stage is often designated the "presumptive" test. McCrady's tables (one of which is quoted on p. 291) are now referred to, and according to the various combinations of positive and negative results, the probable number of coliform bacilli in 100 ml. of the water can be stated.¹

¹ See Report cited in footnote, p. 283.

To ascertain whether the coliform bacilli detected in the presumptive test are typical, sub-inoculations are made on MacConkey plates from those bottles which show acid and gas production. The plates are incubated for twenty-four hours, red colonies are picked off on to agar slopes, and the resulting pure growths are put through the following tests :—

Fermentation of *Lactose*

” ” *Inositol*

Production of *Indole*

Voges-Proskauer reaction

Methyl-red reaction

Citrate utilisation

Liquefaction of *Gelatin*

(pp. 153, 427)

The fermentation of lactose has often been assumed from the presumptive test, but should always be confirmed with the isolated organism.

The common types of coliform bacilli of direct excretal origin show the following reactions :—

Lactose	+	(acid and gas)
Inositol	-	
Indole	+	
Voges-Proskauer reaction	-	
Methyl-red reaction	+	
Citrate utilisation	-	
Gelatin	-	

A method has been advocated for counting typical or “faecal” coliform bacilli in water without the necessity for plating and examining pure cultures as described above. This depends on the ability of these organisms to produce gas when growing in a bile-salt-lactose-peptone-water at 44° C., the atypical coliform bacilli lacking this property (p. 429, Eijkman reaction). Either of the following procedures may be adopted. (1) The various quantities of water are added to the bile-salt-lactose medium, as described above, *in duplicate*, one set of bottles being incubated at 37° C., the other at 44° C., for two days. The former will yield a count for coliform bacilli irrespective of their type ; from the latter a count can be made similarly for typical coliform bacilli. (2) After the usual presumptive test, subcultures are made from all the bottles showing acid and gas into tubes or bottles of the same medium (single strength) as used for the original test, and these are incubated at 44° C. for two days.

Those yielding gas may be regarded as containing typical coliform bacilli, and a computation of the number in 100 ml. of water can be made as before.

It has been stressed that incubation at 44° C.¹ must be carried out by keeping the bottles or tubes in a thermostatically controlled water-bath which does not deviate more than 0·5° C. from 44° C. An incubator regulated at 44° C. is not considered satisfactory for maintaining the cultures at this temperature.

It has been shown that certain "atypical" coliform bacilli are also capable of producing gas at 44° C. though these are comparatively rare in water supplies in this country. They can be distinguished from the "typical" organisms by their inability to produce indole at 44° C.¹

It should be noted further that certain anaerobes, e.g. *Cl. welchii*, may also give a positive Eijkman reaction; this fallacy can be obviated by adding a sufficient concentration of brilliant green to the medium used for the Eijkman test in order to inhibit these anaerobes without affecting the typical coliform bacilli (2 ml. 1 per cent. w/v brilliant green in 1500 ml. of medium).

It must be recognised that it is hardly possible to set up fixed bacteriological standards for water purity from the hygienic standpoint, and it has been emphasised that the aim should rather be to establish a standard for each water supply on the basis of frequent examinations, any later deviation from which would be viewed with suspicion. It is necessary also in reporting on a water supply for the first time to make several examinations under different weather conditions, including an examination after heavy rainfall; thus, a sudden increase in the number of coliform bacilli after rain indicates potential danger as water-borne disease is often associated with flood-water.

A rule originally laid down by Houston has generally been accepted in the past: that a water *from a suspicious source* with a *typical* coliform bacillus in 10 ml. or less should be condemned for drinking purposes. When a *typical* coliform bacillus is found in 1 ml. or less, a water may certainly be regarded as unsafe for drinking. On the other hand, waters of high purity usually show absence of coliform bacilli from 50 ml., and an efficiently chlorinated water should be free from them in 100 ml.

In the Ministry of Health report (cited in footnote, p. 283)

¹ Mackenzie *et al.*, *J. Gen. Microbiol.*, 1948, **2**, 197.

it is recommended that piped water supplies (sampled on entering the distribution system) should be classified as follows :—

	Count of coliform bacilli in 100 ml. by the presumptive test.
Class 1. Highly satisfactory	Less than 1
.. 2. Satisfactory	1-2
.. 3. Suspicious	3-10
.. 4. Unsatisfactory	Greater than 10

Throughout the year 50 per cent. of samples of non-chlorinated supplies should fall into Class 1 ; 80 per cent. should not fall below Class 2 ; and the remainder should not fall below Class 3. In chlorinated supplies the water should be Class 1. In the case of non-chlorinated waters examined at intervals throughout the year an occasional drop to Class 3 need not be regarded as significant of danger, but if the specimens are frequently in Class 3, or if a specimen falls into Class 4, the water may be regarded as definitely below the safety level. The determination of the type of coliform bacillus becomes specially important when there is a fall to Class 3 ; if this is due to atypical coliform bacilli the result is of less significance from the hygienic standpoint, though it has been found that the appearance of such atypical coliform bacilli in a water supply may be the forerunner of a more serious contamination. In the case of waters from a deep source which are usually highly satisfactory (Class 1), a fall to Class 2 would have to be considered significant. If a chlorinated water drops to Class 2, the question would arise as to the efficiency with which the process is being carried out.

In the examination of samples taken on the consumer's premises, if comparison of the water before and after distribution shows an increased number of typical coliform bacilli this would suggest some pollution in distribution and would require immediate investigation to detect the source of the contamination. It should be emphasised that in all cases the results of bacteriological examination must be closely correlated with topographical observations by health officers and water engineers on the gathering ground and sources of the water, reservoirs and other waterworks, and the distribution system generally.

Examination for Streptococci.—The type of streptococcus indicative of faecal pollution is the enterococcus (p. 338). This organism grows in the medium used for the test for coliform bacilli (*vide supra*) and by itself ferments the lactose but without gas production. Its presence

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	2	0	5
	1	2	1	7
	1	2	2	7
	1	2	3	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.

According to the older system of notation, these results can be stated alternatively as "coliform bacillus present in . . . ml.", the number of 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."

in water can therefore be determined by further examination of the contents of the bottles showing acid or acid and gas fermentation in the above-mentioned test. For this purpose 1 ml. of the primary culture is mixed with 9 ml. of sterile water in a stoppered tube, which is then kept in a water-bath at 60° C. for fifteen minutes. The object is to kill the coliform bacilli, the enterococcus remaining alive in virtue of its resistance to heat. A large drop is then transferred with a sterile capillary pipette to a plate of MacConkey's medium and streaked out or spread on the surface (p. 210). After incubation the enterococcus, if present, grows in the form of small red colonies. The identity of the organism can be confirmed by subculturing single colonies and examining further.

Another method recommended is to make subcultures on a lactose-tellurite medium from the bottles showing fermentation in the test for coliform bacilli. This medium contains 1 per cent. peptone, 0·5 per cent. lactose, 0·2 per cent. anhydrous dipotassium hydrogen phosphate, 0·5 per cent. sodium chloride, and is solidified in the usual way with agar; potassium tellurite is added to make a 1 in 15,000 concentration and should be added to the medium after the latter has been sterilised. The enterococcus produces on this medium small bluish-black colonies (see Ministry of Health report cited above).

Examination for Clostridium welchii.—50 ml. of water are added to 100 ml. of sterile milk in a stoppered bottle of suitable size. The bottle is then heated at 80° C. for fifteen minutes. Sterile liquid paraffin is run on to the surface of the medium to maintain anaerobiosis. On incubation the occurrence of the "stormy-clot" reaction is indicative of the presence of *Cl. welchii* (p. 507). To examine larger quantities of water, additional bottles of milk are inoculated each with 50 ml. of the specimen and treated as above. Varying quantities of water can be tested likewise.

For methods of isolating *Vibrio cholerae* from water, see p. 474.

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 to 100 millions.

Typhoid-paratyphoid bacilli may be isolated from communal sewage

by methods of selective culture. For this purpose, one of the enrichment methods is used (pp. 184, 440).

BACTERIOLOGICAL EXAMINATION OF MILK

In hygiene work the bacteriological examination of milk generally consists in :—

- (1) An estimation of the number 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 30 minutes). The test depends on the fact that by heating to the degree necessary for sterilisation the heat-coagulable proteins are precipitated ; and 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*” and “*Accredited*” milks 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.

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 referred to above (the technique of this test is detailed in the Regulations).

Scotland—

The special designations which may be used in Scotland are “*Certified*,” “*Tuberculin-tested*,” “*Standard*,” “*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*” and “*Standard*” milks 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. 298.

“*Sterilised*” milk must satisfy the turbidity test referred to on p. 298 (the technique of the test is detailed in the order cited).

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 taking a specimen. 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, and sixteen to eighteen hours in the case of evening milk before being examined. Other samples should be tested immediately after arrival in the laboratory, but if there should be any unavoidable delay in testing they should be kept at a temperature of 0° to 5° C.; no sample shall be kept for longer than 24 hours.

Technique of estimating the number of viable bacteria.—The medium recommended in the Scottish regulations is practically the same as that described by G. S. Wilson (*The Bacteriological Grading of Milk*, Medical Research Council, Special Report Series, No. 206, 1935, p. 63).

The composition of such medium is as follows:—Yeastrel (Brewers' Food Supply Co. Ltd., Edinburgh) 3 grams, good quality bacteriological peptone 5 grams, washed shredded agar 15 grams, fresh whole milk 10 ml., distilled water to 1 litre. The yeastrel and peptone are dissolved in distilled water in a steamer and the reaction is adjusted to pH 7·4. The agar is washed and excess water expressed and then it is added along with the milk to the broth. The medium is autoclaved at 15 lbs. pressure for twenty minutes and filtered hot through paper-pulp. The pH is adjusted to 7·0 at 50° C. and the medium is tubed in 10 ml. quantities. These are autoclaved. The final reaction at room temperature should be pH 7·2.

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 the other designated milks 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.

In dealing with a milk of unknown quality it is advisable to plate 1 ml. of each dilution.

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 count calculated ; this is multiplied by the dilution and is reported as the "number of viable bacteria per millilitre." The count is made with an artificial illuminant and a hand-lens or a colony illuminator (p. 285). 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.

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. 294 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 (as in the testing of water, p. 286). The range of amounts that require to be tested depends on the likely degree of contamination. In the case of ordinary milk 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 ($6 \times \frac{1}{8}$ 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 decoloured up to within 5 mm. of the surface. The time of complete decolorisation is recorded if within the prescribed period (standards given on p. 294).

Phosphatase Test for Pasteurised Milk¹.—This test determines the 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 standard methods of pasteurisation. The presence of the enzyme is detected by 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; thus a deep blue colour, i.e. over 6 units, indicates a large amount of phenol; a pale blue, i.e. under 2.3 units, a small amount.

Reagents required :—

(1) Buffer substrate tablets (disodium phenyl-phosphate and sodium barbitone)²: 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 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

¹ Kay, Aschaffenburg & Neave, "The phosphatase test for control of efficiency of pasteurisation." *Imperial Bureau of Dairy Science, Technical Communication*, No. 1, October, 1939.

² Obtainable from British Drug Houses, Ltd.

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 glass-ware, 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.

Examination for Tuberclle 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. (A microscopic examination of the sediment for tubercle bacilli may be made at this stage, but is quite unreliable—*vide infra*; before staining, the dried films should be treated with ether for some minutes to remove the fat.) 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. 405). 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.

An alternative method is to centrifuge 100 ml. as above and suspend the deposit in 2·5 ml. of saline. The resultant suspension is injected intramuscularly into the thighs of two guinea-pigs. The animals are kept for five weeks, when both are killed and examined.

Some of the cream from the centrifugalised milk may also be used for the inoculation.

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 necessary 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. sporing anaerobic bacilli. This difficulty may be obviated by treating the sediment with antiformin (p. 403) before injection. 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.

The direct microscopic examination of milk may reveal acid-fast bacilli other than the tubercle bacillus (p. 409). The absence of tubercle bacilli in films does not exclude their presence in the specimen. The microscopic test, therefore, is not a valid method of demonstrating tubercle bacilli in milk.

The method of *direct cultivation* described on p. 404 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 (pp. 184, 440). For the diphtheria bacillus, plates of tellurite medium are inoculated with the sediment (p. 186).

Br. abortus may be demonstrated in milk by inoculating two guinea-pigs as in the test for the tubercle bacillus (*vide supra*), but without any antiformin or similar treatment of the sample. The animals are killed and examined after four and eight weeks respectively, cultures are obtained from the spleen by the appropriate method (p. 488) 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.

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 (*Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service*, 1947, 6, 60; 1948, 7, 84; 1949, 8, 155 and 1950, 9, 231).

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. 296) 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. 297), 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. $2\frac{1}{2}$ to 4 hours
- Grade 3. $\frac{1}{2}$ 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.

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-resistant 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 gm. and the presence of coliform bacilli in 0.01 gm. would indicate faults in the manufacture and handling of ice-cream.

TESTING OF ANTISEPTICS

The terms "antiseptic," "germicide," "bactericide" and "disinfectant" are practically synonymous and are applied to substances which destroy micro-organisms, though "antiseptic" was originally

applied to substances which inhibit bacterial growth. Most substances, however, which inhibit growth are germicides if used in high enough concentration or if the exposure is sufficiently long. The term "bacteriostatic" is now commonly applied to inhibition of growth without immediate killing of the organisms.

The "INHIBITION COEFFICIENT" is the lowest percentage concentration of a particular antiseptic that will completely inhibit growth in nutrient medium, e.g. 0·25 per cent. phenol for *S. typhi*.

One of the best methods of determining this 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 surface of the medium must be free from condensation fluid.

The plates are incubated for forty-eight hours, when observations can be made.

Instead of solid medium, tubes of broth (carefully standardised as regards pH) 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 "INFERIOR LETHAL COEFFICIENT" expresses the concentration of an antiseptic and the time of exposure required to kill non-sporing bacteria, e.g. 1 : 60 phenol usually kills haemolytic streptococci in five minutes.

It can be determined by preparing a series of concentrations of the substance in a fixed quantity of diluent (e.g. sterile water), adding a constant quantity of bacterial suspension and transferring at intervals a certain amount of the mixture to some suitable culture medium (e.g. broth, or melted agar at a temperature of 45° C., which, after the transfer, is poured in plates).

The quantity of antiseptic carried over to the culture medium must be less than the inhibition coefficient.

The "SUPERIOR LETHAL COEFFICIENT" expresses the concentration

of a germicide and the time of exposure required to kill bacterial spores, e.g. 4 per cent. potassium permanganate has been found to kill *B. anthracis* spores in twenty minutes.

The "PHENOL COEFFICIENT" expresses the germicidal power of a particular substance as compared with pure phenol.

The method of estimating this coefficient is known as the RIDEAL-WALKER test.¹

Its chief application is for testing disinfectants composed of coal-tar derivatives which are water-soluble or water-miscible.

Materials Required :—

(1) Standard broth, made as follows :

Lab.-Lemco	20 grams
Peptone (Eugepton) ²	20 "
Sodium chloride	10 "
Distilled water	1 litre

Boil for thirty minutes, cool, and make up to 1 litre with freshly boiled distilled water. Make neutral at 37° C. to phenol phthalein with normal sodium hydroxide solution. Steam for thirty minutes to precipitate phosphates, and filter whilst hot. Cool, and adjust pH to 7.6 with normal hydrochloric acid. Distribute in 5 ml. amounts into tubes and sterilise in the autoclave or steamer.

(2) 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.

(3) Phenol. Pure phenol having a crystallising point of 40.5° C. must be used.

(4) Culture of *S. typhi*. It is of the utmost importance to use always a standard culture.³

Subcultures should be made in the standard broth at twenty-four hours' intervals, three times before the test is carried out, and a twenty-four hours' broth culture used for the test proper.

Method of Testing.—

(1) Determine beforehand the inhibition coefficient of the particular germicide for the standard strain of *S. typhi* and make up a series of five graded concentrations, the lowest being slightly greater than the inhibition coefficient (*vide infra*).

The strongest concentration in the series is made up in a sterile stoppered flask with sterile distilled water as the solvent or diluent, and from this the remaining concentrations are prepared by appro-

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 Allen & Hanbury.

³ 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.

priate dilution with sterile water in sterile flasks or tubes. The necessary measurements are made with sterile graduated pipettes.

(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.

These dilutions must not be used if more than a week old.

(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 hours' 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 of 4 mm. diameter, and transfer to tubes of 5 ml. standard broth.

(5) and (6) Carry out with the phenol solutions the same procedure as in (3) and (4).

The bacterial suspension in (3) should be added to the tubes of antiseptic in succession at definite intervals, e.g. 30 seconds. The loop-transfers to broth from each tube, after 2½, 5, 7½ and 10 minutes respectively, can then be accurately timed.

(7) Incubate the broth tubes for forty-eight hours and note those in which growth has occurred.

(8) The coefficient is calculated as follows.

Divide the figures indicating the degree of dilution of the disinfectant which shows life in 2½ and 5 minutes but no life thereafter by that figure indicating the degree of dilution of phenol which shows life in 2½ and 5 minutes but no life thereafter.

The following results illustrate the test :—

Dilution	Time in Minutes			
	2½	5	7½	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.

A specification of the standard technique of Garrod's modification has been prepared by a Committee of the British Standards Institution¹ and should be consulted for full details.

The test is somewhat similar to that of the Rideal-Walker test, the difference being that yeast is present and that the dilutions of disinfectant and phenol are allowed to act on the test organism for a fixed time (thirty minutes) instead of varying times. The standard broth and *S. typhi* culture, however, are the same.

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. 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 :—

$$\text{Phenol coefficient} = \frac{1.9}{0.434} = 4.4 (\pm 10 \text{ per cent.}).$$

TESTING OF SENSITIVITY OF BACTERIA TO SULPHONAMIDE COMPOUNDS, PENICILLIN AND OTHER ANTIBIOTICS

In sulphonamide therapy it may be found that the clinical response is unsatisfactory, and this may be due to the fact that the particular infecting strain, e.g. of *Strept. pyogenes*, is sulphonamide-resistant. The same may apply in penicillin and other antibiotic therapy. A convenient means of testing the sensitivity of bacterial strains to these

¹ Modified Technique of the Chick-Martin Test for Disinfectants, British Standards Specification, No. 808, 1938; see footnote, p. 303.

chemotherapeutic agents is required, and this can now be accomplished by various methods. A technique originally used by Fleming is as follows :—

Sulphonamides.—A strip of agar about $\frac{1}{2}$ in. wide is cut from the centre of a plate of suitable medium (usually blood agar) and discarded. Agar (5 ml.) containing an appropriate amount of the sulphonamide (20 mgm. per cent., i.e. 1 in 5000) is melted in a tube and cooled to about 50° C., mixed thoroughly with 0.25 ml. of horse blood and pipetted into the gutter in the plate of medium. The plate is then placed in the refrigerator overnight in order to allow diffusion of the sulphonamide into the medium, after which it is inoculated with a suitable dilution of the organism, e.g. *Strept. pyogenes*, to be tested. A satisfactory inoculum is a small loopful of a twenty-four hours' culture in serum broth diluted 1 in 2000. The inoculation is made by stroking across the plate at right angles to the gutter; it is desirable to control the test by similarly inoculating with known sensitive and/or resistant strains. With sensitive organisms growth does not occur right up to the edges of the gutter but is inhibited for a distance varying with their sensitivity to the sulphonamide under examination; resistant organisms are not inhibited (or only to a much smaller extent).

Penicillin.—The gutter may conveniently be cut near the edge of the plate (agar or blood agar) which is inoculated with the test organisms and also with a strain of *Staph. aureus* of known sensitivity to penicillin. It is unnecessary to leave the plate overnight in the refrigerator after penicillin is added to the gutter (*cf.* sulphonamides). Inoculation is carried out at right angles to the gutter across the plate, starting at the edge of the gutter which is then almost filled with a solution of penicillin containing about 1-2 units per ml. When a fluid preparation (as above) is used in the gutter it is an advantage, before introducing the solution, to seal the lower edges of the gutter to the Petri dish by contact with a hot metal rod. After incubation for eighteen to twenty-four hours at 37° C. with the lid of the dish uppermost, the plate is examined for inhibition of growth as in the tests with sulphonamides. Penicillin-sensitive strains of *Staph. aureus* are inhibited for a distance of approximately $\frac{1}{2}$ inch under the conditions outlined above.

The penicillin may be incorporated with melted agar cooled to 50° C. and the mixture used to fill the gutter, but this is not essential. If desired, the control organisms may be of the same species as that isolated from the patient (e.g. a streptococcus, etc.), but reliable comparative results may be obtained by using as a control a *Staph. aureus* of known sensitivity to penicillin.

The size of the inoculum is relatively unimportant in testing penicillin sensitivity, and approximately similar results have been obtained by using a loopful of an undiluted fluid culture, or of a 1 in 400 dilution (in broth) of the culture, or by taking the inoculum from a single colony of a plate culture.

When testing for sensitivity either to penicillin or to sulphonamides the medium used should not be less than 3 mm. in thickness.

More detailed and accurate observations upon sensitivity to penicillin (*e.g.* possible changes in sensitivity of the infecting organisms during penicillin treatment) may be carried out by observing repeatedly the range of inhibition of the organism with graded dilutions of penicillin.

Filter-Paper Method of Testing the Sensitivity of Bacteria to Penicillin

Divide an agar plate with a pencil line on the glass. On the one half spread an inoculum of the organism to be tested. On the other half spread a known control organism such as the Oxford strain of staphylococcus (p. 24). Make up a solution of penicillin containing about 100 units per ml. Dip into it a strip of sterile filter-paper or blotting-paper about $3\frac{1}{2}$ in. long and $\frac{1}{16}$ in. wide, and wipe the lower end of the paper on the bottle or tube of penicillin solution so that there is no drip. Lay the strip on the culture medium across the centre of the plate at right angles to the pencil line. Incubate the plate and note the inhibition of growth on either side of the filter-paper as compared with the standard. A non-sensitive organism will grow right up to the edges of the paper.

The Use of Impregnated Filter-Paper Disks for Determining the Sensitivity of Bacteria to Antibiotics¹

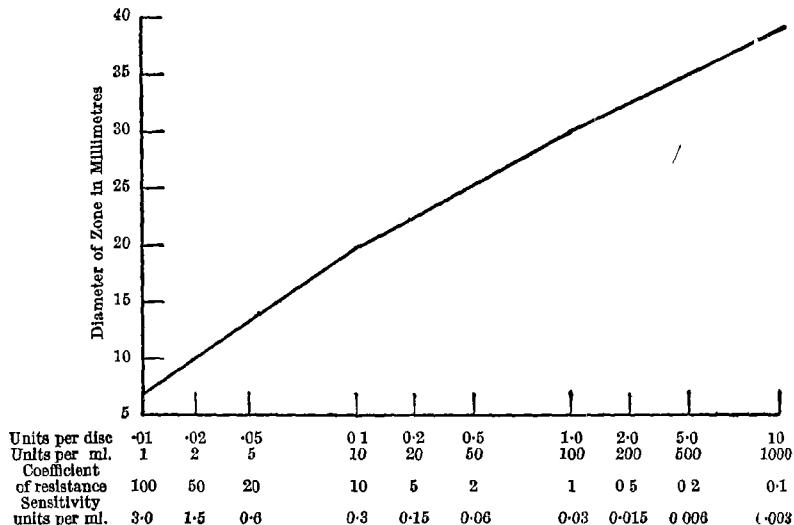
This constitutes a simple and reliable technique specially applicable in routine bacteriological work. It consists in impregnating small disks of a standard filter-paper with given amounts of an antibiotic, placing them on plates of culture medium inoculated with the organism to be tested, and after incubation determining the degree of sensitivity by the easily visible and measurable 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 1 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 3 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.

¹ Gould, J. C., and Bowie, J. H., *Edin. Med. J.*, 1952, 59, 178.

Standard graphs for each antibiotic are prepared by testing disks containing varying amounts with a standard organism of known sensitivity, e.g. *Staph. aureus*. The organism is grown for 18 hours in broth and diluted to approximately 100 million bacterial cells per ml. by comparison with opacity standards (p. 275). 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 30 minutes. 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. Along the abscissa the sensitivity in units or μg . per ml. is denoted (see graph for penicillin).



Standard Graph for Penicillin

(Reproduced by permission of the *Edinburgh Medical Journal*)

In this way according to the measured areas of inhibition in tests with unknown organisms a reading can be made from the graph of the degree of sensitivity as units or μg . per ml.

In testing unknown organisms a suitably diluted broth culture is used for inoculating the surface of a plate (as already described) and disks containing the following amounts of antibiotics are placed on the surface : penicillin 1 unit ; streptomycin 10 μg . ; chloromycetin 25 μg . ; aureomycin 50 μg . ; terramycin 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 areas of inhibition which are easily measurable.

A provisional determination of sensitivity adequate in many cases for clinical purposes can be made by placing disks on plates suitably inoculated directly from the clinical specimen ; this obviates the delay involved in tests with pure subcultures.

For full details of the method and its experimental basis the paper cited should be consulted ; this paper also includes standard graphs for the other antibiotics referred to.

It should be added that the testing of multiple antibiotics on the same plate by the technique discussed necessitates some means of identifying the disks containing the respective antibiotics. Bowie and Gould¹ have adopted a system of denoting the antibiotic by the coloration of the particular disk. For this purpose a series of cotton dyes (obtained from T. B. Ford Ltd.) are used. These are "fast" in the paper, do not interfere with the activity of the antibiotic and in the concentrations recommended do not exhibit antibacterial properties. For details of this system the paper cited should be referred to.

Method of testing sensitivity of tubercle bacillus to streptomycin.

Streptomycin has a beneficial effect in cases of tuberculosis infection if the organism is sensitive to the drug. Unfortunately, however, tubercle bacilli may rapidly become resistant to streptomycin. It is essential, therefore, during treatment to test at intervals the sensitivity of the infecting organism, so that adequate doses may be given or the administration of the drug may be stopped if no inhibitory effect is being exerted on the tubercle bacilli present.

A. A method has been described in a Report by the Pathological Sub-Committee of the Streptomycin in Tuberculosis Trials Committee of the Medical Research Council.² The details are given in full and the article should be consulted by those wishing to carry out the test. The tubercle bacillus is first isolated on Löwenstein-Jensen medium (p. 178), as indicated on p. 404, either direct from the sputum or other exudate, or from tuberculous lesions in inoculated guinea-pigs. The growth on Löwenstein-Jensen medium is much too tough and tenacious to be emulsified into a homogeneous suspension. This culture is transferred to Dubos' medium (p. 187), in which the tubercle bacillus grows quickly and diffusely, the "Tween 80" in the medium having a dispersive effect on the granular growth.

The growth of the isolated organism is compared with a standard human virulent strain (H37Rv) in a series of bottles of Dubos' medium to which known varying amounts of streptomycin have been added. The tests are performed in duplicate. The sensitivity of the strain is expressed as the lowest concentration of streptomycin per ml. giving

¹ Bowie, J. H., and Gould, J. C., *J. Clin. Path.*, 1952, 5, 356.

² Medical Research Council Sub-Committee Report, *Lancet*, 1948, 2, 862.

complete inhibition of growth. The relationship of this end-point to the end-point of the standard strain is also noted, and the "number of times less sensitive than the standard strain" determined. Thus a strain of tubercle bacilli may be expressed as being "three times less sensitive than H37Rv."

B. The following method is recommended for general routine purposes.¹ A modification of Herrold's medium² is used: add 20 ml. glycerol to 1 litre of melted nutrient agar; allow the glycerol-agar mixture to cool to 50° C. and then add 10 ml. 2 per cent. malachite green and the broken yolks of eight fresh eggs. After the addition of the egg-yolk it is important to maintain the temperature of the medium at about 50° C. until all manipulations are complete and to have all containers, measuring cylinders, filtering gauze and distributing funnel warm. The medium is sloped in screw-capped bottles.

Four sets of media are prepared, containing (1) 10 µg., (2) 100 µg., (3) 1000 µg. of streptomycin per ml., (4) no streptomycin (for control purposes). The streptomycin is added before the final bottling of the medium.

Direct cultivation from the pathological specimen is carried out in bottles of media containing the different concentrations of streptomycin, along with the control, and these are examined after 2, 3 and 4 weeks. If growth is scanty or irregular, the control culture is suspended in 0.5 ml. sterile distilled water and inoculated on a further series of media (as above).

Tubercle strains which grow on the control medium and not on the others are reported as streptomycin-sensitive. Those which grow on the medium containing 100 µg. as abundantly as on the control are reported as streptomycin-resistant. Those strains which yield a diminished growth on media containing 10 µg. or 100 µg. are reported as partially resistant; in such cases streptomycin treatment may still be effective.

C. Sensitivity tests can be carried out by incorporating varying amounts of streptomycin in the Löwenstein-Jensen medium when it is being made.³ After coagulation and sterilisation of the medium only about a third of the streptomycin remains. It is convenient to add 10 µg. per ml. to the egg mixture, so that approximately 3 µg. per ml. are present in the finished product. Part of the same egg mixture should be solidified without streptomycin, to act as controls. The streptomycin in the medium should last for 2-3 months at room temperature.

Procedure.—Inoculate control and streptomycin bottles of Löwenstein-Jensen medium direct from the specimen, if culture results are likely to be positive. On incubation a streptomycin-resistant tubercle bacillus will grow in both bottles. A streptomycin-sensitive strain will show only on the control medium, or a very sparse growth may

¹ Personal communication by Dr. A. T. Wallace.

² Herrold, R. D., *J. Inf. Dis.*, 1931, 48, 236.

³ Holt, H. D., and Cruickshank, R., *Month. Bull. Min. Hlth. and P.H.L.S.*, 1949, 8, 103.

appear late on the streptomycin medium. If the organisms are scanty, as in cerebro-spinal fluid, grow first on plain Löwenstein medium until colonies appear. Then inoculate control and streptomycin bottles.

An *alternative procedure* is to cover the surface of the finished Löwenstein-Jensen medium with streptomycin. Prepare a solution of streptomycin so that 4 drops equal 4 µg. per ml. of the medium; that is, 5 ml. of medium needs 20 µg. of streptomycin in the 4 drops. First remove the water of condensation from the bottle of medium, place 3 drops on the surface, and one at the base in place of the condensation water. Leave the bottles in the horizontal position for half an hour before inoculating. Inoculate these and control bottles from each sample as above.

A further modification of these procedures is to place the streptomycin at the foot of the slope, thus allowing for its vertical diffusion in the medium. The usual condensation fluid is replaced with 0·5 ml. of a solution containing 50 µg. of streptomycin. The slope is inoculated from the top down to the level of the fluid. Resistant strains will grow practically to this level, whereas sensitive strains show a well-marked zone of inhibition extending upwards from the fluid. A control test with a known sensitive strain should be made simultaneously for purposes of comparison or contrast.

Method of testing sensitivity of tubercle bacillus to isoniazid (isonicotinic acid hydrazide).

A series of slopes of Löwenstein-Jensen medium, containing 0, 0·2, 1, 5, 10 and 50 µg. of isoniazid per ml., is prepared. (The isoniazid solution used for the purpose is sterilised by Seitz filtration.) The inoculum is obtained from the culture to be tested by shaking for 15 minutes a loopful of the growth in 0·3 ml. sterile distilled water with two glass beads in a sterile $\frac{1}{4}$ -oz. screw-capped bottle and a loopful of the suspension is spread on each slope of the series. The cultures are incubated for 14 and 28 days. Readings at both these periods are recorded. A parallel control test with the strain of tubercle bacillus H37Rv (p. 310) is also made. If growth occurs to any extent on the slopes containing 1 µg. or more the strain is regarded as resistant (see Interim Report, Tuberculosis Chemotherapy Trials Committee, M.R.C., *Brit. Med. J.*, 1952, 2, 735).

TITRATION OF PENICILLIN ACTIVITY IN BLOOD (OR OTHER BODY FLUIDS) DURING TREATMENT¹

When systemic treatment with penicillin is carried out its activity in the blood may be estimated to ascertain whether an adequate content of penicillin is being maintained.

¹ For micro-methods of estimating penicillin in serum and other body fluids, the reader should consult the following papers: Fleming, A., *Lancet*, 1944, 2, 620; and Fleming, A., Young, M. Y., Suchet, J., and Rowe, A. J. E., *Lancet*, 1944, 2, 621; Fleming, A., and Smith, C., *Lancet*, 1947, 1, 401.

The following method, which has been used in the Bacteriology Department, Edinburgh University, is recommended.

Blood is withdrawn by vein puncture and the serum separated in the usual way, being centrifuged if necessary so that it is completely free from suspended red cells. In drawing blood and carrying out the necessary manipulations all the necessary precautions should be taken to avoid contamination.

The test is carried out as soon as possible after withdrawal of the blood, but reliable results can be obtained with serum kept at 0°–4° C. for periods up to twenty-four hours.

To each of a series of sterile stoppered tubes ($3 \times \frac{1}{2}$ in.) 0.3 ml. of varying concentrations of the serum is added as follows :—

1	2	3	4	5
Serum : undiluted.	dil. 1 in 2	1 in 4	1 in 8	1 in 16

The dilutions are made in sterile broth and are prepared with graduated pipettes as in serological tests involving the preparation of a series of doubling dilutions (p. 245 *et seq.*) ; pipettes used must be sterile, the usual technique being adopted to ensure sterility of all materials employed. A control tube containing 0.3 ml. of broth is included in the series. To each tube is then added a large loopful (loop of 3 mm. diameter) of a 1 in 300 dilution (in broth) of a twenty-four hours' broth culture of a standard strain ("Oxford H") of *Staph. aureus*.

The tubes are incubated for eighteen to twenty-four hours, when readings of the resulting growth, or absence of growth, are made according to the visible turbidity or absence of turbidity.

In this way the "bacteriostatic" level of the blood serum can be determined within twenty-four hours. If necessary, these results can be confirmed or checked by taking a loopful from each tube after thorough shaking of the contents and by making single-stroke inoculations in parallel on a 4-in. plate of agar or blood-agar, the position of each stroke being indicated by dividing the outside of the plate into divisions with a grease pencil. The plate will accommodate six such divisions, one for the stroke inoculation from each tube. The presence or absence of growth in the tubes and the relative amount of growth when there is partial inhibition are thus conclusively determined after twenty-four hours' incubation of the plate.

It should be noted that normal serum may produce some degree of bacteriostatic action, though this is usually slight, and it is therefore necessary to test the serum (as above) before the commencement of penicillin treatment. This result serves as a basis for comparison.

It may be said that, in general, satisfactory systemic administration of penicillin will yield results in the test of the order of those indicated below, the relative amount of growth being indicated by the number of + marks.

1	2	3	4	5
Serum : undiluted.	dil. 1 in 2	1 in 4	1 in 8	1 in 16
—	—	to	to	to
		++	+++	+++

With normal serum the *usual* results are as follows :—

1	2	3	4	5
+				
or	+++	+++	+++	+++
++				

An approximate estimate of the penicillin content of undiluted patient's serum may be obtained from consideration of the following data : one unit of penicillin in 20 ml. of nutrient broth usually inhibits completely the growth of the standard strain of *Staph. aureus* used in the above test and also in assay tests on penicillin solutions. If it is assumed that one unit of penicillin in 20 ml. of patient's serum has approximately the same inhibitory power against the standard strain of *Staph. aureus* as one unit of penicillin in 20 ml. of broth, it may be stated that when undiluted serum completely inhibits the growth of the test organisms in the bacteriostatic test, then there is approximately $\frac{1}{20}$ th of a unit of penicillin present in 1 ml. of the serum ; when the inhibition titre is 1 in 2 there is approximately $\frac{1}{40}$ th of a unit in 1 ml. of serum ; when the titre is 1 in 4, $\frac{1}{80}$ th unit, and so on. Estimations of the penicillin content of serum based on the considerations outlined above must be regarded as approximate only since factors such as the inhibiting power of the serum itself against the test organisms may obviously influence the result.

ESTIMATION OF STREPTOMYCIN IN SERUM, CEREBRO-SPINAL FLUID AND OTHER BODY FLUIDS

Method 1.

This method,¹ recommended by a Medical Research Council Sub-Committee, can be used when a rapid clinical assay only is required. When a more precise determination is necessary, method 2, of Mitchison and Spicer (*vide infra*), should be employed.

Test Organism.—A strain of Pneumobacillus (*Klebsiella pneumoniae*, 3, Nat. Coll. Type Cults.).

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

Indicator System.—Boil 5 ml. of the indicator medium in a test-tube. Cool. Add 0·1 ml. of a 24-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.

¹ Fielding, J., *Brit. Med. J.*, 1947, 1, 136; May, J. R., Vourekas, A. E., and Fleming, A., *Brit. Med. J.*, 1947, 1, 627; Medical Research Council Sub-Committee Report, *Lancet*, 1948, 2, 862.

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 30 minutes.

Flame the surfaces of two waxed slides (p. 342) 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 24 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 ml.

Method 2.

This method, devised by Mitchison and Spicer,¹ gives very accurate determinations of the streptomycin content. The details of the method are important and should be adhered to.

The principle of the method is that agar inoculated with *Staphylococcus aureus* is placed in a narrow tube sealed at one end ; the fluid

¹ Mitchison, D. A., and Spicer, C. C., *J. Gen. Microbiol.*, 1949, 3, 184.

to be tested is pipetted on to the surface of the agar column, and the tube is incubated; the streptomycin diffuses through the agar and causes a zone of inhibition of growth, the depth of which is related to the amount of streptomycin present; from the depth of this zone, compared with similar tubes in which known amounts of streptomycin have been added, the concentration of streptomycin in the fluid to be tested can be accurately determined.

Materials required.

Test Organism.—*Staphylococcus aureus* (strain No. 7361, Nat. Coll. Type Cults.) is used, although the "Oxford" strain, used for testing penicillin, can also be employed. The staphylococcus is maintained by daily subculture in Lemco broth (peptone 1 per cent., Lab-Lemco 1 per cent., sodium chloride 0·5 per cent.). An 18-hours broth culture is used, and if kept in the refrigerator can be used up to one week.

Agar Medium.—Add digest-broth agar (2 per cent. Japanese agar) to an equal volume of 1 per cent. peptone, and adjust the pH to 7·8-8·0. Distribute in 19 ml. amounts in 1-oz. screw-capped bottles and sterilise by autoclaving.

Glass Tubes.—Select glass tubing with an internal bore of 3 mm. and cut into 7-8 cm. lengths. Seal one end in the flame and sterilise in bulk at 160° C. for 1 hour. After use the tubes are cleaned by boiling in "Kin-Ray" solution,¹ then boiled in dilute hydrochloric acid and finally rinsed in distilled water.

Microscope.—The zone of inhibition is read with a microscope having a vernier scale fitted to its mechanical stage, and a cross-wire in the eye-piece.

Procedure.

- (1) Melt a suitable quantity of agar and keep melted in a water bath at 45° C.
- (2) Prepare the fluids to be tested and make up the controls as follows:—Serum and cerebrospinal fluid need no further preparation, but urine samples have to be diluted, since urine usually contains a high concentration of streptomycin. Adjust the urine samples to pH 7·8, using aqueous cresol red as the external indicator and dilute 1 in 10 or 1 in 100 in M/15 phosphate buffer solution at pH 7·8.

For controls two different streptomycin concentrations are used, and where the approximate result is known an upper and lower limit can be determined. If the range of values is unknown, make up controls of 4 and 64 µg. per ml. When testing serum make up the controls in human serum; with cerebro-spinal fluid in distilled water; with urine in the pH 7·8 buffer solution.

¹ See footnote, p. 125.

- (3) Place a roll of plasticine on the bench, and insert vertically 4 tubes for each unknown specimen, and 6 tubes for each control.
- (4) Grow a culture of test *Staphylococcus aureus* in broth overnight at 37° C., and add 0·1 ml. to 10 ml. of sterile water. Add 1 ml. of this to 19 ml. of melted agar at 45° C., thus giving a final dilution of 1 in 2000.
- (5) Shake the agar vigorously to mix thoroughly the inoculum, and with a wide-bore capillary pipette distribute the agar rapidly into the tubes so that the column of agar is 2–3 cm. long. If a little agar is sucked back as the pipette is withdrawn, an evenly shaped meniscus will be obtained.
- (6) Allow the agar to set for at least 5 minutes, and it is important that the agar is thoroughly set. Pipette gently the control and test fluids as described under (2) on to the tops of the agar tubes. The quantity added is immaterial provided it produces a layer 2 mm. deep.
- (7) Incubate the tubes overnight at 37° C. in a rack. The depth of inhibition is not altered if the incubation time is longer.
- (8) Read the depth of the zone of inhibition, using the millimetre vernier scale on the mechanical stage of the microscope. Make a small ridge of plasticine on each end of a microscope slide. Place this slide on the mechanical stage, and lay the tubes in succession across the two ridges. Focus the cross-wire of the eye-piece on the centre of the meniscus between fluid and agar, using the $\frac{1}{2}$ -in. objective and reduced lighting. Take the reading on the scale to the nearest 0·1 mm. Traverse the stage until the crosswire is at the edge of the growth. Measurements end where the colonies end abruptly. The difference between the two readings gives the depth of the zone of inhibition.
- (9) Take the figure so obtained for each tube. Find the average for each group of tubes. Square this figure. Prepare a graph with the squares of the average zone of inhibition as the ordinate and the \log_{10} of the streptomycin concentration as the abscissa. Plot the two control points on this graph and join them with a straight line. Use this line for reading the \log_{10} of the streptomycin concentration in the unknown fluid. The actual value is obtained by conversion, using an antilog table.

By this method an assay of streptomycin content can be obtained to an accuracy of ± 5 per cent.

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 No. 6346, Nat. Coll. Type Cults.) 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 (pp. 24, 312).

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 14 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 48 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 . . .

the container or packet shall be opened and the sample removed with aseptic precautions ;

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 ;

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.;

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; . . .

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;

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. 192) 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. 209).

For details of present requirements in the application of sterility tests to Surgical Catgut reference should be made to the latest Therapeutic Substances Regulations (1952).

COLLECTION AND FORWARDING OF SPECIMENS FOR BACTERIOLOGICAL EXAMINATION

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. by $\frac{3}{8}$ 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. 321 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 (*vide supra*) is suitable. A small "spoon" made of tin plate 3½ in. \times ¾ 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. 439). 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. 160) 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 lbs. pressure.

Urine.—For small quantities of urine—*e.g.* from cases of enteric fever, cystitis, etc.—the Universal container (*vide supra*) 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 hours' specimens, the 20-oz. screw-capped bottles are convenient (p. 148).

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. 149) may be used.

Blood for Blood Culture.—Blood can be placed in a sodium citrate "biochemical bottle" (*vide infra*) or preferably added directly to the medium in the special blood-culture bottle described on p. 224.

Biochemical bottles.¹—For biochemical examinations of blood it is convenient to place the specimen in a 1-oz. screw-capped bottle (p. 148) 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 mgm. 10 mgm.	Red label	For blood sugar (venous blood), inorganic phosphorus, uric acid, and non-protein nitrogen tests
Neutral potassium oxalate	40 mgm.	Blue label	For blood urea, and the majority of blood tests
Sodium citrate .	0·3 ml. of 20 per cent. solution (about 60 mgm.)	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 mgm. 15 mgm.	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

¹ See McCartney, J. E., and Ayling, T. H., *Lancet*, 1935, 1, 1388.

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.

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.

<i>Anticoagulant</i>	<i>Amount</i>	<i>Distinguishing mark</i>
Sodium fluoride 10 mgm. Thymol 1 mgm.	11 mgm.	Red label
Neutral potassium oxalate .	10 mgm.	Blue label
Sodium citrate 15 mgm.	1 drop of 20 per cent. solution	Mauve label
Sodium fluoride 5 mgm. Potassium oxalate 4 mgm.	9 mgm.	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 peldorf 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 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. 127). 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 meningococcal carriers and for the early diagnosis of whooping cough (p. 498).

West's post-nasal swab is described on p. 367.

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 dysentery cases, especially in 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 *H. pertussis*, it is advantageous to place about $\frac{1}{2}$ 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

¹ Cockburn, W. C., and Holt, H. D., *Monthly Bull. Min. Hlth. and P. H. Lab. Service*, 1948, 7, 156.

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. 373.

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.

Material in formol-saline or other fixative for sections should be sent in any of the above screw-capped containers.

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. 204) 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.

P A R T III

The Pathogenic and Commensal Micro-Organisms (including the Filterable Viruses) and Bacteriological Diagnosis

CHAPTER X

THE PYOGENIC COCCI AND OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS; DIPLOCOCCUS PNEUMONIAE

PYOGENIC COCCI

THESE organisms are found in various inflammatory and suppurative conditions. The most important are *Staphylococcus aureus* and *Streptococcus pyogenes*.

STAPHYLOCOCCUS AUREUS or MICROCOCCUS PYOGENES var. AUREUS

Morphology and Staining.—Gram-positive spherical cocci arranged in irregular clusters, the individual cells being approximately $0.8\text{--}0.9\mu$ in diameter. Single forms and pairs may also be noted.

Cultural Characters.—Aerobe and facultative anaerobe ; temperature range, $10^{\circ}\text{--}42^{\circ}$ C. ; optimum, about 36° C. ; growth occurs on ordinary nutrient media.

Agar stroke—thick, opaque, moist, shiny, “oil-paint”-like growth which develops a characteristic golden- or orange-yellow colour.

Colonies on agar—circular disks, relatively large even after 24 hours (2–4 mm. in diameter) and presenting the same characters as the stroke culture.

Blood-agar—colonies similar to those on agar but somewhat larger ; definite orange-yellow colour ; marked zones of haemolysis (*vide infra*) on sheep or rabbit blood agar, particularly in an atmosphere containing 25 per cent. carbon dioxide.

Gelatin—growth in this medium is associated with liquefaction due to a proteolytic enzyme (gelatinase).

Broth—uniform turbidity, with subsequent orange-coloured deposit.

Coagulated serum is softened or liquefied.

Various carbohydrates are fermented (with acid but without gas production)—e.g. glucose, lactose, sucrose, mannitol.

Potato medium—growth is similar to that on agar ; pigmentation is specially marked.

Milk—acid formation and coagulation.

Nitrates and methylene blue are reduced, and urea is hydrolysed.

MacConkey's medium—colonies show a characteristic colour, the yellow pigment being tinted pink by the acid change of the indicator (neutral red).

A product designated *coagulase* is characteristic of many strains of *Staph. aureus*; it appears to be the precursor of a thrombin-like substance and coagulates blood plasma; it can be tested for by adding one drop of a broth culture to citrated rabbit plasma and incubating the mixture at 37° C. for three hours. The occurrence of this product is generally accepted as indicative of the pathogenicity of the strain (*vide infra*).

Another method of testing (Fisk) which can be recommended is as follows : (1) dilute citrated human plasma 1 in 10 with normal saline ; (2) place 0.5 ml. of diluted plasma in two small test-tubes ; (3) to one tube add 5 drops (approx. 0.125 ml.) of an overnight broth culture or a broth suspension of an agar culture (opacity equal to that of the broth culture) ; (4) incubate tubes at 37° C. ; (5) examine the tubes after half an hour and at intervals for six hours. Clotting usually occurs within an hour. The second tube serves as a control and should show no clotting.

Phosphatase test.—It has been found that there is a certain degree of correlation between phosphatase and coagulase production by staphylococcal strains. The detection of the former in direct plate cultures has been suggested for the exclusion of non-virulent strains as a substitute for the coagulase test, e.g. cultures from the nose of possible carriers of pathogenic staphylococci. For the purpose an agar medium is used incorporating phenol-phthalein diphosphate. Organisms producing phosphatase liberate free phenol-phthalein which can then be detected by exposing the plate culture to ammonia vapour, the growths becoming bright pink (p. 159). For details of the method, medium and results see Barber, M., and Kuper, S. W. A., *J. Path. Bact.*, 1951, 63, 65.

Viability.—The thermal death-point is about 62° C., but some strains are more resistant to heat, e.g. withstanding even 70° C. for a short time. Laboratory cultures survive for months. The organism is fairly resistant to drying, e.g. surviving for months in the dry state in the absence of light. It is killed within a few minutes by 2 per cent. phenol. Like many other Gram-positive organisms, *Staph. aureus* is very sensitive to various organic dyes, e.g. crystal violet, brilliant green and the flavine dyes, e.g. proflavine. Thus, brilliant green is bactericidal in a concentration of 1 : 10,000,000, though in the presence of albuminous matter, e.g. the serous exudate of a wound, its activity is greatly reduced. The antiseptic action of proflavine, however, is maintained in the presence of serum and under these conditions it is bactericidal in a concentration of 1 : 200,000. These

substances are only slightly toxic and proflavine can be used as a wound antiseptic in a 1 : 1000 aqueous solution.

As a general rule *Staph. aureus* is exceedingly sensitive to penicillin, a highly purified preparation of which may prevent growth even in a dilution of 1 : 30,000,000. Relatively resistant strains, however, are frequently met with.

OTHER TYPES OF STAPHYLOCOCCI

Staphylococcus albus.—Growth similar to that of *Staphylococcus aureus*, but unpigmented and white in colour. This type is generally less active than the *aureus* strains in the liquefaction of gelatin, fermentation of sugars and haemolysin production; on blood agar, may or may not show a zone of haemolysis. Many strains are not coagulase-producers and are quite non-pathogenic.

It should be noted, however, that colonies of the *albus* type may occur as a variant in cultures of *Staph. aureus*; and non-pigmented strains are sometimes coagulase and toxin producers, and actively pathogenic. These have been designated by American systematists *Micrococcus pyogenes* var. *albus*. Non-pathogenic strains of staphylococci corresponding to *Staph. albus* e.g. those occurring as commensals on the skin, have been named *Micrococcus epidermidis*.

***Staphylococcus citreus* or *Micrococcus citreus*.**—An uncommon and mainly saprophytic type; differentiated from others by lemon-yellow colour of growth; does not generally liquefy gelatin.

“*Staphylococcus ascoformans*.”—This organism is associated with the condition of “botryomycosis” occurring in equines. In the tissues the cocci are frequently capsulate and, especially in chronic lesions, occur in zoogloea-like masses or clusters. These aggregates may resemble in naked-eye appearance actinomycetes “granules” (p. 527). 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.

Anaerobic staphylococci have occasionally been described as “commensals, and in pyogenic infections.

PATHOGENICITY AND OCCURRENCE OF STAPHYLOCOCCI

Toxins.—Certain strains of the *aureus* and *albus* types are markedly toxicogenic. Culture-filtrates are haemolytic (when mixed with blood suspensions), kill leucocytes (when added to a preparation of separated leucocytes), produce necrosis of tissue (when injected into the skin) and exert a rapidly lethal effect on intravenous injection. Thus, *haemolysin*,

leucocidin, *necrotoxin* and a *lethal toxin* can be demonstrated in cultures.

Different types of staphylococcal haemolysin can be recognised ; the "α" type produces rapid lysis of rabbit and sheep red cells at 37° C. ; the "β" type 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). A third haemolytic factor, designated γ, has also been described, and a fourth haemolysin, δ, has recently been defined. According to Elek and Levy the γ and δ factors are probably identical. These haemolysins can be demonstrated by testing with horse or human erythrocytes. The α, β and δ factors are antigenically distinct. The α haemolysin, however, is probably identical with the necrotoxic and lethal factors referred to above. The α and δ haemolysins seem to be characteristic of strains of human origin ; the β haemolysin is produced by strains isolated from animals. These three haemolysins are generally associated with coagulase production, and coagulase-negative strains do not produce them (Elek and Levy).

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's peptone, 0.2 per cent. potassium dihydrogen phosphate and 0.03 per cent. magnesium sulphate ; the pH is adjusted to 6.8. 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 M/15 potassium dihydrogen phosphate solution) ; the culture is finally incubated for three days in 25 per cent. carbon dioxide as before.

Certain strains present in lesions of the human subject produce *fibrinolysin* ; and *hyaluronidase* is formed by strains possessing marked invasive properties. These bacterial products are referred to later in relation to the streptococci (p. 336).

Cases of *food-poisoning* have been reported which are due to an exotoxin of staphylococci growing in the incriminated article of food, and pronounced local and general toxic effects have been observed in experimental animals under certain conditions after the introduction of staphylococcal culture-filtrates into the stomach. In certain cases milk and milk-products, *e.g.* cream cakes, custard, ice-cream, have been responsible for such poisoning, in other cases preserved meats, fish and gravies. The occurrence of staphylococci on the skin and in the nose and throat provides frequent means of extraneous contamination of foods by such organisms, but

when milk and milk-products are responsible for the poisoning, the staphylococci may have been derived from the milk itself, e.g. from an udder lesion in the cow. The production of toxin will depend, however, on conditions favouring the growths of the organism in the food, e.g. keeping the food at room temperature (particularly in the warm season of the year) for a considerable time before consumption. This *enterotoxin* is different from the other staphylococcal toxins. It is highly thermostable and can resist boiling for a short period.

It has been claimed that strains producing this toxin can be identified by injecting a culture-filtrate intraperitoneally in a kitten, the haemolysins having been first inactivated by heat. The animal develops symptoms of gastro-enteritis, vomiting and diarrhoea, within a few hours. Intravenous injection in a cat may be substituted for this test. The complete reliability of these tests for the purpose of identifying enterotoxic strains has been questioned.

The production of enterotoxin is generally associated with the formation of coagulase and haemolysin.

Experimental Inoculation.—Strains of *Staph. aureus* isolated from 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 a pyaemic condition with multiple abscesses in the kidneys, lungs, myocardium and other organs. Mice and guinea-pigs can also be successfully infected but are somewhat less susceptible than rabbits.

Occurrence.—Localised abscesses, wound suppuration, mastitis, skin pustules, furuncles and carbuncles, pemphigus neonatorum, blepharitis, mucous catarrhs, acute osteomyelitis and periostitis, septicaemia, pyaemia, urinary sepsis, etc., *Staphylococcus aureus* is the type usually present in pyogenic lesions, though *albus* strains are sometimes found to be pathogenic.

The staphylococci generally are common commensals of the skin, mouth, nose (*particularly anterior nares*) and throat, especially the *albus* type, and are found in air and dust and on clothing, and on eating and drinking utensils. The *aureus* type is the most virulent; the others are either non-pathogenic or of relatively low virulence.

Occurrence in domestic animals.—Staphylococci occur as commensals in animals and also produce inflammatory and suppurative lesions similar to those found in the human subject, though staphylococcal infections are on the whole less frequent in animals. These organisms are responsible for such conditions as botryomycosis (*vide supra*) and pyaemia of lambs (associated with tick infestation), and they may be found in certain cases of bovine mastitis.

The natural classification of the staphylococci presents some

difficulty. There is now a good deal of evidence that coagulase production is characteristic of the pathogenic strains, among which both *aureus* and *albus* forms are represented. These strains are usually mannitol-fermenters and produce haemolysin. It has been suggested that they should be designated *Staphylococcus aureus*, irrespective of the colour of the growths, on the basis that white colonies may occur as variants from the typical pigmented organism (Shaw, Stitt and Cowan), while coagulase-negative strains should be classified as *Staphylococcus saprophyticus*. Reference has been made above to the nomenclature of the American classification.

It has also been shown that among the pathogenic strains, several types and subtypes can be recognised by agglutination tests with antisera, whereas non-pathogenic strains cannot be assigned to these serological types and are more heterogeneous. Further, a specific polysaccharide is stated to be characteristic of the pathogenic strains and absent from other staphylococci; it can be identified by the precipitation reaction with an extract of culture and a specific antiserum.

Bacteriophage Types.—Strains of staphylococci may carry a phage which is lytic to other strains; by means of potent phage preparations strains can be typed as in the case of typhoid and paratyphoid bacilli (p. 436) and this has proved to be of practical value in epidemiological investigations for tracing the spread of the organism.

These staphylococcal phages are not specific for individual types of staphylococci (*cf.* typhoid bacillus, p. 436), but by means of selected phages a "pattern" as it were of positive and negative results can be elicited and from this the phage-type of a particular strain can be determined. (For details of the methods applicable, see Williams, R. E. O., and Rippon, J. E., *J. Hyg.*, 1952, **50**, 320.)

Immunisation.—For the treatment of chronic or recurrent staphylococcal infections, stock and autogenous vaccines have been extensively applied in the past with somewhat variable success. Staphylococcal toxoid has also been advocated for immunisation in such cases. Staphylococcal antitoxin has been used in the treatment of the more acute and severe infections. These methods of therapy have been superseded by antibiotic treatment.

Antibiotic Therapy.—Highly successful therapeutic results are obtained with penicillin in the treatment of even severe staphylococcal infections provided the strains of staphylococci responsible for these cases are sensitive to the antibiotic, but as indicated above a proportion of strains isolated from carriers and from lesions are relatively resistant. In such cases another antibiotic may be used, *e.g.* aureomycin, terramycin or chloromycetin. It is specially important that in a particular case the strain should be tested for sensitivity to penicillin or other antibiotic it is proposed to use.

STREPTOCOCCUS PYOGENES

Morphology and Staining.—Gram-positive, spherical or oval cocci, $0.7\text{--}1\mu$ in diameter, in chains of variable length; involution forms may be observed. By relief methods of staining, virulent strains can be shown to have a capsule (p. 88).

Cultural Characters.—Aerobe and facultative anaerobe; temperature range, generally $22^{\circ}\text{--}42^{\circ}$ C.; optimum, 37° C.; grows on ordinary media, but better on blood or serum media.

Agar stroke—growth consists of small, circular, discrete, semi-transparent, slightly convex disks, about 1 mm. in diameter after twenty-four hours' incubation.

Blood-agar—colonies larger than on agar; clear zones develop round them due to the diffusible haemolysin produced by the organism (*vide infra*).

Variation in colony characters may be observed, e.g. the "matt" and "glossy" types of colony, the former type being that noted in recently isolated strains from pathological lesions, the latter representing an avirulent variant. A "mucoid" type of colony may also be observed; in virulence it corresponds to the "matt" form.

Broth—growth usually forms as a granular sediment in the culture tube.

Biochemical Reactions.—Tested by growing in Hiss's medium with 1 per cent. of fermentable carbohydrate, and an indicator of acidity (litmus, Andrade's indicator, or phenol red, p. 172); alternatively, on serum-peptone-water-agar slopes containing the particular carbohydrate and an indicator (pp. 164, 174). The reactions are usually as follows:—

Glucose	Lactose	Sucrose	Mannitol	Salicin	Raffinose	Inulin
• ⊥	⊥	⊥	—	⊥	—	—

(⊥ = acid, no gas; — = no fermentation.)

Other biological characters of *Strept. pyogenes* will be referred to in relation to the classification of streptococci generally.

Viability.—The thermal death-point is about 54° C. The organism can survive for some time in air and dust. Ordinary cultures do not long maintain their viability, but survive best if kept at a low temperature, e.g. 0° C. A convenient method of keeping laboratory cultures is to grow the organism in cooked-meat medium (p. 192) and store the cultures at 0° C. Cultures which have been rapidly desiccated *in vacuo* and kept *in vacuo* in the dark at low temperatures remain viable for long periods (p. 221). Like staphylococci, *Strept. pyogenes* is sensitive to the antiseptic dyes, e.g. proflavine. It is highly sensitive

to the various sulphonamide compounds (though occasionally resistant strains are encountered) and to penicillin and certain other antibiotics.

Toxic Products.—In addition to *haemolysin*, *leucocidin* can be demonstrated in culture filtrates. Recently isolated virulent strains yield also a diffusible product which rapidly lyses human fibrin *in vitro* (*fibrinolysin*). This is probably the activator of a serum protease. Another important toxic product is the *erythrogenic* or *scarlatinal toxin* (p. 346).

Two antigenically different haemolysins are produced, designated "O" and "S" (Todd). "O" is developed when the organism is growing in serum-free broth and is oxygen-sensitive. "S" is not present in serum-free broth and is not oxygen-sensitive, though very susceptible to heat.

Culture-filtrates of some strains also contain an enzymic substance capable of producing marked increase in the permeability of tissues ("diffusion factor"). This product (*hyaluronidase*) hydrolyses hyaluronic acid, which forms the cement substance of tissues. (Various other organisms also yield a similar factor.)

Experimental Inoculation.—Recently isolated cultures are usually virulent to rabbits, guinea-pigs and mice. Subcutaneous inoculation may produce a local inflammatory and suppurative lesion and in some instances a generalised septicaemic infection. Intravenous injection in rabbits produces a septicaemia, or a pyaemic condition with suppuration in joints.

Serological classification.—*Strept. pyogenes* corresponds to Lancefield's Serological Group A (p. 339).

CLASSIFICATION OF STREPTOCOCCI; AND THE VARIOUS TYPES OF THESE ORGANISMS

The streptococci constitute a somewhat heterogeneous group, and, besides the common pathogenic strains, e.g. *Strept. pyogenes*, include types which are normal inhabitants of the mouth, throat and intestine.

These organisms have been broadly classified in medicine according to the *appearance of growths on blood-agar*. Thus, in the case of *Strept. pyogenes*, wide zones of complete haemolysis develop round the colonies (*vide supra*), and this is a characteristic feature. The designation "haemolytic" or " β (beta)" has been applied to this type of streptococcus.

The most reliable method of identifying a haemolytic streptococcus is to grow it in 20 per cent. serum broth for about six hours and then to add varying amounts (e.g. 0.1 to 1 ml.) of the culture to 0.5 ml. of a 5 per cent. suspension of ox or horse red blood corpuscles. The mixtures are incubated at 37° C. for one and a half hours, when haemolysis can be observed.

Certain varieties occurring as commensals in the mouth and throat are non-haemolytic or only partially haemolytic, and their colonies on blood-agar show a zone of greenish coloration, generally regarded as due to peroxide formation and the resultant production of methaemoglobin. The colour change is most marked when the organism is growing on a medium containing heated blood (*e.g.* "chocolate agar") and in this case is probably due to an oxidation product of haematin. Streptococci producing such changes on blood media are designated "*Streptococcus viridans*," or the "*a* (*alpha*)" type. Varieties which have been named *Streptococcus salivarius* and *Streptococcus mitis* are of this category.

It has been claimed that the *viridans* form may under certain conditions be derived as a variant from a haemolytic organism. This may in some cases be a weakly haemolytic derivative, and if the organism is cultivated anaerobically, the variant exhibits the typical β haemolysis.

A third group found as commensals in the mouth, throat and bowel produces no obvious change in blood media and is sometimes designated the " γ (*gamma*)" type.

Morphological differences are of little significance in classification, though the faecal streptococci ("enterococcus" or *Strept. faecalis*) are usually oval in shape, or even lanceolate like a pneumococcus (*q.v.*), and occur in pairs or short chains.

Streptococci producing a mucoid capsule have been described. "*Streptococcus mucosus*."—On blood-agar it yields slimy colonies with dark-green coloration of the blood; it has been noted in various suppurative conditions, *e.g.* otitis media. "*Streptococcus epidemicus*."—This organism is of the haemolytic type, but produces a mucoid capsule and its growth on medium is likewise mucoid. Otherwise it corresponds to *Strept. pyogenes*. It has been reported in milk-borne outbreaks of sore throat.

Biochemical reactions have been extensively utilised for the differentiation of streptococci, but these cannot be regarded as characters on which an accurate classification can be based. Certain biochemical tests, however, are of some significance in the general grouping of the streptococci. Thus, the faecal streptococci are generally mannitol- and aesculin-fermenters: the salivary types usually ferment raffinose. It has been pointed out that among the haemolytic streptococci those of human origin, *e.g.* *Strept. pyogenes*, ferment trehalose but not

sorbitol, whereas strains fermenting sorbitol but not trehalose are usually of animal origin (*vide infra*).

Streptococci differ considerably in their *resistance to heat*, and this has been utilised as a basis of classification. Thus, the faecal strains (*enterococcus*) are able to withstand 60° C. for thirty minutes, whereas this temperature kills the haemolytic streptococci and also the common mouth types.

The reaction of the fluid culture tested for heat-resistance should be neutral.

Other differential features.—The enterococcus is usually able to grow between 10° and 45° C. (*cf. Strept. pyogenes*) and has considerable viability in culture. It can be cultivated also in the presence of 6·5 per cent. sodium chloride and at a pH of 9·6, *i.e.* conditions under which other streptococci cannot grow. The final pH of a growth in 1 per cent. glucose broth is 4·0–4·2, whereas *Strept. pyogenes* yields a final pH of 5·2 (p. 162).

The enterococcus is also capable of growing on media containing bile-salt (*e.g.* MacConkey's), which is inhibitory to other types. A certain proportion of strains of this organism differ from other streptococci in liquefying gelatin. These are sometimes designated *Streptococcus liquefaciens*.

The *Streptococcus lactis* found in soured milk is biologically similar in some respects to the enterococcus, but represents a separate serological group (*vide infra*). It does not grow at pH 9·6 and is not resistant to heat at 60° C. It differs also from the enterococcus in the absence of sucrose fermentation.

Anaerobic or micro-aerophilic streptococci are not infrequently met with. They correspond in their general biological characters to the aerobic forms. Micro-aerophilic haemolytic strains have been found to be associated particularly with spreading necrotic infections of the skin. Most of the anaerobic streptococci are non-haemolytic, and foetid gas formation in culture is a common characteristic of these. Such organisms have been observed in septic lesions and in gangrenous conditions of mucous membranes. They are sometimes present in puerperal sepsis and may produce a blood infection. They have been found normally on mucous surfaces.

The following Table summarises the main differential features of the three medically important groups of aerobic streptococci.

	<i>Pyogenic streptococci</i>	<i>Mouth and throat streptococci</i>	<i>Enterococcus (faecal streptococci)</i>
Morphology .	Chains of moderate length ; cocci tend to be spherical	Chains of pronounced or moderate length ; cocci often elongated	Usually in pairs but may occur in short chains ; cocci oval or lanceolate
Type as regards effect on blood (<i>vide supra</i>)	β	α or γ	usually γ
Mannitol-fermentation	Usually —	—	+
Heat resistance (<i>vide supra</i>)	—	—	+
Inhibition of growth by bile-salt	+	+	—

SEROLOGICAL CLASSIFICATION OF STREPTOCOCCI

LANCEFIELD'S GROUPS.¹—By means of precipitation reactions with antisera, Lancefield distinguished a number of groups of streptococci, and it is now possible to correlate this grouping to some extent with that based on other characters including pathogenicity to man and animals. These precipitation reactions depend on group-specific haptens ("C" substances), which have been defined as carbohydrates.

Group A.— β -haemolytic on blood-agar ; produces O and S haemolysins (p. 336) ; ferments trehalose but not sorbitol (*vide supra*) ; does not hydrolyse sodium hippurate (*cf.* Group B). The strains are of human origin and are associated with infections of the human subject, often of a severe nature. This group includes all Griffith's serological types (*vide infra*) with four exceptions, types 7, 16, 20 and 21. It is the most important group from the medical standpoint, and strains belonging to it must be regarded as pathogenic or potentially pathogenic. Organisms of this group correspond to the classical type described as *Strept. pyogenes*.

Group B.—Includes both haemolytic (β) and non-haemolytic (α and

¹ See Lancefield, R. C., *J. Exper. Med.*, 1933, 57, 571-95; Hare, R., and Colebrook, L., *J. Path. Bact.*, 1934, 39, 429-42; Lancefield, R. C., and Hare, R., *J. Exper. Med.*, 1935, 61, 335-49.

γ) strains as judged by effects on blood-agar; the β -haemolytic strains produce the S haemolysin, but this property is a less frequent feature of Group B than Group A; hydrolyses sodium hippurate. Found in bovine mastitis and milk. Some strains from the human vagina and the throat also belong to Group B. This group is rarely pathogenic to the human subject, but has been recorded in a few cases of puerperal infection assuming a pyaemic form. Streptococci of Group B correspond to the organism designated *Streptococcus agalactiae* (p. 349).

Group C.— β haemolytic and produces the S haemolysin (but α and γ strains also included in group); does not hydrolyse sodium hippurate; many strains ferment sorbitol but not trehalose (cf. Group A). This group is found mainly in animals, but may also occur in human infections, including puerperal sepsis. *Streptococcus equi* of strangles in horses is assigned to this group, but does not ferment sorbitol, trehalose or lactose. Griffith's serological types 7, 20 and 21 belong to Group C (*vide infra*).

Group D.—Originally described as β -haemolytic on blood-agar. Such strains were isolated from human faeces and the vagina, and from cheese, and their relationship to the enterococcus was recognised. The S haemolysin has been demonstrated in this group. It now seems likely that all strains of the enterococcus, irrespective of their haemolytic action, belong to Group D. Griffith's type 16 belongs to this group (*vide infra*).

Group F.— β -haemolytic on blood-agar, and the S haemolysin has been demonstrated (but α strains also included in group). Colonies are minute and growth is slow. Strains have been found mainly in infection of the throat and upper respiratory passages. It has been noted that Group F infection may be associated with nephritis (p. 346).

Group G.—Produces the O and S haemolysins like Group A. The majority of strains have been found as commensals in the human subject, though the group includes pathogenic representatives.

Groups designated *E*, *H* and *K* have been defined. These may occur in the human subject as commensals and occasionally as pathogens, mainly in the upper respiratory passages. Group E has been found in milk. *Groups L* and *M* have been found mainly in dogs and pigs, rarely in man. *Streptococcus lactis* represents a further group designated *N*. This group is quite non-haemolytic. An *O* group has also been defined exemplified by strains found in the human subject.

It will thus be seen that of the strains infecting the human subject the great majority belong to Group A. The value of differentiating these groups is illustrated in relation to puerperal fever in which, with a few exceptions, the infecting strains of streptococci belong to Group A, whereas the haemolytic streptococci which may occur normally in the birth canal belong to other groups.

When a β -haemolytic streptococcus is found either in the parturient woman or attendants, the first point to determine is whether it produces a filterable haemolysin in fluid culture, and then its particular serological group is ascertained.

The *precipitating serum* is prepared by injecting rabbits with known strains of the particular groups. Broth cultures are centrifuged and the deposited organisms killed with 0·2 per cent. formalin, and diluted with this fluid so as to contain 2000 million per ml. The rabbits are given a series of four daily intravenous injections of 1 ml. followed by four days' rest. When the appropriate group serum and a bacterial extract (*vide infra*) containing the specific soluble substance are mixed a profuse flocculation occurs within a few minutes, easily visible to the naked eye.

It is important to secure a potent serum, as the precipitate is soluble in excess of antigen (bacterial extract). A weak serum will give a feeble precipitate which is at once re-dissolved. The antigen is at first tested undiluted; if no reaction occurs it should be re-tested in dilutions of 1 in 3 and 1 in 9. Alternatively, the three concentrations can be tested simultaneously. With a weak serum, a reaction may only be seen with the highest dilution of antigen.

Methods for carrying out the Precipitation Test

Before proceeding with the test the haemolytic property of the strain should be confirmed. Sub-inoculate a haemolytic colony from the plate into 2 ml. of 20 per cent. horse-serum broth and incubate for six hours or overnight. Add one volume (0·5 ml. is a convenient amount) of the culture to one volume of 5 per cent. suspension of horse red corpuscles in saline and incubate the mixture at 37° C. for two hours. Haemolysis is usually complete within thirty minutes. If the streptococcus is haemolytic, an antigenic extract is then prepared.

From the serum-broth culture inoculate 50 ml. of 0·05 per cent. glucose-broth and incubate overnight. Centrifuge the culture and pour off the supernatant fluid so that the sediment of organisms is as dry as possible.

Add 1 ml. of N/20 HCl and two drops of 0·2 per cent. Congo red solution and re-suspend the sediment. Add one or two drops of N/1 HCl until a slate-blue colour is obtained. Transfer the contents to a conical centrifuge tube to which is attached a small piece of adhesive plaster on which the name or number of the culture should be written. Immerse the centrifuge tube in boiling water for ten minutes. The colour of the material should now change to pink. Cool the tube under running water. Centrifuge and remove carefully the supernatant fluid, the sediment being discarded. Add one drop of phenol red solution and neutralise cautiously with N/1 NaOH and re-centrifuge. The resultant clear supernatant fluid constitutes the antigenic extract.

The method of testing is to add varying concentrations of the antigenic extract (*vide supra*) to a given volume of undiluted serum in narrow tubes so that the extract is superimposed on the serum. Precipitation is first observed at the interface of the two reagents usually within five minutes, but becoming more pronounced when the tubes are incubated for two hours and then allowed to stand for a time at room temperature.

If no reaction occurs with Group A serum, the procedure should be repeated with Groups C and G sera, and if necessary with other group sera.

An alternative method which can be recommended is that in which formamide is used in the preparation of the antigenic extract (*Fuller's method*). Add 0·1 ml. of formamide to the bacterial sediment from 5 ml. of culture in horse-digest broth (p. 152), shake the tube thoroughly and place in an oil bath at 150° C. for fifteen minutes. (The metal cup of a thermos flask makes a simple oil bath, olive oil or liquid paraffin being used.) Cool and add 0·25 ml. of acid-alcohol—95 parts of absolute alcohol with 5 parts of 2N hydrochloric acid (1 part of concentrated acid to 4 parts of water). Mix and centrifuge to separate the resulting precipitate. Pipette off the supernatant fluid into a small tube and add 0·5 ml. acetone. Shake the tube, centrifuge and discard the supernatant fluid. Add 1 ml. of saline and a drop of phenol red indicator and neutralise the solution with a trace of sodium carbonate. This constitutes the antigen for the test.

In order to conserve serum, the test may be made in a capillary tube 2 in. long. This form of tube is best obtained by purchasing the capillary tubes used for vaccine lymph and cutting them in two. Make a mark on the capillary tube, about $\frac{1}{2}$ in. from the end, draw up antigen to this mark and expel the drop on a paraffined slide (made by dipping a microscope slide into melted paraffin wax and allowing it to cool in the vertical position). Place a similar volume of Group A serum beside it, and mix with a fine, straight inoculating wire. Hold the slide at an angle, touch a drop of the mixture of antigen and serum with the capillary tube held horizontally, when the fluid will enter the tube. Seal one end of the tube in the flame. Place the tube in a piece of plasticine, or in a block of wood with a number of holes made with a No. 42 Morse drill. Macroscopic precipitation should occur within a few minutes and certainly within half an hour in the incubator. If left overnight, cross-reactions may occur. The procedure can be repeated with different dilutions of the antigen (*vide supra*) and if no reaction occurs the same test is carried out with antisera for Groups C and G, and if necessary, other groups.

The following method devised by Maxted¹ for the preparation of streptococcal extracts is simpler than those described above. A *Streptomyces albus* has been isolated which produces a potent proteolytic substance, presumably an enzyme, capable of dissolving β haemolytic streptococci and so liberating the soluble antigen used for the Lancefield grouping. A culture of this *Streptomyces* is obtainable from the National Collection of Type Cultures.²

(1) Prepare an agar medium as follows :—

" Eupeptone No. 2 "	.	.	0·5	per cent.
" Yeastrel "	.	.	0·3	" "
Glucose	.	.	0·2	" "
Dibasic sodium phosphate (anhydrous)	.	0·2	"	"
Fildes' extract (p. 184)	.	2·5	"	"
Agar fibre	.	1·25	"	"
in distilled water.				

¹ Maxted, W. R., *Lancet*, 1948, 2, 255.

² See footnote, p. 303.

- Place suitable amounts (75–100 ml.) in Roux bottles, or 20-oz. medical flats. Sterilise and set in the usual way. pH before inoculation should be 7·2–7·4.
- (2) Suspend in saline spores from a culture of the *Streptomyces*, and run the suspension over the surface of the agar medium in the Roux bottles.
 - (3) Incubate at 30°–37° C. for 4–6 days.
 - (4) Place the bottles in a –10° C. refrigerator for 12 hours, and then allow to thaw out. This permits the bulk of the fluid to be recovered from the agar gel.
 - (5) Test the pH of this fluid, which contains the active lytic substance (usually about pH 9·0), and adjust to pH 7·5 by adding N/1 hydrochloric acid.
 - (6) Filter through a Seitz disk.
 - (7) Test for effectiveness of the filtrate by adding 0·1 ml. of a heavy suspension of heat-killed Group A streptococci to 0·4 ml. of the proteolytic extract in a $3 \times \frac{1}{2}$ in. tube. Place in a water bath at 50° C. As a control put up a similar mixture, but with heated filtrate (to destroy the enzyme). An active filtrate will lyse the streptococcal suspension in about 30 minutes.
 - (8) Store in the cold with 0·5 per cent. phenol as preservative. The filtrate keeps well and is active over a pH range of 5·6–9·6.

The streptococcal extracts are then prepared as follows :—

- (1) Strains of haemolytic streptococci to be grouped are inoculated by spreading on segments of a blood-agar plate which is incubated overnight.
- (2) Into each of a series of Dreyer's tubes place 0·25 ml. of *Streptomyces* filtrate. Take up a loopful of the blood-agar growth and suspend it in the fluid to make an even suspension. Place the tubes in a water bath at 50° C. until quite clear (approximately 1½ hours). The clear solution is the antigen used to carry out the precipitin tests as already described.

GRIFFITH'S TYPES.—By agglutination and agglutinin-absorption tests streptococci have been found to be exceedingly heterogeneous. Thus, Griffith separated thirty serological types of haemolytic streptococci which he considered of epidemiological significance. Type-specificity depends on a nucleo-protein antigen, designated "M," and another antigen probably of protein nature, "T." Of these types the commonest are 1, 2, 3, 4, 6 and 8. All but types 7, 16, 20 and 21 belong to Group A.

The antisera, which are obtained by injecting rabbits with killed cultures of known type-strains, usually show cross-reactions and have to be purified by absorption with other types. Because of this, such type-sera are difficult to prepare. The agglutination test is carried out on a slide as described on p. 250, the agglutination being visible

in a few minutes to the naked eye. The organisms are grown in serum-broth overnight, the supernatant fluid is removed, and the resultant dense suspension of streptococci used directly. Frequently the streptococcus is "rough," the resultant suspension showing visible clumps of organisms, and the test cannot be carried out. The streptococcus has then to be subcultured repeatedly until a homogeneous suspension is obtained.

It is convenient to do a preliminary test with pooled sera, each representing four to six types. Having found agglutination with a certain pool of sera, the streptococcus is tested with the individual type-sera comprising the pool.

The following alternative procedures can be recommended.

In place of serum-broth for cultivating the streptococci, horse-flesh-digest medium (p. 152) or the glucose-phosphate broth described by Todd and Hewitt (p. 189) may be used. The organisms are cultivated either at 37° C. or 22°–25° C., the latter temperature being preferable for the purpose of obtaining homogeneous or uniform suspensions suitable for the agglutination test. The culture is centrifuged and all but about 0·5 ml. of the supernatant fluid medium is removed. The deposited organisms are re-suspended in the remaining medium. This suspension should be homogeneous, but if it is granular, two drops of B.D.H. Universal Indicator (p. 159) and two drops of pancreatic extract (Cole and Onslow, p. 151) are added. Sufficient N/5 NaOH is then added to turn the indicator pale green, and the suspension is incubated at 37° C. This treatment may render it homogeneous in the course of 24 hours, but if not the streptococcus has to be subcultured repeatedly until homogeneity is obtained.

Slide agglutinations (p. 250) with the various type-sera are carried out and the use of a lens aids the recognition of the results. Cross-reactions sometimes cause difficulty, and it has been found that a precipitation test will then assist in the exact determination of the type; for this test immune sera containing antibodies for the "T" antigens are used (see Swift, H. F., Wilson, A. T., and Lancefield, R. C., *J. Exp. Med.*, 1943, **78**, 127).

The typing of streptococci by Griffith's method is useful in studying outbreaks of scarlet fever or sore throat, in which all the cases show the same type of streptococcus. If a carrier or suspected source of infection yields the same type it is strong presumptive evidence of the origin of the outbreak. It has been shown that the complications of scarlet fever are due, in the majority of cases, to a secondary infection with a different type of streptococcus.

THE PATHOGENICITY AND OCCURRENCE OF STREPTOCOCCI

The pathogenic effects of the streptococci depend on the virulence of the particular strain and the susceptibility of

the host. Thus, a strain of low virulence may produce only a localised inflammation, whereas strains of high virulence may be associated with spreading inflammation often followed by septicaemia.

There is usually a sharp contrast between the virulence of the β type to animals and the low degree of pathogenicity of the α and γ types; thus, animals may withstand inoculation of large doses of cultures of the latter types without obvious effects; but it is noteworthy that after intravenous injection with these organisms localisation may occur in the endocardium with a resultant subacute endocarditis, or in the wall of the gall-bladder with subacute cholecystitis. Such effects are of significance in relation to the causation of subacute endocarditis and cholecystitis in the human subject (*vide infra*).

Haemolytic streptococci (β type) are found in the following conditions:—

Wound suppuration; localised abscesses; spreading inflammatory conditions—*e.g.* erysipelas, cellulitis, lymphangitis, lymphadenitis; septicaemia (*e.g.* puerperal septicaemia) and pyaemia; suppurative otitis and mastoiditis; meningitis, usually secondary to some other lesion, *e.g.* otitis; ulcerative endocarditis; inflammation of the fauces, tonsils, pharynx and larynx—*e.g.* acute follicular tonsillitis, the angina of scarlet fever (*vide infra*); broncho-pneumonia secondary to influenza, measles, etc.

In septic lesions, mixed staphylococcal and streptococcal infections are not infrequent.

Streptococci may also occur as secondary invaders, *e.g.* in diphtheria, etc.

In the great majority of human infections the β streptococci belong to Lancefield's Group A; the occurrence of other groups has been referred to on p. 340.

It should be noted that virulent haemolytic streptococci may be found in the throats of healthy persons; from such carriers may originate cases and outbreaks of various forms of streptococcal disease—*e.g.* tonsillitis, scarlet fever, puerperal infection, wound sepsis, etc.

The frequent association of the *acute rheumatic state* with *antecedent* infection of the throat or upper respiratory passages by haemolytic streptococci is now well recognised, but in the past there has been no evidence that this condition is due to infection of the blood by these organisms or that the joint and cardiac lesions are the results of localisation of streptococci in the affected tissues. More recently, however, in some fatal cases of acute rheumatism with endocarditis, haemolytic streptococci have been demonstrated, by cultivation in a

rich digest broth (p. 152), to be present in the vegetations on the heart valves. It has been claimed that the essential clinical and pathological manifestations of the rheumatic state represent an allergic reaction of the tissues to the products of these organisms in specially predisposed individuals.

The occurrence of *acute nephritis* following streptococcal infections has also been supposed to be due to a similar allergic condition.

Chemotherapy.—Acute infections by *Strept. pyogenes* usually respond well to treatment with the sulphonamide compounds. These substances interfere with the synthetic metabolism of the streptococcus and inhibit its growth so that the natural defences of the body come into full play in destroying the organism (p. 21). Infections by *Strept. pyogenes* are also amenable to therapy by penicillin and certain other antibiotics, e.g. aureomycin.

Non-haemolytic types (α and γ) may occur as pathogens in lesions of a less acute form than those produced by the haemolytic varieties—e.g. tonsillitis, otitis media, dental abscesses, pyorrhoea, broncho-pneumonia, cholecystitis, subacute bacterial endocarditis. The last-mentioned condition is usually due to the *viridans* type of streptococcus.

Chemotherapy of infections by non-haemolytic streptococci. The α type streptococci are susceptible to penicillin, aureomycin and certain other antibiotics ; penicillin is the first choice for the therapy of infections by these organisms. Infections by the enterococcus are best treated with aureomycin, this organism being less susceptible to penicillin, though in certain cases, e.g. bacterial endocarditis, the two antibiotics may be combined.

SCARLATINA (or SCARLET FEVER)

It has long been recognised that haemolytic streptococci are constantly present in the throat in early cases of scarlet fever, and that the majority of the complications of this disease are streptococcal. In 1923 G. F. and G. H. Dick produced scarlet fever by inoculating the throat of a volunteer with a culture of haemolytic streptococcus isolated from the disease ; and later they demonstrated that in culture filtrates of scarlatinal streptococci there is present a toxin (erythrogenic or scarlatinal toxin) which in suitable dilutions produces a local erythema when injected intradermally into persons susceptible to the disease, while convalescents generally show no reaction. The test, designated the *Dick test*, is made by injecting intradermally into the skin of one forearm 0.2 ml. of a dilution (e.g. 1 in 1000) of filtrate from a broth culture, while into the other forearm is similarly injected, as a control, a like amount of the same diluted filtrate which has previously been heated

to destroy the toxin present. The toxin is remarkably stable and requires prolonged heating for its inactivation, *e.g.* four hours at 96° C. In a positive reactor a red erythematous patch appears in four to sixteen hours, at the site of injection of the toxin. The redness remains for about twenty-four hours and then fades. The control injection, *i.e.* of heated toxin, should produce only a transient redness just after inoculation, and no erythema when readings of the test are made. For the reaction to be recorded as positive, the diameter of the erythematous area should be at least 1 cm.; a typical positive usually shows an area 2-3 cm. in diameter. Pseudo-reactions, in which both toxin and control injections produce an erythematous reaction, may occasionally occur in adults, but such results are relatively uncommon in children. The pseudo-reaction generally appears later than the true reaction, *e.g.* after about 24 hours. A positive Dick reaction indicates that the subject is sensitive to the erythrogenic toxin and that there is an absence of natural or acquired immunity. The reaction is usually positive in the early stages of scarlet fever, being visible over the redness of the rash, but becomes less intense as the disease progresses, and is usually negative after the third or fourth week of the disease. It may be stated broadly that a positive Dick reaction signifies susceptibility to scarlet fever and an absence of immunity to the toxin. Up to the present it has been found difficult to standardise this scarlatinal toxin accurately as laboratory animals do not normally react to it in the same way as they do to many other bacterial toxins. Cutaneous reactions can be obtained, however, in rabbits with high concentrations of the toxin. By tests in known susceptible persons the toxin has been assayed in terms of the "skin-test" dose (S.T.D.), *i.e.* the minimum amount which, injected into the skin, produces in twenty-four hours an erythematous reaction of at least 1 cm. diameter.

The toxin is prepared by growing a strain of scarlatinal streptococcus in a digest-broth medium, *e.g.* Hartley's broth, for forty-eight hours at 37° C., the medium being in a bottle kept in the upright position, *i.e.* with minimal exposure of the culture to air. The culture is then centrifuged at high speed and the supernatant fluid decanted and passed through a tested earthenware (Berkefeld) or Seitz filter. The filtrate constitutes the "toxin." Phenol (0·5 per cent.) is added as a preservative, and for use the toxin is diluted with normal saline or preferably the buffer solution used for the Schick

test reagents (p. 384) so that 0·2 ml. of the dilution contains one skin-test dose.

It has been shown that serum from a convalescent scarlet fever patient (0·2 ml. of a 1 in 10 dilution), when injected intradermally in an early case of scarlet fever, causes a blanching or extinction of the rash within 8 to 24 hours around the site of injection. This is termed the *Schultz-Charlton reaction*, and shows that convalescent serum contains a neutralising substance (antitoxin) for the scarlatinal toxin. A similar result is obtained with antitoxic serum from an immunised animal (*vide infra*). This test may be employed clinically for diagnosis in doubtful cases of scarlet fever.

Active immunisation in susceptible individuals (*i.e.* Dick-positive) is carried out by injecting saline dilutions of toxin made up so that a certain volume, *e.g.* 1 ml., contains a given number of "skin-test" doses. Successive and increasing doses are given at weekly or fortnightly intervals, ranging from 500 to 80,000 or more "skin-test" doses. This treatment usually causes the Dick reaction to become negative. It may be noted that toxoid cannot be obtained from scarlatinal toxin since treatment with formalin (*cf.* diphtheria toxin) destroys the antigenicity of the toxin.

By the immunisation of horses with scarlatinal toxin, an antitoxic serum is obtained which can be used for therapeutic purposes. The antitoxin is concentrated and refined in the same manner as diphtheria antitoxin (*q.v.*). It is difficult to standardise accurately owing to the relative insusceptibility of laboratory animals to the toxin.

Standardisation was first attained by neutralisation tests in Dick-positive reactors, mixtures of serum and toxin being injected intradermally. Thus, in the U.S.A. the antitoxic unit adopted was that amount of serum which just neutralised 50 skin-test doses of toxin; and 6000 units were recommended as an initial therapeutic dose. Another method of assay, based on cutaneous reactions in rabbits (*vide supra*) and the neutralisation of these by antitoxin, has been used in Great Britain and certain other countries.

The dose is from 3000 to 10,000 U.S.A. units given intramuscularly or, in severe cases, intravenously. This antitoxin may also be of therapeutic value in certain other conditions due to haemolytic streptococci, *e.g.* erysipelas and cellulitis.

The scarlatinal streptococci belong to Lancefield's Group A (p. 339). They can be assigned by agglutination reactions to the various types

defined by Griffith (p. 343). The most frequent types are Nos. 1, 2, 3 and 4. There is no serological distinction between scarlatinal strains and haemolytic streptococci from other lesions in the human subject.

THE OCCURRENCE OF STREPTOCOCCI IN DISEASES OF THE DOMESTICATED ANIMALS

Streptococci are relatively infrequent in suppurative lesions of sheep and swine, but may be met with more commonly in cattle, and occur also in horses. Conditions regarded as due to these organisms are mastitis of cattle, strangles and contagious pleuropneumonia of horses, omphalophlebitis of new-born animals with metastases in joints, septicaemia of chickens (*Strept. gallinarum*).

Bovine mastitis.—The most prevalent organism in this condition is the streptococcus, and the type commonly found, especially in the chronic form of the disease, has been designated *Strept. agalactiae*: characterised as a long-chained streptococcus producing a flocculent type of growth in broth, acidifying and coagulating milk within forty-eight hours, fermenting glucose, lactose, sucrose and salicin but not acting on mannitol or inulin; the strains vary in their action on blood-agar, being of the α , β or γ form. The final pH of growths in glucose broth is 4.2–4.6 (pp. 162, 338). Methylene blue in milk is not reduced. This organism exhibits the property of hydrolysing sodium hippurate. This is tested by growing the organism in broth containing 1 per cent. sodium hippurate and incubating for five days; to 1 ml. of the supernatant fluid of the culture is added 0.2–0.4 ml. 12 per cent. ferric chloride solution containing 2 per cent. hydrochloric acid (by volume), the tube being immediately shaken; a precipitate which remains insoluble is taken to indicate the presence of benzoate, which in turn signifies hydrolysis of the hippurate. Four main serological types distinguished by agglutination reactions have been described. *Strept. agalactiae* belongs to Lancefield's Group B (p. 339).

Besides *Strept. agalactiae* two other "groups" of streptococci occur in bovine mastitis. "Group II" is distinguished by the absence of haemolysis, reduction of methylene blue in milk and absence of fermentation of mannitol, salicin and aesculin. These organisms have sometimes been designated "*Strept. dysgalactiae*"; they belong serologically to Lancefield's Group C. "Group III" is non-haemolytic, reduces methylene blue, produces acid in milk at 10° C., and ferments mannitol, salicin and inulin. This group is sometimes named "*Strept. uberis*".

Exceptionally strains of streptococci isolated from mastitis are indistinguishable from the haemolytic forms found in pyogenic lesions of the human subject (Group A), and it has been assumed that such organisms are of human origin. The milk of cows suffering from such streptococcal infection may give rise to outbreaks of sore throat or scarlatina in the human subject, the type of streptococcus being

actively haemolytic. This organism has sometimes been designated *Strept. epidemicus* (*vide supra*).

Bacteriological diagnosis of bovine mastitis.—This can be carried out by plating centrifuged deposits from milk samples on a medium which is relatively selective for streptococci, viz. crystal-violet-blood-agar. It consists of 1 litre Lemco agar (pH 7.4) with 2 ml. 0.1 per cent. crystal violet, 50 ml. defibrinated ox blood and 1 gm. aesculin.¹ Many of the other organisms present in milk are inhibited by the crystal violet, and those which are dye-resistant usually produce black colonies in the presence of aesculin and so can be differentiated at sight from streptococci. Deep plating in the medium may be an advantage for detecting haemolytic colonies. Pure cultures of the streptococci present are obtained from single colonies and these are then identified by further tests (*vide supra*).

Reference to streptococci occurring in animals has also been made on p. 340 in relation to Lancefield's serological grouping of the streptococci.

GAFFKYA TETRAGENA

(*Micrococcus tetragenus*)

Morphology and Staining.—Gram-positive spherical cocci in tetrads, about 0.7μ in diameter, and capsulated when growing in the tissues.

Cultural Characters.—Aerobe and facultative anaerobe; optimum temperature, 37° C.; grows well on ordinary media.

Agar—growth resembles that of *Staphylococcus albus*.

Gelatin—not liquefied.

Occurrence.—Suppuration in region of mouth and neck—e.g. dental abscess, cervical adenitis, pulmonary abscess, etc.

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.

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, e.g. being derived from air and dust. Some resemble staphylococci in morphology, others appear in the form of tetracocci or 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, C., Stitt, J. M., and Cowan, S. T., *J. Gen. Microbiol.*, 1951, **5**, 1010.)

¹ Edwards, S. J., *J. Compar. Path.*, 1933, **46**, 211.

OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS

PSEUDOMONAS AERUGINOSA (or PYOCYANEA)

(*Bacillus pyocyaneus*)

Morphology and Staining.—Straight rods; 1·5 to 3 μ by 0·5 μ (average); motile with one to three terminal flagella; non-sporing; Gram-negative.

Cultural Characters.—Aerobe and to some degree a facultative anaerobe; temperature range, 18°–43° C.; optimum, 30°–37° C.; grows on ordinary media.

Agar—stroke-inoculation produces an abundant, moist, greenish-blue fluorescent growth; the pigment ("pyocyanin"), on which the colour depends, also diffuses through the medium. Pigment is most abundantly produced at room temperature.

A similar but purely saprophytic organism is *Ps. fluorescens*, which is commonly found in water and soil.

Gelatin—liquefied, with a greenish-blue coloration.

Potato medium—growth is similar to that on agar, but the pigment alters, as a result of oxidation, to a brownish colour.

Occurrence.—Suppurating wounds, usually in mixed infection with pyogenic cocci, otitis media, inflammatory conditions of the urinary tract, etc. This organism may occur as a commensal in the intestine of man and animals. In young subjects it may give rise to enteritis and even produce a general infection. Cases of pneumonia and endocarditis have been reported due to this organism.

Antibiotic Therapy.—Many strains of *Ps. aeruginosa* are susceptible to polymyxin and some to streptomycin and chloromycetin, and these antibiotics may be used in the treatment of infections by this organism, e.g. urinary tract infections.

PROTEUS

(*Bacillus proteus*)

Morphology and Staining.—Straight rods about same size as *Ps. aeruginosa* (*vide supra*); pleomorphic; motile with numerous lateral flagella; non-sporing; Gram-negative.

Cultural Characters.—Grows aerobically on ordinary media. Optimum temperature about 25° C., but grows well at 37° C. A single stroke-inoculation on agar produces a moist, trans-

lucent, greyish-white growth, which tends to spread all over the available surface of the medium—hence the designation “spreader” often applied to this organism (p. 355). This is due to the fact that as colonies form the bacilli migrate from their periphery and “swarm” over the medium. Spreading tends to occur in successive waves and this is manifested by the successive zones of growth which are visible on the medium. Gelatin is usually liquefied; this property, however, may be lost after the organism has been artificially cultivated for some time. Coagulated serum is liquefied by some strains.

A non-motile variant type of *Proteus* may be met with which does not spread on culture medium; it corresponds to the “O” variants of other motile bacteria (p. 45).

Biochemical Reactions.—*Proteus* includes biological types which differ somewhat in biochemical reactions; the varying reactions are indicated as follows:

Glucose	Lactose	Dulcitol	Sucrose	Mannitol	Maltose	Indole production	Serum liquefaction
+	-	-	±	-	±	±	±

(+ under carbohydrates signifies acid and gas production)

H_2S is formed, and urea is actively converted into ammonia. This latter reaction is illustrated in the use of certain media for the identification of *Proteus* (e.g. Christensen's and Elek's media, pp. 193, 194).

Strains which ferment sucrose and maltose and produce indole are assigned by some systematists to the classical species *Proteus vulgaris*, but subdivision into species on the basis of these reactions is of doubtful validity.

Some non-liquefiers of gelatin are now assigned to the *Proteus* group, e.g. Morgan's bacillus, *Proteus morganii* (p. 457).

Occurrence.—Septic infections—e.g. suppurating wounds, urinary sepsis, otitis media, etc.—usually along with other pyogenic organisms. It may be present in faeces, particularly in children, and occurs frequently as a saprophyte in decomposing organic matter. It has been reported as the causative organism in cases of acute bacterial food-poisoning. Organisms assigned to the *Proteus* group have been described in ozaena (e.g. “*Coccobacillus foetidus ozaenae*” of Perez), but this organism does not liquefy gelatin. These are not primary causal organisms in this condition.

Antibiotic Therapy.—*Proteus* infections, e.g. of the urinary tract, are amenable to treatment with chloromycetin, streptomycin and polymyxin.

Weil-Felix Reaction.—In typhus fever the blood serum agglutinates a *Proteus* type (“X19”) and to a lesser degree another type (“X2”),

originally isolated from the urine in this disease. The reaction with X19 is utilised as a diagnostic test (Weil-Felix reaction), but the organism has no aetiological relationship to the disease (p. 559). Strains of the X19 type are serologically different from other strains of *Proteus*. The agglutinin responsible for the Weil-Felix reaction is of the "O" type (p. 45).

The Weil-Felix reaction has been explained on the basis that *Proteus* X19 and the organism of typhus fever possess a common polysaccharide, which enters into the constitution of their antigens (p. 39).

Another type designated "Kingsbury" or "XK" is agglutinated by the serum of patients suffering from the typhus-like disease of the Far East often named "Scrub typhus," and is used in the diagnosis of this condition (p. 563). Maltose fermentation is absent as compared with the X19 type. It does not liquefy gelatin.

Another group of organisms frequently found in inflammatory and suppurative lesions is that of the coliform bacilli comprising the genera *Escherichia* and *Aerobacter*, and closely related to these *Klebsiella* (*Pneumobacillus*). The biological characters and pathogenic relationships of these organisms are described in Chapter XV.

BACTERIOLOGICAL DIAGNOSIS OF PYOGENIC INFECTIONS

COLLECTION OF SPECIMENS AND MICROSCOPIC EXAMINATION

Films are made on microscope slides from the pus or inflammatory exudate, dried and fixed by heat. The films should, if possible, be made directly from the lesion, a sterile wire loop or sterile capillary pipette (p. 207) 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 (p. 319). 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 in it. Swabs (p. 322) 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 should be taken, if possible, with a sterile wire loop, and films (and also cultures) are made at once, or the exudate is collected in capillary tubes or on swabs.

In the case of *pleural and peritoneal fluids*, the fluid should be withdrawn into a 1-oz. screw-capped "biochemical bottle" containing four drops of 20 per cent. sodium citrate solution (p. 321). This is to avoid coagulation, which renders cytological and bacteriological examination difficult. The material is centrifuged and films are made from the deposit.

Urine should be drawn, with aseptic precautions, by means of a catheter smeared with a sterile lubricant, e.g. glycerol-jelly. No antiseptics are to be used. The specimen is passed directly into a sterile stoppered bottle or 8-oz. screw-capped pot (p. 320), and should be submitted for investigation without any delay. In the female this procedure is absolutely necessary to avoid contamination. In the male, however, a fairly satisfactory specimen may be obtained by cleansing the urinary meatus and, after a portion of the urine has been voided, collecting a sample directly into a sterile bottle. The urine is centrifuged, and films are made from the deposit. After the films have been dried and fixed they should be gently washed in water to remove crystalline deposit.

Sputum should be expectorated directly into a suitable sterile container as described on p. 320. Films are made as in the case of pus.

The films are stained by Gram's method.

CULTIVATION

Successive stroke-inoculations are made on blood-agar in a Petri dish, and it may sometimes be advantageous to duplicate the culture, one being incubated aerobically, the other anaerobically, e.g. if microaerophilic streptococci are present. A proportion of the growth will be represented by separate colonies, and the colony characters of the organisms can be recognised. Films are also made from colonies and stained by Gram's method. In this way the organism present can generally be identified. If a mixed growth results, single colonies can be subcultured on blood-agar slopes, so that pure cultures are available for any further examination required.

Coliform bacilli can generally be recognised at once when cultures are also made on MacConkey's agar (p. 180).

For the rapid identification of *Proteus*, Christensen's or Elek's media may be used (pp. 193, 194).

If *septicaemia* or *pyaemia* is suspected, blood culture is carried out (p. 222).

A difficulty frequently encountered in the bacteriological examination of pus, particularly from infected wounds, is the overgrowth of pyogenic cocci, e.g. streptococci, by other organisms present, such as coliform bacilli, *Proteus* and *Ps. aeruginosa*. This may sometimes be obviated by making use of the selective bacteriostatic effect of potassium tellurite incorporated in the medium employed for the primary culture. Thus, if microscopic examination of the pus reveals large numbers of Gram-negative bacilli, tellurite may be added to the agar used for plating, in a 1 in 50,000 concentration (0·2 ml. of a 1 per cent. solution in 100 ml. agar). Coliform bacilli and *Ps. aeruginosa* are inhibited and the cocci, if present, can be more easily isolated. *Proteus*, however, is sometimes resistant to this concentration, and the spread of this organism on plates often makes the isolation of other organisms difficult if not impossible. Sodium lauryl sulphate is an effective agent for preventing the spreading growth of *Proteus*; for this purpose, Lominski has recommended an agar medium with a concentration of 1 : 6000 of this substance, along with 1 per cent. lactose and half the amount of neutral red usually incorporated in MacConkey's medium. Sodium lauryl sulphate may also be used in blood-agar, but it lyses the blood and this is a disadvantage when the medium is being used for the recognition of haemolytic cocci. If a non-lytic agent is required in blood-agar to prevent spread of *Proteus*, "dispersol LN" (made by Imperial Chemical Industries) can be used. This is kept as a 5 per cent. aqueous solution (which need not be sterilised), and for 10 ml. agar containing 10 per cent. oxalated horse blood (p. 176) add 0·65 ml. The reagent is mixed with the agar and then the blood is added, frothing being thus avoided. (Note: in weighing this substance avoid any of it getting into the air as it causes sneezing.)

The use of 6 per cent. agar has proved one of the most convenient methods of preventing spread of *Proteus* on blood-agar plates.

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 in Chapter XVIII.

DIPLOCOCCUS PNEUMONIAE (*PNEUMOCOCCUS*)

The causative organism of Lobar Pneumonia.

Morphology and Staining.—The typical appearance is that of an oval or lanceolate Gram-positive coccus in pairs with the rounded ends opposite; it is about 1μ in its long diameter; shows a thick capsule, which may appear as an unstained zone round the organism, unless positively stained by special methods (p. 102). In culture it is not so typical, being

less lanceolate and more rounded ; the capsule is not so evident, and the cocci may occur in chains.

Cultural Characters.—Aerobe and facultative anaerobe ; optimum temperature, about 37° C. ; does not grow below 25° C. ; grows on ordinary media, but best in the presence of blood or serum.

The addition of glucose (*e.g.* 0·1 per cent.) to culture media promotes the growth of the pneumococcus. It should be noted in the preparation of a broth for the cultivation of this organism that it may be inhibited by an oxidised constituent of the peptone. This difficulty can be obviated by adding the peptone to the medium before heating so that it is later subjected to the reducing action of the meat infusion (p. 150). Commercial peptones may also contain metallic impurities which are responsible for inhibitory effects in the cultivation of the pneumococcus (p. 150).

It is sometimes advantageous to grow the organism in an atmosphere of 5 per cent. carbon dioxide as some strains grow better under these conditions than in the ordinary atmosphere.

Agar—growth consists of small (about 1 mm. diameter), delicate, semi-transparent, dewdrop-like colonies, which tend to remain discrete, *i.e.* like colonies of streptococci.

Blood agar—non-haemolytic, or only partially lytic ; growth produces the same greenish coloration as "*Streptococcus viridans*." Colonies are at first flat and smooth, but later may develop elevated margins and concentric ridges (likened to a draughtsman).

Under anaerobic conditions the pneumococcus may be more actively haemolytic. The haemolysin is readily inactivated by oxidation.

Cultures may show transformation of the colonies from the typical smooth (S) type to the rough (R) form ; this variation is associated with loss of capsule formation, absence of type-specificity (*vide infra*) and loss of virulence.

Broth—growth shows at first a uniform turbidity, but later forms a granular deposit in the tube.

Biochemical Reactions.—Ferments various carbohydrates (*e.g.* glucose, lactose, sucrose) and differs from many strains of streptococci in its fermentation of inulin. Hiss's serum-water, made with 0·1 per cent. of peptone-water, is a convenient medium for these tests (p. 172). The agar medium used for testing the biochemical reactions of the gonococcus also gives good results (p. 174).

Bile Solubility.—It is sharply differentiated from the streptococci by its solubility in bile. This test consists in

adding 1 part of sterile ox bile or 1 part of a sterilised 10 per cent. solution of sodium taurocholate in normal saline to 10 parts of a broth culture. The test may also be carried out by using a 10 per cent. solution of sodium desoxycholate, 0·1 ml. being 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.

Viability.—The thermal death-point is about 52° C. The ordinary laboratory cultures lose viability rapidly, especially under aerobic conditions, and require to be subcultured at short intervals (*e.g.* about fourteen days). Cultures survive for a longer period in a semi-solid agar containing blood. If it is desired to maintain cultures over a considerable time without subculturing they should be preserved by the method of rapid drying *in vacuo* (p. 221).

Experimental Inoculation.—Pathological material containing the pneumococcus (*e.g.* pneumonic sputum) or a virulent culture, injected subcutaneously into rabbits or mice, produces a rapidly developing septicaemia, fatal in twenty-four to forty-eight hours; at autopsy, typical capsulated diplococci are present in large number in the heart blood. The virulence for animals rapidly decreases if the organism is grown on media without blood. By drying the spleen of an infected mouse in a desiccator, the virulence of the organisms present may be maintained for about a month. (The spleen should be removed immediately after death.)

Serological Types.—Thirty-two types have been generally recognised, differing in their reactions with specific antisera, though cross-reactions have indicated that such classification is incomplete. These types have been designated numerically, I, II, III, etc. Types I and II are the so-called "epidemic" types, being responsible as a rule for over 50 per cent. of acute lobar pneumonia. Type III is recognised also by its cultural characters; it is the "*Pneumococcus mucosus*," and produces raised mucoid or slimy colonies.

At one time only Types I, II and III were serologically defined, all other strains being classified as "Group IV." It was noted that the pneumococci occurring normally as mouth and throat commensals mostly belonged to this group.

The *identification of the type* is carried out by using the agglutination test with specific sera, or by the direct method, in which "capsule-swelling" is the criterion of specific interaction between the organism and the appropriate serum.

Agglutination Method.—For rapidity of obtaining a suspension of the

organism advantage is taken of the extreme susceptibility of the mouse. The animal is inoculated intraperitoneally with about 0·5 ml. of the sputum and when it appears ill or moribund is killed and the contents of the peritoneal cavity are washed out and suspended in 1 ml. of saline. This suspension of organisms is usually dense enough for the test, but it may be centrifuged and re-suspended in a smaller quantity of saline if necessary. A direct agglutination test is done by the slide method as described on p. 250. Specific antisera for the various types are required and for preliminary tests these sera are pooled in groups (*vide infra*). The antisera are produced by immunising rabbits. A drop of bacterial suspension is placed on the slide and a drop of pooled serum "A" (*vide infra*) placed beside it and the two mixed. If the result is positive, visible clumping will be observed easily with a lens or the low power of the microscope. If pooled serum "A" gives a negative result, the other pooled sera are used in turn until a positive result is obtained. When a pooled serum gives a positive result, the pneumococcal suspension is then tested with the type-sera comprising the pool until the actual type is identified. It is usually possible to obtain a result at once by this method. Sometimes the suspension from the peritoneal cavity is granular so that agglutination cannot be observed; in this case, cultures are made on blood agar and in glucose-broth, and the latter culture is used for typing as above. The blood-agar culture serves to identify the organism present as a pneumococcus or otherwise, if no result is obtained from the typing with the glucose-broth culture.

Direct Method of Typing the Pneumococcus (Neufeld reaction).—It has been shown that when the pneumococcus, present in sputum from a case of pneumonia, is acted on by a specific serum its capsule appears to swell and the organism exhibits a clear refractile capsular zone. This reaction, which occurs within three minutes, has been utilised as a direct method of typing. The procedure is as follows. An emulsion of the sputum is prepared in physiological saline solution and separate drops of this emulsion, deposited on slides, are mixed with double the amount of the respective undiluted type-antisera. (A similar drop mixed with saline serves as a control.) If the sputum is not tenacious, flecks taken up with a wire loop may be used instead of the drops of emulsified material. The mixtures are covered with No. 1 cover-slips and examined with an oil-immersion lens, the substage condenser being suitably lowered and the diaphragm reduced in aperture. The physical change in the capsules when acted on by the particular type-serum can readily be observed within a few minutes. It should be noted that the sharpness of outline of the capsule is more significant than its apparent enlargement. Some workers prefer to add a drop of methylene-blue solution to the preparations; the capsules remain unstained and present a ground-glass appearance. The sputum should be examined within one hour after expectoration.

This is the method which has been generally used for determining the type of pneumococcus in clinical cases. A set of type-sera for the

various types is required, and to simplify the determination a preliminary test is done with sera pooled in groups, e.g. as follows: "A" for Types I, II and VII, "B" for Types III, IV, V, VI and VIII, "C" for Types IX, XII, XIV, XV and XVII, and three further mixtures comprising the other types. After the organism has been assigned to one of these groups, it can be tested with the individual sera of the group.

Since the recognition of the numerous types of the original Group IV, as mentioned above, the occurrence of cross-reactions has been emphasised, and it has been found that precise determination of types may only be made by means of sera absorbed with cross-reacting strains. Further serological analyses have thus resulted in sub-types and new types being defined and have considerably extended the total number of serological varieties.¹

Specific Substances of the Pneumococcus.—Type-specificity is dependent on polysaccharide haptens (p. 39) contained in the capsule of the organism. Loss of the capsule-forming property and R transformation of the colonies (*vide supra*) are associated with absence of the specific carbohydrate and of type-specificity and also loss of virulence.

It has been shown that the specific polysaccharides of Types I, II and III are "excreted" in the urine in the majority of cases of pneumonia due to these types, and can be demonstrated by a precipitin reaction with type-sera. In the early stages of the disease the polysaccharide is present in the urine in a proportion of cases only, but these have a graver prognosis than cases in which it is not found.

Occurrence.—In lobar pneumonia the pneumococcus is present often in considerable number 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 pathological complications of pneumonia—e.g. pleurisy, empyema, endocarditis, pericarditis, meningitis, arthritis, etc.

The pneumococcus is found in broncho-pneumonia, simple catarrhal conditions of the throat and respiratory tract, conjunctivitis, otitis media, primary meningitis, primary peritonitis.

As noted above, this organism may be present normally in the mouth and throat secretions.

In this country Types I and II together have been found to be responsible for the majority of cases of acute lobar pneumonia, e.g. 50 per cent. or over. Type III is less frequent, e.g. 10 per cent. or

¹ U.S. *Publ. Health Reps.*, Washington, 1944, 59, 449, 451, 1041.

less. The other types considered collectively (*Group IV*) are found in a varying proportion of cases, usually under 30 per cent. The relative prevalence of types varies in different localities and at different times. The mortality statistics of *pneumonia* indicate that Type III is the most virulent, and that Type II is more virulent than Type I. Types I and II are, however, more invasive than the others, as shown by their prevalence in primary meningitis and peritonitis, and the occurrence of Type III and *Group IV* in otitis media and conjunctivitis. *Group IV* comprises a large proportion of the strains found in primary broncho-pneumonia in young subjects.

Therapeutic Antisera.—Prior to the introduction of sulphonamide therapy, monovalent antisera for the various types of pneumococcus were extensively used with successful results, especially in cases of pneumonia due to Types I, II, V, VII, VIII and XIV. Such sera were concentrated and refined, which allowed a large dose of antibodies to be administered intravenously without inconvenience and with little risk of serious shock due to the foreign protein. The sera were standardised according to their power of protecting mice against virulent pneumococci, and the unit was that amount of serum which protected a mouse against 1,000,000 lethal doses of pneumococcus culture. Repeated doses of 10,000–40,000 such units were administered. For all practical purposes these sera are now obsolete.

Chemotherapy.—Pneumococcal infection is very amenable to treatment with sulphonamide compounds, e.g. sulphapyridine. These substances seem to act on the organism *in vivo* in the same way as they affect the haemolytic streptococci (*q.v.*). Successful therapeutic results are also obtained with penicillin, which among present antibiotics is the first choice, with aureomycin and terramycin as alternatives.

DIAGNOSIS OF PNEUMOCOCCAL INFECTION

A specimen of sputum is obtained (p. 320), and films are stained by Gram's method. Cultures are made by successive strokes on a blood-agar plate. It is an advantage to incubate in an atmosphere of 5 per cent. carbon dioxide. The characteristic colonies can be recognised among the other organisms that constantly develop from sputum, and are subcultured on blood-agar slopes for the purpose of isolation. The fermentation of inulin and bile-solubility are determined with the pure cultures obtained.

The occurrence of septicaemia with characteristic diplococci in the heart blood on inoculation of a mouse with the sputum or the culture isolated is conclusive proof of the identity of the organism present, even though the diplococci noted in the specimen or cultures may not be entirely typical.

The serological type can be determined by the methods described above.

In a case of acute pneumonia, blood culture is of value in determining the presence or absence of septicaemia, which is of prognostic significance.

Repeated blood-cultures may be of additional value in ascertaining whether the blood infection is progressive or not. In this case it is advisable to add a given volume (*e.g.* 1 ml.) or varying quantities of blood to melted agar, so as to obtain a count of the number of colonies.

In cases under sulphonamide treatment *p*-aminobenzoic acid should be incorporated in the blood-culture medium (*p.* 225).

CHAPTER XI

NEISSERIA GROUP:

MENINGOCOCCUS, GONOCOCCUS AND OTHER GRAM-NEGATIVE DIPLOCOCCI

NEISSERIA MENINGITIDIS

(*MENINGOCOCCUS*)

CAUSATIVE organism of Epidemic Cerebro-spinal Meningitis ; may also produce septicaemia (without clinical meningitis), and this is sometimes chronic.

Morphology and Staining.—Oval diplococci with opposed surfaces flattened or concave ; sometimes in tetrads ; cocci are about $0\cdot8-1\mu$ in diameter ; the long axes of the cocci in pairs are parallel, not in line as in the case of the pneumococcus (*q.v.*) ; Gram-negative. Morphological capsules are not evident, but when the organisms react with specific anti-serum, capsule-like structures become apparent, this effect corresponding to the “capsule-swelling” reaction of the pneumococcus (p. 358). 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 sometimes obtained most readily in an atmosphere containing 5 per cent. of carbon dioxide; temperature range is $25^{\circ}-42^{\circ}$ C., and the optimum is about 37° C.; does not generally grow on ordinary media and requires blood or serum for growth; 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. (p. 177).

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.

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 (pp. 164, 165). The medium described on p. 174 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. 373).

Viability.—When first cultivated artificially the meningococcus tends to die quickly in culture, e.g. within two or three days. In culture it persists best at incubator temperature. Cultures remain viable for a longer time on an egg medium. For maintaining cultures, 1 per cent. agar in digest-broth *plus* 20 per cent. serum gives good results. When desiccated under ordinary atmospheric conditions the meningococcus usually dies within two hours, but cultures can be preserved by rapid drying *in vacuo* (p. 221).

Serological Types.—Gordon recognised two main types (Types I and II) differentiated by agglutination and agglutinin-absorption reactions with antisera, and representing over 80 per cent. of all cases occurring in this country. Other less frequent serological types (e.g. III and IV) were also identified.

These types are not sharply differentiated, and Griffith classified strains into two serological groups, one of which (*Group I*) includes Gordon's Types I and III, and the other (*Group II*), Types II and IV. He also showed that the antigenic structure of individual strains may be complex and made up of multiple constituents.

The majority of cases of cerebro-spinal meningitis at the present time are due to Group I; whereas many strains found in the naso-pharynx of persons who have not been in contact with cases belong to Group II (*vide infra*). It has been concluded that Group II is of lower pathogenicity than Group I.

Agglutinating antisera are prepared by immunising rabbits with successive and graded doses of dead cultures (p. 251) injected intravenously. The initial dose should be 500–1000 million organisms. The serum of the immunised animal may agglutinate the homologous organism in dilutions up to 1 in 400 or 1 in 800. In carrying out the agglutination test, the general method described in Chapter VIII may be followed; the tubes should be placed in a water-bath at 50° C. for twenty-four hours, when the results can be read.

Parallel serological results may be obtained by using the "capsule-swelling" reaction (*vide supra* and p. 358).

Experimental Inoculation.—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.

Toxin.—The meningococcus is actively toxicogenic, and potent toxic 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. Some evidence has been brought forward that it is antitoxinogenic, but this is doubtful.

Occurrence.—As already mentioned, the meningococcus may produce a septicaemic condition and this is sometimes quite apart from clinical manifestations of meningitis; such septicaemia may assume a chronic form; rare types of meningococcal infection are: primary conjunctivitis, hepatitis and pneumonia. Complications of the typical disease are: labyrinthitis, arthritis and tenosynovitis.

Therapeutic Antisera.—Polyvalent antisera and also monovalent sera for individual serological groups and types have been used—administered by spinal injection and intravenously. Such sera are *antibacterial* in their properties (p. 41), but it has been claimed that their efficacy depends also on antitoxin (*vide supra*). Serum treatment has now been superseded by sulphonamide and antibiotic therapy.

Chemotherapy.—Meningococcal infections respond well to treatment with the sulphonamide compounds, e.g. sulphydryidine, sulphathiazole and sulphadiazine. Therapeutic results are also obtained with penicillin, and combined therapy with a sulphonamide and penicillin may be advantageously employed.

DIAGNOSIS OF MENINGOCOCCAL INFECTION

In the early stages of cerebro-spinal meningitis the organisms are present usually in considerable numbers in the cerebro-spinal fluid and can be recognised by microscopic examination. At a later stage they may be scanty and even apparently absent.

Lumbar Puncture.—The specimen of fluid is obtained by lumbar puncture with a special nickel or stainless steel needle fitted with a stilette. The patient is placed on the right side with the knees drawn up and the left shoulder thrown forward. The skin over the lumbar region, the operator's hands and the needle are sterilised by appropriate methods, and a sterile container (p. 319) should be ready for collection of the fluid. The puncture is made in the middle

line between the 3rd and 4th, or 4th and 5th lumbar vertebrae, the needle being inserted, with the stilette in position, in a forward and slightly upward direction. In the adult it is introduced to a depth of about 5-6 cm. In a young child the depth is about 1 cm. The stilette is withdrawn and the fluid is allowed to flow into the container. In a case of cerebro-spinal 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. 91). Cultures should also be made on one of the blood or serum media referred to above. Films are made from the resulting colonies or 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 is identified by agglutination tests with the appropriate antisera.

For clinical diagnosis the microscopic examination is generally sufficient—*i.e.* if Gram-negative, intracellular diplococci with the characteristic shape of the meningococcus are observed. While the meningococcus is present in large number usually at an early stage of the untreated illness, it may become relatively scanty in the cerebro-spinal 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 cerebro-spinal 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 culture should be carried out.

In making cultures from blood or cerebro-spinal fluid taken from cases *already treated* with a sulphonamide compound, the medium should contain *p*-aminobenzoic acid (p. 146). It should also be remembered that in such cases the organisms may not be observed either by micro-

scopical or cultural examination of the cerebro-spinal fluid. The same applies in cases which have been treated with penicillin.

Other Meningeal Infections.—In routine investigations of meningitis, other causal organisms must be considered—*e.g.* pyogenic cocci, pneumococcus, tubercle bacillus, etc. If 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. 94). In tuberculosis the cellular exudate is mainly lymphocytic at an early stage of the illness, as compared with the polymorph exudate in meningococcal and pneumococcal 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, though this is rarely found in early cases; further, in tuberculous meningitis polymorph leucocytes may sometimes be fairly numerous. In young children, acute meningitis may be caused by *Haemophilus influenzae* (*q.v.*). This organism usually develops elongated filaments in such infections and these are seen among the polymorph leucocytes of the exudate in the spinal fluid. It should be noted that, the virus of poliomyelitis may produce an inflammatory condition of the meninges, indicated by the presence of a leucocytic exudate in the spinal fluid. In such cases no visible organisms are present. In acute lymphocytic chorio-meningitis (p. 587) there is also a lymphocytic exudate and absence of cultivable organisms.

DIAGNOSIS OF MENINGOCOCCUS CARRIERS

During an epidemic of cerebro-spinal meningitis healthy contacts may become carriers, and the meningococcus is found in the nasopharynx. The organism may also be found in the naso-pharynx of healthy persons who have not been in contact with cases and during non-epidemic times. The recognition of carriers has sometimes been utilised in the past for controlling the spread of the disease. The practical utility, however, of this measure is most doubtful. During an epidemic the carrier-rate among contacts may be very high and the segregation of carriers may present great difficulty. Moreover, it has been found where outbreaks occur in groups of persons living together, *e.g.* troops, that spread can be checked by increasing the space per person in sleeping quarters and by improved ventilation and environmental hygiene. In a limited community, when a case of cerebro-spinal meningitis occurs, it may be of some value to swab the nasopharynx of immediate contacts and to isolate any carriers detected in this way. The detection of a carrier depends on cultivating the meningococcus from the naso-pharyngeal secretion.

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 (p. 323), with a longer wire-holder than the usual throat swab and with the terminal $\frac{1}{4}$ in., carrying the cotton-wool plectget, bent through an angle of about forty-five degrees. The swab with wire-holder is enclosed in a stoppered test-tube of sufficient width to admit the bent end. It is necessary that cultures should be made immediately after swabbing and the medium incubated at once owing to the feeble viability of the organism apart from the body.

The tongue is depressed, and the swab is passed behind the soft palate and introduced into the naso-pharynx. Before withdrawal, the swab is also rubbed over the posterior wall of the naso-pharynx. The swab must be introduced and removed from the mouth without touching the tongue.

West's Post-Nasal Swab is specially adapted for the purpose ; it consists of a curved tube containing a flexible wire with a wool plectget as in the case of a throat swab. The tube is introduced into the mouth and by means of its curve can be passed up behind the palate ; from this end the swab is protruded into the naso-pharynx. It can then be retracted, and the tube is withdrawn from the mouth.

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. It is advisable to use agar containing a digest basis. 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 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. It is stated that under these conditions the meningococcus may remain viable for twenty-four hours (Downie). In the laboratory the swab is used to inoculate a blood-agar plate, and the swab-tube is also incubated.

Suspected colonies are examined by means of Gram-stained films, and subcultures from single colonies are made on serum- or blood-agar slopes.

The oxidase reaction may be used to assist in the recognition of meningococcus colonies (p. 373), but it must be remembered that other members of the *Neisseria* group also give the reaction.

The resulting pure cultures are then available for identification. The differentiation of other Gram-negative throat diplococci from the meningococcus is considered below. The absence of growth at room temperature, the inability to grow on ordinary meat-infusion medium, the sugar reactions and the readiness with which the growths emulsify in saline, are important features in the identification of the meningococcus.

The final identification is carried out by means of a polyvalent agglutinating anti-meningococcus serum or antisera to the different groups or types.

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.

NEISSERIA CATARRHALIS (*Diplococcus 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 (*vide infra*). Cultures when emulsified in saline tend to be auto-agglutinable.

N. catarrhalis is not agglutinated by meningococcus antisera.

NEISSERIA FLAVA AND RELATED TYPES (*Diplococcus pharyngis*)

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 p. 370.

They are not agglutinated specifically by meningococcus antisera.

Neisseria flavaescens

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 *Neisseria flava*.

Neisseria sicca (Diplococcus pharyngis siccus)

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.

Biochemical reactions (*vide Table*).

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.

Biochemical reactions (*vide Table*).

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.

FERMENTATIVE REACTIONS OF NEISSERIA GROUP

		<i>Glucose</i>	<i>Maltose</i>	<i>Lactose</i>	<i>Sucrose</i>
Meningococcus . .	.	—	—	—	—
Gonococcus . .	.	—	—	—	—
<i>N. catarrhalis</i> . .	.	—	—	—	—
<i>N. flava</i> and related types	.	—	—	—	—
<i>N. crassa</i>	—	—	—	—

(— = acid ; — = variation in reaction among different types.)

NEISSERIA GONORRHOEAE

(GONOCOCCUS)

The causative organism of Gonorrhoea.

Morphology and Staining.—Oval diplococci with opposed surfaces flattened or concave. The diameter of the coccus is about $0\cdot8-1\mu$. It stains Gram-negatively. Morphologically the gonococcus is identical with the meningococcus. In inflammatory exudates the intracellular position of the organism is characteristic and pus cells often appear to be almost filled with ingested diplococci. In culture, involution forms are frequent.

Cultural Characters.—Aerobe; temperature range, $30^{\circ}-39^{\circ}$ 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. 173) 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.

Care should be taken to ensure that the medium used is sufficiently moist and is kept moist during incubation. For this purpose, plate cultures may be incubated inside a closed jar containing a pad of cotton wool moistened with water.

Many strains grow better in an atmosphere containing carbon dioxide (e.g. 5 per cent.) than in ordinary air (p. 218).

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.

Two different types of colonies have been described : a large, thin irregular colony on which papillae develop, and a small round colony which is more raised and without papillae.

In primary cultures, colonies may be slow in developing and growth may not appear for two or three days.

Biochemical Reactions.—Can be tested for as in the case of the meningococcus, the medium described on p. 174 being used ; ferments glucose but not maltose (*vide Table supra*).

Viability.—The thermal death-point is about 55° C. This organism is very susceptible to drying and when desiccated under ordinary conditions dies within two hours. It 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 cultivated, 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, e.g. two to three weeks. Cultures die at room temperature in two days.

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, vesiculae seminales, epididymis, bladder mucosa and peri-urethral tissue (producing a peri-urethral abscess).

In the *female* the urethra and cervix uteri are infected, but rarely the vaginal mucosa. The vestibular glands, the endometrium and the Fallopian tubes may be invaded, and even the peritoneal cavity.

Blood invasion may result from primary gonorrhoeal infections, and arthritis may occur as a complication. 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 (*cf.* acute rheumatism in relation to streptococcal infection, p. 346). 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 pro-

duce a persistent vulvo-vaginitis with involvement also of the rectum.

In new-born infants gonorrhoeal ophthalmia may result from direct infection at birth.

Chemotherapy.—The sulphonamide compounds have proved effective chemotherapeutic agents in gonococcal infections though sulphonamide-resistant strains may be met with. Penicillin therapy has been applied with conspicuous success. Gonococcal infections are also amenable to treatment with certain other antibiotics, e.g. aureomycin, though this would be a second choice to penicillin.

DIAGNOSIS OF GONORRHOEA

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 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—p. 91—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 already described.

In *chronic infections*, particularly in the female, the coccii 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 from the centrifuged urinary deposit or the discharge after prostate 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 (*vide supra* and Table p. 370); 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 (*vide supra*), 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, the following method for maintaining the viability of the organism, as recommended by Cooper *et al.*,¹ may be adopted : a swab on a wooden applicator is employed and is contained in a narrow test-tube with 1 ml. of "crystal-violet-blood" made up of 1 ml. 1 per cent. crystal violet, 150 ml. glass-distilled water and 15 mgm. *p*-aminobenzoic acid (sterilised in the autoclave), to which is added an equal volume of sterile citrated rabbit blood. The swab is inserted into the crystal-violet-blood at the foot of the tube after impregnation with the discharge or exudate. (Thallium acetate, 1 : 5000, can be substituted for crystal violet.)

It should be remembered that in cases already treated with a sulphonamide compound or 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, 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 develop a purple colour (oxidase reaction). If subcultures are required from the colonies, these should be made immediately ; after ten minutes it may not be possible to subcultivate them. This method is specially useful in dealing with heavily contaminated material containing only scanty gonococci.

Serum Diagnosis.—The complement-fixation test is applicable for diagnosis and in some cases may be of value to the clinician. The technique of the test is described on p. 273.

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 the appendix, pyorrhoea, pulmonary lesions, etc., and regarded as potentially pyogenic.

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°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.

¹ Cooper, V. E., *et al.*, *Brit. J. Venereal Dis.*, 1950, 26, 16.

CHAPTER XII

THE CORYNEBACTERIA : DIPHTHERIA BACILLUS AND BIOLOGICALLY ALLIED ORGANISMS

CORYNEBACTERIUM OVIS AND CORYNEBACTERIUM
PYOGENES ; LISTERIA MONOCYTOGENES

CORYNEBACTERIUM DIPHTHERIAE (*BACILLUS DIPHTHERIAE*)

THE causative organism of Diphtheria.

Morphology and Staining.—Slender rod-shaped organism, straight or slightly curved ; the average size is 3μ by 0.3μ , 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. 375). By Neisser’s method (p. 97) 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. Appearances also vary among strains ; certain strains exhibit very short forms with poorly developed granules (e.g. *gravis* type, p. 375).

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.

By electron microscopy the volutin granules can be demonstrated as well-defined structures and are not merely the products of the staining method generally used for showing them.

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 in diameter 3–4 mm. 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 (*vide infra*).

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. 185–187).

On blood-tellurite media the colonies tend to show distinctive appearances. Three types of colony have been recognised and considered 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.

The colony characters on Hoyle's tellurite medium and blood-agar (pp. 388, 389) are dealt with later in relation to the diagnosis of diphtheria.

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 have been emphasised as 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. 172) 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.)

In addition to the biological types originally described as *gravis*, *mitis* and *intermedius*, certain additional types have been described in cases of diphtheria and carriers. Two of these, however, are avirulent forms.

The following Table illustrates the classification of these types.

Type	Colony form	Starch fermentation	Virulence to guinea-pig	Haemolysis Ox Rabbit Blood
I (<i>mitis</i>) .	mitis	-	+	+
II (<i>inter-</i> <i>medius</i>) .	inter- medius	-	+	- -
III (<i>gravis</i>) .	gravis	+	+	- +
IV . .	"	-	+	- -
V . .	"	+	-	- -
VI . .	"	-	-	- -
VII . .	mitis	+	+	. .

Strains are also occasionally met with which cannot be exactly classified.

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.

Carter has described a dissociating form of the organism which shows colonies similar to the *intermedius* type, but ferments starch ; from the colonies papillae are developed, and these, when subcultured, yield typical colonies of the *gravis* type.

Serological classification.—By agglutination reactions with antisera *gravis* strains have been classified into thirteen types (Hewitt), 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).

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 (H. A. Wright).

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. The bacilli in dried membrane kept at room temperature and in the dark have been found to be alive and virulent after several months. Diphtheria bacilli may remain alive and virulent for a considerable period in the dust of premises.

Occurrence.—The bacilli are present in large numbers in the false membrane and in the throat secretions. They do not invade the lymphatics to any extent and there is generally no blood infection. In nasal diphtheria the organisms can be detected in the nasal discharge. Infection of wounds, the conjunctiva, vulva and vagina may occasionally occur. A diphtheritic paronychia is sometimes met with.

It is generally agreed that the *gravis* type tends to be associated with a more severe and toxic form of diphtheria than the *mitis*, but the *intermedius* may practically equal the *gravis* type in pathogenicity to the human subject. The relative prevalence of these types varies in different areas and varies also at different times.

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 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 Mueller 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 what would appear to be a pure 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 mgm. for a 250 grams guinea-pig [10,000,000 lethal doses per gram] (Pappenheimer).

For large-scale production, 5-litre bottles (D.W.Q.) containing 500-700 ml. of Hartley's broth (p. 151) or preferably that of Pope and Smith¹ are inoculated with the Park-Williams 8 strain and incubated at 34° C. for six to ten days. The bottles are placed in a horizontal position to secure maximum surface for growth and to allow free access of oxygen which is important for toxin production. The cultures are removed from the incubator after ten days and the organisms are killed by the addition of toluene. Filtration through paper and then through an earthenware or Seitz filter yields a bacteria-free filtrate which constitutes the crude toxin.

The toxic substance so produced is somewhat 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 loss of toxic action 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 determined by injecting the material into guinea-pigs; when 5 ml. injected subcutaneously or intraperitoneally produce no symptoms, the change is regarded as complete. Toxoid produced through the action of formalin in this way is a valuable immunising agent.

Diphtheria toxin is a powerful poison, particularly for guinea-pigs in which very small amounts, e.g. 0.00025 ml., of a culture-filtrate may be fatal within five days. At the site of inoculation there is 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 amount of toxin formed varies with the strain of organism used and the composition of the medium, and each batch of toxin has to be tested by animal experiment.

The same pathogenic effects as those described above are produced by the subcutaneous injection of a living

¹ Pope, C. G., and Linggood, F. V., *Brit. J. Exper. Path.*, 1939, 20, 303.

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.

Virulence of the Diphtheria Bacillus.—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. 186), are recommended for the easy isolation of the diphtheria bacillus. Monckton's enrichment method (p. 187) may also be used, and makes it possible to complete a virulence test in forty-eight hours after the throat swab has been received. 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 500,000,000 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. 232. 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. Several 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. It is not advisable to carry out more than ten simultaneous tests on an animal. Four hours afterwards the test guinea-pig (*i.e.* the one that did not previously receive antitoxin) is now injected with 100 units of antitoxin. Alternatively, the test guinea-pig may be injected immediately with $\frac{1}{10}$ 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.

*Recognition in vitro of Toxigenic Strains of the
Diphtheria Bacillus*

The following method by Elek¹ shows the production of toxin in a Petri dish culture and can be used as a reliable substitute for the animal test described above. This *in vitro* test depends on the formation of a flocculent precipitate when diphtheria toxin interacts with its homologous antitoxin in appropriate neutralising amounts, Ramon reaction (*vide infra*—diphtheria antitoxin). Where toxin and antitoxin so interact in culture medium an opaque line of precipitate is observed.

Culture Medium.

A. Peptone (Difco proteose)	4 grams
Maltose	0·6 gram
Lactic acid (B.P.)	0·14 ml.
Distilled water	100·0 ml.

Adjust pH to 7·8.

B. Agar	3 grams
Sodium chloride	1 gram
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 30 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 45 minutes in the incubator.

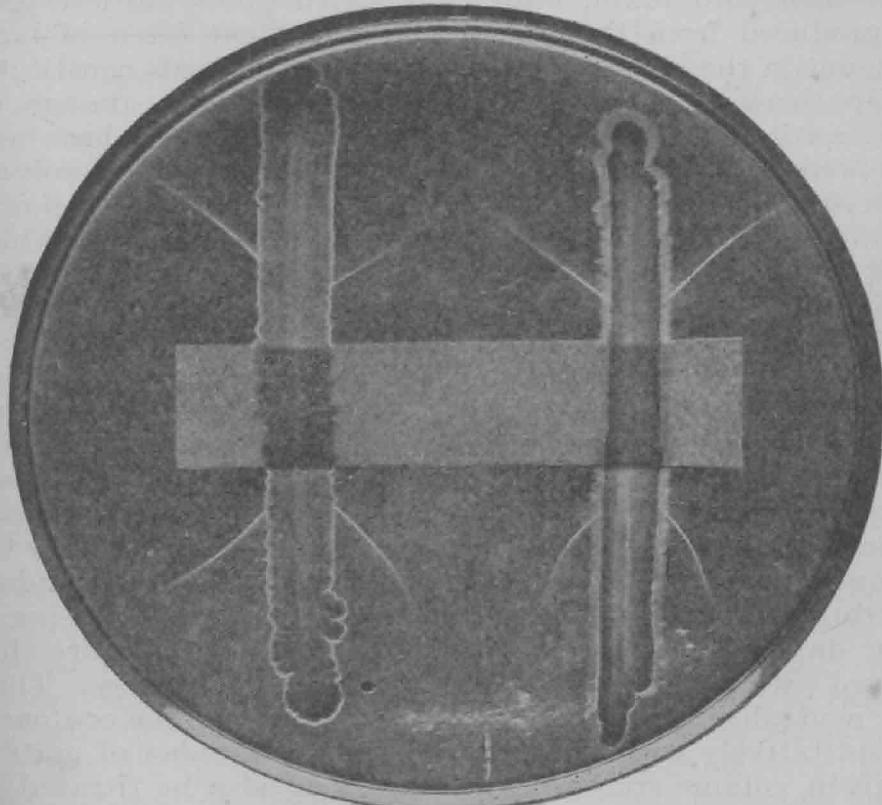
¹ Elek, S. D., *Brit. Med. J.*, 1948, 1, 493; Ouchterlony, Ö., *Lancet*, 1949, 1, 346.

(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 24 and 48 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



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.

appears at about 24 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 48 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 flocculent 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 grams 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, and, as a preliminary to the *in vivo* tests, by the flocculation reaction in mixtures of toxin and antitoxin; flocculation occurs most quickly at the neutrality point.

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 is removed. This was the method originally used, but "refined" anti-

toxins are now available, prepared by treatment of the serum with proteolytic enzymes, *e.g.* pepsin, which, under the conditions applied, digests albumin, leaving the antitoxin-globulin unaltered. The volume to be injected is thus reduced, and the same number of units represents less than half the amount of protein present in the concentrated sera previously used. The incidence of serum sickness has also been much lessened.

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, however, 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 Park-Williams 8 strain, which is so generally employed for the production of toxin in serum laboratories, produces this substance in large amount; 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

Schick Reaction.—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. It should be noted, however, that there are 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 prepara-

¹ O'Meara, R. A. Q., *Lancet*, 1947, 1, 212.

tion 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. It is usually possible to determine without difficulty whether the result is positive or negative.

Pseudo-reactions are more common in older children and adults, because they have apparently been sensitised by exposure to diphtheria bacilli. They are usually immune to diphtheria, as such exposures have also given rise to the production of specific antitoxin.

Children between six months and eight years of age are generally non-immune and rarely suffer from reactions after prophylactic immunisation. While in older children a preliminary Schick test may be carried out to ascertain whether immunisation is required, up to the age of eight years the Schick test may be omitted before immunisation.

Under the Therapeutic Substances Regulations the reagents of the Schick test are designated "Schick test toxin" and

¹ A mixture of 57 grams crystal bórax, 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.

"Schick control," and the methods of manufacture must be in accordance with these regulations.

Diluted toxin prepared with the proper buffer solution and kept in the cold will remain fully active for many months.

Diphtheria Prophylactics.—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. 378). The recommended dosage is as follows : for adults, three injections of 0·1 ml., 0·2 ml. and 0·3 ml.; for children, 0·2 ml., 0·4 ml. and 0·6 ml. The interval between doses is three weeks, and injections are made intramuscularly. As the reactions are likely to be severe, except in young children, 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 anti-toxin are mixed in appropriate "neutralising" amounts. Its tendency to cause reactions is slight, and a high degree of immunity follows the injection of three doses, each of 1 ml., given at intervals of four weeks.

(3) Alum-precipitated toxoid (A.P.T.): a suspension of the washed precipitate produced by the addition of a small amount of alum 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. Even a single injection of 0·5 ml. gives a fair degree of immunity, but it is preferable to inject two doses, each of 0·5 ml. in children or 0·2 ml. followed by 0·5 ml. in adults; at an interval of four weeks. The interval may with advantage be lengthened, e.g. to three or even six months. Adolescents or adults who show a local reaction after 0·2 ml. may receive three injections of 1 ml. of T.A.F. at intervals of four weeks to complete their immunisation.

Another prophylactic which has been advocated is a suspension of purified toxoid adsorbed on hydrated aluminium phosphate (P.T.A.P.). It is a very good antigen, but its efficacy compared to other preparations still awaits final assessment.

The testing of all diphtheria prophylactics must be done in accordance with the Therapeutic Substances Regulations.

Whatever prophylactic is used, a subsequent Schick test should be carried out eight to twelve weeks after the last injection to confirm the production of a satisfactory immunity.

(For further details of the preparation of toxin, toxoid and antitoxin, see *Bacterial and Virus Diseases*, H. J. Parish, Edinburgh, 1951.)

DIAGNOSIS OF DIPHTHERIA

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 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 Albert's modification (p. 97). Albert's method can be specially recommended.

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 over-grow 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. 379, 380.

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* type, in bacteriological practice *in this country*, virulence has been generally assumed without resorting to an animal 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. 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. 375), 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

¹ See Wright, H. A., *Edin. Med. J.*, 1944, 50, 737.

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 of inhibitory effect on the diphtheria bacillus, as shown by the smallness of colonies after 12 hours' incubation, the swab may be plated on digest-agar containing 10 per cent. horse blood. 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*). The colony appearances on blood-agar are as follows:—

Gravis type.—Size about 2 mm. in diameter (where the colonies are not crowded on the plate); flattish, slightly dull or matt, with very slightly irregular edge, and greyish-white in colour; a "dry" granular type of colony, and can be pushed over the surface of the medium entire, but sticking to the plate when attempts are made to remove it. When a colony joins with another as a result of spreading on the plate, a definite straight line of demarcation may be observed. Some strains show slight haemolysis.

Intermedius type.—Size about 1 mm. in diameter; readily recognised by its even growth and pinkish-red colour; a hand-lens will show a somewhat opaque centre; can be picked off easily from the plate, leaving a mark where it has been removed. If a film is made from a colony and examined microscopically, the typical large "barred" bacilli are seen.

Mitis type.—Size about $1\frac{1}{2}$ mm. in diameter (*i.e.* slightly smaller than the *gravis* type); greyish-white in colour, smooth, shiny, "moist," with a perfectly rounded, shiny entire edge; well raised above the surface of the medium; can be picked off easily; the colonies "run into" each other and do not remain discrete like the *gravis* type. Some strains show slight haemolysis.

It should be noted, however, that the colony appearances are not constant, and variations from the above may from time to time be noted.

The *diphtheroid* group shows several kinds of colony formation: (1) moist, shiny colony like *mitis*, but white; (2) small, pin-point, pinkish colony; (3) dull brown colony about 2 mm. diameter; (4) *C. xerosis* type (*vide infra*)—small colony, often with serrated edges; (5) Hofmann's bacillus (*vide infra*)—two types have been noted: dull white, matt in appearance, and a sticky, shiny colony.

Further differentiation between *gravis* and *mitis* strains may be made as follows:—

The organism is heavily inoculated on a blood-agar plate. A strip of filter-paper 60×15 mm. is then dipped in a 1 : 3000 solution of copper sulphate, allowed to drain and then placed on the surface of the plate. After incubation, the *mitis* colonies grow up to the filter-paper, whereas the *gravis* colonies are inhibited, only minute colonies being observed in the vicinity of the paper.

Monckton's Enrichment Method¹

The swab or a Löffler's serum culture is inoculated into blood-copper-sulphate-tellurite broth (B.C.T. broth)—p. 187—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.

(1) Inoculate a bottle of B.C.T. broth directly with the swab or Löffler's serum culture. Rotate the swab in the fluid and squeeze it against the upper part of the bottle to remove absorbed broth. In the case of the serum culture emulsify all the growth in the small amount of condensation fluid usually present in the bottom of the tube (if none is present, add two drops of sterile saline solution). Add a full loopful to the broth. If a direct plating on blood-agar has been

¹ Monckton, J. C., *Bull. Inst. Med. Lab. Tech.*, 1947, 13, 2.

made, some of the confluent growth can be inoculated into B.C.T. broth.

- (2) Incubate the inoculated B.C.T. broth for six hours.
- (3) Plate a full loopful on ordinary blood-agar.
- (4) Incubate the plate overnight and examine next morning.
- (5) Pick off typical colonies, verify by staining films if necessary, make a suspension in broth and from this inoculate Hiss's serum-water media—glucose, sucrose and starch.

(6) If desired, carry out a virulence or toxigenicity test with the broth suspension.

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.¹ *If the clinician considers a case to be diphtheria it is 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 increases with the delay in administering anti-toxin, 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 in accordance with the directions given above, and precise details as to the nature and source of the material should be furnished.

Allowance being made for the possible limitations of the

¹ *A System of Bacteriology*, Medical Research Council, London, 1930, 5, 100.

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.

BACTERIA BIOLOGICALLY ALLIED TO THE DIPHTHERIA BACILLUS (DIPHTHEROID BACILLI)

CORYNEBACTERIUM PSEUDODIPHTHERITICUM or HOFMANNI (*Bacillus of Hofmann*)

A commensal of the throat.

Morphology and Staining.—Compared with the diphtheria bacillus it is shorter (about 2μ) 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 method.

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. 389, 390.

Biochemical Reactions.—Table, p. 393.

It is non-pathogenic to laboratory animals.

CORYNEBACTERIUM XEROSIS

(*Bacillus xerosis*)

A commensal in the conjunctival sac. Closely resembles the diphtheria bacillus, and may show volutin granules.

For appearance of colonies on blood-agar, see p. 390.

Can be differentiated from the diphtheria bacillus by its production of acid in sucrose (*vide* Table, p. 393) and by its non-pathogenicity to laboratory animals.

CORYNEBACTERIUM ACNES

(*Acne bacillus*)

An organism associated with acne, but its aetiological relationship to the disease is doubtful. It is Gram-positive, rod-shaped, and measures about 1.5μ by 0.5μ . It is markedly pleomorphic and frequently shows a beaded appearance, like other diphtheroid bacilli.

In primary culture grows under anaerobic conditions or as a micro-aerophile if a fermentable carbohydrate such as glucose is present, or aerobically if the medium contains serum or blood and is definitely acid (pH 6·2-6·8).

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. 97), 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 naso-pharynx, the external ear, conjunctival sac, the skin, lymph glands (apart from disease) and other tissues, pus, wounds, etc. Compared with the true diphtheria bacillus they are of low virulence to laboratory animals.

Barratt has described diphtheroid bacilli in the naso-pharynx 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. Jebb has reported somewhat similar organisms which ferment starch and might seem to be intermediate between *C. diphtheriae gravis* and *C. ovis*. (See Cook, G. T., and Jebb, W. H. H., *J. Clin. Path.*, 1952, 5, 161.)

BIOCHEMICAL REACTIONS OF *C. DIPHThERIAE* AND CERTAIN ALLIED TYPES

	Glucose	Sucrose	Dextrin
<i>C. diphtheriae</i>	+	- ¹	+
<i>C. pseudodiphtheriticum</i> .	-	-	-
<i>C. xerosis</i>	+	+	-

(+ = acid production ; if a serum-water medium is used, e.g. Hiss's, the acid also produces clotting.)

CORYNEBACTERIUM OVIS

(*Bacillus pseudo-tuberculosis ovis*—*Preisz-Nocard bacillus*)

The causative organism of caseous lymphadenitis and pseudo-tuberculosis 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.
Morphology and Staining.—Non-motile, slender rod-shaped organism

¹ It has been stated that occasionally strains may ferment sucrose.

1-3 μ in length by 0.4 μ 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.

Broth—a granular growth occurs with sometimes a surface pellicle.

Gelatin—slow liquefaction occurs.

Glucose, maltose and dextrin are fermented, but usually not sucrose, lactose or mannitol. A haemolysin is produced in culture medium.

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. 423). Subcutaneous injection is followed by lymphatic gland involvement, the glands showing the characteristic caseation. In rats, inoculation produces a fatal septicaemia. Sheep and goats are also susceptible to experimental inoculation.

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. muri-septicum*) produces a septicaemic disease in mice.

CORYNEBACTERIUM PYOGENES

(*Bacillus 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. Growth on coagulated serum produces small pits of liquefaction. Gelatin is also liquefied. This organism is haemolytic when growing on blood-agar, and a filterable haemolytic toxin can be demonstrated in suitable culture medium.¹ In milk, acid and clot result in three days and after a time the clot is digested. Glucose, lactose and, in some cases, sucrose are fermented. Mannitol is not usually 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 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. Glucose is fermented; some strains also ferment laevulose and mannose.

Corynebacterium equi has been reported as the causative organism of pneumonia in colts. It differs from other members of the diphtheroid group in its profuse viscid growth and the production of a red pigment. Carbohydrates are not fermented.

LISTERIA MONOCYTOGENES

(*Bacterium 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 mono-

¹ See Lovell, R., *J. Path. Bact.*, 1941, 52, 295.

cytosis in the blood. It was originally isolated from these animals ; it has been found in gerbilles, sheep and certain other animals, and in meningitis of the human subject. It has been reported recently in human cases presenting the features of infectious mononucleosis ; this disease, however, has generally been regarded as of virus origin. The organism has been found in foetuses from cases of abortion in sheep.

It occurs as a Gram-positive non-sporing bacillary organism, $2\text{-}3\mu$ by $0\cdot5\mu$ (average), often in pairs end-to-end at an acute angle. It is feebly motile and possesses a single terminal flagellum. Sometimes elongated filaments may be observed.

Cultures can be obtained at 37°C . under aerobic conditions on ordinary media, but growth is better on media containing liver extract 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. Gelatin is not liquefied. Glucose is regularly fermented ; lactose and sucrose may be fermented, though slowly ; mannitol is not acted on.

When cultures are inoculated into rabbits and guinea-pigs, monocytosis results (*vide supra*), and if the dose is considerable, localisation may occur in the myocardium, meninges and liver with associated focal necrosis.

The species appears to be serologically somewhat heterogeneous, and four types have been recognised by means of agglutination tests.

CHAPTER XIII

THE MYCOBACTERIA : TUBERCLE BACILLUS AND OTHER ACID-FAST BACILLI

MYCOBACTERIUM TUBERCULOSIS (*BACILLUS TUBERCULOSIS*)

THE causative organism of Tuberculosis in man, mammals and birds.

Under this designation are included different types—the “human,” “bovine” and “avian” types, so called in virtue of their occurrence in man, cattle and birds respectively.

An organism occurring in a tubercle-like disease among voles represents a further type of tubercle bacillus.

HUMAN TYPE

(*Mycobacterium tuberculosis* var. *hominis*)

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. 94, 96). 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. 177). 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. 177–179); 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. 187) 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 about 60° C. While many individual bacilli die when desiccated, a certain proportion survive for several weeks or months. The organism is relatively resistant to injurious chemical substances: it can survive in putrefying material, it withstands gastric juice, and *in sputum* may resist 5 per cent. phenol or antiformin (p. 403) for several hours. It is highly susceptible to sunlight and ultra-violet radiation. Cultures remain viable usually for several months.

Experimental Inoculation.—The guinea-pig is 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 general tuberculosis is noted ; the spleen is enlarged and contains greyish-white tuberculous nodules or larger necrotic lesions. The liver and also various other organs present 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, tuberculous nodules may be present only in the spleen and on the peritoneum.

Animals can be infected also by inhalation and by feeding.

Occurrence in Animal Tuberculosis.—The human type of tubercle bacillus, apart from its occurrence in human disease, has been found also in tuberculosis of monkeys, pigs and dogs.

BOVINE TYPE

(*Mycobacterium tuberculosis* var. *bovis*)

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 laboratory 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 mgm. of dried bacilli (from a culture). The bovine type produces an acute generalised tuberculosis, and the animals usually die within two months ; in the case of the human type the animals survive, or die only after two months, with slight lesions confined usually to the lungs and kidneys.

The differentiation may also be made by injecting 10 mgm. 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 (Griffith).

Recently it has been pointed out that voles are highly susceptible to the bovine type of tubercle bacillus, whereas they are resistant to the human type; and a dose of 0.001 mgm. moist weight of culture injected intraperitoneally into voles will distinguish between the two types.

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 OF TUBERCLE BACILLI IN HUMAN LESIONS

Both the human and bovine types are met with, though the former is the more prevalent. The latter is infrequently found in adults, but a proportion of tuberculous conditions in young subjects is due to this type, particularly cases resulting from alimentary infection by milk from tuberculous cows.

The percentage frequency of the bovine type of tubercle bacillus, was reported some years ago by Griffith as follows: tuberculosis of cervical glands, 91.3 in England and 65 in Scotland in children under five years, and 50 in England and 52.6 in Scotland at all ages; bones and joints, 19.7 in England and 30.7 in Scotland; genito-urinary system, 17.4 in England and 31 in Scotland; lungs, 1.4 in England and 5.4 in Scotland; meninges, 24.6 in England and 29.6 in Scotland; skin (lupus), 48.7 in England and 69.2 in Scotland.

More recent evidence pertaining to the incidence of the bovine type infection in non-pulmonary tuberculosis is given in a publication of the National Association for the Prevention of Tuberculosis¹ which should be consulted for further information on the part played by the bovine tubercle bacillus in human tuberculosis in Great Britain.

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 infections 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.

¹ *Non-pulmonary Tuberculosis of Bovine Origin in Great Britain and Northern Ireland*, by G. S. Wilson, J. W. S. Blacklock and L. V. Reilly, London, 1952.

In the lesions they are usually found free from cells, but intracellular bacilli may be noted.

In phthisis, tubercle bacilli are present in the sputum, and often in large numbers if the pulmonary lesion is active and rapidly breaking down.

In tuberculosis of the urinary system the bacilli may be found in the urine by microscopic examination of the deposit after centrifuging, but, as a general rule, they are relatively scanty, and may not be observed in film preparations.

In intestinal tuberculosis the bacilli may, in some cases, be found in films from the faeces.

In tuberculous meningitis, tubercle bacilli may be seen in films from the spinal fluid after centrifuging, or in films from the coagulum which forms in the fluid after it is withdrawn. In early cases, however, microscopic examination may yield negative results.

Immunity.—Dead cultures of the tubercle bacillus have generally been found to have little immunising effect. A certain degree of resistance can be conferred, however, by introducing into the body living organisms of attenuated virulence. For this purpose, Calmette and Guérin advocated the use of a bovine strain attenuated by prolonged growth on a bile-glycerol-potato medium. This strain is generally designated "B.C.G." (*Bacille Calmette-Guérin*) and is practically non-pathogenic. It has been used as a vaccine for immunising children and cattle, and claims have been made that it is effective even by oral administration in young subjects, three doses of 10 mgm. at forty-eight hours' intervals being recommended. Alternative methods of administration are subcutaneous and intracutaneous injection; in the former, one dose of 0.005 to 0.02 mgm. is given; in the latter, one dose of 0.05 to 0.3 mgm. Recently, cutaneous administration by "multiple puncture" has been recommended; and an instrument which makes forty punctures at one stroke has been devised for the purpose. This method overcomes a serious difficulty in B.C.G. vaccination, namely, the occurrence of an "abscess" at the site of injection. The practical results have been difficult to assess, but the statistical evidence indicates that the vaccine lowers morbidity and mortality from the disease. It has been proved by experiments in calves that a substantial degree of immunity follows intravenous injections of B.C.G., especially if repeated at six-monthly intervals.

It may be noted that *experimental* immunisation against human and bovine types of the tubercle bacillus has been achieved by inoculation of living cultures of the vole type in animals (*e.g.* guinea-pig, calf), to which this type is only slightly virulent.

Chemotherapy.—The tubercle bacillus is generally very susceptible to streptomycin, and this antibiotic has now been extensively used in the treatment of particular forms of

tuberculosis. The results have been encouraging, but streptomycin therapy has proved to be subject to a serious drawback, namely, that under treatment the organism may become relatively resistant to the antibiotic and that certain strains of the tubercle bacillus are naturally resistant. If *p*-aminosalicylic acid is combined with streptomycin the likelihood of streptomycin-resistant strains developing in the course of treatment is reduced.

Recently it has been found that isonicotinic acid hydrazide ("isoniazid") is a highly effective anti-tuberculosis agent, but is subject to the same disadvantage as streptomycin, viz., the emergence of drug-resistant strains in the course of treatment (p. 311).

DIAGNOSIS OF TUBERCULOUS INFECTION

Direct microscopic examination.—Sputum: a film is prepared from the purulent portion of the sputum and stained by the Ziehl-Neelsen method (p. 94). The use of the $\frac{1}{2}$ -in. oil-immersion objective is particularly recommended (p. 56). *The oil is dropped on the film*, and the dipper of the oil-bottle should never touch the film, otherwise acid-fast bacilli may be transferred to the oil container. After each examination the front of the objective should be wiped free from oil. The bacilli may be more easily recognised, particularly when they are scanty, by using a blue-green screen, Wratten H (No. 45), in front of a high-intensity illuminant. The bacilli appear black, while if the counterstain is malachite green the background of cells, etc., almost disappears, rendering the bacilli more easily recognised. In case of doubt the screen can be removed, and the organisms verified with the $\frac{1}{2}$ -in. objective. A prolonged examination may be necessary in some cases where the bacilli are relatively scanty, and one examination with a negative result by no means excludes tuberculosis.

Fluorescence microscopy after staining with auramine as described on p. 77 may be substituted for ordinary microscopy with Ziehl-Neelsen stained films.

In young children who swallow their sputum, stomach contents obtained by gastric lavage should be examined, or coughing may be induced by inserting a swab into the throat, the secretion or expectoration on it being then used to prepare a film for microscopic examination. Repeated examination of faeces may also be resorted to as an alternative to examination of gastric contents.

Urine, pleural and peritoneal fluids are centrifuged, films are made from the deposit and stained by the Ziehl-Neelsen method.

If a specimen of urine has not been taken with a catheter, it is essential to treat the film with alcohol (two minutes) after decolorisation with acid in the Ziehl-Neelsen process, in order to exclude if possible smegma bacilli (p. 96). In examining urine it is advisable to obtain the sediment from a twenty-four hours' specimen and treat it with antiformin (*vide infra*).

Cerebro-spinal fluid is allowed to stand in a stoppered tube for an hour or longer, when a "spider-web" coagulum usually forms in the fluid. The clot is carefully decanted into a watch-glass, and a cigarette paper (held with forceps) is laid over it, gentle pressure being exerted with a wire loop so that the clot adheres to the paper, on which it is then transferred to a slide. By firm blotting the clot is spread on the slide, to which it remains adherent after the paper is removed. The preparation is dried and stained by the Ziehl-Neelsen method. In the absence of clotting the fluid is centrifuged and the deposit examined in the usual way.

Pus and faeces : direct films can be made and stained by the Ziehl-Neelsen method, but it is advisable to treat with antiformin (*vide infra*).

In the case of tissues, sections are stained by the Ziehl-Neelsen method (p. 96).

Antiformin method for the detection of scanty tubercle bacilli in sputum, pus, tissues, etc.—By this method the bacilli can be concentrated in the material examined, and it is particularly valuable where the bacilli are scanty. The method is, of course, supplementary to the ordinary examination, and is quite unnecessary where bacilli can be seen in direct film, unless there should be any doubt as to the identity of acid-fast bacilli observed by the usual method.

"Antiformin" consists of equal parts of liquor sodae chlorinatae (B.P.) and 15 per cent. caustic soda. It has the property of dissolving cells and other bacteria, leaving tubercle bacilli intact.

A quantity of sputum or other material is treated with three or four times its bulk of antiformin diluted 1 in 6 with water, and the mixture is shaken and allowed to stand at 37° C. till it becomes thoroughly liquefied. This usually takes about an hour, but it is sometimes necessary to add more diluted antiformin to complete the solution. The mixture is then centrifuged and the supernatant fluid is removed. Water is added and mixed with the sediment. After centrifuging again, thick smears are made from the sediment, dried, fixed and stained by the Ziehl-Neelsen method.

Cultivation of the Tubercle Bacillus from Pathological Material.—If the tubercle bacilli are likely to be present in pure culture in the material, screw-capped bottles containing slopes of egg medium can be inoculated directly. At least four, and preferably six, bottles of medium should be used. If other organisms are present, e.g. in sputum, the antiformin method or one of the procedures described below can be used and cultures made from the sediment; in this case the centrifuge tubes, water used for washing the sediment, etc., must be sterile.

Petroff's method (modified).—Sputum is mixed thoroughly with three to four times its volume of 4 per cent. caustic soda and placed in the incubator at 37° C. for thirty minutes, the container being shaken from time to time. The mixture is centrifuged at 3000 r.p.m. for thirty minutes and the supernatant fluid poured off. The deposit is neutralised with 8 per cent. hydrochloric acid, which is added drop by drop, the reaction of the mixture being tested by adding a drop of phenol red solution to the tube. The deposit is then inoculated on egg medium containing 1 : 10,000 crystal violet (Petroff's medium) which inhibits the growth of other organisms, or preferably on the Löwenstein-Jensen medium (p. 178). Dubos medium (p. 187) can also be used with advantage as regards rapidity of growth for direct cultivation of the tubercle bacillus from pathological material after treatment, as above, to remove other bacteria.

An alternative method is to treat with 6 per cent. sulphuric acid for the same time. In this case, neutralisation of the centrifuged deposit with caustic soda is carried out before the culture medium is inoculated. In dealing with specimens of sputum some workers prefer Petroff's method in which tenacious mucus is dissolved by the alkali.

Jungmann's method of homogenising tuberculous material¹:—Prepare the following solutions :

Solution A.

Ferrous sulphate	20 grams
Concentrated sulphuric acid	20 ml.
Distilled water	180 ml.

Solution B.

Hydrogen peroxide (20 vols.)	5 ml.
Distilled water	95 ml.

Solution A can be made up in bulk and keeps indefinitely. Solution B must be made up fresh on each occasion. For use place 2 ml. of

¹ Nassau, E., *Tubercle*, 1942, 23, 179.

sputum in a Universal Container. Add 1·2 ml. solution A and 1·2 ml. solution B. Shake the container for 30 seconds and allow to stand on the bench for 20 minutes, shaking at intervals. Centrifuge the bottle at 3000 r.p.m. for 30 minutes and discard the supernatant fluid. Fill the container to the shoulder with sterile saline, shake vigorously, and again centrifuge. Decant the supernatant fluid and for cultivation of the tubercle bacillus inoculate the deposit on Löwenstein-Jensen medium. It is unnecessary to neutralise the deposit.

Note.—In applying the method of direct cultivation the possibility must be borne in mind of non-pathogenic acid-fast bacilli (*vide infra*) occurring in cultures from the various materials examined, these organisms sometimes yielding growths not unlike that of the tubercle bacillus. Careful scrutiny of the culture should therefore be made, and if there is any doubt an animal inoculation test 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 20 minutes, and then washing thoroughly with sterile distilled water, and finally placing the slides in a suitable fluid medium, e.g. citrated human blood diluted with three parts of distilled water. 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 streptomycin (p. 309). (See : Rosenberg, K. S., *Lancet*, 1943, 1, 615 ; Oeding, P., *Act. Tubercul. Scand.*, 1951, 25, 208.)

A slower method of obtaining pure cultures is to inoculate a guinea-pig with the material (p. 398), and to make cultures from the lesions in glands or infernal organs (e.g. spleen) after active or generalised tuberculosis results. Cultures can be obtained from the inguinal glands if the lesions have not ulcerated through the skin. When this has occurred, cultures may be made from the pelvic glands.

In laboratory diagnosis, where tubercle bacilli cannot be detected in specimens by microscopic examination, *direct cultivation* or *guinea-pig inoculation* should be resorted to, as these procedures often yield positive results when microscopic examination is negative.

The usual method of carrying out the guinea-pig inoculation test is to inject material subcutaneously in the flank or thigh or intramuscularly in the latter situation. 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.

The *complement-fixation reaction* has sometimes been applied for diagnosis and for gauging the activity of lesions, but its practical value is very limited. The method has been referred to in Chapter VIII. The reaction is most frequent and most marked in the case of chronic but active lesions. It is negative in many arrested cases and may be negative also in rapidly progressive tuberculous conditions.

Haemagglutination Test.¹—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. 40); 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. The test has received recently a good deal of study and clinical trial, but it is still premature to make any statement as to its practical value for gauging tubercle infection.

Tuberculin is a preparation containing the specific protein of the tubercle bacillus. It was originally obtained from a six-weeks-old culture in glycerol-broth, evaporated to one-tenth of its volume, sterilised by heat and filtered (Koch's "Old Tuberculin"). Various methods have been employed, however, in its preparation. Thus Koch's "New Tuberculin" is derived by grinding the bacilli (obtained from a growth on solid medium) in 50 per cent. glycerol. Tuberculin has also been prepared from cultures grown in a synthetic medium and has the advantage of being free from extraneous protein material, which may give non-specific reactions. Moreover, the specific protein can be separated from other constituents and products of culture, and thus purified. The process of isolating this protein involves ultra-filtration and precipitation by trichloracetic acid.

This "Purified Protein Derivative" (Tuberculin P.P.D.) is preferable to Old Tuberculin as it is constant in composition and potency. Moreover, 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. Tuberculin P.P.D. is used in the same way as Old Tuberculin.

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.

Tuberculin has been used in the diagnosis of tuberculosis, and for full details one of the larger works should be consulted. The diagnostic application depends on the fact that the tissues of a person or animal infected with tuberculosis exhibit hypersensitivity (allergy) to the specific protein of the bacillus. Thus, the subcutaneous injec-

¹ Middlebrook, G., and Dubos, R., *J. Exp. Med.*, 1948, 88, 521; Scott, N. B., and Smith, D. T., *J. Lab. Clin. Med.*, 1950, 35, 203.

tion of tuberculin in a tuberculous subject may lead to local, focal and general reactions. Cutaneous allergy can be elicited by the *cutaneous reaction of von Pirquet*, in which Old Tuberculin is applied to a small abraded area of skin, or by the *intracutaneous test of Mantoux*, which consists in the intradermal injection of this tuberculin (0·1 ml. of a 1 : 1000 dilution) by means of a syringe with a fine needle. In both cases, in a tuberculous subject, an area of erythema and swelling appears at the site of inoculation within a few hours and attains a maximum in twenty-four to forty-eight hours. It is to be noted, however, that such reactions do not necessarily denote active tuberculosis, and a high percentage of adults give a positive reaction, probably due to previous sub-clinical infection. In infants and young children the reaction is of more significance from the diagnostic standpoint.

The tuberculin reactions have been utilised also in the recognition of tuberculosis in cattle, and are of great importance in testing milch 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* : 0·1 ml. Old Tuberculin (or one of the newer preparations—*vide supra*) is injected intradermally in a shaved area of skin in the neck, and the thickness of a fold of skin in this area is measured with callipers before the injection. The result is observed after forty-eight hours and a positive reaction is indicated by the occurrence of a hot, tender, diffuse and ill-defined swelling, the increased thickness of the fold of skin being 7-17 mm.; in non-tuberculous animals there may be some degree of infiltration, which is usually well-defined and small in area; if the result after the first injection seems negative or inconclusive, a second dose is given at the same site and the result noted after twenty-four hours. A positive reaction is indicated by the same type of inflammatory swelling as that described above, the increased thickness of the skin fold being stated as 13-40 mm.

By the use of the purified protein derivative described above a single intradermal test can now be substituted for the double test. 0·1 ml. of a standard preparation is injected intradermally in the neck; a visible infiltration following the injection is considered positive independently of any increase in the thickness of the skin. An increase of 4 mm. or more in the thickness of the skin at the site of injection would also denote a positive result, but lesser degrees of swelling are to be regarded as negative or of doubtful significance.

It is also well recognised now that cattle may give positive reactions in the absence of demonstrable tuberculosis *post mortem*, possibly as the result of inapparent infection by the avian type of tubercle bacillus or some other acid-fast organism.

By using in parallel bovine and avian type tuberculins, this fallacy can be overcome; an animal with tuberculosis reacts more strongly

to the bovine than to the avian tuberculin, whereas a stronger reaction to the avian tuberculin would not be of diagnostic significance. In the case of positive or doubtful reactions to both tuberculins a re-test is carried out with preparations of half-strength.

By the Regulations under the Therapeutic Substances Act, the term tuberculin is restricted and applies to preparations of fluid media in which the tubercle bacillus has been grown in artificial culture and which have been freed by filtration from the bacilli. These regulations stipulate that Old Tuberculin preparations shall be tested by an approved method for specific toxicity to guinea-pigs or other animals infected with the tubercle bacillus. A standard preparation of Old Tuberculin is kept in the National Institute for Medical Research, London, and Old Tuberculin shall not be issued if its activity differs from the standard preparation to such an extent that the difference is revealed by the test.

Mycobacteria of Ulcerative Lesions in the Human Subject.—Recently ulcerative lesions of the skin have been reported in Australia and Sweden from which mycobacteria, distinguishable from the tubercle bacillus, have been isolated. The Australian strains were found to be pathogenic to mice and rats but not guinea-pigs. In culture the optimum temperature was 33° C. The strains described in Sweden were likewise pathogenic to mice, though active disease was not produced in rats; in guinea-pigs only a local abscess resulted; in rabbits inoculation of an abraded area of the skin produced ulcers similar to the human lesions, which were described as "tuberculoid" in nature. The Swedish strains grew at 31° C. but not 37° C. (See: MacCallum, P., et al., *J. Path. Bact.*, 1948, **60**, 93; Norden, A., and Linell, F., *Nature*, 1951, **168**, 826.)

Mycobacterium avium (*Avian tubercle bacillus*).—The causative organism of a tuberculosis-like disease 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 mgm. 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 evidence available suggests that the vole tubercle bacillus is practically non-pathogenic to man. Inoculation of the organism in guinea-pigs and calves brings about some degree of immunity to virulent tubercle bacilli.

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 to mammals or birds, but produce lesions on experimental inoculation in frogs, fish, etc.

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. These organisms are less resistant to antiformin than the tubercle bacillus.

Acid-fast bacilli have been frequently demonstrated in the deposits from the interior of laboratory taps. This possibility 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-corks, such as are used for stoppering 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. 96), which has no effect on the tubercle bacillus. It is also less resistant to antiformin than the tubercle bacillus.

MYCOBACTERIUM LEPRAE (*BACILLUS 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. The attempted cultivation of this organism is not within the scope of routine practical bacteriology, and for further information on this subject the text-books and other literature should be consulted.

Occurrence and Distribution.—Leprosy is an infective granuloma, developing as (1) the "nodular" type, in which nodules of granulation tissue 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 leprosous 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.

DIAGNOSIS OF LEPROSY

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.

The films or sections are stained by the Ziehl-Neelsen method, substituting 5 per cent. sulphuric acid for 20 per cent. (It should be noted that 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.

As a routine measure, 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.

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 leprae murium.—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 or PARATUBERCULOSIS (*Bacillus of Johne's Disease*)

The causative organism of a chronic enteritis of cattle, and a similar disease of sheep.

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 2μ), 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 refractory.

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 orange-coloured pigment.²

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 (see paper cited in footnote 1).

¹ Taylor, A. Wilson, *J. Path. Bact.*, 1950, **62**, 647.

² *Ibid.*, 1951, **63**, 333.

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.

CHAPTER XIV

THE ANTHRAX BACILLUS AND ALLIED ORGANISMS; THE GLANDERS BACILLUS

BACILLUS ANTHRACIS

THE causative organism of Anthrax in animals and man.

Morphology.—A non-motile, straight, rod-shaped, sporing bacterium, rectangular in shape and of relatively large size —4–8 μ by 1–1.5 μ . 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 consists of a polypeptide of *d*-glutamic acid which probably renders the organism resistant to proteolytic enzymes. 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.—Gram-positive. The spore is unstained by the ordinary methods, but can be stained differentially by special methods (p. 99).

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. 87), 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.

Colonies on agar—white, granular, circular disks (about 3 mm. in diameter after 24 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. 421), which are markedly lytic.

Glucose, sucrose and maltose are fermented (without gas production).

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 thermal death-point of the vegetative form is about 60° C. Spores can withstand 100° C. (moist heat) for five to ten minutes, and resist desiccation for an indefinite period. They are destroyed by 4 per cent. potassium permanganate in fifteen minutes. For 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.).

It has also been pointed out that animal hair and bristles can be disinfected, as regards the spores of the anthrax bacillus, by six hours' treatment with 0.25 per cent. formaldehyde solution at 60° C., and that 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 spores*, and laboratory infection from handling such material has been recorded. It is claimed that fixation of films by 1 : 1000 mercuric chloride for 5 minutes kills the spores and does not interfere with staining reactions (Soltys).

Occurrence in Animal Lesions.—The anthrax bacillus produces an epizootic disease in herbivorous animals, particularly among sheep and cattle. 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 in the spleen, which is enlarged, soft and diffuent.

Infection in Animals.—Animals may be infected from pasture which has become contaminated with *B. anthracis* spores derived from previous cases of the disease in the particular area, or from effluents of tanneries and wool-factories. The disease is also spread by imported artificial foodstuffs, e.g. oil-cake. Bitting flies, e.g. *Stomoxys*, may convey the organism, though in an entirely mechanical way.

Experimental Inoculation.—Guinea-pigs and mice are highly susceptible. If a guinea-pig is injected subcutaneously with pathological material containing the bacilli, or with 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 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 internal organs. They are specially numerous in the spleen, which is enlarged and soft.

Occurrence in Human Lesions.—Transmission of the infection to man is from an animal source :

- (1) Infection may occur through the skin—e.g. in persons handling infected animals, carcasses or hides, from shaving brushes, etc.—the resulting lesion being described as a “malignant pustule,” i.e. an area of intense inflammation, with a central slough and with surrounding inflammatory oedema. Lymphatic spread may occur, and even septicaemia.
- (2) 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 may also 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.

(3) Infection may occur by the intestine, but this is relatively uncommon in man.

Prophylactic Immunisation.—Pasteur's vaccine has been extensively applied in the prevention of the disease among animals. The vaccine is a culture of the bacillus whose pathogenicity has been reduced by continuous growth at 42°–43° C. for certain lengths of time. The degree of attenuation is, however, difficult to regulate. Besredka advocated the intradermal inoculation of the vaccine (as opposed to the subcutaneous injection of the older method) and claimed that a solid immunity could be produced in this way. A "combined" method of prophylactic immunisation has also been applied—viz. injection of Pasteur's vaccine along with an immune serum, and a *spore vaccine* prepared from a strain attenuated by Pasteur's method, administered along with immune serum, has been widely used with successful results.

Therapeutic Antiserum.—The serum of artificially immunised animals is able to confer passive immunity, and has been used in the treatment of human anthrax. Doses of 50–100 ml. given intravenously and repeated daily if necessary have been recommended.

Antibiotic Therapy.—The anthrax bacillus is sensitive to penicillin, which can be used for therapeutic purposes in human anthrax. Aureomycin and terramycin have also been used successfully.

DIAGNOSIS OF ANTHRAX

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 examin-

ing 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. 236 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, i.e. 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 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 18 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 3–4 hours with occasional shaking. Squeeze the material if possible and heat the supernatant fluid to 70° C. for 10 minutes. Add different volumes (0·2 ml.–2·0 ml.) of this fluid to melted agar and pour plates (p. 211); incubate at 37° C., from evening to early morning. It is essential to examine the plates early. Examine for typical deep colonies, as surface ones may be misleading. A rich culture medium is essential and plates should not be too crowded with colonies. Confirmation is obtained by inoculation subcutaneously in the mouse.

The selective medium of Pearce and Powell¹ may be used with advantage in isolating anthrax bacilli. Its constituents are :

¹ Pearce, T. W., and Powell, E. O., *J. Gen. Microbiol.*, 1951, 5, 387.

- (1) 400 µg. haemin/ml. in 0·01N NaOH, which is autoclaved at 5 lbs. per sq. in. for 3 mins.
- (2) 600 µg. lysozyme/ml. in 0·01N acetic acid, heated to 55° C. for 15 mins. before use.
- (3) Peptone agar, made from peptone (Evans) 2 per cent., NaCl 0·5 per cent., agar 2 per cent.; final pH 7·4.

Melt 8 volumes of agar, cool to 60° C., add 1 volume haemin solution, 1 volume lysozyme solution and pour plates immediately.

Incubate plates at 37° C. or 40° C. The higher temperature makes the medium more selective, as *B. mycoides* (*vide infra*) does not grow, but there may be a 20 per cent. diminution in the number of *B. anthracis* spores that will germinate. At 37° C. 95 per cent. of soil spores capable of growing on peptone agar will be suppressed. Organisms that do grow at 37° C. can readily be distinguished from *B. anthracis* by their colony appearance.

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 15 minutes. Discard the supernatant and inoculate the residue intramuscularly into a guinea-pig which has been passively immunised 24 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 2-3 days. Death from gas-gangrene (usually due to *Cl. septicum* or *Cl. bif fermentans*) 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 carcasses, and may be applicable even in the case of putrefied material. It depends on the occurrence of a specific precipitin (p. 46) 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.

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 types tend to resemble the anthrax bacillus and young forms are Gram-positive. Some are morphologically identical with *B. anthracis* and occur in similar chain formation. Others 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 (e.g. *B. subtilis*, *B. mycoides*), sub-terminal or terminal. It may be relatively small, not exceeding 0.8 μ (e.g. *B. mesentericus*), or large, up to 1.8 μ (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 appearances of growths vary 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 on broth is a frequent character. Generally, gelatin is liquefied and proteolytic action is well developed. Some types ferment carbohydrates. Starch may be hydrolysed.

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. Growths on blood agar are usually haemolytic (cf. anthrax bacillus). It should be noted that if a large dose of culture 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. 414).

Phospholipinase Reaction of Certain Aerobic Sporing Bacilli

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

¹ *J. Gen. Microbiol.*, 1948, 2, 334.

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.

Method.

Separate the yolk from the white of an egg, weigh it and make up a 5 per cent. w/v mixture in nutrient broth. To each 100 ml. add 2 grams of kieselguhr, stir, filter through paper pulp on a Buchner funnel and sterilise by passing through a Seitz disk. Add equal portions of this egg-yolk broth to 4 per cent. nutrient agar at 45° C. and immediately pour plates. The medium is thus ordinary nutrient agar containing 2·5 per cent. egg-yolk.

The organisms which produce phospholipinase are marked by a thick opaque zone extending several millimetres from the colony.

B. cereus is a saprophytic widely-spread organism commonly found in heat-treated milk; as it has the property of reducing methylene blue (p. 296), it may be necessary to identify it, and this is easily done on the egg-yolk-agar plate. It is almost indistinguishable from *B. mycooides* and the two organisms probably represent varieties of one species. Most of the strains generally classified as "*B. antracoides*" (p. 421) are in reality *B. cereus*. The power to split lecithin is very much greater in *B. cereus* than in *B. anthracis* in which it is only slight, and the egg-yolk-agar plate will distinguish easily *B. cereus* from the other saprophytic aerobic sporing bacilli and from *B. anthracis* itself.

MALLEOMYCES or LOEFFLERELLA MALLEI

(*Bacillus 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.

Morphology.—Straight or slightly curved bacilli with rounded ends, about 2-3μ by 0·4μ. Short forms are frequently noted and also longer filaments. Bacilli with club-shaped ends, and even branched forms have been observed. In old cultures, swollen irregular involution forms are numerous. The bacilli occur singly or in pairs. They are non-motile and non-sporing.

Staining.—Gram-negative. An important feature of the organism, as seen in the tissues and inflammatory exudate, is its granular or beaded appearance.

Cultural Characters.—Aerobe and weakly facultative anaerobe; optimum temperature, 35°-38° C.; does not grow below 20° C.; grows on ordinary media, but the addition of glycerol (4 per cent.) assists growth. Blood or serum also enriches cultures. Primary cultures are generally slow in developing, e.g. only after two days.

Agar stroke—uniform, white, semi-transparent, moist band of

growth along wire track, which later becomes opaque, somewhat slimy and yellowish brown in colour.

Colonies on agar—about 1 mm. diameter after two to three days' growth; round and convex with the same physical characters as the confluent growth described above. Colony variants presenting a dry and wrinkled appearance have been observed.

Gelatin is not liquefied, but the glanders bacillus does not grow well at room temperature at which gelatin cultures are incubated for testing liquefaction.

Potato—at first a transparent yellowish growth ("honey-like"), later becoming opaque and of a chocolate-brown colour. Care should be taken to ensure that the potato is alkaline (p. 179).

Glucose is fermented, but the reaction is slight and not regular in occurrence. A slight effect on salicin has been recorded. No other carbohydrates are fermented, but it is stated that growth in milk produces slight acidity and slow clotting.

In primary culture, growth does not occur readily and the organisms die out quickly, but after several subcultures they become adapted to a saprophytic existence, and may live for two months.

Occurrence.—Glanders is an infective granuloma, with a marked tendency, however, to suppurative change. It is essentially a disease of horses, asses and mules, and is only occasionally transmitted to man, usually by direct infection from an animal source.

In acute and subacute animal glanders, ulcerating nodules occur in the nasal mucosa and later in the lungs and internal organs. The bacilli are present in considerable numbers in all the lesions, situated for the most part extracellularly.

In chronic animal glanders ("farcy"), where the superficial lymph glands and vessels are involved, the bacilli are less numerous.

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.—In equidae, the typical disease can be reproduced by subcutaneous injection of recently isolated cultures; asses are most susceptible. 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). Even when pathological material containing the glanders bacillus in mixed infection is injected intraperitoneally, the specific organism seems to flourish best in the tunica, and can be isolated more easily from this situation than from the peritoneum.

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. It should be noted that the bacilli are not easily demonstrable in preparations from the lesions.

Cultures are also made on glycerol-agar, and if a mixed growth results, pure cultures are obtained from single colonies. The chocolate-coloured growth on potato is an important criterion in identification.

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 culture isolated; in two or three days an enlargement of the testis results, and the animal subsequently dies, showing the lesions of acute glanders (*vide supra*). If pyogenic organisms are also present in the material injected, and a septic peritonitis results, the glanders bacilli will be found more numerous in the tunica vaginalis. 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 Test.—In the diagnosis of glanders in horses, the agglutination test has been used. The technique followed is that described on pp. 243-249, with certain minor differences. The culture selected should be one which has been proved suitable for the agglutination reaction by previous tests with positively-reacting sera. The bacterial suspension is prepared from young glycerol-agar slope cultures, and is then heated at 65° C. for one hour to kill the organisms. For personal safety it is necessary to use dead cultures. Serum dilutions ranging from 1 in 100 to 1 in 4000 are tested. The agglutination tubes are incubated for two hours at 37° C. and are then kept in a refrigerator overnight before readings are made. Normal horse serum, however, may agglutinate the bacillus, and only agglutination in high dilutions, 1 in 1000 or more, can be accepted as definite proof of infection. Agglutination in lower titres does not, of course, exclude the existence of the infection. Any spontaneous agglutination in the control would, of course, invalidate the result. Some workers include parallel tests with normal horse serum as an additional control.

A complement-fixation test (pp. 45, 254, *et seq.*) has also been employed as a serum diagnostic method. It has been regarded as more satisfactory than the agglutination test, especially in chronic cases.

The antigen is prepared in the same way as the bacterial suspension for the agglutination test. The concentration to be used in the actual test should be free from anti-complementary action. This can be determined by a preliminary titration, varying dilutions being mixed with 2 M.H.D. of complement and incubated for one and a half hours at 37° C., when the haemolytic system is added. After further incuba-

tion for one hour, the lowest dilution which exhibits no anti-complementary action (*i.e.* complete lysis) is selected for the complement-fixation test.

Mallein is a preparation analogous to tuberculin, and the allergic reaction to it has proved of great value in the diagnosis of glanders infection. A four weeks' culture in glycerol broth is sterilised at 100° C., and then filtered. The filtrate which contains the heat-stable soluble products of the organism is concentrated to one-tenth of the original volume and constitutes the preparation "crude mallein," which can readily be preserved and utilised for diagnostic purposes in a manner analogous to tuberculin. Subcutaneous injection of 1 ml. diluted mallein (containing 0·1 ml. of the crude preparation) in an infected animal produces a local reaction (a swelling reaching a diameter of five inches after twenty-four hours) and a general reaction manifested by a definite elevation of temperature. A conjunctival reaction may be elicited readily by introducing mallein into the conjunctival sac, and is a more satisfactory test than that in which the mallein is injected subcutaneously. Another method which has been employed is the *intradermal-palpebral test*, in which 0·1 ml. of crude mallein diluted 1 in 4 is injected intradermally near the margin of the lower eyelid. A pronounced swelling of the eyelid, with conjunctivitis continuing for three or four days, constitutes a positive reaction.

Malleomyces or Loefflerella pseudomallei

(*Bacillus whitmori*)

The causative organism of Melioidosis—a glanders-like disease occurring in the Malay States, where it is epizootic among rodents, *e.g.* rats, and from these animals transmissible to man. The disease has been reported also in other parts of the Far East. 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 polar, usually two but sometimes more, and occasionally situated at both poles. Mucoid and corrugated types of growth on agar have been described, and a brown growth on potato similar to that of the glanders bacillus. Glucose, lactose, dulcitol, sucrose and mannitol are fermented (without gas production). Susceptible animals (*e.g.* guinea-pig, rat) may be infected experimentally. In the male guinea-pig the Straus reaction occurs as in animals injected with the glanders bacillus. *Malleomyces mallei* and *pseudomallei* are serologically related.

CHAPTER XV

THE GRAM-NEGATIVE AEROBIC BACILLI OCCURRING AS COMMENSALS OR PATHO- GENS IN THE INTESTINE; ENTAMOEBA HISTOLYTICA AND OTHER INTESTINAL PROTOZOA; LACTOBACILLI

THE GRAM-NEGATIVE INTESTINAL BACILLI¹

THESE organisms comprise the following main groups :—

1. The coliform bacilli: which include the common commensal aerobic bacteria of the colon of man and animals.
2. The *Salmonella* group: which includes the typhoid-paratyphoid bacilli (of enteric fever) and the organisms of bacterial enteritis or food poisoning.
3. The *Shigella* or dysentery group.

General Characters.—Gram-negative, non-sporing bacillary organisms, about $2\text{--}4\mu$ by 0.5μ (average)—aerobes and facultative anaerobes—growing best about 37° C.—fermenting various carbohydrates, but not usually liquefying gelatin or coagulated serum.

The various groups and species are differentiated by their cultural, biochemical and serological characters.

COLIFORM BACILLI

This group includes a considerable number of different species or types, in the past designated by the term *Bacillus coli*. Certain of these types are of regular occurrence in the colon and faeces, and are present in large numbers; they have been described as "typical" or "faecal" coliform bacilli. Others are less frequent and less numerous in faeces and have sometimes been referred to as "atypical"; since these latter may be found often apart from the animal body, they have also been described as "non-faecal."

American systematists have assigned these organisms to two main genera, *Escherichia* and *Aerobacter*, and their special characters will be referred to later along with those of certain well-recognised species. The "typical" or "faecal" coliform

¹ For detailed information on the biological characters of these organisms the following monograph should be consulted: *Enterobacteriaceae*, by F. Kauffmann, Copenhagen, 1951.

bacilli belong to the genus *Escherichia*. (The generic name *Bacterium* is also given to the coliform bacilli, e.g. "*Bacterium coli*.")

Morphology and Staining.—Gram-negative bacilli, about $2\text{--}4\mu$ by 0.5μ , but filamentous forms up to $8\text{--}10\mu$ in length may occur, and short cocco-bacillary forms are not infrequent. Types vary in motility. The motile varieties show a peritrichous arrangement of their flagella. Some forms possess a mucoid capsule and certain produce slime (p. 8).

Cultural Characters.—Aerobes and facultative anaerobes. Temperature range, $10^\circ\text{--}45^\circ\text{ C.}$; optimum, 37° C. Grow abundantly on ordinary media.

Colonies on agar—relatively large, thick, greyish-white, moist, circular disks; the opacity and size vary with different strains; capsulate or slime-producing strains exhibit colonies and growth of a viscid or mucoid consistence; the margin of the colonies may be circular or wavy. "Rough" colony variants may be observed.

Gelatin stab—a white line of growth develops along the track of the inoculating wire, with a disk at the top on the surface of the medium; gas spaces form in the gelatin due to the fermentation of the natural muscle sugar of the meat infusion, which is the basis of the medium; no liquefaction occurs, unless in the case of certain atypical forms, e.g. *Aerobacter cloacae*.

Broth—a uniform turbidity usually results.

Potato medium—white moist growth, later assuming a brownish colour.

Biochemical Reactions.—The most prevalent type of coliform bacillus in the intestine is *Escherichia coli* and exhibits the following reactions:—

Glucose	Lactose	Dulcitol	Sucrose	Adonitol	Inositol	Indole	Voges-Proskauer reaction	Methyl-red reaction	Citrate utilisation
+	+	±	±	-	-	+	(vide infra)	(vide infra)	(vide infra)
Motility ±							Eijkman reaction + (vide infra)		

(+ under various carbohydrates signifies acid and gas formation.)

(Vide p. 165 for method of testing, and Table, p. 468a.)

The biochemical characters of other coliform bacilli are referred to later.

Indole Production.—This can be tested for by growing the organism in peptone water, and, after two days, withdrawing with a sterile pipette 2 or 3 ml. into a test-tube. An equal volume of Ehrlich's rosindole reagent is then added:—

Para-dimethyl-amido-benzaldehyde	·	·	·	4 grams
Absolute alcohol	·	·	·	380 ml.
Pure hydrochloric acid	·	·	·	80 ml.

A rose 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 about 1 ml. of ether to the culture, which is then vigorously shaken. The ether extracts the indole, and after it has separated, the culture having been allowed to stand for a few minutes, about 0·5 ml. of Ehrlich's reagent is added.

Voges-Proskauer Reaction.—This reaction is exhibited by certain "atypical" varieties of coliform bacilli (*vide infra*). About 1 ml. of a 10 per cent. solution of caustic potash are added to 5 ml. of a two days' glucose-broth culture (*vide infra*, and p. 181), and the culture is then allowed to stand at room temperature for some hours. If the reaction is positive an eosin-like colour develops, due to a reaction between diacetyl (formed by oxidation of acetyl-methyl-carbinol) and a guanidine residue in the peptone.

This test is rendered more delicate by adding a trace of creatine to the culture and 2·5 ml. 40 per cent. sodium hydroxide. A pink colour develops in a few minutes if the reaction is positive.

Barritt's modification is highly sensitive and gives the colour reaction more rapidly than the original test:—To 5 ml. of a two-days' glucose-phosphate-broth culture (*vide infra*) add 3 ml. of a 5 per cent. solution of *a*-naphthol in absolute alcohol and 1 ml. 40 per cent. potassium hydroxide solution. A positive reaction is denoted by a bright pink colour which appears at the surface of the culture in 5 to 15 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 a 0·5 per cent. peptone-water medium containing 0·5 per cent. glucose and 0·5 per cent. dipotassium hydrogen phosphate (p. 181). (The same medium may be used for the Voges-Proskauer reaction.) To approximately 5 ml. of culture, one drop of a methyl-red solution is then added (0·1 gram methyl-red dissolved in 300 ml. alcohol and then made up to 500 ml. with distilled water). A red colour, denoting a high hydrogen-ion concentration (pH 4·5 or less), is described as "positive"; yellow, signifying a low concentration, is "negative." The prevalent types of coliform bacilli are "methyl-red positive."

It has been found that the optimum temperature of growth for the methyl-red and Voges-Proskauer reactions is about 30° C.

Citrate-utilisation Reaction.—This test has also been used for separating typical and atypical forms of coliform bacilli in the examination of water supplies. It depends on the ability of such coliform bacilli as *Aerobacter aerogenes* to utilise citrate as a source of carbon¹ and to

¹ Brown, H. C., *Lancet*, 1921, 1, 22.

grow in a synthetic medium containing this salt, the typical forms being unable to grow under these conditions. The medium recommended by Koser¹ consists of 1·5 grams sodium ammonium hydrogen phosphate (microcosmic salt), 1 gram potassium dihydrogen phosphate, 0·2 gram magnesium sulphate and 2 grams sodium citrate in 1 litre distilled water. (See also p. 181 for alternative formula.) *Aerobacter* types grow and produce a visible turbidity in the medium.

Other Biochemical Reactions common to the typical Coliform Bacilli.—On MacConkey's bile-salt neutral-red lactose agar—the colonies are rose-coloured, due to the action on the neutral red of the acid produced from the lactose.

Litmus milk—acid and clot produced.

Fluid media containing neutral red—a green fluorescence may develop, especially under anaerobic conditions.

Media containing nitrates—nitrates are reduced to nitrites.

Carbon dioxide and hydrogen are produced by the fermentation of glucose in the following proportions :—CO₂ : H₂—1 : 1–2.

Eijkman Test.—Typical strains of coliform bacilli produce gas in a lactose medium when growing at 44° C.; some atypical forms exhibit no gas production, and this difference has been used as a criterion in distinguishing "faecal" from "non-faecal" strains (p. 426).

Haemolysis.—Some strains, particularly those isolated from urinary infections, are haemolytic towards human red cells. This property can be elicited best by growing the organism in a fluid medium, to which blood has been added.

ATYPICAL COLIFORM BACILLI

(1) *Aerobacter*. The organism originally named *Bacillus lactis aerogenes* belongs to this genus (*Aerobacter aerogenes*). In current literature it is also designated "*Bacterium aerogenes*." *Aerobacter* types are non-motile and are generally characterised by their large, raised, slimy or viscid colonies, the bacilli being capsulate and producing slime. They ferment inositol and adonitol, and usually exhibit the Voges-Proskauer reaction. The citrate-utilisation reaction is positive, the organism growing in Koser's citrate solution (*vide supra*). Indole production is usually absent, and the methyl-red reaction is negative. The CO₂ : H₂ ratio (*vide supra*) may differ from that of the typical coliform bacilli, being approximately 2 : 1.

These organisms are closely related to the pneumobacillus and similar types (p. 432) which have been assigned by American systematists to the genus, *Klebsiella*; it is now proposed by Kauffmann and others that *Aerobacter* and *Klebsiella* species should be placed in one genus with the latter designation (*Klebsiella*).

¹ Koser, S. A., *J. Bact.*, 1923, 8, 493; *ibid.*, 9, 69.

(2) Atypical varieties are met with, which in biochemical reactions seem to occupy an intermediate position between *Aerobacter* and the typical forms as defined above. Among these are strains which differ from the typical coliform bacilli in utilisation of citrate (e.g. *Escherichia freundii*), adonitol fermentation or absence of indole production. These are less frequently found in faeces than the typical forms and some are only occasionally observed.

(3) Certain non-lactose-fermenters. Such organisms have sometimes been described as "paracolon" bacilli (*Paracolobactrum* or "*Bacterium paracoli*"). Some of these may ferment lactose after spontaneous variation in culture, e.g. the type originally designated *Bacillus coli mutabilis* (Table, p. 468a).

(4) Non-gas-producing types are occasionally met with, originally named *Bacillus coli anaerogenes* (Table, p. 468a).

Aerobacter cloacae (*Bacillus cloacae*).—This organism is generally regarded as closely related to the group of coliform bacilli, but differs in respect of its liquefaction of gelatin (Table, p. 468a). In other characters it tends to resemble *Aerobacter*. It has been found in sewage and polluted water.

Wilson's classification of the coliform bacilli serves a useful purpose in assessing the significance of strains of these organisms found in water and milk. This classification is as follows :—

Type	Methyl-red reaction	Voges-Proskauer reaction	Citrate utilisation	Indole production	Eijkman reaction	Gelatin liquefaction
" <i>Bact. coli</i> " I	+	—	—	+	+	—
II	+	—	—	—	—	—
" Intermediate " I	+	—	+	—	—	—
II	+	—	+	+	—	—
" <i>Bact. aerogenes</i> "						
I	—	+	+	—	—	—
II	—	+	+	+	—	—
" <i>Bact. cloacae</i> "	—	+	+	—	—	+
" Irregular " I	+	—	—	+	—	—
II	+	—	—	—	+	—
III	+	—	—	—	—	+
IV	+	—	+	—	—	+
V	—	+	—	—	—	—
VI	—	+	+	—	+	—
VII	—	—	+	+	—	—
VIII	—	—	—	—	—	—

"*Bact. coli*" type I is the commonest type found in human and bovine faeces. It is the "typical" coliform bacillus already described. For full information regarding the occurrence of the various types, see : Wilson, G. S., *Med. Research Council, Spec Rep. Series*, No. 206, 1935, p. 156.

Serological Classification.—The coliform bacilli are exceedingly heterogeneous in their serological characters. Kauffmann has classified them accordingly to their H and O antigenic components and also another form of antigen ("K") which is a somatic surface antigen which may cause inagglutinability by a homologous O antiserum. It is possessed by strains which are more frequently isolated from pathological conditions than from normal faeces. A considerable number of different serological types have now been defined in terms of their antigenic characters, and antigenic analysis is carried out as in the *Salmonella* group (*q.v.*). It may be noted that the "K" antigens have been found to be of different kinds described as "L," "A" and "B." (See : Kauffmann, F., *J. Immun.*, 1947, **57**, 71.)

The *Test for coliform bacilli in Water Examination* is referred to on p. 286.

Occurrence of Coliform Bacilli.—Reference has already been made to their occurrence normally in the intestinal contents, where they are present in very large numbers. These organisms are also potential pathogens. They are found most frequently in pyogenic infections of the urinary tract (pyelitis, cystitis, etc.) either in pure culture or mixed with pyogenic cocci, cholecystitis and cholangitis, appendix abscess, peritonitis, septic wounds (mixed with other pyogenic organisms).

The occurrence of numerous cases and outbreaks of gastro-enteritis in infants from which cultures of the faeces show only the usual coliform bacilli has opened up the question whether these organisms or particular types may possess pathogenic properties in the alimentary tract particularly in young subjects. It has been long recognised that coliform bacilli may be pathogenic to calves, as in the condition of "white scours." There is now evidence which points to certain serological types of coliform bacilli as causative agents of gastro-enteritis in the human subject, and these types possess the "B" surface antigen as described by Kauffmann, who has defined them in terms of their antigenic formulae as follows :—

- | | |
|-------------------------------------------------------------------------------|----------------------------------|
| (1) O 111 ; B 4 (non-motile)
or O 111 ; B 4 ; H 2
or O 111 ; B 4 ; H 12 | } sometimes called the "α type." |
| (2) O 55 : B 5 ; H 6 | |

Biochemically these organisms conform to the reactions of the "typical" coliform bacilli without special distinguishing features.

The types so defined are of course lactose-fermenters and cannot

be differentiated at sight from other coliform bacilli on plates of neutral-red-lactose-agar, e.g. MacConkey's medium. The colonies have to be recognised in the first place by slide-agglutination tests with appropriate O, B and H antisera (p. 431), the results being confirmed with subcultures in quantitative agglutination tests.

Antibiotic Therapy.—Aureomycin and chloromyctin are regarded as the first choice among the various antibiotics in the treatment of infections by the coliform bacilli. These infections are also amenable to treatment with terramycin.

DIAGNOSIS OF INFECTIONS BY COLIFORM BACILLI

In film preparations from pus, urinary sediment, etc., stained by Gram's method, *coliform bacilli* can be recognised as Gram-negative bacilli of the average dimensions described above, but the morphological characters of these organisms are similar to those of many other related species. If cultures are made on a differential medium containing lactose and an indicator of acidity, their identity can be established at once *in the case of the typical forms*, by the characteristic colour change in the indicator, e.g. on MacConkey's medium by the rose-pink colour of the colonies. It must be remembered, however, that certain strains of even those which ferment lactose in fluid medium may at first produce pale colonies on MacConkey's medium, so that for identification it may be necessary to isolate the strain and test its reactions in detail.

KLEBISELLA PNEUMONIAE

Pneumobacillus of Friedländer ; Bacterium pneumoniae)

Originally described as a causative organism of pneumonia.

Morphology.—A small non-motile, non-sporing, Gram-negative bacillus with rounded ends and varying greatly in size— $1\text{--}4\mu$ by $0\cdot5\text{--}1\mu$. The shorter forms simulate cocci. The bacilli occur usually in pairs, but also singly, and in short chains. They are typically capsulate, especially when seen in the tissues.

Cultural Characters.—Generally resembles the *Aerobacter* subgroup of coliform bacilli. The colonies exhibit a characteristic mucoid or viscid consistence, already referred to on p. 427.

Biochemical Reactions.—Different strains vary. Some correspond closely with *Aerobacter aerogenes* (Table, p. 468a). Others are non-lactose fermenters. Some strains form gas

slowly, others apparently fail to produce gas in fermentation tests. With the majority of strains the indole reaction is negative ; the methyl-red reaction is frequently positive. The Voges-Proskauer reaction is, in some cases, positive.

This organism may be regarded as biologically related to the group of coliform bacilli, though its habitat as a commensal or pathogen is generally in the upper respiratory passages.

It is proposed by Kauffmann that organisms previously classified as *Aerobacter* (p. 429) and Pneumobacillus should be assigned to the genus *Klebsiella* and he has pointed out that the serological features of all these organisms depend on three factors : capsular (K), somatic R and O antigens.

Occurrence in Human Disease.—The pneumobacillus is only rarely responsible for cases of lobar pneumonia. It may be associated, however, with catarrhal conditions of the respiratory tract, suppuration in nasal sinuses, meningitis, conjunctivitis, empyema, etc.

Serological Classification.—Three serological types were originally defined (analogous to the types of pneumococcus) ; a certain proportion of strains, however, could not be identified with these types, and formed a serologically heterogeneous group analogous to Group IV of the pneumococcus. Type-specificity depends on a polysaccharide substance contained in the bacterial capsule. It is of special interest that the specific substance of one of these types has been identified serologically with the specific substance of Type II pneumococcus.

Kauffmann has classified organisms of the *Klebsiella* group according to their O and K antigenic constituents. They can be grouped first according to their O antigens, and such groups can be subdivided into types by the serological reactions of their K antigens. (See *Act. Path. et Microbiol. Scandin.*, 1949, 26, 381 ; *Enterobacteriaceae*, by F. Kauffmann, Copenhagen, 1951.)

Klebsiella rhinoscleromatis (*Bacillus of Rhinoscleroma*).—This organism closely resembles the pneumobacillus in morphology and cultural characters, but produces no gas from glucose, and does not ferment lactose. It does not grow on media containing bile, and does not give the indole and Voges-Proskauer reactions. It is stated that strains are serologically homogeneous with characteristic capsular (K) and somatic (O) antigens. The organism is associated with a chronic granuloma of the mucous membrane of the nose, mouth or throat. The bacilli can be seen in the lesions, situated intracellularly. The disease is prevalent in South-Eastern Europe.

Klebsiella ozaenae (*Bacillus ozaenae*).—Closely resembles the pneumobacillus, but is non-gas-producing in glucose. It is found associated with ozaena, but it is not to be regarded as the causative agent of this condition.

TYPHOID-PARATYPHOID OR ENTERIC FEVER BACILLI

These include *Salmonella typhi* (typhoid bacillus), *Salmonella paratyphi A* and *Salmonella paratyphi B* (paratyphoid A and B bacilli).

An organism with similarities to *S. paratyphi A* and *B* has also been noted in cases of enteric fever, and designated *Salmonella paratyphi C* (p. 444). Certain other types belonging to the *Salmonella* group have occasionally been reported in cases of enteric fever (Table, p. 449).

It is convenient to classify the typhoid and paratyphoid infections together as "enteric fever."

SALMONELLA TYPHI or TYPHOSA

(Bacillus typhosus)

The causative organism of Typhoid fever.

Morphology and Staining.—A Gram-negative non-sporing bacillus, about $2\text{--}4\mu$ by $0\cdot5\mu$, actively motile, with numerous long peritrichous flagella as observed in specially stained preparations; elongated forms of the organism are common.

It has been pointed out by Pijper that when the typhoid bacillus is examined by solar dark-ground illumination, two flagella only are observed, one on each side, and that in the movement of the organism these become conjoined to form a tail-like process from the posterior pole of the bacillus.

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—like those of coliform bacilli, but smaller, thinner and more transparent; stock laboratory cultures may show a mixture of "smooth" and "rough" colonies (p. 29).

Gelatin stab—like coliform bacilli, but the growth is less abundant, and there is no gas formation. No liquefaction occurs.

Colonies on MacConkey's medium—smaller than those of coliform bacilli and "pale" or colourless, the typhoid bacillus being a non-lactose-fermenter.

Desoxycholate-citrate medium—colonies also "pale" or colourless (as compared with coliform bacilli).

Biochemical Reactions.—Ferments glucose and mannitol with acid, but no gas formation; does not ferment lactose or sucrose and does not produce indole. Some strains exhibit slow fermentation of dulcitol (Table on p. 468a).

Strains differ as regards the fermentation of xylose and arabinose, and "types" have been differentiated according to the presence or absence of these reactions.

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 occur for six weeks or longer. In culture the organism persists for long periods, e.g. several months.

Occurrence.—This organism shows a selection for lymphoid tissue—e.g. the Peyer's patches and lymphoid follicles of the intestine, the mesenteric glands and spleen. Thus, it attacks the Peyer's patches of the small intestine, and leads to an acute inflammatory reaction and infiltration with mono-nuclear cells, followed by necrosis, sloughing and the formation of the characteristic typhoid ulcers. It is present in large number in the inflamed tissue, in the ulcers, and is found in the intestinal contents and the dejecta. The mesenteric glands show similar inflammatory changes. At an early stage of the disease (during the first week or ten days) and in relapses, a condition of bacteraemia exists, and the typhoid bacillus can be demonstrated by blood culture. It is also present in the spleen, occurring in clumps. The bacillus is frequently present in large number in the gall bladder. It may localise in the kidney and appear in the urine, sometimes producing a marked bacilluria. Localisation may also occur in bone-marrow.

The bacillus is found in other lesions occurring as complications or sequelae of typhoid fever—e.g. acute suppurative osteitis, abscess of the kidney, acute cholecystitis, bronchopneumonia, empyema, ulcerative endocarditis. Even in the suppurative lesions it may be present in pure culture.

Infection is by ingestion; from the small intestine the organism passes by the lymphatics to the mesenteric glands, and invades the blood probably *via* the thoracic duct; the liver, gall bladder, spleen, kidney, bone-marrow, etc., then become infected from the circulation; from the gall bladder a further invasion of the intestine results, and the bacilli may appear in the faeces in appreciable numbers (p. 438).

In 2 to 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 foci in the kidney, and are excreted in the faeces or urine.

The typhoid bacillus cannot grow as a saprophyte in

nature, but it can survive long enough to be transmitted by some vehicle of infection—*e.g.* sewage and polluted water, shell-fish and vegetables contaminated with human excretal matter, house flies, articles of food contaminated by carriers, etc. Milk may serve as a culture medium for the typhoid bacillus, so that contamination of milk, *e.g.* by carriers engaged in dairy work, is a likely source of outbreaks of the disease.

Serological Reactions.—The serum of animals immunised with typhoid bacilli contains agglutinating antibodies (p. 44) which are specific for the characteristic antigens of the organism, and agglutination reactions are therefore employed in the identification of this species (p. 448).

The *antigenic structure* of this organism in relation to allied species is shown in the Table on p. 449.

Vi Antigen and Agglutinin.—Recently isolated strains of the typhoid bacillus have been shown to possess a somatic antigen designated "Vi," which is associated with virulence (judged experimentally by inoculation of mice) and renders the organism relatively inagglutinable by an O antiserum. By continued cultivation the Vi antigen is lost, the strains become susceptible to O agglutination, and along with these changes there is also loss of virulence.

This antigen is labile at 100° C. and is partially labile to phenol. It seems to be developed best when the organism is cultivated in a soft ascitic-fluid-agar. A Vi agglutinating antiserum is obtained by immunising animals with living typhoid bacilli known to contain the Vi antigen, and by absorbing H and O agglutinins from the serum with strains representing H and O antigens, but devoid of the Vi component. This absorbed serum can be used in testing strains for Vi antigen. The agglutination is of the granular type. For the detection of Vi agglutinin in serum (*e.g.* in a case of typhoid fever) a selected strain giving a pure Vi reaction may be used, the suspension consisting usually of living organisms. The diagnostic applications of this test are dealt with on p. 443.

Bacteriophage Typing of the typhoid bacillus.—Craigie and Yen 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, eleven types of typhoid bacilli were recognised, but, more recently, twenty-four types and five provisional types have been identified. In this way, freshly isolated strains possessing Vi antigen can be classified, and the method has proved valuable in epidemiological studies of typhoid infections, *e.g.* in correlating cases and in tracing the source of an outbreak and its mode of spread. The method used is briefly as follows. An agar plate is heavily inoculated with a young broth culture of the organism to be typed, and allowed to dry. A drop of each specific phage at its critical

test-dilution is then placed on the inoculated plate, the various drops and their positions being identified by appropriate pencil marks on the glass dish. The drops are allowed to dry and the plate is incubated overnight. A clear spot on the grown culture indicates phage activity, and the type causing this is noted. Special attention to certain technical details is required in this work, e.g. the propagation, preservation and dilution of the phages. Standard cultures and type-specific phage preparations are obtainable from Dr. A. Felix, Central Public Health Laboratory, London. The detailed technique of the method is described in the following papers : Craigie, J., and Yen, C. H., *Canadian Pub. Health J.*, 1938, 29, 448 and 484; Craigie, J., and Felix, A., *Lancet*, 1947, 1, 823.

SALMONELLA PARATYPHI A (*Bacillus paratyphosus A*)

AND

SALMONELLA PARATYPHI B or SCHOTTMÜLLERI

(*Bacillus paratyphosus B*)

Causal organisms of paratyphoid fever, which is, for all practical purposes, clinically similar to typhoid.

The morphology and general cultural characters are identical with those of the typhoid bacillus.

Their fermentative and biochemical reactions are detailed in the Table on p. 468a. Important reactions are : lactose—no change ; glucose and mannitol—acid and gas ; sucrose—no change ; the paratyphoid bacilli can be distinguished from the typhoid bacillus by their gas production. *S. paratyphi A* ferments dulcitol but often slowly ; *B* usually ferments dulcitol, but strains may vary in this character ; *B* ferments xylose and, in culture, produces hydrogen sulphide, whereas *A* does not ferment xylose and strains vary in the production of sulphide. The ultimate identification depends on agglutination reactions.

Their occurrence and distribution in the body of an infected person are the same as in the case of the typhoid bacillus.

S. paratyphi A is more common in the East, whereas *S. paratyphi B* is the commoner in Europe. In recent times, in Great Britain, paratyphoid *B* infections have assumed a greater relative prevalence than formerly and have tended to be more frequent than true typhoid infections. Many of the cases are of a relatively mild type.

Serological Reactions.—These organisms possess different antigens, and agglutinating antisera for known strains are used for the identification of the respective types. The paratyphoid bacilli are similarly differentiated from the typhoid bacillus.

The antigenic structure of these organisms in relation to other members of the *Salmonella* group is shown in the Table on p. 449.

Types of Salmonella paratyphi B

Bacteriophage typing of this organism has been used for epidemiological studies, as in the case of the typhoid bacillus. Ten types have so far been identified. This procedure has proved of value in correlating cases and tracing sources and spread of outbreaks (p. 436; Felix, A., and Callow, B. R., *Brit. Med. J.*, 1943, 2, 127).

DIAGNOSIS OF ENTERIC INFECTIONS¹

The bacteriological 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 stage of the illness, blood culture is a 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 possibility of demonstrating typhoid-paratyphoid bacilli in the blood lessens as the disease progresses. The method is referred to on p. 222. If the result is positive the strain is isolated in pure culture, and identified by morphological, cultural and biochemical characters (Table, p. 468a), and by testing it with agglutinating antisera to known typhoid and paratyphoid bacilli according to the biochemical reactions observed. H and O agglutinating antisera for these organisms can be obtained from the Central Public Health Laboratory, London. Further reference to their serological identification is made later (p. 448).

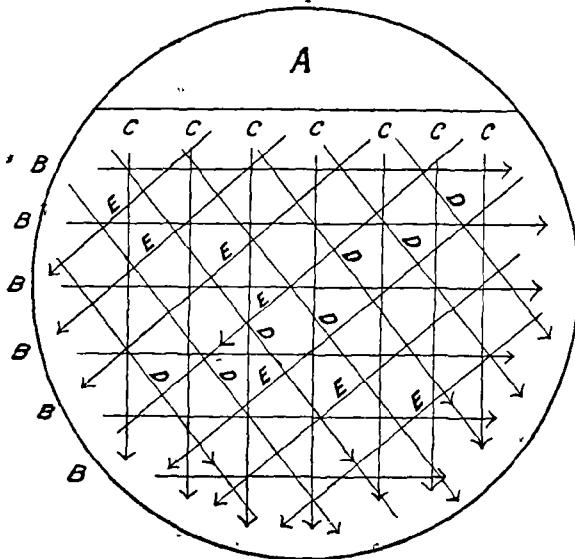
Bone Marrow Culture.—Aspiration of bone marrow by sternal puncture sometimes enables the organism to be isolated when blood culture has been unsuccessful.

FAECES.—Typhoid and paratyphoid bacilli can be isolated from the faeces and are most frequent at the end of the second week or during the third week, but may be detected at all stages of the disease. Examination of faeces, however, may yield negative results unless repeated, and the isolation of typhoid-paratyphoid bacilli from this source is often

¹ See *Laboratory Control of the Enteric Fevers*, by Felix, A., *Brit. Med. Bull.*, 1951, 7, 153.

rendered difficult owing to their being relatively scanty as compared with coliform bacilli.

In submitting specimens of faeces for culture, if there is likely to be a delay of some hours before the laboratory cultivation can be carried out, 2 volumes of 30 per cent. neutral glycerol in buffered 0·6 per cent. sodium chloride solution (p. 320) should be added to 1 volume of the faeces and thoroughly mixed with it. This obviates the overgrowth of the typhoid-paratyphoid bacilli by the other intestinal organisms present. The glycerol solution is apt to become acid on keeping, in which condition it is quite unsuitable for use. To check



this, it may be tinted with phenol red, and if this indicator changes to a yellow colour the fluid should be discarded and a fresh solution used.

Direct Plating of Faeces.—A medium is required which will differentiate colonies of typhoid-paratyphoid bacilli from those of the ordinary coliform bacilli; in the past, MacConkey's bile-salt neutral-red lactose agar (p. 180) has been extensively used for this purpose, but recently has been generally superseded by other selective media which are inhibitory to coliform bacilli, including desoxycholate-citrate agar which, like MacConkey's medium, contains lactose and neutral red (p. 182). On both these media typhoid-paratyphoid bacilli produce "pale" colonies as compared with the pink colonies of coliform bacilli. (MacConkey's medium may still be used for the direct cultivation of faeces in other infections where col-

form and paracolon bacilli have to be examined, e.g. in cases of enteritis, pp. 431, 457.) *It is essential that the surface of the medium should be sufficiently dry before inoculation.* If even a small amount of condensation water is present on the medium, a confluent growth may result instead of separate colonies.

When *desoxycholate-citrate* medium is used, the inoculation is made as follows.

Three loopfuls of the specimen (liquid faeces or a dense emulsion in saline from solid or semi-solid faeces) are smeared over area A of the plate (diagram, p. 439). The loop is sterilised in the 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, D and E, 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. The method described allows a heavy inoculation to be made, with the resulting colonies well separated except, of course, in area A.

When *MacConkey's medium* is used a lighter inoculation is required and instead of using one 4-in. plate the inoculum may with advantage be distributed over two plates.

After eighteen to twenty-four hours' incubation the colonies are usually sufficiently large for sub-inoculating those that are considered likely to be typhoid-paratyphoid bacilli. The plates may also be incubated for longer periods if no suspicious colonies are noted after twenty-four hours, and occasionally this procedure is of value. The colonies of the typhoid-paratyphoid bacilli present a pale or colourless appearance (as compared with the typical coliform bacilli), but other intestinal organisms may also produce colourless colonies. Several of these colonies are therefore subcultured on agar slopes, and for this purpose a straight wire is used for sub-inoculating them from the 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 (p. 183) and is regarded by some as superior to desoxycholate-citrate agar for the isolation of the typhoid bacillus.

Enrichment Methods.—These have proved specially valuable for isolating typhoid-paratyphoid bacilli when present in relatively small numbers in faeces. Fluid media are used, incorporating substances which inhibit coliform bacilli, while

allowing the typhoid-paratyphoid bacilli to flourish. In this way an enriched culture of the latter is obtained and, sometimes, an almost pure growth. These special media include tetrathionate broth, and selenite F medium which gives very satisfactory results (p. 185).

In the examination of faeces from enteric cases 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, direct plating on desoxycholate-citrate medium and the selenite or tetrathionate enrichment methods may be used together, or Wilson and Blair's medium may be substituted for or used in addition to desoxycholate-citrate agar. For isolating the paratyphoid B bacillus tetrathionate enrichment gives somewhat better results than selenite.

URINE.—Typhoid-paratyphoid bacilli may also be isolated from urine. The specimen is centrifuged, several loopfuls of the deposit are inoculated on a plate of desoxycholate-citrate medium, and successive strokes made in the usual way so that isolated colonies are obtained. In enteric fever there appear to be transient bacilluric periods, and repeated examinations of the urine are of particular value where the isolation of the causative organism is aimed at and where other methods have been unsuccessful.

Examination of Bile.—Another method which may be used for bacteriological diagnosis in the later stages of the illness is the aspiration of bile by means of the duodenal tube, cultures being made from the bile on desoxycholate-citrate medium.

WIDAL Reaction.—The technique is described in Chapter VIII. It is customary to test the serum with standard H and O suspensions (in parallel) of each enteric fever organism likely to be encountered—*e.g.* in this country, *Salmonella typhi* and *Salmonella paratyphi B*. As a rule, both O and H agglutinins are developed, but, in some cases, only one of these agglutinins is demonstrable, particularly at an early stage.

The reaction becomes definitely manifest *usually* about the seventh to the tenth day. Occasionally, it is earlier in development (*e.g.* fifth day), but may be delayed. A negative result at an early stage of the illness therefore may be inconclusive. 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 in diagnosis.

For determining the type of infection, H agglutination is more to be relied on than the O, since the enteric organisms have common O antigenic components, e.g. XII (Table, p. 449); thus, in a typhoid infection O agglutination may sometimes be pronounced with both the typhoid and paratyphoid B bacilli, while in a paratyphoid B infection marked O agglutination may also occur with both organisms. However, both H and O agglutinins should be tested for routinely.

Enteric carriers usually exhibit a positive reaction (*vide infra*).

It must be remembered that normal serum may agglutinate the typhoid and paratyphoid bacilli in low dilutions, and no diagnostic significance can be attached to the Widal reaction unless the titre is beyond the range of such normal reactions. In this country the following are the usual limits of normal agglutination (the figures refer to tests with standard suspensions): *S. typhi*, H 1 in 30, O 1 in 50; *S. paratyphi B*, H 1 in 30, O 1 in 50; *S. paratyphi A*, O and H, 1 in 10. However, normal agglutination of these organisms may vary 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. Later results may show higher titres and are therefore more conclusive.

It should be noted that the enteric fever bacilli and other members of the *Salmonella* group may sometimes contain a non-specific antigenic component ("x") for which there may be an agglutinin in normal human serum. This may cause a fallacy in the Widal reaction unless the standard strains used for the agglutination test are known to be free from this antigen.

Persons inoculated with typhoid-paratyphoid vaccine also show specific agglutinins in their sera, and this complicates the interpretation of the Widal reaction in such persons.

In previously vaccinated cases a definitely rising titre for any one of the enteric group has been regarded as significant from the diagnostic standpoint; but non-specific factors, such as a non-enteric febrile condition, may bring about increase of agglutinins already present as a result of vaccination, and enteric fever infection by one organism may lead to an increased agglutination titre for the others.

It has been claimed also 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. In such cases, however, a reliable diagnosis cannot be made merely by the demonstration of O agglutinins in the serum, since vaccination

may give rise to O agglutinins as well as H, though the former have usually a lower titre and decline more rapidly.

Thus, in the application of the Widal reaction in vaccinated subjects, the results are generally 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 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. Such suspension in concentrated form can be obtained from the Central Public Health Laboratory, London. 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. 245), 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.

DIAGNOSIS OF TYPHOID AND PARATYPHOID CARRIERS

In a considerable proportion of carriers the Widal reaction is positive, and the test is of some value as a preliminary one. A negative Widal reaction does not, of course, exclude the carrier state. In detecting carriers of the typhoid bacillus the Vi agglutination test is of particular value (*vide supra*).

The proof that a person is a carrier depends on the isolation of a typhoid or paratyphoid bacillus from the faeces or urine, and *at least* three successive examinations should be made before the result is declared negative.

As the bacilli are likely to be most numerous in the bile and in the contents of the small intestine, three grains of calomel followed by a saline purgative may be given, and after catharsis, the second or, preferably, the third stool is used for the examination. The specimen should be cultured as soon as possible after it is passed. The methods used are as described above. It is essential that at least two different methods should be used together, including an enrichment method.

The urine is examined as in the diagnosis of enteric fever.

Typhoid-paratyphoid (T.A.B.) Vaccine

This is prepared from selected "S" cultures of *S. typhi*, *S. paratyphi A* and *B* according to the method described on p. 274. In the past, the vaccine has usually been sterilised at 60° C. (thirty minutes) and 0.5 per cent. phenol has been added as a preservative. For prophylactic use, two doses are usually given subcutaneously at intervals of seven to ten days.

	1st dose	2nd dose
<i>S. typhi</i>	500 millions	1000 millions
<i>S. paratyphi A</i>	250 or 375 millions	500 or 750 millions
<i>S. paratyphi B</i>	250 or 375	500 or 750

The prophylactic value of this vaccine has been well established.

Salmonella paratyphi C may also be incorporated in the vaccine, which is then designated "T.A.B.C."

It has been emphasised that the full immunising potency of typhoid-paratyphoid vaccine depends on the use of virulent strains containing adequate O and Vi antigen, but sterilisation by heat and preservation with phenol tends to destroy the Vi antigen. For this reason, Felix and his co-workers have advocated treatment of the cultures with alcohol in place of heating and phenolisation, e.g. killing the organisms with 75 per cent. and preservation with 22.5 per cent. alcohol. A vaccine prepared in this way from strains selected as above mentioned stimulates the formation of Vi antibody in a substantial proportion of cases.

Antibiotic Therapy.—Chloromycetin has been used with success in the treatment of enteric fever.

Salmonella paratyphi C or hirschfeldi (Bacillus paratyphosus C)

This organism has been reported in cases of enteric fever in different parts of the world. It is closely related to *S. cholerae-suis*, though exhibiting certain differences from the latter (Tables on pp. 449, 468a).

It can be distinguished serologically from the other paratyphoid bacilli.

Enteric infections due to the Kunzendorf monophasic type of *S. cholerae-suis* have also been observed (Tables, pp. 449, 468a).

Isolation of Typhoid and Paratyphoid Bacilli from Sewage

Various methods have been used for the purpose. The following procedure as reported by Moore¹ may be adopted. A piece of gauze, 4 ft. in length and 6 in. wide, is folded into a pad of eight thicknesses and a long piece of stout string is attached firmly to one end. The gauze pad is immersed in flowing sewage through a sewer manhole, the string being fixed under the manhole cover. The pad is left in the sewage for 48 hours. It is then removed and transported to the laboratory in a suitable sealed container. In the laboratory the pad is covered with an enrichment medium, e.g. tetrathionate or selenite F medium and incubated overnight. Subcultures are then made on a selective solid medium, e.g. desoxycholate-citrate agar or Wilson and Blair's medium. In this way the presence of carriers can be demonstrated in urban communities.

ORGANISMS OF BACTERIAL ENTERITIS OR FOOD POISONING

These organisms, which are related to the typhoid-paratyphoid bacilli, and also belong to the *Salmonella* group, are associated with cases of "meat" or "food poisoning," in which the illness usually takes the form of an acute gastro-enteritis with marked toxæmia. Different types of such organisms vary to some extent in their invasiveness. In some cases, bacteraemia or septicaemia may occur, and meningitis has been recorded not infrequently as a result of the infection. Cholecystitis may also result. The incubation period may be short, symptoms occurring even a few hours after the ingestion of the contaminated food. Preserved meats (e.g. sausage, brawn, etc.) are frequently responsible. The food may not show any obvious signs of bacterial contamination. The *Salmonella* organism growing in the food produces an active endotoxin which may possibly be responsible for the early symptoms in cases in which the incubation period is short. This toxin is generally stable at 100° C. for thirty minutes.

Occurrence.—These organisms are found in the intestinal contents during the disease, and, in some cases, in the blood.

As regards the source of the infection : the animal from

¹ Moore, B., *Monthly Bull. Min. Hlth. and P.H.L.S.*, 1948, 7, 241.

which the flesh is derived may have been infected during life, or the meat may have been contaminated from some extraneous source. Thus, organisms of this group occur in cattle and pigs. Cow's milk and milk products may be a source of infection. *Salmonella* organisms also produce epizootic infections in rats, mice and other rodents, and in certain birds. They have been found in the eggs of infected pigeons, ducks and hens, and cases of food poisoning in the human subject have occurred after the consumption of infected eggs. In recent years, infection in Great Britain has resulted from imported American spray-dried eggs. Human carriers have been noted, and these may sometimes be responsible for contamination of foods. Attention has also been drawn to *Salmonella* infections of man derived from dogs and cats.

SALMONELLA ENTERITIDIS

(*Bacillus enteritidis* of Gaertner)

This organism is generally similar to *S. paratyphi B* in its various cultural and biochemical characters (Table, p. 468a), but does not ferment inositol. Its serological identification is dealt with on p. 448 (see also Table, p. 449).

Four varieties of *S. enteritidis* have been described, including the original form which may be designated the *Gaertner* variety. These differ in certain biochemical reactions. Strains of *S. enteritidis* have been found in cattle, pigs, rats, field-mice, ducks and chickens. The organism is extremely virulent to laboratory animals by parenteral inoculation, and even by enteral administration of culture produces a haemorrhagic enteritis and septicaemia. The *Damyssz* variety has been used as a "virus" for the destruction of rats, producing in these animals a fatal epizootic disease. The possibility of the organism being transmitted sometimes in this way to man has to be considered.

SALMONELLA TYPHI-MURIMUM

(*Bacillus aertrycke*)

In general resembles *S. paratyphi B* in cultural and biochemical reactions (Table, p. 468a) and is also closely related to it in serological characters. The two organisms possess certain antigenic constituents in common, though each in the specific phase contains a different flagellar (H) antigen (Table, p. 449).

The identification of *S. typhi-murium* and other *Salmonella* types is carried out by the method of antigenic analysis which is dealt with later (p. 448).

S. typhi-murium produces epizootic enteritis in a wide variety of animals, e.g. mice ("mouse typhoid"), guinea-pigs, rats, pigs, cattle, sheep, cats, ducks, hens and certain other birds.

Phage typing of strains of *S. typhi-murium* can be used in epidemiological work, as in the case of *S. typhi* and *paratyphi B* (Felix).

OTHER TYPES OF THE SALMONELLA GROUP FOUND IN FOOD POISONING

S. typhi-murium has been the commonest cause of food poisoning in this country, with *S. enteritidis* second in prevalence. Other types, designated "Thompson" and "Newport," have been not infrequent in occurrence. Less common types are *S. cholerae-suis*, *S. morbillifrons (bovis)*, and the so-called "Dublin," "Stanley," "Derby," "Potsdam," "Senftenberg var. Newcastle," "Eastbourne," "London" and "Aberdeen" types. Since 1940 a considerable number of additional types, not previously recorded in Great Britain, have been found in outbreaks and cases, e.g. "Oranienburg" and "Montevideo" types and *S. anatis* and *S. meleagridis*. It would appear that the importation of spray-dried egg from America has been responsible for these infections.

A very large number of types of the *Salmonella* group has now been described in various parts of the world (Table, p. 449). These organisms exhibit the general biological characters of the group and are differentiated by their biochemical and serological characters.

Salmonella cholerae-suis (*Bacillus suisfester*—Bacillus of hog-cholera) was originally regarded as the causal agent of swine fever or hog-cholera, which is now known to be due to a filterable virus. The biochemical reactions of this organism are given in the Table on p. 468a, and reference to it has been made on p. 444 in relation to *S. paratyphi C*. Identification is established by the method of antigenic analysis described on p. 448 (Table, p. 449). Two forms of this organism have been described: a diphasic type, and a variety designated "Kunzendorf," which has been regarded as monophasic, occurring only in the group or non-specific phase, but this is now doubtful since, under certain conditions, it has been transformed to the specific phase.

DIAGNOSIS OF SALMONELLA FOOD POISONING

The stools are plated on desoxycholate-citrate medium as in the diagnosis of enteric fever, "pale" colonies are subinoculated, and the resulting cultures are tested and identified. It is advisable also to employ selective enrichment as in enteric

fever, e.g. selenite medium (p. 185). Detailed agglutination tests are necessary for identification of the *Salmonella* type present (*vide infra*).

Blood culture may, in some cases, yield positive results, and should be carried out as a routine measure. The culture is made by the same method as in enteric fever.

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 sometimes be applied retrospectively in convalescent cases (previously undiagnosed) for purposes of epidemiological investigation. The serum is tested with specific H suspensions of *S. typhi-murium*, *S. paratyphi C*, *S. enteritidis*, Newport and Stanley types (for the last *S. typhi* H suspension may be used—*vide* Table, p. 449). The suspensions can be obtained from the Central Public Health Laboratory, London. If a reaction occurs with one of these in dilutions above 1 in 50, this may be considered significant. These suspensions, however, only represent the commoner members of the *Salmonella* group. A suspension consisting of mixed, monophasic, non-specific *S. typhi-murium* and *cholerae-suis* types can also be obtained for such retrospective tests, but a reaction with it will only indicate *Salmonella* infection without any determination of the type. A reaction with this suspension in a titre of over 1 in 50 should be regarded as significant. Of course previous typhoid-paratyphoid vaccination invalidates these diagnostic results.

Bacteriological examination of suspected articles of food, if available, should be carried out.

IDENTIFICATION OF TYPES OF THE SALMONELLA GROUP

When a non-lactose-fermenting bacillus, presenting the general characters of this group, has been isolated from a case, its precise identification involves 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 carefully noted and a complete set of sugar media is inoculated (including xylose, arabinose, trehalose, inositol and rhamnose) along with lead acetate agar. Christensen's urea medium (p. 193) is also inoculated to exclude organisms of the *Proteus* group. The biochemical reactions are of value when considered in association with the antigenic structure. Other biochemical reactions which may be of value are the utilisation of d-tartrate, l-tartrate, i-tartrate, citrate and mucate.

As will be seen from the Table the identification of the O

antigen provides a means of placing any member of the group in one of a number of subgroups. To assign the unknown bacillus to its subgroup, sera prepared by immunising animals with heated (100° C.) suspensions of representative members of each subgroup are used in agglutination reactions with an alcohol-treated (O) suspension of the unknown. A convenient, though of course limited, set of such O sera are those for *S. paratyphi A*, *S. paratyphi B*, *S. cholerae-suis* (monophasic), Newport type and *S. enteritidis*. Small-flake agglutination to the titre of the serum would indicate the nature of the O antigen present. The results of the direct agglutination reaction may be confirmed by an agglutinin-absorption test.

SOME REPRESENTATIVES OF THE SALMONELLA GROUP¹

(Kauffmann-White Classification)

Arranged in Subgroups with common O antigens

Sub-group	Type Designation and Associated Disease	O antigen	H antigen	
			Phase 1 (mostly specific)	Phase 2 (mostly non-specific)
A	<i>paratyphi A</i> —Enteric fever . . .	(I), II, XII	a	—
B	<i>paratyphi B</i> —Enteric fever . . . <i>typhi-murum</i> Stanley Food poisoning Reading (gastro-enteritis) Derby . . . <i>abortivo-equina</i> —Equine abortion . . . <i>abortus-ovis</i> —Abortion of sheep . . .	(I), IV, (V), XII (I), IV, (V), XII IV, V, XII IV, XII (I), IV, XII IV, XII IV, XII	b i d e, h f, g — c	1, 2 1, 2 1, 2 1, 5 e, n, x 1, 6
C	<i>paratyphi C</i> (Hirschfeld)—Enteric fever . . . <i>typhi-suis</i> —Infection of pigs . . . <i>cholerae-suis</i> Thompson Food poisoning Potsdam (gastro-enteritis). Montevideo Kunzendorf monophasic Oranienburg (phase 2) variety Newport of <i>S. cholerae-suis</i> <i>morbificans</i> reported in enteric fever . . .	VI, VII, (Vi) VI, VII VI, VII VI, VII VI, VII VI, VII VI, VIII VI, VIII	c c c k l, v g, m, s m, t e, h r	1, 5 1, 5 1, 5 1, 5 — e, n, z, — 1, 2, 3 1, 5
D	<i>typhi</i> —Enteric fever . . . <i>enteritidis</i> Food poisoning (gastro-enteritis). Eastbourne and Dublin types reported in enteric fever. <i>gallinarum</i> —Fowl typhoid . . . <i>tullorum</i> —Bacillary white diarrhoea of chicks . . .	IX, X ² , (Vi) (I), IX, XII I, IX, XII (I), IX, XII , IX, XII I, IX, XII	d g, m g, p e, h (non-flagellate) (non-flagellate)	— — — 1, 5 — —
E	<i>London</i> <i>S. anatum</i> <i>S. meleagridis</i> Senftenberg var. Newcastle Food poisoning (gastro-enteritis)	III, X III, X III, X I, III, XIX	l, v e, h e, h g, s, t,	1, 6 1, 6 1, w —
F	Aberdeen—Gastro-enteritis . . .	XI	l	1, 2

(1) This component may sometimes be wholly or partly absent.

The antigenic components given in the table do not necessarily represent the complete antigenic formulae.

1 See *The Diagnosis of Salmonella Types*, by F. Kauffmann, 1950, Springfield, Illinois, U.S.A., *A Manual for Exteric Bacteriology*, by P. R. Edwards and W. H. Ewing, Communicable Diseases Center, Atlanta, Ga., U.S.A.

Before proceeding to identify the H antigen or antigens of the organism it is necessary to ascertain whether the culture (if diphasic) is in the specific or non-specific phase (p. 45). An H-agglutinating serum prepared against the non-specific monophasic variety of *S. cholerae-suis* is a convenient reagent for this purpose, its H agglutinin being of "group" character. If a formolised suspension of the unknown organism is agglutinated in large floccules to any extent by this serum, it is in the group phase, and an effort must be made to secure a specific-phase subculture of it before further identification is attempted.

The original method was as follows. Plates were inoculated so as to yield discrete colonies, and a number were examined. Separate colonies were touched with an inoculating wire and some of the growth in each case was emulsified in a drop of a low dilution of monophasic *cholerae-suis* serum on a slide. A colony, organisms from which did not agglutinate with the group serum, was subcultured as being probably in the specific phase.

A more convenient method for obtaining the specific form of a *Salmonella* organism is as follows: 5 ml. amounts of nutrient agar containing 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 group-serum (filtered to ensure sterility) are added, giving final concentrations of 1 in 50 and 1 in 25 respectively. The medium is allowed to solidify in the upright position. The agar *inside* the inner tube is inoculated by the stab method with the group-phase culture. The specific forms can then be separated by incubating, and subcultivating 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. (See Tulloch, W. J., *J. Hygiene*, 1939, **39**, 324.) Another useful procedure for the same purpose is referred to later.

When the specific phase has been obtained, formolised suspensions are used in agglutination reactions with pure specific-phase H sera prepared against the *Salmonella* types in the subgroup to which the already determined O antigen belongs. Agglutinin-absorption tests can be used to confirm the results of direct agglutination reactions. (It should be noted that in some diphasic types the two phases are both relatively specific.)

In many cases the evidence thus obtained, in association with the biochemical reactions (Table, p. 468a), will serve to identify the unknown organism if it is one of the usual types.

When the strain in question cannot be identified in this way a full antigenic analysis is required with selectively absorbed monospecific sera for the various O and H antigenic components of the *Salmonella* group, and in laboratories specialising in the study of the *Salmonella* group such antigenic analysis is the general procedure adopted. A

system is given below for the recognition of the commoner *Salmonella* types met with in Great Britain and this is applicable to the routine of the ordinary bacteriological laboratory.

It should be noted that where the bacillus under investigation has undergone variation to the "rough" form its O antigen can no longer be identified, while a non-motile variant can be allocated only to its appropriate subgroup, since it lacks the H (flagellar) antigens necessary for complete serological identification.

In the Table on p. 449 the numbers and letters designate various antigenic components which have been defined in the types referred to. The Table, which includes only a limited representation of *Salmonella* types, illustrates the extreme complexity of antigenic structure and yet close inter-relationship of these organisms and also indicates the problem of the ultimate identification of an organism which has given rise to an outbreak of food poisoning.

For further information, readers should consult the monographs cited on p. 449.

The following system may be recommended for the recognition of the commoner types met with in ordinary bacteriological routine. After an unknown organism has been placed in the *Salmonella* group by its morphological, cultural and biochemical characters, slide agglutinations (p. 250) are used for the serological determination of the type (*vide infra*), but these must be confirmed by quantitative tests in tubes to ensure that the organism is agglutinated to the titre of the standard sera. Thus, slide tests may show low-titre reactions due to the presence of normal agglutinins in the sera for organisms outside the *Salmonella* group, e.g. paracolon bacilli which may simulate this group in biochemical reactions.

The set of standard agglutinating sera recommended is the following :—

- (1) Polyvalent *Salmonella* serum, composite H specific and non-specific.¹
- (2) Polyvalent, *Salmonella* serum, H non-specific only.¹

O sera—

- (3) Polyvalent O serum, subgroups A-E.²
- (4) *S. paratyphi A*, antigen II, subgroup A.¹
- (5) *S. typhi-murium*, antigens IV, V, subgroup B.¹
- (6) *S. paratyphi C*, antigens VI, VII, subgroup C.¹
- (7) Newport type, antigen VIII, subgroup C.¹
- (8) *S. typhi*, antigen IX, subgroup D.¹
- (9) *Salmonella* subgroup E-O, antigen III [X, XV, XIX].²
- (10) Aberdeen type, antigen XI.²

¹ Obtainable from the Standards Laboratory, Central Public Health Laboratory, London, N.W. 9.

² Obtainable from Burroughs Wellcome & Company, London.

H sera—

- (11) *S. paratyphi A*, antigen a.¹
- (12) *S. paratyphi B*, „ b.¹
- (13) *S. paratyphi C*, „ c.¹
- (14) *S. typhi*, „ d.¹
- (15) Newport type, „ e, h.¹
- (16) Derby type, „ f, g.²
- (17) *S. enteritidis*, „ g, m.¹
- (18) Dublin type, „ p.²
- (19) *S. typhi murium* „ i.¹
- (20) Thompson type, „ k.²
- (21) London type, „ l, v.² •
- (22) Oranienburg type, „ m, t.²
- (23) *B. morbificans (bovis)*, „ r.²
- (24) Bareilly type, „ y.²

Having determined that the culture is motile (sometimes when isolated from differential media, such as desoxycholate-citrate agar, the organisms are deficient in flagella, but soon become motile on subculture), slide agglutinations are carried out :—

(a) With the polyvalent H specific and non-specific serum (1) to confirm that the organism is a member of the *Salmonella* group ; (b) with the O sera (3-10) to determine the somatic antigens ; (c) with the H specific sera (11-24) to determine the characteristic flagellar antigen or antigens, the sera selected for these tests depending on the O antigenic subgroup to which the organism appears to belong.

Where it is possible that the organism is diphasic, test with H non-specific serum (2) ; if no agglutination is obtained, proceed to final identification with H specific sera. If the organism is in the non-specific phase it cannot be further identified until the specific phase has been obtained. The following method has been found convenient for the purpose : a subculture is made into 1 ml. of nutrient broth to which is added 1 drop of undiluted non-specific H serum (2), giving approximately a dilution of 1 in 20. Continue subculturing in broth with non-specific serum, until the culture no longer agglutinates, then subculture on an agar plate. The colonies should not agglutinate with non-specific sera and the bacilli should be motile. The organism is now in the specific phase, and can be tested with H specific sera.

In this way it should be possible to state the type : e.g. if the ascertained antigens are IV, V, i, the organism may be identified as *S. typhi-murium* ; if VIII, e, h, it may be identified as the Newport type (Table, p. 449).

Salmonella pullorum (Bacillus pullorum)

The causative organism of "white diarrhoea" of chicks.

In general characters this organism belongs to the *Salmonella* group, but is non-motile. Its biochemical reactions are given in the Table (p. 468a) ; gas formation is not abundant. The organism is present

^{1, 2} See footnotes on previous page.

in the faeces, blood and internal organs, e.g. liver, spleen, and, at autopsy, can be isolated readily on nutrient agar. It has also been found in the ovaries of adult birds (which have become carriers), in egg yolks and in the yolk-sacs of developing chicks. The serum of infected birds agglutinates the bacillus, and this reaction has been utilised in controlling the spread of the disease among flocks. Agglutination by a serum dilution of 1 in 50 is diagnostic. An agglutinating antiserum is employed in identifying strains. This organism shows a close serological relationship to *S. typhi*, possessing the somatic antigen which is also common to *S. enteritidis* (Table, p. 449).

Salmonella gallinarum (*Bacillus gallinarum*,
BACILLUS OF FOWL TYPHOID)

This organism, associated with a disease of fowls characterised by severe anaemia, is difficult to distinguish from *S. pullorum* though it generally fails to produce gas in fermentation reactions; however some strains of *S. pullorum* are also non-gas-producing. It has the same somatic antigen as *S. typhi* and *S. pullorum*, and is serologically indistinguishable from the latter (Table, p. 449).

GROUP OF DYSENTERY BACILLI—SHIGELLA

The causative organisms of an acute form of dysentery most prevalent in tropical and sub-tropical countries, but occurring also in temperate climates. The condition called "asylum dysentery" and some cases of infantile diarrhoea are due to these organisms.

Morphology and Staining.—Non-motile, non-sporing, Gram-negative bacilli about $2\text{--}4\mu$ by 0.5μ , but often showing a tendency to shorter cocco-bacillary forms.

Cultural Characters.—Resemble the *Salmonella* group. Gelatin is not liquefied.

Biochemical Reactions.—The dysentery bacilli ferment glucose without gas production, and in the case of sugar fermentations generally are non-gas producing. (The "Newcastle" dysentery bacillus—*vide infra*—is an exception to this rule.) The dysentery group can be subdivided into species or types according to the fermentation of lactose, dulcitol, sucrose, mannitol and other carbohydrates, the production of indole from peptone and agglutination reactions with specific antisera.

Classical Dysentery Bacilli.—The dysentery bacilli first described were those which are now designated *Shigella shigae* or *dysenteriae*, the Shiga type of dysentery bacillus, and *Shigella flexneri* or *paradysenteriae*, the Flexner type or subgroup.

The biochemical reactions are shown in the Table on p. 468a. It will be noted that the Flexner strains differ from the Shiga type in their fermentation of mannitol.

The Shiga type is also identified by agglutination with an antiserum to a known Shiga strain, and is clearly differentiated in serological characters from the other types of dysentery bacilli.

Organisms with the biological characters of the Flexner subgroup possess a common antigenic constituent, but can be differentiated into six types at least, each containing a distinctive specific antigen (Boyd). These may be designated I, II . . . VI. Type VI is serologically similar to the organism which has been called the *Newcastle* dysentery bacillus (*vide infra*), but has the biochemical characters of the classical Flexner type with the exception that it may slowly ferment dulcitol.

Types I, II and III correspond to those originally named by Andrewes V, W and Z respectively, and types IV, V and VI to types named by Boyd "103," "P119" and "88."

It has been shown that, by variation, the type-specific antigen may be lost, the group antigen remaining; and probably the organism described as the "Y" type by Hiss and Russell is of this nature. It has long been recognised as possessing serological characters common to the whole Flexner subgroup and, in the past, a Y agglutinating antiserum proved a useful reagent for identifying this subgroup.

It may be noted that an X type was described by Andrewes, but it seems doubtful whether it is distinctive.

The *Newcastle* bacillus (*vide infra*) which is serologically related to type VI of the Flexner subgroup has been included by some writers in this subgroup, but presents distinct differences in biochemical reactions from the other members (p. 456).

Experimental Inoculation.—Cultures of dysentery bacilli are generally non-pathogenic when introduced by the mouth in laboratory animals. Intravenous injection produces a haemorrhagic enteritis, and, if the animal survives, muscular paralysis may result. These effects are specially marked in the case of the Shiga type, which forms a potent diffusible toxin.

Other Dysentery Bacilli.—Besides these types, other dysentery bacilli are met with which do not conform to the characters of the Shiga or Flexner organisms and yet have the general features of the dysentery group (as stated above). These

were at one time designated "atypical" dysentery bacilli. Some of them have been clearly proved to be pathogenic; the pathological relationships of others have been questioned.

Strains are met with possessing all the biological characters of the Flexner subgroup but serologically quite distinct from the recognised forms of this subgroup. Serological types with these characters have been described by Boyd. The different types may be designated "Boyd I," "II," etc. Types I, II and III correspond to those denoted by him "170," "288" and "D1" respectively.

The question arises whether such types should be classified separately from the Flexner types. Certain writers have placed them in the Flexner subgroup and have described further serological types of this subgroup, but all exhibiting the biochemical characters of the Flexner bacillus.

Shigella sonnei (*Sonne type*).—This organism is a frequent causal agent of dysentery in this country. It represents a distinct serological type. In biochemical reactions it resembles the Flexner subgroup, but produces late fermentation of sucrose and frequently lactose. Indole production is absent. The colonies of this organism on a neutral-red lactose medium, e.g. MacConkey's or desoxycholate-citrate agar, are at first "pale" and similar to those of other dysentery bacilli. In some cases, red papillae develop on the colonies after several days' incubation, and the late fermentation might appear to be a function of biological variation (cf. "*B. coli mutabilis*").

Shigella ambigua (*Schmitz type*).—Similar to the Shiga type, but differs in producing indole and in serological characters. Strains of this type are serologically homogeneous. Some strains are highly toxic and, in this respect, resemble the Shiga type.

Shigella alkalescens (*Bacillus alkalescens*).—Differs biochemically from the Flexner subgroup in its fermentation of dulcitol. It produces indole and alkalines milk. Sucrose fermentation has been reported in some strains. Apart from dysentery, it has been found in urinary infections and suppurative conditions. Strains are not serologically uniform and different types have been described.

It has been pointed out that this organism differs from most dysentery bacilli in its production of acid in glucose-broth at 45.5° C., the formation of hydrogen sulphide, haemolysis of sheep red cells, agglutination of human red cells, and that serologically it is more related to the coliform group than to *Shigella*.

Shigella dispar.—This type ferments lactose and sucrose (and sometimes dulcitol) as well as glucose and mannitol. It also forms indole and coagulates milk. There has been some doubt whether it is a

dysentery-producing organism. Like *Sh. alkalescens* it is serologically related to the coliform bacilli.

It is difficult to separate the lactose-fermenting types of dysentery bacilli from non-motile "anaerogenes" types of the coliform bacilli (p. 430), though the latter may occur quite independently of any pathological condition.

Newcastle type.—This designation is applied to an organism which ferments glucose, maltose and usually dulcitol, but has no action on lactose, sucrose or, as a rule, mannitol. Under certain conditions it appears to be non-gas-producing, but in a suitable broth medium, gas is formed from the carbohydrates it ferments. Indole is not produced. Strains of this type are serologically uniform. (Strains fermenting mannitol have sometimes been named "Manchester type".)

As stated above, type VI of the Flexner subgroup is serologically similar to this organism.

In our experience, strains which may be classified as the Newcastle type ferment glucose, but not lactose, sucrose or mannitol. Most strains ferment dulcitol slowly. A small amount of gas may be produced from the fermented carbohydrates even in the ordinary peptone water medium.

Parashiga type.—This term has been given to strains corresponding generally in biochemical characters to the Shiga or Schmitz type, but differing serologically from these organisms. Various serological subtypes have been described.

Classification of dysentery bacilli may be summarised as follows :—

Classical.—*Shigella shigae* or *Shiga type*—non-mannitol-fermenter. *Shigella flexneri* or *Flexner subgroup*—mannitol fermenters—at least 6 distinctive serological types, I-VI, the last being serologically similar to the Newcastle dysentery bacillus (*vide infra*).

Other dysentery bacilli.—"Boyd's types"—like Flexner subgroup, but serologically quite distinct—serological types, I, II, etc.

Sonne type—like Flexner subgroup, but ferments (slowly) sucrose and frequently lactose; serologically distinctive.

Schmitz type—like Shiga type, but produces indole; serologically distinctive.

Shigella alkalescens—like Flexner subgroup, but ferments dulcitol.

Shigella dispar—like Flexner subgroup, but ferments lactose and sucrose.

Parashiga type—like Shiga or Schmitz type, but serologically distinct from these.

Newcastle type differs from other dysentery bacilli in its gas production from fermented carbohydrates, glucose and, in some cases, dulcitol and mannitol. It is serologically related to Flexner subgroup.

Occurrence.—The dysentery bacilli are found in large numbers in the stools at an early stage of the illness, even in practically pure culture, but become progressively less

numerous, until, at a later stage, they are apparently absent. They do not invade the blood stream as a rule. The Shiga type is associated mostly with the acute and severe forms of dysentery, many of the atypical bacilli with the milder cases, and the Flexner subgroup occupies an intermediate position as regards the severity of illness with which it is associated. It may be noted here that the Shiga type differs from other varieties in its production of a powerful diffusible toxin which, like the exotoxins, is antitoxinogenic. The Schmitz type, however, has been shown to produce a somewhat similar exotoxin.

Concomitants.—When dysentery bacilli tend to disappear from the stool they are found along with, or, as it were, replaced by, certain other unusual organisms.

The commonest are :—

Morgan's bacillus (<i>Proteus morganii</i>) and related types	Gram-negative, non-sporing, gelatin-non-liquefying, aerobic bacilli.
Paracolon bacilli (p. 430)	
<i>Alcaligenes faecalis</i> (<i>Bacillus faecalis alkaligenes</i>)	

Their biochemical reactions are shown in the Table on p. 468a. They are non-lactose-fermenters and produce pale or colourless colonies on neutral-red lactose media, resembling, in this respect, the dysentery bacilli.

Though Morgan's bacillus does not liquefy gelatin, it is now classified with *Proteus*. Thus, on 1 per cent. agar at 25° C., it shows the spreading character of the growth of *Proteus* and it is also serologically related to some strains of this organism.

Alcaligenes faecalis colonies on neutral-red lactose medium may be relatively large, and around each colony may be noted a broad light-yellow zone due to the alteration of the neutral red by alkali production.

While strains corresponding in general biological characters to this organism have been described with peritrichous flagella, similar organisms are frequently met with which have terminal flagella like a vibrio, and show curved vibrionic forms. Many strains produce a brown growth on potato like the *Brucella* group, and *Alcaligenes faecalis* resembles these organisms in its lack of fermentative properties. The biological relationships of this organism are still a matter of doubt.

These organisms are also found in cases of non-dysenteric diarrhoea. Cases of *Alcaligenes faecalis* bacteraemia have been recorded. Morgan's bacillus and allied types are considered to be responsible for some cases of infantile diarrhoea.

The *Diagnosis of bacillary dysentery* is dealt with later (p. 462).

Therapeutic antisera for the dysentery bacilli.—In the past polyvalent antisera have been extensively used, these being prepared by immunising horses with the main types of dysentery bacilli. They possessed antibacterial properties (p. 41), but were also antitoxic towards the toxin of the Shiga type. The best therapeutic results were obtained in Shiga-type infections. It is now generally accepted that only an antitoxic serum for the toxin of the Shiga type is of therapeutic value, and this serum may be used in Shiga-type infections. Large doses should be administered, and in severe cases by intravenous injection.

Bacteriophages for dysentery bacilli (p. 628) have been advocated for the treatment of bacillary dysentery, but their therapeutic value is doubtful.

Chemotherapy.—Considerable success has been obtained in bacillary dysentery from the use of certain sulphonamide compounds, e.g. sulphaguanidine.

Shigella equirulis (*Bacillus pyosepticus equi*).—This organism causes purulent nephritis in horses and some cases of "joint ill" in foals. It has also been described as a rare cause of pyaemia in young pigs. It has been regarded as allied to the dysentery bacilli, though its true relationships may be with the pneumobacillus group. It is non-motile and Gram-negative. In the tissues the organisms are found in masses with capsular material around them. Growth is favoured by the presence of serum, but the organism can grow on ordinary media. On agar it produces two types of colonies: mucoid and non-mucoid. In broth, it produces a slimy sediment and a viscous supernatant fluid. Glucose, lactose, sucrose and mannitol are fermented (without gas); indole is not produced; gelatin is not liquefied.

THE INTESTINAL PROTOZOA

ENTAMOEBA HISTOLYTICA

The causative organism of one form of dysentery occurring mainly in tropical and subtropical areas.

Biological Characters.—The vegetative forms are large, rounded, elongated or irregular amoebae, varying in diameter from $10-50\mu$, the average size being about $20-25\mu$. 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 move-

ments 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 more or less spherical, with a thin, hyaline, refractile cyst wall, which gives them a distinct double contour. The contents are finely granular. The average diameter is 9–14 μ . 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."

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 septic infection occurs.

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.

Cyst carriers may also be found who have no history of previous dysentery, but it seems likely that such persons have small lesions of the bowel insufficient to produce obvious clinical signs of the disease.

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.

Cultivation.—This organism can be cultivated artificially by the method of Boeck and Drbohlav or a modification of their method (p. 199).

Experimental Inoculation.—Infection can be produced in kittens by feeding them with material containing cysts; a condition resembling the human disease results. Rats can also be infected.

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 ectoplasm. 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* (Dobell). Bacteria are ingested often in large numbers. The cysts are larger ($15\text{--}30\mu$) 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 10μ 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 BUTSCHLII 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 to these conditions. It is about $10\text{--}20\mu$ in diameter and resembles *Ent. histolytica* in many respects, showing active amoeboid movement and differentiation of the cytoplasm into ecto- and endo-plasm; the nucleus is indistinct in unstained preparations; the organism possesses the property of ingesting free cells, e.g. leucocytes.

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, $9\text{--}15\mu$ 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 *Trichomonas hominis* may occur in the vagina, and has been named *Trichomonas vaginalis*. It may be found in cases of vaginitis, and though regarded by some as pathogenic its aetiological relationships have not been fully established. For its recognition, "wet" preparations of vaginal secretion should be examined first with the lower power of the microscope and then with a $\frac{1}{2}$ -in. lens. 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 8μ 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-

¹ See *Protozoology*, Wenyon, London, 1926; *Handbook of Medical Protozoology*, Hoare, London, 1949.

shaped ; bilaterally symmetrical ; 10–18 μ 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 10–15 μ long, with two or four nuclei (the cyst containing two organisms formed by subdivision) ; the parallel axostyles are observable.

For further information regarding *other intestinal protozoa*, reference should be made to works on protozoology.

DIAGNOSIS OF DYSENTERY

Collection of specimens of stools.—The stool should be examined as soon as possible after being passed, and 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 very satisfactory alternative is the Universal container described on p. 320. If the stool contains both faecal matter and mucus, a portion of the latter should be included in the specimen.

When considerable numbers of cases have to be examined for dysentery bacilli, specimens may be conveniently obtained by rectal swabs (p. 323). This procedure is specially suitable when examining children.

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. 90). A loopful of the stool or the mucous discharge 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 $\frac{1}{6}$ -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. 71). 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 amoeboid movement and the inclusion in the cytoplasm of numerous red corpuscles (*vide supra*). On the other hand, immobile vegetative amoebae without ingested corpuscles present considerable difficulty in their identification. The presence of cysts facilitates diagnosis owing to the more striking differences between the encysted *Ent. histolytica* and other amoebae (*vide supra*). In the iodine preparation the nuclei of the cysts are distinctly seen.

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.

Other intestinal protozoa that may be present can also be detected in unstained preparations (*vide supra*).

Demonstration of Amoebic Cysts by the Flotation Method.—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.

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. Any leucocytes present are not so degenerate as in bacillary dysentery. 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.

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. 115).

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. 115).

Cultivation.—When the clinical and microscopical data point to a bacillary infection, cultures are made from the stool on desoxycholate-citrate medium as in the direct culture of enteric specimens (p. 439). In the past MacConkey's medium was extensively used, but has been generally replaced by the desoxycholate-citrate medium which facilitates the detection of dysentery bacilli owing to its inhibitory effect on coliform organisms. In general, however, if cases are examined within the first two or three days the infecting organism can be isolated without difficulty, even on a medium such as MacConkey's which does not inhibit coliform organisms. At a later stage the dysentery bacilli become less numerous and "concomitants" (*vide supra*) are present, often in large numbers. For the complete bacteriological investigation of a case of bacillary dysentery, therefore, it is necessary to consider all the unusual organisms present

in the stool. These are for the most part non-lactose-fermenters, and their colonies, like those of the dysentery group, are of the pale type on a neutral-red lactose medium. Certain varieties of dysentery bacilli (*e.g.* the Sonne type) may ferment lactose in fluid medium, but in primary culture from stools present pale colonies at twenty-four hours.

Subcultures on agar slopes should be made from each type of pale colony ; and since colonies which appear to be similar may represent different organisms, at least three pale colonies should be subcultured.

Sufficient growth is usually obtained after twelve hours' incubation to proceed with the examination of the agar-slope cultures. Tubes of the following media are inoculated :—

- (1) peptone water
- (2) glucose peptone water
- (3) lactose „ „ „ } with phenol red or Andrade's indicator,
- (4) sucrose „ „ „ } and Durham tube (p. 166).
- (5) mannitol „ „ „ }

and these are incubated for twelve to twenty-four hours. The peptone-water culture is examined after six to seven hours for motility of the organisms.

In this way, one can ascertain to what subgroup or biological type the various cultures belong. If a culture has the following characters :—

	<i>Motility</i>	<i>Glucose</i>	<i>Lactose</i>	<i>Sucrose</i>	<i>Mannitol</i>
Gram-negative bacillus	—	—	—	—	— or —

(— = acid; no gas)°

—*i.e.* corresponding to the classical dysentery bacilli, Shiga type or Flexner subgroup (Table, p. 468a)—the final identification is made by tests with specific agglutinating sera—*i.e.* an antiserum to the Shiga type, and a polyvalent anti-serum for the Flexner subgroup. The Central Public Health Laboratory, London, supplies two polyvalent Flexner sera for this purpose : (1) prepared by immunising animals with types I, II and III and the X type of the series V-Z referred to on p. 454, and (2) prepared by immunising with types IV and V and the Newcastle dysentery bacillus which is antigenically the same as type VI (p. 456).

It should be noted that the Newcastle bacillus after twenty-four hours usually shows fermentation of glucose only, often with slight

gas production. It is identified by means of a monovalent agglutinating serum. It reacts, of course, to the No. 2 polyvalent serum of the Central Public Health Laboratory.

If it is considered necessary for the epidemiological study of outbreaks, monovalent agglutinating sera for each of the Flexner types may be used.

If the unknown strain fails to react to the appropriate antiserum, its biological characters should be thoroughly studied : dulcitol medium should be inoculated, and the peptone water culture is tested for the presence of indole (p. 427) after forty-eight hours, and if negative, also after seven days. All fermentation tubes should be incubated for several days before final readings are made. A gelatin stab, or, alternatively, a coagulated-serum culture, should also be made to test for liquefaction. Christensen's medium (p. 193) is specially useful for excluding *Proteus*.

If the strain still appears to have the characteristic reactions of the Flexner dysentery bacillus, the possibility of its belonging to one of the additional types, e.g. those described by Boyd, would have to be considered ; their identification would be made by specific antisera for known strains.

The Sonne and other biological types can be identified by their characteristic biochemical reactions and, in the case of the Sonne type, agglutination with a specific antiserum. The Schmitz type can also be identified by means of a specific serum.

Other unusual intestinal organisms (p. 457) can be identified by completing the cultural tests as in the investigation of the atypical dysentery bacilli. Their reactions are shown on the Table, p. 468a.

Agglutination Tests with Patient's Serum.—In cases where the causative organism cannot be isolated, agglutination tests have a limited application in diagnosis.

Normal serum may agglutinate the dysentery bacilli in low dilutions (Shiga type in a titre of 1 in 25, Flexner types in a titre of 1 in 50), and in cases of bacillary dysentery the specific agglutinating effect of the serum towards the actual causative strain isolated from the case may be relatively weak. Normal serum reactions introduce, therefore, a limitation to the diagnostic test. In the case of the Sonne type, however, agglutination in a titre of 1 in 20 is said to be diagnostic. -

As in the Widal reaction, the agglutination reaction is only applicable after five to seven days from the onset of the illness.

In general, the test is carried out on the same principle as the Widal reaction (p. 243); known strains of the different types of dysentery bacilli are tested in parallel series with varying dilutions of the patient's serum. Standard suspensions of various types are obtainable from the Central Public Health Laboratory, London.

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 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 an acid medium (pH 4·0 or less). It occurs in faeces, saliva and milk. In 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 coecal forms. Some are about 1μ 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. 194), 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 out-growths ("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°–62° 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 hydrochloric acid is absent or deficient and *Döderlein's Bacillus*, which is found normally in the vagina.

Lactobacillus bifidus (*Bacillus bifidus*)

Derives its name from the apparently bifid appearance described by the original observers. This organism is found in large number in the faeces of breast-fed infants. Its average dimensions are 4μ by 0.5 – 0.7μ , but it shows 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° C. in tubes of neutral lactose-broth containing a piece of sterile rabbit kidney (p. 195), 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 organism may ultimately become micro-aerophilic. Glucose, sucrose, maltose and various other sugars are fermented with acid production, but no gas.

TABLE SHOWING BIOCHEMICAL REACTIONS OF VARIOUS ORGANISMS DESCRIBED IN CHAPTER XV^a

	Motility	Glucose	Lactose	Dextrose	Sucrose	Mannitol	Maltose	Xylose	Arabinose	Rhamnose	Adonitol	Inositol	Voges and Proskauer reaction	Indole production	Utilization of sodium citrate	Blackening of lead acetate agar ¹ due to H ₂ S	Litmus Milk	Gelatin
1 <i>Esch. coli</i> var. <i>communis</i> —a typical rodiform bacillus	+	+	+	+	—	+	+	+	+	—	—	—	—	—	—	A.C.	—	1
2 <i>Aero. aerogenes</i> —an atypical coccus bacillus	+	+	+	+	+	+	+	+	+	—	—	—	+	+	—	A.C.	—	2
3 A type of <i>B. coli anaerogenes</i>	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	A.C.	—	3
4 A Pneumobacillus type	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A.C.	—	4
5 “ <i>B. coli mutabilitas</i> ” (Massini)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	(late)	—	5
6 <i>Aero. eltor</i> type	+	+	+	+	+	+	+	+	+	—	—	—	—	—	—	—	slow	6
7 A type of paracolon bacillus	++	++	++	++	—	—	—	—	—	—	—	—	—	—	—	practically unchanged	—	7
8 <i>S. typhi</i> .	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	8
9 <i>S. paratyphi A</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	—	—	9
10 <i>S. paratyphi B</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	Alk.	10
11 <i>S. enteritidis</i> (Gaertner)	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	11
12 <i>S. typhimurium</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	12
13 <i>S. cholerae-suis</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	13
14 <i>S. paratyphi C</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	14
15 <i>S. pullorum</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	15
16 <i>S. shigae</i>	+	+	+	+	—	—	—	—	—	—	—	—	—	—	—	+	++	16
17 <i>Sh. flexneri</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	17
18 <i>Sh. sonnei</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	A.C. (late)	—	18
19 Schmitz type of dysentery bacillus	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	19
20 <i>Sh. albusseus</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	20
21 Newcastle type of dysentery bacillus ⁴	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	21
22 Morgan's bacillus and allied types	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	22
23 <i>Alcaligenes faecalis</i>	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	Alk.	23

Note.—Only the more important reactions are signified in the Table.

+ in motility column=motile; + in gelatin column=liquefaction; + under various carbohydrates=acid and gas production; — under various carbohydrates=acid and no gas.

¹ Lead acetate agar—nutrient agar containing 0·05 per cent. basic lead acetate; inoculated by stab method.

² Non-gas-producing strains of *S. pullorum* occur.

³ The monophasic type produces H₂S, but strains of the diphasic form vary.

⁴ Gas-production slight.

⁵ Some strains stated to ferment sucrose.

⁶ For full details of the biochemical reactions of these organisms and the various types of the *Salmonella* group, reference should be made to *Enterobacteriaceae*, by F. Kauffmann, Copenhagen, 1951.

CHAPTER XVI

CHOLERA VIBRIO AND ALLIED ORGANISMS; PLAQUE BACILLUS AND PASTEURELLA GROUP

VIBRIO CHOLERAE

or VIBRIO COMMA

THE causative organism of Asiatic Cholera.

Morphology and Staining.—Curved or “comma-shaped” rod (vibrio) with rounded or slightly pointed ends, about $1\cdot5$ – 3μ by $0\cdot5\mu$. 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. Dieudonné'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 a “rugose” colony; in the last mentioned, the corrugated growth is due to a gelatinous intercellular substance or a definite capsule (White).

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.

Potato medium—at first, a white layer of growth forms, and later a brownish-yellow or pinkish colour is developed; to obtain this growth the reaction of the potato medium must be alkaline.

Broth—a uniform turbidity results, and a characteristic pellicle forms on the surface.

Biochemical Reactions.—The fermentative reactions are as follows :—

Glucose	Lactose	Dulcitol	Sucrose	Mannitol	Maltose	Mannose	Arabinose
—	—	—	—	—	—	—	—
(some strains \perp after several days' growth)							
(\perp =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.

The cholera vibrio does not give the Voges-Proskauer reaction (p. 428; cf. *El Tor vibrio infra*).

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; observations are then made (Greig).

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 coagulated blood media, e.g. "chocolate agar," in the same way as ordinary blood-agar.

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 the organism may survive for a considerable period, e.g. eighteen days or longer. The cholera vibrio is readily distributed by water supplies.

Serological Reactions.—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.

Transformation of a *V. cholerae* strain to the "rough" form is associated with loss of the specific O antigen.

It may be noted that within the serological subgroup represented by the classical *V. cholerae* and with a common O antigen, serological types can be recognised, distinguished by agglutination and absorption tests with O antisera; this difference depends on a subsidiary O antigenic component characteristic of the type; the stability of these types is doubtful. In India two such types have been recognised and designated according to the names of standard strains "Inaba" and "Ogawa." It is possible that a third such type also exists possessing the characteristic components of both the above-mentioned types (Gardner, A. D., and Venkatraman, K. V., *Lancet*, 1935, 1, 265).

It is also of interest that similar type differences occur among haemolytic (El Tor) vibrios.

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. There is great epithelial desquamation, and the stools contain white flakes consisting of epithelial cells and mucus. 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 is frequently infected.

Convalescents may remain carriers, and the organism may persist in the gall bladder.

Cholera Vaccine has been used in the prophylaxis of the disease. It is prepared from a twenty-four hours' culture on nutrient agar

and the bacterial suspension is killed by heat at 55° C. (one hour) and then standardised (p. 274). Two doses are given, 5000 and 10,000 million organisms respectively, at an interval of seven days.

DIAGNOSIS OF CHOLERA

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.

Procedure for Cultivation and Isolation

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. M/5 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. 190) 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. 191) and desoxycholate-citrate agar (p. 182) 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. In examining possible carriers, the stool, if solid or semi-solid, is thoroughly emulsified in sterile salt solution and several

loopfuls are added to the medium. The peptone water used is 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. In general, however, the fuchsin-stained film can be relied on alone for the detection of vibrios. 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 a 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.

Where vibrios are relatively scanty, and are not cultivated directly on a Dieudonné plate, the organism can be isolated after enrichment in either one or two peptone water cultures.

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. 251). 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.

If a direct growth from the stool is obtained on Dieudonné's medium it is, as a rule, practically pure, and the bacteriological diagnosis can be expedited by carrying out the agglutination reaction at once from the plate culture. 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 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 (p. 137) and by using the disk as the inoculum for a peptone water culture.

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*).

When tested in the form of plain saline suspensions they do not react specifically with an agglutinating anti-cholera serum, and represent a number of serological types, differing in their agglutination reactions with antisera.

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. foetus* *V. jejuni* and *V. metchnikovii*.

V. foetus 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 susceptible to experimental inoculation.

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 appears to be related to *V. foetus*.

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 paracolera 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.

PASTEURELLA PESTIS

(*BACILLUS PESTIS*)

The organism of Oriental Plague.

Morphology and Staining.—In its most characteristic form this organism is a short, oval bacillus with rounded ends—i.e. cocco-bacillary—about 1.5μ by 0.7μ and occurring singly and in pairs. In the tissues a typical capsule may be observed; in cultures grown at 37°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 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.

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.

Viability.—The thermal death-point is about 55° C. Dies within one to two days when subjected to drying. Laboratory cultures remain viable for long periods (e.g. months) if kept moist and at low temperatures.

Experimental Inoculation.—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.

Toxin.—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 is apparently of the intracellular type.

Occurrence in Human Lesions.—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.

In *Septicaemic Plague* the condition is a general infection without definitely localised lesions.

Infection.—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 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 secretion 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.

Antigens.—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. (*vide supra*). 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, is stated to have greater immunising properties 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 (Schütze). Heating the culture for half an hour at 56° C. before addition of phenol does not affect the antigenic value of the resulting vaccine. Strains of *P. pestis* are serologically homogeneous.

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. One dose of 4 ml. is injected subcutaneously.

Living non-virulent cultures have also been employed as vaccines.

Therapy.—Sulphonamide compounds have been applied with some success. Among the antibiotics streptomycin has been employed in the treatment of the disease. Therapeutic results have also been obtained with antisera.

DIAGNOSIS OF PLAGUE

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, etc., 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. 222).

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.

OTHER ORGANISMS OF PASTEURELLA GROUP

The plague bacillus is only one species in a biological group (*Pasteurella*) which includes the organisms of "haemorrhagic septicaemia" in various animals, and *Pasteurella pseudo-tuberculosis* of rodents. 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 *Pasteurella* organisms isolated from haemorrhagic septicaemia have been generally named according to the animal in which they occur and they will be described here according to this system, but they are possibly members of the same species differing, perhaps, in their parasitic adaptations to particular hosts.

Pasteurella avicida (*Bacillus avisepticus*) is the causative organism of "fowl cholera" and septicaemia in certain birds. In the blood the bacilli are present usually in considerable numbers and show characteristic bipolar staining. Under experimental conditions this organism is virulent to fowls, pigeons and rabbits, but in guinea-pigs the infection may remain localised to the site of inoculation.

Pasteurella suilla (*Bacillus suisepcticus*).—The organism of swine plague which takes the form of a rapidly fatal septicaemia or a pneumonia with enteritis. Various animals are susceptible to experimental inoculation—e.g. swine, rabbits, cats, cattle, sheep; guinea-pigs and pigeons are stated to be less susceptible. This type of organism has been noted by various observers as a commensal in the respiratory passages of swine, and the question whether it is the primary causal agent of swine plague is not entirely settled.

Pasteurella bovicida (*Bacillus bovisepcticus*).—The organism of haemorrhagic septicaemia of cattle and of septic pleuropneumonia of calves. On experimental inoculation this organism is highly pathogenic to cattle, rabbits and mice.

Another recognised organism of the group is *Pasteurella cuniculicida* (*B. lepisepcticus*) of rabbit septicaemia and snuffles (p. 240). Similar infections also occur in horses, sheep, goats, etc. *Pasteurella pseudo-tuberculosis* (*B. pseudo-tuberculosis rodentium*) occurs in pseudo-tuberculosis and septicaemia of rats and certain other rodents.

The organisms of haemorrhagic septicaemia can be differentiated from *P. pestis* by the inhibition or absence of growth on a taurocholate medium (e.g. MacConkey's) on which the plague bacillus is able to grow. *P. pseudo-tuberculosis*, which might be confused with *P. pestis* if isolated from wild rats, can be distinguished by its motility when growing at 22° C. and its feeble pathogenicity to white rats.

Different serological types of *P. pseudo-tuberculosis* have been recognised by agglutination reactions. The organism possesses three antigenic constituents: (1) flagellar, (2) somatic and type-specific, and (3) 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. pseudo-tuberculosis* and *P. avicida* (representing the haemorrhagic septicaemia subgroup) may be differentiated :

	Motility at 22° C.	Sucrose	Milk	Indole	Growth on Bile-salt Media	Patho- genicity to White Rat
<i>P. pestis</i>	—	—	No change	—	+	+
<i>P. pseudo-tub.</i>	+	±	-Alk.	—	+	—
<i>P. avicida</i>	—	±	No change	+	—	+

— = acid; no gas. ± = strains vary in their reaction

In freshly isolated culture *P. pestis* can be differentiated from *P. pseudo-tuberculosis* and other *Pasteurella* organisms by adding very small inocula (from dilutions of the culture) to rabbit-blood agar and incubating at 37° C.: *P. pseudo-tuberculosis* 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 (*vide supra*).

The name *Pasteurella multocida* or *septica* has been proposed for all the typical organisms of haemorrhagic septicaemia, and the term *Pasteurella haemolytica* has been applied to atypical strains of bovine or ovine origin which are avirulent to rabbits and differ from the typical strains in their haemolytic properties and inability to produce indole. *P. septica* has been subdivided by fermentative and agglutination reactions into two main subgroups with possibly a third intermediate subgroup. Group I comprises all strains of avian origin; morphology is uniform; the colony presents a fluorescent appearance; growth in broth exhibits a uniform turbidity; arabinose and dulcitol are usually fermented but not xylose. Group II contains strains from a variety of animals; morphology is less regular; colonies are mostly non-fluorescent; growth in broth is of a mucoid type; xylose is fermented but not arabinose and dulcitol (see Rosenbusch, C. T., and Merchant, I. A., *J. Bact.*, 1939, **37**, 69).

Rare cases have been recorded of human infection by *P. septica* and *P. pseudo-tuberculosis*.

PASTEURELLA TULARENSIS

(*Brucella tularensis*; *Bacterium tularensense*)

By some writers this organism has been classified with the *Pasteurella* group; by others it has been placed in the *Brucella* group, particularly in view of its serological relationships to recognised members of this latter group. Its taxonomy, however, must be regarded as somewhat doubtful.

In the Western States of America it produces a plague-like disease (tularacmia) 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. The organism is a small rod-shaped structure not usually exceeding 0.7μ in length, and sometimes capsulated. It is present in large numbers in the spleen and liver of infected animals. The occurrence of specially large numbers inside cells in these organs has suggested that it may multiply as an intracellular parasite (*cf. Rickettsia*, p. 560). *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.

This infection is also transmissible to man—*e.g.* from handling infected animals (*e.g.* rabbits and hares), and from laboratory cultures (*which retain an extremely high degree of infectivity*). 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.

Aureomycin and terramycin can be used in the treatment of the infection.

CHAPTER XVII

BRUCELLA GROUP; HAEMOPHILIC BACTERIA (HAEMOPHILUS INFLUENZAE AND RELATED ORGANISMS)

BRUCELLA GROUP

THE generic name *Brucella* is now applied to a group of pathogenic bacteria which include the organism of classical Malta fever, *Brucella melitensis*, and that of bovine contagious abortion, *Brucella abortus*. These organisms have generally been classified as separate species but are similar in many of their features, and additional *Brucella* types can be recognised 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 abortion is an outstanding result of infection though an infected animal 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 similar to Malta fever, though the severity varies with the type : *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

(*Bacillus melitensis*)

The causative organism of undulant fever of the Mediterranean littoral and islands (Malta Fever), 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 0.4μ in diameter. Definite bacillary forms ($1-2\mu$ in length), however,

may be observed. The organism occurs singly, in pairs, or even short chains. It is non-motile and non-sporing.

Cultural Characters.—Grows under ordinary aerobic conditions. Optimum temperature, 37° C. Grows even at 20° C. It can be cultivated on ordinary nutrient media, but a better growth is obtained on liver-infusion agar (pH 6·6–6·8) —p. 191.

Colonies on agar in primary growth may not appear for two or three days; they are small transparent disks 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.

Br. melitensis exhibits no fermentative properties demonstrable by the ordinary methods.

Differentiation from other members of the *Brucella* group and serological characters are dealt with on pp. 487, 489.

Viability.—The thermal death-point is about 60° C. Resists drying for two to three months. Laboratory cultures remain viable for several months.

Experimental Inoculation.—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 and an autopsy carried out, necrotic areas are found in the liver and spleen, in which the living organisms are present.

Occurrence.—The organisms are present in the blood, especially at an early stage. In some cases (about 10 per cent.) they may be demonstrated in the urine.

Post mortem they are found in considerable numbers in the spleen and also in various organs. In Malta and the Mediterranean littoral, infection 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 carcases. Thus, the human infection may result either from the ingestion of milk or by contact with animals. It may be noted that recently *Br. melitensis* infection of cattle has been reported in England, but unassociated with any overt cases of the human infection.

A "rough" variant of *Br. melitensis* can be recognised differing in its agglutination reactions with antisera, though otherwise similar. This type was originally designated "*Bacillus paramelitensis*."

DIAGNOSIS OF MALTA FEVER

Blood culture should be carried out 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. Cultures may also be obtained by spleen puncture.

In some cases the organism may be isolated from the urine.

The agglutination test with patient's serum and known strains of *Br. melitensis* is carried out as a routine procedure.¹

Suspensions for the agglutination test are prepared from a number of smooth strains and are heated at 55° C. for 1 hour; 0.2 per cent. formalin can be added as a preservative. The mixtures of serum dilutions and suspensions are incubated in agglutination tubes at 37° C. or 50° C. for about 18 hours before final readings are made.

The agglutination reaction may be elicited after five days from the onset of the illness. It has to be noted that apparently normal serum may agglutinate *Br. melitensis* in low dilutions. In cases of Malta fever, however, the serum often agglutinates *Br. melitensis* in high dilutions, e.g. 1 in 1000. In a suspected case, if the reaction occurs only with low dilutions, the result cannot be regarded as conclusive. When the test is repeated, a "rising titre" may be observed and a more conclusive result obtained.

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

¹ A standard suspension for the agglutination test is obtainable from the Standards Laboratory, Central Public Health Laboratory, London.

serum at 56° C. for 15–30 minutes. Other methods of obviating this "blocking" effect in diagnostic tests have also been described. (See Griffitts, J. J., *Pub. Hlth. Rep.*, Washington, 1947, **62**, 865; Schuhardt, V. T., *et al.*, *J. Bact.*, 1951, **61**, 299.)

It may be noted that agglutinins for *Brucella* organisms may be present in the serum of cases of tularaemia.

Post mortem, the organism can be cultured from the spleen.

In goats, the infection can be recognised by using the agglutination test with the animal's serum and by cultivating the organism from the milk.

BRUCELLA ABORTUS AND BRUCELLA SUIS

(*Bacillus abortus*)

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 about 10 per cent. carbon dioxide (p. 218). 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 and is not dependent on such a high carbon dioxide content in the atmosphere as *Br. abortus*. This is an important distinguishing feature. 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 a suitable atmosphere for the growth of this organism in a tube is simply to seal the mouth of the tube with paraffin-wax after flaming the stopper. This procedure usually results in a sufficiency of carbon dioxide in the contained air.

As in the case of *Br. melitensis* the most suitable medium for these organisms is a liver-infusion agar (p. 191); 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 growth like that of *Br. melitensis*. They exhibit no obvious fermentative reactions.

Both *Br. abortus* and American strains of *Br. suis* form sulphuretted hydrogen (the latter more markedly). This can be tested for 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* do not produce sulphuretted hydrogen. *Br. melitensis* likewise does not produce sulphuretted hydrogen or only a slight amount on the first day of growth.

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 (*vide* Table, p. 488). 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 by incorporating the dyes in liver-infusion agar, pouring plates and then making stroke inoculations, the inoculum being a loopful of a dense suspension of an agar culture. Several strains can of course be tested simultaneously on the same plates. A plate of liver-infusion agar without dye is also inoculated for control purposes. The plates are incubated in an atmosphere containing 10 per cent. carbon dioxide.

Alternative method as described by Cruickshank.¹—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 10 per cent. carbon dioxide for 2–3 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.

Serological characters of *Br. abortus* and *suis* are dealt with on p. 489.

¹ Cruickshank, J. C., *J. Path. Bact.*, 1948, **60**, 328.

	<i>CO₂</i> requirement	<i>H₂S</i> production	<i>Growth in presence of</i>	
			<i>Basic fuchsin</i> 1 : 25,000	 <i>Thionin</i> 1 : 30,000
<i>Br. melitensis</i> . .	—	— or slight	+	+
<i>Br. abortus</i> . .	+	+	+	—
<i>Br. suis</i> (American strains)	—	++	—	+

Danish strains of *Br. suis* are similar to the American strains but do not produce *H₂S*.

Br. abortus can be isolated from the stomach contents, heart blood and tissues of the aborted foetus and from the uterine exudate, and in such materials it may also be demonstrated microscopically by appropriate methods. It may be present in the udder and excreted in the milk. Its specific relationship to the disease has been established by the experimental production of abortion in pregnant animals following intravenous injection of cultures or inoculation into the vagina. Similar experimental effects have also resulted from administration of cultures by the mouth.

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. 299).

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.

A "rough" variant of *Br. abortus* may occur in culture. This variant was originally designated "*Bacillus para-abortus*."

Diagnosis in Animals.—The agglutination test with the serum of supposed infected animals and known cultures of *Br. abortus* 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 is by means of 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 40–50 minutes, *i.e.* sufficient time for the cream to rise.

The stained antigen¹ 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 30 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 5 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 microscopically in the uterine discharge 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* 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.

RELATIONSHIP OF BR. ABORTUS AND BR. SUIS TO BR. MELITENSIS, AND THEIR OCCURRENCE IN UNDULANT FEVER

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 differ-

¹ See Hamilton, A. V., and Hardy A. V., *Amer. J. Pub. Hlth.*, 1950, **40**, 321.

ence in 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*.

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.

It is now well established that *Br. abortus* may, under certain conditions, produce undulant fever ("abortus fever") in man, infection being derived from cow's milk, or through contact with cattle; likewise contact with pigs infected by *Br. suis* may lead to human infections by this organism. *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. *Br. abortus* infection in man may be either a typical undulant fever or an irregular febrile illness which is mild in type.

Diagnosis of Br. abortus Infection in Man.—The diagnostic procedures are similar to those described in undulant fever due to *Br. melitensis* (p. 485); blood cultures, however, should be incubated in an atmosphere of 10 per cent. carbon dioxide; the serum of cases agglutinates *Br. abortus*, often in high dilutions. The minimum titre which can be regarded as significant is 1 in 80, and the result of a single test should not be reported as definitely positive unless the titre is 1 in 320 or higher. A rising titre on repeated testing is more conclusive. Reference has been made on p. 485 to the possible occurrence of a serum constituent which "blocks" agglutination or causes "zone" phenomena.

Some workers have recently claimed success in overcoming this difficulty by using an antiserum for human globulin, as in the Coombs' test for *rhesus* antibody. (See Wilson, M. M., and Merrifield, I. V. O., *Lancet*, 1951, 2, 913.)

The complement-fixation reaction has also been advocated for diagnostic purposes.

Castaneda's Method of Blood-culture in Brucella Infection.—3 per cent. melted agar is allowed to set on one of the narrow sides of a 120 ml. flat rectangular bottle with a perforated screw cap (p. 224); 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 48 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. (See *Proc. Soc. Exper. Biol. Med.*, 1947, **64**, 115.)

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*.

Further types deviating from the standard characters of the three recognised species have also been described.

Antibiotic Therapy of Brucella Infections in the Human Subject

The *Brucella* organisms are highly susceptible to aureomycin and chloromycetin and either of these antibiotics may be used in the treatment of acute infections. Successful results by combined therapy with streptomycin and sulphonamides have also been claimed.

THE GROUP OF HAEMOPHILIC BACTERIA (HAEMOPHILUS)

Haemophilus influenzae, Pfeiffer's influenza bacillus, originally described as the causal organism of epidemic influenza, 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 (*vide infra*). These growth factors, however, are not restricted to blood, but are present also in certain vegetable tissues. The Koch-Weeks bacillus (of conjunctivitis) shows the same growth requirements as *Haemophilus influenzae*, and may appropriately be grouped with it. *Haemophilus pertussis* (of whooping-cough) has also been placed in the "haemophilic" group, but its growth requirements are different from those

of the influenza bacillus. 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

(*Bacillus influenzae* of Pfeiffer)

Morphology.—A very small slender bacillus, usually about $1\cdot5\mu$ by $0\cdot3\mu$, with rounded ends, occurring singly or in pairs; non-motile; non-sporing. Shorter oval coccobacillary forms are also noted, and in culture there is marked pleomorphism. Some strains, particularly 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 (p. 177).

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 (p. 213). It has been found that the X factor can be dispensed with under anaerobic conditions. It is claimed also that cysteine can replace haematin under aerobic conditions, since, in the presence of this substance, hydrogen peroxide would be reduced and catalase would not be required. The other factor, designated V, is more easily destroyed by heat. It has been identified as coenzyme I (cozymase) 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, etc. This is due to the fact that these organisms synthesise the V factor. Thus, in a mixed culture the growth of the influenza bacillus is more marked

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. In culture, transformation to the "rough" type of colony may be observed.

Glucose and various other carbohydrates are fermented. Indole is produced by some strains.

Haemolytic type.—Certain strains have been found to differ from the typical form in their haemolytic properties and these are sometimes coarser in microscopic appearance. They also tend to develop elongated threads. While the typical influenza bacillus requires for its growth both the X and V factors, most of these haemolytic strains require only the V factor.

Some non-haemolytic strains also are independent of the X factor and require only the V substance ; and all strains which require only this factor have been designated "*Para-influenza bacillus*." Strains of this type occur mainly in inflammatory conditions of the pharynx, and have been described in some cases of ulcerative endocarditis.

Haemophilus canis, 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.

A somewhat similar organism has also been described recently in endocarditis of the human subject. This type of *Haemophilus* requires carbon dioxide for its growth.

Haemophilus suis.—This organism has been found in an influenza-like disease of pigs associated with a filterable virus (p. 598). It differs from *H. influenzae* in the lack of fermentative action.

Experimental Inoculation.—It has not been found possible to produce in laboratory animals a condition corresponding to influenza by experimental inoculation with this organism. According to certain observers a soluble toxin is formed in culture which, on inoculation in laboratory animals, produces pulmonary lesions like those in epidemic influenza.

Occurrence.—Found in the sputum, nasal and throat secretions in a considerable proportion of cases of epidemic influenza with inflammatory conditions of the respiratory system. In the inflammatory exudate the bacilli are often seen inside leucocytes. In the sputum they may occur in exceedingly large numbers, but usually along with the pneumococcus, streptococci and other organisms associated with inflammation of the respiratory passages. They may be found in the pulmonary lesions in influenzal pneumonia, in empyema and other complications, and have also been noted in the blood in some cases.

It must be remembered that *H. influenzae* may occur in catarrhal conditions of the respiratory system apart

altogether from epidemic influenza, and it may also occur in the mouth and throat in healthy persons.

To facilitate the isolation of *H. influenzae* from sputum, etc., penicillin (p. 22) has been used (Fleming). In culture medium it permits the growth of the influenza bacillus, while inhibiting that of various Gram-positive cocci. A suitable concentration for the purpose is 6 units (p. 24) for 12 ml. of medium in a 4-in. plate. The penicillin solution should be added half an hour before inoculating the plate and spread evenly with a sterile glass spreader (p. 210), and the surface of the medium is then dried in an incubator.

Though the influenza bacillus is not the primary cause of epidemic influenza, it may play an important part in the pathogenesis of the pulmonary and other complications.

Haemolytic strains (*vide supra*) have been found in cases of primary meningitis occurring in young subjects, mostly between the ages of six months and one year.

It may be noted here that aureomycin is considered the first choice among the antibiotics in the treatment of meningitis due to *Haemophilus influenzae*. Terramycin is also used with successful results.

Antigenic Structure.—Recently isolated "S" forms of *H. influenzae* have been classified into a number of serological types possessing specific polysaccharides as capsular constituents. In culture the organism readily undergoes transformation to the "R" 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. It was found that organisms isolated from meningitis belonged to type b. Straker in sixty cases of meningitis found that fifty-eight were due to type b organisms and the other two to types a and f respectively.

HAEMOPHILUS CONJUNCTIVITIDIS

(*Koch-Weeks Bacillus*)

Associated with an acute and often infectious form of conjunctivitis.

Morphology and Staining.—Short, slender, Gram-negative rods about $1-1.5\mu$ 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 it is, in fact, identical with the influenza bacillus. On blood-agar, growth develops in the form of minute dewdrop-like colonies.

Its exact relationship to, or differentiation from *H. influenzae* has not been clearly defined.

MORAXELLA LACUNATA

(*Diplobacillus of Morax*)

Associated with sub-acute or chronic conjunctivitis.

Morphology and Staining.—Gram - negative, rod - shaped organism measuring about 2μ by 1μ , 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° - 37° 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.

DIAGNOSIS OF CONJUNCTIVITIS

Films should be made from a loopful of conjunctival exudate, and stained with dilute carbol fuchsin, or by Gram's method. Cultures are made on blood-agar. Pure cultures should be obtained from single colonies and the biological characters of the particular organism determined.

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 PERTUSSIS

(*Bacillus pertussis*)

Generally accepted as the causative organism of Whooping-cough (Pertussis).

Morphology and Staining.—A very small, oval cocco-bacillus, slightly larger than *H. influenzae*, often showing polar staining ; definite bacillary forms are noted, but it is generally more oval in form than the influenza bacillus, and is more uniform in size and shape ; non-motile ; non-sporing ; Gram-negative.

Cultural Characters.—Aerobe. Compared with *H. influenzae*, it is not so strictly haemophilic. 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. For primary culture the special medium of Bordet and Gengou or a modification of this (p. 189) should be employed. The colonies develop slowly, and are smaller but thicker and more opaque than those of *H. influenzae*, and have a "pearly" appearance; growths may become sticky or slimy. Stroke subcultures have been likened to "streaks of aluminium paint."

The organism has no fermentative properties.

It has been pointed out that the growth-promoting effect of blood depends on the albumin fraction which acts by absorbing substances toxic to the organism, possibly unsaturated fatty acids (Pollock).

H. pertussis differs also from the influenza bacillus in its continued viability at low temperatures (0° – 10° C.) and its inability to grow, when first isolated, on agar containing heated blood.

Certain strains from cases of whooping-cough first described in America have been named *Haemophilus parapertussis*; these are reported as forming a brown pigment on culture medium. They are serologically distinct from *H. pertussis*. On Bordet-Gengou medium *H. parapertussis* grows more rapidly than *H. pertussis* and produces the characteristic pearly colonies (*vide supra*) in two days; later the medium undergoes blackening under the growth. *H. parapertussis* can grow on agar without blood or serum.

Experimental Inoculation.—Intranasal inoculation of *H. pertussis* cultures in mice anaesthetised with ether produces an interstitial pneumonia which may be fatal. Young rats can be infected readily in the same way; in these animals, paroxysmal coughing may occur and continue for a considerable period if the infection is not fatal. A condition similar to clinical whooping-cough has been produced in monkeys by introduction of cultures into the respiratory tract.

H. pertussis forms a potent endotoxin which can be extracted from cultures. This toxin is lethal to guinea-pigs on intravenous injection. In rabbits a dermonecrotic action can be demonstrated.

Serological Properties.—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.

Occurrence.—Present in sputum, especially in the early stages. It would appear from the evidence available that *H. pertussis* is the specific causal agent of whooping-cough.

It may be noted here that lymphocytosis is characteristic of this disease.

Haemophilus pertussis Vaccines.—There is evidence that vaccination confers some increased resistance to the disease, and this procedure is advocated for prophylaxis in young children. The form of vaccine which has been generally employed is a killed suspension of recently isolated cultures of the organism (in the original S phase). While 0·5 per cent. phenol has been used for killing the organisms some workers prefer to use thiomersalate. Alum precipitation is recommended to increase the immunising effect. The vaccine is standardised to contain 10,000 million organisms per ml. and is injected in three graduated doses amounting in all to 2 ml.¹

DIAGNOSIS OF PERTUSSIS INFECTION

For detecting and isolating the organism the "cough-plate" method may be used : a plate of the Bordet-Gengou medium is held four inches in front of the mouth of the patient while coughing ; the plate is thus inoculated directly with the droplets of sputum. After three days' incubation the "pearly" colonies of *H. pertussis* can frequently be recognised without difficulty, and identified by microscopic, cultural and serological methods.

In using this cough-plate method it is essential to avoid over-inoculating the plate ; otherwise the medium becomes overcrowded with colonies and *H. pertussis* is then difficult to detect and isolate ; moreover, certain other organisms present, e.g. staphylococci, may inhibit growth of *H. pertussis*. Generally a plate with not more than 100 colonies is suitable. It is recommended that two plates should be exposed during the spasm. Penicillin, as used for the isolation of *H. influenzae* (p. 494), is of value in controlling the growth of staphylococci on the plates and may even yield a pure growth of *H. pertussis*. When colonies with the characters of *H. pertussis* develop on the medium, these are spread with an inoculating wire and the plate is re-incubated. From this larger quantity of growth a nigrosin preparation (p. 88) is examined and if the organisms show the characteristic morphology some of the growth is emulsified in a drop of saline on a slide, and a drop of anti-pertussis rabbit serum appropriately diluted is added. Agglutination of the organism confirms the diagnosis. A patient yielding two negative results by the cough-plate method in a week may be regarded as non-infective.

¹ See *Bacterial and Virus Diseases*, II. J. Parish, 1951, Edinburgh, p. 121.

The cough-plate method has undoubtedly proved of practical value in the recognition of early cases of pertussis before the typical clinical manifestations have declared themselves, but, while applicable in hospital cases, is less convenient in general medical practice. More recently it has been recommended that a specimen for bacteriological diagnosis can be obtained more conveniently by using a pernasal swab. For this purpose, the 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. Cruickshank¹ has successfully used the ordinary throat swab bent at the end (pp. 323, 367) and introduced behind the soft palate so as to collect secretion from the naso-pharynx. In cultivating the organism 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 over-growth of the plate by other organisms and to render colonies of *H. pertussis* more easily detectable and so facilitate isolation of pure growths, 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—p. 494). It is claimed that more numerous colonies are obtained from the nasopharyngeal swab than by the cough-plate method, and it has also been shown that if swabs are kept moist by means of a small amount of saline-agar at the foot of their glass containers, the organisms remain viable for twenty-four hours (p. 323).

Serum Diagnosis.—Both complement-fixation and agglutination tests have been applied. Such reactions are likely to be most pronounced at a later stage of the disease, when the organisms are less easily demonstrated in the sputum, and may be useful in corroborating the diagnosis of atypical cases.

Antibiotic Therapy.—Chloromycetin, aureomycin and terramycin have been used in the treatment of pertussis and though *in vitro* the specific organism is sensitive to these antibiotics, the evidence of their therapeutic effectiveness remains somewhat doubtful.

Haemophilus bronchisepticus* or *Brucella bronchiseptica
(Bacillus bronchisepticus)

Was originally described in canine distemper and has been considered to have relationships to the *Brucella* group. It is generally classified in the genus *Haemophilus*, but is independent of the X and

¹ *Lancet*, 1944, 1, 176.

V growth factors and can grow on ordinary media without blood; it is related, however, in its antigenic characters and its toxin to *H. pertussis*, but differs from this organism in its motility and its possessing peritrichous flagella. In canine distemper it represents a secondary infection, but is frequently responsible for a broncho-pneumonic condition in rodents; it may be found in snuffles of rabbits.

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 (as above).

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.

CHAPTER XVIII

THE GROUP OF CLOSTRIDIA:

TETANUS BACILLUS AND OTHER ANAEROBES OF INFECTED WOUNDS; CLOSTRIDIUM BOTULINUM; CLOSTRIDIUM CHAUVOEI (OF BLACKLEG).

CLOSTRIDIUM TETANI

(*BACILLUS TETANI*)

THE causative organism of Tetanus.

Morphology.—Straight, slender, rod-shaped organism, $2\text{--}5\mu$ by $0\cdot4\text{--}0\cdot5\mu$, with rounded ends ; shorter forms and longer filaments are also noted ; motile, with numerous, long, wavy flagella, peritrichous in arrangement ; but movement is not markedly active. Characteristic spores are developed—spherical, two to four times the diameter of the bacillus, and situated terminally, they produce the “drum-stick” appearance which is a striking morphological feature of the organism.

Staining.—Gram-positive, but different individuals may show variation in the reaction to Gram’s stain ; by the ordinary staining methods, only the periphery of the spore is stained.

Cultural Characters.—An obligatory anaerobe, but growths can be obtained in the presence of minimal traces of oxygen ; temperature range, $14\text{--}43^\circ\text{C}$. ; optimum, 37°C . ; grows on ordinary nutrient media ; the meat medium described on p. 192, and media prepared from a peptic digest of blood (p. 190) are specially suitable for the growth of the tetanus bacillus. For methods of anaerobic cultivation see p. 213 *et seq.*

On agar or blood-agar, 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 feathery appearance, and the whole growth may spread over the surface of the medium by means of the branching processes from the colonies.

Non-motile variants may produce quite isolated colonies without these characteristic feathery processes.

Agar stab—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.

Milk—usually no coagulation.

Gelatin—slowly liquefied.

Coagulated serum—rendered more transparent and softened, but is not usually liquefied.

Meat medium—gas production, but only slight digestion and blackening of meat.

No carbohydrates are fermented.

Cultures have an unpleasant odour which is not markedly putrefactive.

Viability of Spores.—The spores possess a high resistance to adverse agents. From some strains they may withstand boiling water for forty to sixty minutes, and even longer. They may resist dry heat at 150° C. for one hour, 5 per cent. phenol and 1 : 1000 perchloride of mercury for considerable periods (two weeks or more). Iodine in watery solution and hydrogen peroxide (10 volumes) kill them within a few hours.

Toxin.—This is an exotoxin and can be prepared artificially by growing the organism in broth and filtering through a bacterial filter after five to fourteen days' growth, the optimum time varying with the strain. The essential pathogenic constituent is *tetanospasmin* which acts on the nervous system; another constituent, *tetanolysin*, causes lysis of red blood corpuscles.

Tetanospasmin has recently been separated as a pure crystalline protein. The estimated lethal dose for mice is 0·000,000,1 mgm.

Tetanus toxin is an extremely powerful poison. When injected into guinea-pigs or mice the animals die in twelve to eighteen hours with the typical signs of tetanus. In animals, tetanic spasms may start in the muscles related to the site of injection ("local tetanus"). It has long been supposed on the basis of experimental results that the toxin reaches the central nervous system by passing along the motor nerves, being absorbed probably by the motor end-plates and spreading along the axis cylinders; it seems to act as an excitant to the motor cells in the anterior horn of the spinal cord and may also interfere with the normal inhibition of motor impulses exercised by the upper motor neuron over

the lower, producing increased muscular tonus and tonic spasms. This affords an explanation of local tetanus.

Another view is that in cases of general tetanus the toxin is absorbed by the lymphatics and reaches the central nervous system by the blood-stream, while local tetanus is supposed to be due to a direct action of the toxin on the nerve-endings in muscles.

For the prophylaxis of tetanus, *formol-toxoid* (cf. diphtheria toxoid) was used during the war of 1939-45 to produce active immunity among troops before proceeding on active service. Two doses of 1 ml. of a standard preparation were administered intramuscularly or subcutaneously, at an interval of six to eight weeks. An alum-precipitated toxoid analogous to diphtheria A.P.T. (p. 385) is also an effective antigen.

Antitoxin.—An antitoxic serum can be obtained by immunising horses with toxin or toxoid. This serum is of value in the prophylaxis of tetanus, given immediately after a wound. Its use as a curative agent after the development of tetanus is less reliable than, and not nearly as effective as, the corresponding antitoxin treatment in diphtheria.

Tetanus antitoxin is standardised in Great Britain in terms of the "International Unit (1950)." The method of determining the potency of an antitoxic serum is similar to that used in assaying diphtheria antitoxin, i.e. by comparison with a preserved standard serum. For prophylactic purposes, 1500 units should be given subcutaneously and repeated on one or two occasions at weekly intervals if considered necessary, e.g. wounds with severe sepsis or necrosis of tissue. Larger initial doses, e.g. 3000 or 5000 units, may be given where the wound is a severe one. In the treatment of cases reliance is now generally placed on the administration of a large initial dose intravenously (e.g. 100,000 units), subsequent injections (e.g. of 25,000 units) being given if necessary.

Occurrence.—The spores are widespread in nature, but are specially prevalent in manure and manured soil. Tetanus bacilli may occur naturally in the intestine of certain animals—e.g. horses, cattle, sheep—and have been noted occasionally in the human intestinal contents. It is uncertain whether this organism flourishes as a saprophyte in nature, or is derived entirely from an animal source.

Tetanus is usually the result of a wound contaminated with *Cl. tetani* spores. The infection remains strictly localised, and the tetanic condition is due to the diffusible toxin absorbed into the nervous system.

Certain conditions favour the germination of the spores and the propagation of the organisms in the tissues, e.g. infection by other organisms (*Cl. welchii*, pyogenic cocci,

etc.), foreign bodies such as pieces of clothing carried into the wound, necrotic tissue and effused blood. It has been shown that the spores germinate only under a reduced oxygen tension.

Cl. tetani infection may occur in the uterus, as in cases of septic abortion, and also in the umbilical wound of new-born infants. Cases of post-operative tetanus have been recorded, due to imperfectly sterilised catgut, dressings or glove-powder.

DIAGNOSIS OF TETANUS

Films may be made from the wound exudate and stained by Gram's method, but the appearance of "drum-stick" bacilli is not conclusive evidence of the presence of *Cl. tetani*, as other organisms having terminal spores, which are practically identical in morphological characters, may be present (p. 506). Moreover, it is often difficult or impossible to detect the tetanus bacilli in wounds by microscopic examination.

The more reliable method for diagnostic purposes is to produce tetanus in white mice by subcutaneous injection of a filtrate of an anaerobic fluid culture from the wound. A control test should be included in which tetanus antitoxin has been administered as a prophylactic (*cf.* diphtheria virulence test, p. 379). While significant results may 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 isolating the organism, advantage is taken of the tendency of *Cl. tetani* colonies to spread and extend beyond the growth of other bacteria. In Fildes' method the material is incubated anaerobically in 5 per cent. peptic-blood broth for two to four days at 37° C. (p. 190). The culture is then heated at 65° C. for half an hour to kill spreading non-sporing organisms such as *Proteus*. The condensation water of a peptic-blood agar slope is inoculated from the heated culture, and the tube is incubated under anaerobic conditions. After twenty-four to forty-eight hours the edge of the culture is examined with a low-power binocular microscope or hand-lens, when a growth of tetanus bacilli is seen as a mass of very fine filaments. Subcultures from the marginal growth usually yield pure cultures of *Cl. tetani*. (It is advantageous to keep the peptic-blood agar tubes until the surface of the medium is dry at the top.)

OTHER ANAEROBES OF INFECTED WOUNDS AND GAS-GANGRENE

These organisms, belonging to the genus *Clostridium*, are associated with rapidly spreading inflammatory oedema, necrosis and gangrene of the tissues, and gas production, occurring as a complication of wound infection. They are all sporing organisms, and their source 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.

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 in some cases from the skin, especially in areas of the body that may be contaminated with intestinal organisms.

In nature, these organisms play an essential part in the process of putrefaction.

They have been broadly classified into two types: (1) *saccharolytic*, (2) *proteolytic*.

(1) The saccharolytic organisms are characterised by their rapid and vigorous growth in carbohydrate media with the production of acid and abundant gas. If grown in a medium containing both carbohydrate and protein, e.g. meat medium (p. 192), there is rapid production of acid and gas, but no digestion of the meat. The cultures may have a slightly sour smell, and the meat is often reddened.

(2) The proteolytic type is characterised by the digestion of protein. In meat medium the proteolytic ferments of these organisms decompose and blacken the meat with the formation of foul-smelling sulphur compounds. The protein is also broken up into amino-acids, and small, white, feathery masses of tyrosine crystals may be seen in cultures of certain species.

The more important organisms exemplifying these types are the following :—

Saccharolytic

- Cl. welchii*
- Cl. septicum*
- Cl. tertium*
- Cl. fallax*

Proteolytic

- Cl. sporogenes*
- Cl. histolyticum*
- Cl. tetani*—
- slightly proteolytic
(p. 501)

There is, however, no hard and fast line of demarcation between the two groups. Thus, strains of *Cl. welchii* produce small amounts of amino-acids, and *Cl. sporogenes*, though essentially proteolytic, has saccharolytic properties.

A Table (quoted from the *Medical Research Committee's Special Report*, No. 39, 1919) is given on p. 506, showing the general classification of these organisms.¹

It must be emphasised that the separation and cultivation of these anaerobes is much more difficult than in the case of the aerobes. In wounds there is practically always a mixed infection, so that simple plating alone is frequently not sufficient as in the case of the aerobes; alternate growths on plates and in fluid media have sometimes been found necessary before a pure culture is obtained.

An account is given on p. 513 of the methods in general use for the bacteriological examination of wounds, and this includes an outline of the procedures for isolating and identifying the more important anaerobes, with particular reference to cases of gas-gangrene.

As in the case of tetanus, the presence of foreign bodies, dead tissue and blood clot promotes the occurrence of gas-gangrene. Thus, in war casualties, infection tends to occur where there are deep lacerated wounds caused by irregularly shaped pieces of shell, and into which muddy clothing and particles of earth are carried. Spores are then introduced under most favourable conditions for the development of the organism.

In the contaminated wound the saccharolytic organisms are the first to grow and may spread rapidly in the interstitial substance of lacerated muscles. Toxic products of growth cause death of the muscle fibres. The saccharolytic organisms (usually *Cl. welchii*) may actually multiply within the sarclemma sheath, fermenting the muscle sugar and forming abundant gas. The death of the muscle is assisted by the oedema and gas formation, which tend to cut off the blood supply. The process may spread throughout the length of the affected muscles. There is also marked toxæmia. The proteolytic organisms (usually *Cl. sporogenes*) multiply later, digesting the dead muscle and causing blackening and the foul odour.

Gas-gangrene may also be located in subcutaneous tissue, e.g. where there has been extravasation of blood. Less severe forms of clostridial infection may occur without the typical toxæmia; such wounds have

¹ See also *Medical Research Council War Memorandum*, No. 2, Revised Second Edition, 1943.

TABLE SHOWING CLASSIFICATION OF SPORING ANAEROBES

	<i>Both proteolytic and saccharolytic properties</i>	<i>Slight proteolytic but no saccharolytic properties</i>	<i>Neither saccharolytic nor proteolytic properties</i>	<i>Neither saccharolytic but no proteolytic properties</i>
Proteolytic properties predominating Coagulated serum and gelatin are liquefied	Saccharolytic properties predominating Serum not liquefied; gelatin liquefied	Serum not liquefied; gelatin liquefied	Neither serum nor gelatin liquefied	Neither serum nor gelatin liquefied
<i>Clostridium sporogenes</i> <i>Clostridium parasporeogenes</i> <i>Clostridium histolyticum</i> <i>Clostridium aerofaciendum</i> <i>Clostridium butyfermentans</i>	<i>Clostridium welchii</i> <i>Clostridium septicum</i> <i>Clostridium chauvoei</i> <i>Clostridium cibdellum</i> <i>Clostridium pasteurianum</i>		<i>Clostridium fallax</i> <i>Clostridium multifermensans</i>	
Oval terminal spore .			<i>Clostridium tertium</i>	<i>Clostridium cochlearium</i>
Spherical terminal spore .		<i>Clostridium tetani</i>	<i>Clostridium tetanomorphum</i> <i>Clostridium sphenorum</i>	<i>Clostridium sphacelatum</i>

This Table is quoted by permission of the Controller of H.M. Stationery Office, from the *Medical Research Committee's Special Report*, No. 39. (The original names used in this Report have been changed to the new nomenclature.)

a foul odour, and gas bubbles form in them. Moreover, it must be remembered that potentially pathogenic anaerobes may be cultivated from a wound which never shows any signs of gas-gangrene.

CLOSTRIDIUM WELCHII or PERFRINGENS
(Bacillus welchii)

The most frequent organism and commonest cause of Gas-gangrene.

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. They are oval and subterminal.

Cultural Characters.—Obligatory anaerobe. Optimum temperature about 37° C. . Grows best on carbohydrate-containing media, e.g. glucose-agar.

Surface colonies—large, round, smooth, regular, opaque disks. (Another type of colony is also observed, with an opaque centre and a transparent border which is radially striated.) On blood-agar the colonies are haemolytic.

Milk—acid, clot and gas production result; the gas breaks up the clot, producing the characteristic “stormy-clot” reaction; the culture has a sour, butyric-acid odour.

Gelatin is liquefied.

Coagulated serum—no liquefaction usually occurs. (Some strains, e.g. from animal diseases, have been stated to liquefy serum.)

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.

Toxins.—Filtrates of cultures are strongly haemolytic, e.g. to sheep's erythrocytes, and a lethal exotoxin can be demonstrated by intravenous injection of mice. A local necrotising action on muscle tissue is a property of toxic filtrates, and such filtrates also contain leucocidin, fibrinolysin and hyaluronidase (“spreading factor”). Further details of the toxic factors of *Cl. welchii* are given later in relation to the different types of the organism.

An antitoxic serum for prophylactic and therapeutic use is obtained by immunising horses with toxic filtrates.

Experimental Inoculation.—The virulence varies greatly with different strains. Some are markedly pathogenic to guinea-pigs by subcutaneous injection of culture, and the animal may die within twenty-four hours. At autopsy, a spreading inflammatory oedema with gas production is noted in the subcutaneous tissue, and necrosis 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. Apparently the products of growth of the bacillus increase its aggressiveness.

Pigeons are exceedingly susceptible to experimental inoculation.

Occurrence.—Apart from its pathological relationships, *Cl. welchii* occurs normally in the large intestine of man and animals. 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. Apart from wound infections it may occur in uterine infections (*e.g.* septic abortion) and in infections of the intestinal tract, gall bladder and the urinary system.

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.

Cl. welchii may also occur in gas-oedema of the muscles in cattle and sheep.

Lamb dysentery.—This condition has been shown to be due to a type of *Cl. welchii*, *viz.* type B. (The classical type occurring normally in the intestine of man and animals is designated type A.) Its pathogenicity depends on exotoxins, for which an antitoxic serum can be obtained. This 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. It is supposed that the antibodies produced by such immunisation are conveyed to the lamb in the colostrum.

Another type (C), originally named *Bacillus paludis*, has been found associated with a disease of sheep known as "struck," the condition being essentially a toxæmia due to absorption of the bacterial toxins from the small intestine. A similar disease has been reported in Australia, and designated "infectious entero-toxæmia"; the associated organism, a further type (D), has been designated *Bacillus ovotoxicus*. A disease affecting lambs, called "pulpy kidney disease,"

is of similar nature and aetiology to infectious entero-toxaemia. Appropriate methods of immunisation may be carried out against these diseases, as in the case of lamb dysentery.

Seven different toxic factors, distinguishable by neutralisation tests with antitoxic sera, have been demonstrated among the types of *Cl. welchii*. The neutralisation tests are made by the intravenous injection of mice with mixtures of toxin and antitoxin. The types of *Cl. welchii*, referred to above, differ as regards their production of the several toxins. The differences are indicated in the following Table, and the toxic properties of each factor are also tabulated :—

Type	Toxic Factors							Antitoxin for Type
	α	β	γ	δ	ϵ	η	θ	
A (classical type)	++	—	—	—	—	+	+	A neutralises only type A toxin.
B (Lamb dysentery organism)	+	++	+	\pm	++	—	+	B neutralises types A, B, C and D toxins.
C	+	++	+	++	—	—	+	C neutralises types A and C toxins.
D	+	—	—	—	++	—	+	D neutralises types A and D toxins.

+ toxic factor produced, — not produced, \pm not invariably produced.
++ predominant toxic factor.

α —haemolytic, necrotising and lethal (α lecithinase—p. 518, Nagler's reaction).

β —necrotising and lethal.

γ —lethal.

δ —haemolytic and lethal.

ϵ —necrotising and lethal.

η —lethal.

θ —haemolytic, necrotising and lethal ; also produces a haemorrhagic condition ; not a lecithinase.

Strains of type D may undergo transformation serologically to type A through loss of their specific toxic factor.

A Type E has also been described in enterotoxaemia of calves. It produces a separate toxin designated ι which is lethal and necrotising. Recently a Type F has been reported as the causal agent of a condition occurring in Germany and named "enteritis necroticans." Its main toxins are β and γ but it does not produce δ (cf. Type C).

Cl. welchii also produces a collagenase which breaks down collagen and gelatin and softens muscle tissue ; this has been called κ -toxin. It is characteristic of Types A and C and is produced by some strains of Types B and D. Another somewhat similar substance, λ , is pro-

duced by certain strains of *Cl. welchii*, e.g. Type B ; it does not affect collagen but attacks hide-powder.¹

CLOSTRIDIUM SEPTICUM

(*Vibrio septique*)

Morphology and Staining.—Moderately large bacillus, with rounded ends, about $3\text{--}10\mu$ by $0.6\text{--}1\mu$. 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, lemon-shaped forms, which have been designated "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—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.

Milk—slight acid is formed, and the milk is slowly clotted, but often the change is slight.

Gelatin is liquefied.

Coagulated serum—no liquefaction.

Meat medium—meat is reddened, and not digested.

Various sugars are fermented, e.g. glucose, lactose, maltose and salicin, but not mannitol or sucrose.

Exotoxin with lethal and haemolytic properties can be demonstrated in cultures, and a specific antitoxin can be obtained by immunising animals. Fibrinolysin, hyaluronidase and collagenase are also produced.

Experimental Inoculation.—Subcutaneous injection of cultures in laboratory animals produces a spreading inflammatory oedema, with slight gas formation in the tissue. 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.

Cl. septicum is responsible for *Braxy* in sheep and some cases of *Blackleg* in cattle and sheep (*vide infra*).

A vaccine consisting of formalised culture of this organism has been used with success in the prevention of braxy.

¹ Oakley, C. L., et al., *J. Path. Bact.*, 1948, **60**, 495.

CLOSTRIDIUM OEDEMATIENS or NOVYI*(Bacillus oedematiens)*

This organism resembles *Cl. welchii* in morphology, but is somewhat larger and more pleomorphic. It possesses peritrichous flagella, but its motility is not active. The spores are oval and central or subterminal. Surface colonies are transparent, flat and tend to fuse, forming a spreading film of growth. Deep colonies are small, irregular, "woolly" or "snow-flake"-like balls of growth. In milk, late clotting may occur. Gelatin is liquefied; coagulated serum is not digested. The organism is actively saccharolytic and certain sugars are fermented, e.g. glucose and maltose. In meat medium the meat is reddened, but not digested. Culture-filtrates are highly toxic, and this organism has been found specially associated with a markedly toxic form of gas-gangrene. Such filtrates exhibit haemolytic and lecithinase activity, also necrotising and lethal properties. As in the case of *Cl. welchii* different immunological types have been defined. These differ in the distribution of various toxic factors.¹

Cl. oedematiens causes "Black disease" among sheep in Australia and New Zealand. This condition is activated apparently by the invasion of the liver fluke. A necrotic hepatitis is produced, followed by toxæmia and death. The same infection has recently been described in Scotland. Formolised culture may be used as a prophylactic vaccine.

CLOSTRIDIUM SPOROGENES*(Bacillus sporogenes)*

An anaerobic motile bacillus, with peritrichous flagella, and oval, central or subterminal spores. It is about the same size as *Cl. welchii*, but more slender. It is typically Gram-positive in young cultures, but Gram-negative forms are frequent in older cultures.

A stab culture shows a growth like that of *Cl. tetani*, with lateral radiations or spikes. Surface colonies present a "medusa-head" appearance (*cf. B. anthracis*) if the plate is dry, but may be irregular and feathery if moisture is present. Cultures have an exceedingly putrid odour. The organism decomposes protein, producing amino-acids, ammonia, sulphuretted hydrogen, etc. In milk the casein is precipi-

¹ Oakley, C. L. et al., *J. Gen. Microbiol.*, 1947, 1, 91.

tated and digested. In meat medium the meat is blackened and digested. Coagulated serum is liquefied. Glucose and maltose are fermented, with gas production.

It is non-pathogenic to laboratory animals.

Clostridium fallax (*Bacillus 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 (*Bacillus tertius*).—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. Neither gelatin nor coagulated serum is liquefied, but the organism shows active saccharolytic properties. In milk, acid is formed with gas production and slow clotting. Meat is reddened, but not digested. Its pathogenicity is doubtful, but when present in wounds it may give rise to gas production. No exotoxin is produced.

Clostridium histolyticum (*Bacillus histolyticus*) 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.

Further particulars of these anaerobes are given in the Table on pp. 516, 517.

For information regarding other species and 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.

Gas-gangrene Antisera.—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.

Chemotherapy.—The sulphonamide compounds have been used both in the prophylaxis and treatment of gas-gangrene; they may be administered orally, and as prophylactics have also been applied locally in powdered form. The effect varies with the different organisms and is most evident in the case of *Cl. welchii*.

Local application of penicillin has also been employed as a prophy-

lactic measure; systemic administration is required for the established infection and combined treatment with sulphadiazine and penicillin has been advocated.

DIAGNOSIS OF GAS-GANGRENE AND THE GENERAL BACTERIOLOGICAL EXAMINATION OF INFECTED WOUNDS

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 therefore convenient here to give an account of the complete examination of such wounds, including special reference to the recognition of the anaerobic bacilli.

A great variety of bacteria may flourish in a wound: *Streptococcus pyogenes*, pathogenic staphylococci (coagulase-positive), e.g. *Staphylococcus aureus*, the various sporing anaerobic bacilli (the tetanus and gas-gangrene organisms), streptococci of the *viridans* type, enterococcus, anaerobic or micro-aerophilic streptococci, pneumococcus, *Proteus*, *Ps. aeruginosa*, coliform bacilli, *C. diphtheriae*, haemophilic bacteria, non-pathogenic staphylococci (coagulase-negative) and various saprophytic Gram-positive cocci, various Gram-negative cocci, *Alcaligenes faecalis*, non-pathogenic diphtheroid bacilli, sporing aerobic bacilli (*B. subtilis* group). Though some of these are mainly saprophytic and are of little clinical significance, their presence may delay healing.

When a systematic bacteriological investigation of a wound is called for, and particularly with a view to ascertaining the effects of treatment, repeated examinations are required starting from the time of admission to hospital or just before any initial operative procedure, the second examination being made when the first dressing is carried out and subsequent examinations at weekly intervals or whenever there is any adverse change in the clinical condition.¹

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. 322), rubbed over the wound surface and soaked in the exudate, serve well for the purpose.

Sterile swabs mounted on wooden applicators, 5 in. long, are recommended in the memorandum cited¹; they are supplied to the

¹ Medical Research Council, War Memorandum No. 2, Revised Second Edition, London: H.M. Stationery Office, 1943.

surgical ward or theatre in a sterile container. 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. When specimens are taken from wounds treated locally with a sulphonamide powder, the exudate before cultivation should be suspended in broth containing 0·05 per cent. *p*-aminobenzoic acid; this neutralises any inhibition of growth due to the presence of the sulphonamide in the specimen. Likewise penicillinase (p. 316) should be used if material is cultivated from a wound treated with penicillin.

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, e.g. the morphological types of organisms present (staphylococci, streptococci, Gram-negative or Gram-positive bacilli), 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, though it has been pointed out that *Cl. oedematiens* may appear to be relatively scanty in the wound exudate, even in an active infection. Thick, rectangular, Gram-positive bacilli would suggest the presence of *Cl. welchii*, *Cl. fallax* or *Cl. bif fermentans*; "citron bodies," boat- or leaf-shaped pleiomorphic 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.—The following media should be inoculated: (a) blood-agar plate to be incubated aerobically; should *Proteus* be present it tends to spread all over the plate, making impossible the isolation and recognition of other organisms this may be obviated by using 6 per cent. agar; other methods are referred to on p. 355; (b) blood-agar plate to be incubated anaerobically; the surface should be well dried before inoculation to prevent spreading of colonies of certain anaerobes; (c) plate of MacConkey's medium; (d) 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.); (e) litmus milk, in a long narrow tube, previously boiled and then cooled;

after inoculation a layer of melted vaseline is superimposed on the milk.

After eighteen to twenty-four hours' incubation the aerobic blood-agar culture is examined and the various types of colonies scrutinised with the naked eye, a hand-lens or plate-culture microscope, and films may also be made and stained by Gram's method. If necessary, the incubation may be continued to allow colonies to become more characteristic. In this way, for example, staphylococci and streptococci can be recognised and also the cultural types of these. Pure cultures can be obtained by subculture from isolated colonies, and staphylococci can be tested for the coagulase reaction, while streptococci can be examined, if necessary, for haemolysin production and serological characters. Various other aerobic organisms can also be identified from the blood-agar plate by appropriate methods.

The plate of MacConkey's medium is useful for the recognition at sight of lactose-fermenting coliform bacilli and the enterococcus, and also for ascertaining the relative numbers of coliform bacilli, and *Proteus* which forms pale colonies on this medium without spreading. Colonies of *Staphylococcus aureus* are easily identified on MacConkey's medium by their characteristic colour—the usual yellow pigment being tinted pink by the acid change of the neutral red.

The anaerobic plate is examined after twenty-four and forty-eight hours' incubation. It must be remembered that this plate yields growths of various aerobes (and 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. The difficulties of obtaining pure cultures of these organisms have been referred to on p. 505, and the identification of the more important species is detailed earlier in this chapter. A Table of differential characters is given on pp. 516 and 517.

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
							Saccharose Maltose Sucrose Lactose Glucose
<i>Clostridium welchii</i>	Large, thick, often rectangular bacilli; spores usually absent	Large, circular, with regular outline; haemolytic	Gas, no digestion, 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 welchii</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>Clostridium perfringens</i>	Slender bacilli with round terminal spores	Transparent, with long, fatty spreading projections; usually haemolytic	Slight digestion, blackening and putrefactive odour	Unaltered	(but may be softened)	—	—

<i>Cl. tertium</i>	Long, slender bacilli, with oval terminal spores	Small, transparent with regular outline; non-haemolytic	Gas, no digestion	Acid, gas, slow clotting	-	+	+	+	+	-
<i>Cl. fallax</i>	Resembles <i>C. welchii</i>	Large, opaque, irregular	Gas, no digestion, meat reddened	Acid, gas, slow clotting	-	+	+	+	+	+ when first isolated
<i>Cl. sporogenes</i>	Somewhat slender bacilli; central or subterminal spores	" Medusa-head " formation, or irregular, with feathery projections; haemolytic	Gas, digestion blackening and putrefactive odour	Acid, clot, digestion, later alkaline	+	+	-	+	(+)	-
<i>Cl. histolyticum</i>	Resembles sporogones	Small, transparent, circular or irregular; non-haemolytic	Digestion, blackening and putrefactive odour; deposit of tyrosine crystals	Clot and digestion	+	(+)	-	(+)	-	Usually +
<i>Cl. tetanomorphum</i>	Resembles <i>C. tertium</i> ; round terminal spores	Small and transparent, with irregular outline	Gas; no digestion; no putrefactive odour	Unaltered	- (but may be softened)	+	-	-	-	-

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* and *Cl. fallax*, 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.

§ Under fermentation signifies acid production without gas.

Note.—*Cl. tertium* and *Cl. histolyticum* are not strict anaerobes.

Double symbols in brackets signify variability in reaction among strains.

The litmus milk culture is intended for the rapid detection of *Cl. welchii* by the "stormy-clot" reaction; but all strains do not give the reaction (*vide infra*—Nagler's reaction).

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. This medium is specially valuable for anaerobes which grow slowly in culture and for the detection of small numbers of streptococci. Film preparations also yield further information as to the morphological types of organisms growing in it. For isolating the organisms present, subcultures are made on aerobic and anaerobic blood agar plates, and these are studied in the usual way.

Additional Methods.

If anaerobic jars are not available, 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 have recently been 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. 214), and these may be adopted advantageously in routine work with the anaerobic organisms.

Nagler's reaction.—This depends on the demonstration of the lecithinase activity of the α toxin of *Cl. welchii* in human serum with the formation of a visible precipitate. The effect is specifically neutralised by *Cl. welchii* antitoxin. In the test, equal parts of Fildes' peptic digest broth (p. 190) 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 0·08 ml. of a standard *Cl. welchii* antitoxin has been added. Both tubes are then inoculated with a drop of a 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 the Medical Research Council Memorandum cited, p. 513. 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, 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.

CLOSTRIDIUM BOTULINUM

(*BACILLUS BOTULINUS*)

The organism of Botulism, a fatal form of food poisoning characterised by pronounced toxic effects mainly on the parasympathetic 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 in South Africa, "Limberneck" of fowls. Three main types of *Cl. botulinum* have been differentiated and designated A, B and C, and two further types, D and E, are now recognised.

Morphology and Staining.—A sporing bacillus with rounded ends, about 4–6 μ by 0·9–1·2 μ , 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°–30° C.; more recent observations indicate that the optimum is about 35° C. Grows on ordinary media; meat medium yields abundant growths.

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—type A and some strains of B produce slow liquefaction; other strains of type B, types C and D do not liquefy serum.

Milk—type A and some strains of B precipitate and digest the casein ; other strains of B, types C and D are inactive in this respect.

Cooked meat—type A and some strains of B digest and blacken the meat ; other strains of B, types C and D do not digest meat. (Thus, as on coagulated serum and in milk, some strains of type B are proteolytic, others non-proteolytic.)

Type E like C and D is non-proteolytic.

Ferments glucose and maltose—type A also ferments salicin and glycerol, type B ferments glycerol but not salicin, type C does not act on either of these substances.

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 90° C. for forty minutes.

The toxin of Type A (*vide infra*) 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 0.000,000,033 mgm.

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.

Types A, B and E are associated with botulism in the human subject.

Occurrence.—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 foods responsible for botulism frequently exhibit signs of spoilage.

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.

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 canning foods is an important factor in the causation of this form of poisoning.

In cases of botulism the bacillus may 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.

Experimental Inoculation.—Laboratory animals are susceptible to experimental inoculation and feeding with cultures. The resulting condition resembles in its symptomatology the human disease ; at autopsy, marked congestion of the internal organs, extensive thrombosis and haemorrhages are noted.

Antitoxin can be prepared by immunising animals with toxin preparations, and is used therapeutically. In general, a bivalent serum containing antitoxins to the A and B types of toxin is employed.

Animal Botulism.—The disease occurs in such animals as horses, cattle, sheep and poultry, due to feeding on material in which the organism has been growing. Type C_a is responsible for a paralytic disease of chickens and botulism of ducks. Type C_b 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.

BACTERIOLOGICAL INVESTIGATION OF BOTULISM

As the condition of botulism is essentially a food intoxication, the suspected food calls for investigation.

Gram-stained films may first be examined to ascertain whether sporing bacilli or spores are present. The food is then macerated in sterile salt solution, heated at 65° C. for half an hour to eliminate non-sporing bacteria, and then cultures are made under anaerobic conditions, *e.g.* in meat medium. A culture-filtrate is obtained and its toxicity tested by injection of guinea-pigs or mice. For control purposes, the filtrate is also injected into animals along with

the different type-antitoxins. *Cl. botulinum* can be isolated in pure culture by appropriate plating methods, and identified by its biological characters and its toxicity.

An extract should also be made from the food, sterilised by filtration, and injected subcutaneously into guinea-pigs.

CLOSTRIDIUM CHAUVOEI

(*Bacillus chauvoei*)

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 the *Cl. septicum* and is $3\text{--}5\mu$ in length and $0\cdot5\text{--}0\cdot6\mu$ broad. 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.

Staining.—Gram-positive in young cultures but older forms may be Gram-negative. Stains readily with ordinary dyes.

Cultural Characters.—Strict anaerobe; optimum temperature, 37° C. ; but grows at room temperature. Grows on ordinary medium but a blood or meat medium is preferable.

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.

Coagulated serum is not liquefied.

Meat medium—meat is reddened and is not digested.

Ferments glucose, lactose, sucrose, maltose, but not mannitol, salicin or inulin.

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. 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.

Immunity.—Various methods of prophylactic vaccination have been practised: e.g. the inoculation of a dried powder of the muscles of animals dead of the natural disease, the powder being subjected to a suitable temperature to ensure the attenuation of the virulence of the contained spores; or a mixture of this preparation with specific antitoxin; aggressin (p. 38) has likewise been used for immunisation; a formolised culture of *Cl. chauvoei* in broth has also been applied with successful results. An antitoxic serum has been used for therapeutic purposes.

Cl. septicum may also be responsible for a condition similar to black-leg.

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.

CHAPTER XIX

THE ACTINOMYCETES AND ACTINOBACILLUS;

ERYSIPEROLOTHRIX RHUSIOPATHIAE; ACTINOMYCES
MURIS; FUSOBACTERIUM; ACTINOMYCES NECROPHO-
RUS; BACTEROIDES; DIALISTER PNEUMOSINTES;
ASTEROCOCCUS MYCOIDES

ACTINOMYCES

THE causative organism of Actinomycosis in animals and man.

This organism belongs to the genus which in the older classification and nomenclature was called *Streptothrix* and was placed among the Higher Bacteria (p. 5). The term *Actinomyces* is now applied as a generic name (p. 32).

Morphology.—It tends to grow in the tissues in colonial form as a mycelium or felted mass of branching filaments which are comparatively slender ($0\cdot8\mu$ - 1μ 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 filaments 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 (p. 227) 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, 1 per cent. being substituted for 20 per cent. sulphuric acid.

Cultural Characters.—Two main cultural types have been recognised :—

(1) The “anaerobic” or micro-aerophilic type, first described by Israel and Wolff, which is the prevalent form in animal and human lesions.

(2) The aerobic type, exemplified by Boström's classical strain ; it has been suggested that this type is not pathogenic and that its occurrence in lesions represents a secondary contamination ; similar organisms are common saprophytes in soil, on grain and grasses, and may be found in the mouth and alimentary tract of animals and also man.

*Anaerobic or Micro-aerophilic Type (*Actinomyces israelii*).*—The optimum temperature is 37° C., and growth does not occur at temperatures much below the optimum. The organism requires a reduced oxygen tension for its growth on culture medium, and increased carbon dioxide concentration also favours growth. Blood-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.

Strains from animals (*Actinomyces bovis*) may yield softer and smoother colonies with a more regular outline. Animal strains may also be more oxygen-tolerant than human strains.

A shake culture in a tube of 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.

If the organism is grown in the form of a stab culture in agar, growth is also at an optimum in a similar zone.

It should be noted that the cultural appearances of this type of organism 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.

As compared with the aerobic actinomycetes, this type is less active chemically : it is non-proteolytic, non-haemolytic and does not produce pigment. Saccharolytic action, however, can be demonstrated, various sugars being fermented (without gas).

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.

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, D., *Med. Res. Council Spec. Rep.* No. 240, 1940.

Aerobic Type.—The aerobic mycelial organisms described even in typical cases of actinomycosis have been somewhat heterogeneous in biological characters and probably represent different species.¹ Only one of these will be described here, viz. the classical actinomycetes of Boström.

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

¹ See Erikson, D., *Med. Res. Council Spec. Rep.* No. 203, 1935.

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 the 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.

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.

Chemotherapy.—*Actinomyces* is susceptible to sulphonamides and penicillin and these substances have been used in the treatment of actinomycosis. The organism is likewise susceptible to aureomycin and terramycin.

Actinobacillus actinomyctemcomitans.—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, but its actual relationship to *Actinomyces* is unknown. Experimental inoculation does not produce any specific lesions.

DIAGNOSIS OF ACTINOMYCOSIS

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 granules are allowed to sediment or deposited by centrifuging and then removed with a pipette; this is repeated two or three times so that the granules are thoroughly washed. This procedure is particularly necessary when there is mixed infection. Treatment of the granules with absolute alcohol for two minutes before transferring them to medium has been recommended to facilitate the isolation of the organism by destroying any associated pyogenic cocci. Two blood-agar plates are inoculated with the separated granules. One is incubated aerobically, the other anaerobically.

ACTINOBACILLUS LIGNIERESI

This organism has been cultivated from a large proportion of cases of bovine actinomycosis. To such cases the term "actinobacillosis" has been 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.

Actinobacillus actinoides

This organism has been isolated from a pneumonic condition in calves, and presents some similarities to *Actinobacillus lignieresii*. In the tissues it appears as a slender Gram-negative bacillus. Cultures have been obtained by placing a piece of infected lung tissue in the condensation fluid of a tube of coagulated serum and then sealing the tube, the growth occurring after several days as small white flocculi in the fluid. Microscopical examination of such cultures has revealed filamentous and club-shaped forms. The organism has also been cultivated on the surface of agar containing a piece of sterile guinea-pig spleen and with calf serum added, the colonies being small, coherent and slightly yellowish in colour.

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* in the newer system of classification and nomenclature. Of course, 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 FARCIHICA

(*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. It is Gram-positive and tends to be acid-fast. It grows aerobically at 37° C. on ordinary media, producing raised irregular greyish-white colonies after two to three weeks.

Guinea-pigs are susceptible to experimental inoculation and develop nodular or tubercle-like lesions. In cattle, subcutaneous injection leads to a localised abscess which breaks through the skin and produces a chronic ulcerated 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.

The *black granules* in mycetoma represent true fungi, which have been designated under the generic name of *Madurella*.

The colonies consist of a mycelium of branching, septate hyphae $3\text{--}8\mu$ in breadth, and contain a considerable amount of black pigment.

This type of organism can be cultivated on nutrient agar, and old cultures show the black pigmentation.

A number of different varieties have been described and this group has been regarded as related to the genus *Aspergillus* (p. 633).

LEPTOTHRIX

This term has been generally given to organisms resembling the Actinomycetes but showing absence of 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 *Leptothrix buccalis*. Pathogenic properties have been claimed for it, but its invasive power is probably slight.

Leptothrix types have also been reported in suppurative lesions in the region of the mouth and throat.

ERYSYPELOTHRIX RHUSIOPATHIAE

(*Bacillus rhusiopathiae*)

The causative organism of Swine Erysipelas.

Morphology and Staining.—Slender, Gram-positive, non-motile rod-shaped organism $1\text{--}2\mu$ by $0.2\text{--}0.4\mu$, occurring singly and in chains. In culture media, longer and filamentous forms are observed. True branching has been described.

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.

Experimental Inoculation.—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.—The bacilli can be observed in the characteristic skin lesions, and in internal organs, e.g. lungs, spleen and kidney. In some cases there is a marked 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.

Ery. rhusiopathiae may occur in apparently healthy pigs, and has been isolated from the tonsils, intestines and faeces.

Artificial immunisation against the disease has been carried out by the injection of immune serum immediately followed by injection of a virulent broth culture. Immune serum is also used for therapeutic purposes.

Cases of *human infection* ("erysipeloid") by this organism have been recorded. This is usually contracted through abrasions of the skin when infected carcasses are handled, e.g. by abattoir workers. The lesion is situated on the hand or forearm.

A similar organism, *Erysipelothrix muriseptica*, is responsible for epizootic septicaemia in mice. It is doubtful whether this organism constitutes a separate species, and it would appear that *Ery. rhusiopathiae* has a wide distribution among animals, including birds.

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.

In the diagnosis of human cases, Smeath, *et al.*,¹ have successfully used a method of producing a weal with 1 per cent. procaine at the edge of the lesion, excising a whole-thickness fragment of skin and cultivating aerobically in glucose-broth.

ACTINOMYCES MURIS

(*Streptobacillus moniliformis*)

This organism was reported in France in cases diagnosed as erythema multiforme, and named *Streptobacillus moniliformis*; it was also described in America in a group of cases of multiple arthritis associated with an erythematous eruption (Haverhill Fever—Erythema arthriticum epidemicum). It has been found associated with a disease of mice characterised by multiple arthritis often involving the joints of the feet and leading to swellings of the feet and legs. The organism

¹ *Brit. Med. J.*, 1951, 2, 1063.

may occur in the naso-pharynx of rats, and some cases of "rat-bite fever" are due to it. Certain writers have named it *Actinomyces muris*, though its relationship to the Actinomycetes is doubtful; the generic name *Streptobacillus* is inappropriate, and pending the clarification of its taxonomy the provisional name *Actinomyces muris* is used here.

The organism is a Gram-negative, pleomorphic bacterium, occurring as short rod-shaped forms ($1\text{--}3\mu$ 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. 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.

Branching of the filaments has been described by some writers, but it is questionable if true branching occurs.

Reference is made on p. 536 to the occurrence of forms similar to those of pleuro-pneumonia in cultures of *Actinomyces muris*.

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.

In the human infection the organism has been isolated by blood culture, and from joint fluid in cases with arthritis.

FUSOBACTERIUM PLAUTI-VINCENTI

(*Bacillus fusiformis*; *Fusiformis fusiformis*)

This organism is referred to on p. 545 as a concomitant of a spirochaete in Vincent's angina, and is found in various necrotic 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, $5\text{--}14\mu$ by 1μ .

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.

ACTINOMYCES NECROPHORUS

(*Bacillus necrophorus*; *Fusiformis necrophorus*)

The taxonomy of this organism is still doubtful and its designation here as in *Actinomyces* is provisional. 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, liver abscesses in various domesticated animals.

The organism may appear in the form of elongated slender filaments varying in length and attaining sometimes to 50 or even 100μ . 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.

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.

This type of organism has occasionally been isolated from suppurative lesions in the human subject. It may occur in septic wounds associated with sloughing of the tissue. *Actino. necrophorus* has been found in the mouth, intestine and female genital tract of healthy persons. Strains of human origin appear to be only slightly pathogenic to animals.

BACTEROIDES

Organisms of this genus (Gram-negative, non-sporing, motile or non-motile anaerobic bacilli) may occur in the intestine of mammals 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.

It should be noted that *Bacteroides fragilis* has also been classified by some writers along with the organisms described above as *Fusobacterium* (p. 533). The taxonomy and relationships of these organisms require further study.

DIALISTER PNEUMOSINTES

(*Bacterium pneumosintes*)

This organism was first isolated from the naso-pharynx of cases of epidemic influenza. Its taxonomy is doubtful ; it may have relationships to the organisms classified above as *Bacteroides*, but has been placed by some writers in a separate genus named *Dialister*.

Morphology and Staining.—A minute cocco-bacillary organism measuring 0.15–0.3 μ in length. After prolonged artificial culture, its length may attain 0.5–1 μ . The organisms are usually found singly, but may be in pairs, and even in short chains of three or four individuals. *Dialister pneumosintes* is stained best with well-ripened polychrome methylene blue, when the organism appears deep purple in colour. It is Gram-negative.

In young cultures a certain proportion of the organisms are small enough to pass Berkefeld V and N filters.

Cultural Characters.—Obligatory anaerobe. Optimum temperature, 37° C. Grows well in Smith-Noguchi medium (p. 195), forming a haze in the medium around the fragment of kidney in three to four days. On anaerobic blood-agar plates minute transparent colonies are formed after about six days' incubation.

Occurrence.—Was originally found in the nasal washings of epidemic influenza in the first thirty-six hours of the disease. Similar organisms have been observed in the throats of healthy persons and in other pathological conditions of the respiratory passages.

It is doubtful whether this organism has any pathological relationships, and it is practically non-pathogenic to animals on experimental inoculation.

ASTEROCOCCUS MYCOIDES

This is the organism of Bovine Pleuro-pneumonia. It was originally classified with the filterable viruses in view of its ability to pass the coarser filters ; it is, however, within the range of microscopic visibility and can be cultivated readily on artificial medium. The biological designation given above must be regarded as only a provisional one.

The morphology, which can be studied in impression preparations from cultures (p. 118), 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 cocco-bacillary, not exceeding 0.4 μ 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 Smiles 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 (p. 638), 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.

Cultures can readily be obtained aerobically at 37° C. in serum-broth or on serum-agar (10 per cent. of horse or ox serum). 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. Cultures remain viable for several weeks. Cultivation leads to attenuation of virulence. The organism is killed within one hour by heating at 58° C.

Subcutaneous inoculation of cattle with exudate from the disease, or with a virulent culture leads to a local inflammatory lesion, and in some cases a fatal result, with an inflammatory condition in the interstitial tissue of the lungs and a pleuritic exudate. The disease, as seen naturally, has also been reproduced by intravenous inoculation. Laboratory animals resist experimental inoculation.

Cattle can be immunised against the disease by inoculating infective material or cultures into the point of the tail : injection in this situation produces a non-fatal infection which is followed by immunity for at least a year. The serum of immune animals contains protective antibodies.

Morphological forms similar to those of the pleuro-pneumonia organism have been found as a supposed symbiont in cultures of *Actinomyces muris*, but these are probably derivatives of *Actino. muris*, and do not represent an independent organismal entity (p. 532). Similar forms have been observed also in cultures of other bacteria.

An organism similar to that of pleuro-pneumonia 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 genital tract of male and female subjects (urethra, vagina, cervix uteri), usually in cases with non-specific inflammatory conditions, gonorrhoea or *Trichomonas* infection (p. 461).

Filter-passing organisms isolated by Laidlaw and Elford from sewage are similar to the pleuro-pneumonia 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 pleuro-pneumonia. It can be found in arthritic fluid. The organism can pass the coarser earthenware filters. It can 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.

Donovania granulomatis

This organism, whose biological relationships are still doubtful, is responsible for a chronic granulomatous disease ("granuloma venerum") 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 2μ 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.

(It should be noted that this infection is quite different from lymphogranuloma inguinale—p. 604—and should not be confused with the latter disease.)

CHAPTER XX

THE PATHOGENIC AND COMMENSAL SPIROCHAETES

TREPONEMA PALLIDUM

THE causative organism of Syphilis.

Morphology.—An exceedingly delicate, spiral filament 6–14 μ (average 10 μ) by 0·2 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 (p. 71).

Tr. pallidum was originally described as having terminal flagella. Later it was supposed that the organism was devoid of flagella and the tapering ends were referred to as "terminal filaments." In specially stained preparations terminal flagella have been demonstrated by some observers, and, by electron microscopy, flagella-like structures have also been observed situated at the sides or ends of the organism ; whether these are true flagella remains doubtful.

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. Its progression is relatively slow as compared with many of the motile bacteria.

Multiplies by transverse binary fission.

Division into four and even smaller 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. For the demonstration of this organism in films, Fontana's silver impregnation method (p. 113) is one of the best available in routine work. In tissues, the spirochaetes can be stained by Levaditi's silver impregnation method (p. 114).

Cultivation.—According to the claims of Noguchi and others, *Tr. pallidum* has been grown anaerobically in the Smith-Noguchi medium (p. 195), but most workers have failed to cultivate the organism. Noguchi's technique of isolating pure cultures was as follows. Deep tubes of 2 parts agar and 1 part ascitic fluid were prepared, with a piece of sterile tissue added (e.g. rabbit kidney). The medium was covered with a layer of sterile vaseline. The tubes were inoculated from exudate with a capillary pipette (stab inoculation). Both the spirochaetes and the bacteria present in the material grew along the line of inoculation, but later the spirochaetes spread out from the stab and formed a haze in the medium. The tube was cut, and transplants were made in Smith-Noguchi medium, without carrying over any of the bacterial growth.

Viability apart from 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 easily killed by the usual antiseptics and by heat (even at 41.5° C. in an hour).

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.

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 buboes.

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 gummata, 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.

Spirochaetes have likewise been demonstrated in the placenta.

Experimental Inoculation.—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.

SEROLOGICAL REACTIONS OF SYPHILIS

Wassermann Reaction.—Reference has been made to this manifestation of syphilis on p. 254 *et seq.*, and the technique has been described.

The reaction appears in about two to four weeks after the onset of the primary lesion, but its development may sometimes be delayed. In secondary syphilis the reaction is usually well marked and very constantly present. It is less frequently positive in the latent stage (25–50 per cent. of cases) and in tertiary cases (about 75 per cent.). In general paralysis and in many cases of locomotor ataxia, the reaction is positive both when tested with blood and with cerebro-spinal fluid. In cerebro-spinal syphilis the spinal fluid may react positively even when the blood yields a negative reaction. Active cases of congenital syphilis usually exhibit a strongly positive reaction.

As the result of antisyphilitic treatment the Wassermann reaction may become negative, but often temporarily, and the reaction re-appears when treatment is stopped. The test serves as a gauge of the effectiveness of treatment.

The *Flocculation Reaction* and certain techniques for carrying it out are dealt with on p. 267 *et seq.* It follows very closely the Wassermann reaction in its occurrence, and corresponds quantitatively with the latter. The two reactions, however, are not in all cases parallel in their occurrence and degree.

Reference is made later to the occurrence of "non-specific" or "false positive" reactions; the *Kahn verification test*, described on p. 271, is frequently of value in differentiating these "non-specific" reactions from the true syphilitic effect, the former being often most pronounced at low temperatures, the latter at 37° C.

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, Fontana's staining method can be used.

Tr. pallidum is recognised by its special morphological features, 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 frequently 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. Another method of obtaining exudate is to apply alcohol to the sore for a minute, and allow the surface to dry; this leads to an exudation of serous fluid, which is collected and examined.

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 buboes by means of a syringe.

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.

In dealing with cases of congenital syphilis in young infants, the mother's blood should be tested if there is any difficulty in obtaining a specimen of blood from the child.

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. 271, 541.

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. 543, 544) 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, 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. 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 bring about positive reactions. Blood donation has also been reported as responsible for such reactions in the donor. Various other conditions have likewise been reported by certain writers 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, it 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.

TREPONEMA PERTENUE

The causative organism of Framboesia or Yaws, a tropical disease pathologically and clinically resembling syphilis, though differing in its contagious and non-venereal character.

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, 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*.

Bejel, a disease of Arabia, is another non-venerel disease which is either related to syphilis or is a modified form of that disease.

Treponema herrejoni.—This organism, which is indistinguishable morphologically from *Tr. pallidum*, is associated with a skin disease of Mexico, Colombia, 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 disease has often been regarded as due to fungi found in the lesions. 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.

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. 541). 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 minutum, 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 ($8-10\mu$), 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*.

BORRELIA REFRINGENS AND RELATED SPIROCHAETES

These are large, motile, refractile spirochaetes (about $10\text{--}30\mu$ by $0.5\text{--}0.75\mu$) with irregular wide and open coils, which are relatively few in number. They are easily stained by the ordinary methods, and are Gram-negative. They 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. A spirochaete of this form occurring in the mouth has frequently been designated by the specific name *buccalis*. 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*.

Borrelia vincenti occurs in a pseudo-membranous condition of the throat—Vincent's angina. It resembles *Borr. refringens*, but is sometimes described as smaller ($5\text{--}25\mu$) and more delicate.

By electron microscopy, terminal filaments and lateral flagella-like processes have been recorded. Granular structures in the spirochaete or situated terminally have also been observed, and certain appearances suggest that these are shed as free structures and from them developmental forms of the spirochaete are derived (*cf. Tr. pallidum*).

Borr. vincenti is generally associated with a large fusiform bacillus—*Fusobacterium plauti-vincenti* (p. 533). 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 *Fuso-*

bacterium occurs in balanitis gangrenosa, ulcerative stomatitis and gingivitis, and a chronic ulceration of the skin of tropical countries (*ulcus tropicum*).

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.

Borr. vincenti is highly susceptible to penicillin, and this antibiotic may be used locally in the treatment of Vincent's infection.

THE SPIROCHAETES OF RELAPSING FEVER

BORRELIA OBERMEIERI or RECURRENTIS

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 μ broad, with about five to seven fairly regular coils 2-3 μ long by 1 μ in amplitude. The structure of the organism is regarded as consisting of a spring-like axial filament covered by a layer of contractile protoplasm enclosed in a thin membrane or periplast. Active motility of a rotatory or oscillating type is noted in fresh preparations. Multiplication is by transverse fission.

This spirochaete stains readily with a Romanowsky stain (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 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. 113 *et seq.*).

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 superimposed 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. 204); 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 number 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 re-appear 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.

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 some stage 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. 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. obermeieri*.

Other Relapsing Fever Spirochaetes.—The originally described spirochaete of North American relapsing fever resembles *Borr. obermeieri*, 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. obermeieri*. 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. 106, 641).

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 illumination.

If spirochaetes are not detectable, inoculation of white mice with 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.

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 GALLINARUM

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.

LEPTOSPIRA ICTEROHAEMORRHAGIAE

The causative organism of Infectious Jaundice (Weil's Disease).

Morphology and Staining.—A leptospira (p. 14) about 7–14 μ long by 0·2 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. The spirochaete has a fine, elastic, axial filament and the protoplasm is disposed spirally round this central structure. In culture the organism tends to be longer than in the tissues. Active movement is observed in fresh preparations examined with the dark-ground microscope. The movement is mainly rotatory.

The organisms can be stained by Giemsa's solution (as in the case of *Tr. pallidum*), but the silver impregnation methods of Levaditi and Fontana give the best results.

Cultivation.—*Lept. icterohaemorrhagiae* was first cultivated by Noguchi in a semisolid blood agar (p. 196), and was found to grow just below the surface, *i.e.* like a micro-aerophilic organism. Fletcher's, Stuart's and Korthof's media serve very well for the routine cultivation of this spirochaete (p. 196 *et seq.*). The optimum temperature is from 25° to 30° C., but growth may also occur at 37° C.

Occurrence.—The organisms are present in the blood during the first four to six days of the illness, and though scanty, have occasionally been demonstrated microscopically in blood films. Later they disappear from the blood. They are present in the liver often in considerable numbers, and, particularly during the later stages of the disease, in the kidneys, when they can be detected in the urinary sediment. They have also been found in the cerebro-spinal fluid, after disappearance from the blood.

Lept. icterohaemorrhagiae occurs in wild rats, which act as reservoirs or carriers of the infection. In these animals the spirochaetes are present in the kidneys (being demonstrable microscopically in the tubules) and are excreted in the urine. In this way, soil, water, food, etc., are contaminated. Infection of the human subject may possibly occur sometimes through mucous membranes, *e.g.* alimentary tract, but it has been shown that the organisms can pass through the skin, possibly more readily when there are fissures or abrasions or when it has become sodden by continuous wetting. In Japan, epidemics of infectious jaundice were specially noted among workers in wet mines. Similarly, during the war of 1914-18, outbreaks occurred among troops in wet trenches. The infection may follow bathing in stagnant swimming-pools contaminated by rats, and immersion in canal or river water

has also been the cause of the disease. In this country the disease has affected miners working in wet mines, sewer workers, fish curers, etc. Transmission from the urine of rats harbouring the leptospira is undoubtedly an essential factor in the spread of the disease, and invasion occurs through skin abrasions or even the intact skin, as has been indicated by animal experiments.

Saprophytic leptospirae similar in morphology to *Lept. icterohaemorrhagiae* have been observed in water (e.g. *Lept. biflexa*), and the question has arisen whether these organisms are potentially pathogenic. Infectious jaundice has been produced in experimental animals by inoculation with apparently saprophytic leptospirae, but generally such organisms have proved non-pathogenic.

Lept. icterohaemorrhagiae infects dogs, producing the condition known as "Yellows" which is pathologically similar to human infectious jaundice. The infection has also been observed in foxes. It has been reported in young pigs.

Antibiotic therapy.—*Lept. icterohaemorrhagiae* has been regarded as sensitive to penicillin, and this antibiotic has been used in the treatment of infectious jaundice; its therapeutic efficacy is doubtful. Some observers have claimed successful results with aureomycin and terramycin.

DIAGNOSIS OF LEPTOSPIRAL JAUNDICE

During the first six days of the disease 10 ml. of blood are withdrawn by vein puncture, citrated (p. 321) and injected intraperitoneally into young guinea-pigs. In typical cases the inoculation produces death of the animals in eight to twelve days with marked jaundice and with haemorrhages in the lungs, under the serous membranes and in the muscles. The leptospirae are present in large numbers in the liver and kidneys, and can also be found in various other organs and in the blood. The methods of demonstration in the stained and unstained conditions have been referred to above.

Sometimes individual guinea-pigs, particularly older animals, are somewhat resistant to infection and do not show obvious illness; it is advisable therefore to take the animals' temperature daily and, if found elevated, to withdraw some peritoneal fluid by aspiration with a syringe; this is examined microscopically and injected into another animal. The blood may also be examined for spirochaetes.

In early cases the organism may occasionally be demonstrated microscopically in the blood by dark-ground illumination, and may also be cultivated directly from

the blood in one of the media referred to above: several tubes of medium should be inoculated with varying quantities of blood from 0·1 to 1 ml., and the tubes are incubated for one to three weeks at 25°–30° C.

If a case is met with only at a later stage, e.g. after ten days from the onset, the urine (about 100 ml.) is centrifuged and the sediment examined by the dark-ground microscope. Guinea-pigs should also be inoculated intraperitoneally with the centrifuged deposit.

The organism is present in the urine by the twentieth day in nearly every case; it may persist till the fortieth.

In the urine the organism often presents an atypical appearance and cannot be identified readily as a leptospira; this has been attributed to the bile or acid in the urine, which tends to kill the spirochaete. When examining urine, a neutral reaction should be ensured by immediately adjusting the reaction of the specimen with alkali, and when an animal is inoculated, this should be done within one hour after the specimen has been passed. Inoculation of animals with urinary sediment may not produce the characteristic disease; jaundice may be absent, though lung haemorrhages are a more constant feature. In such animals the leptospirae may be relatively scanty in the tissues.

It may be noted here that the elementary coils of the dying organism may become unwound or opened out, so that it presents an appearance more like that of a treponema.

Serological Diagnosis.—Leptospiral infection not infrequently occurs without the typical signs and symptoms, and a diagnosis can only be made by laboratory methods. In such cases, serum reactions have proved most valuable for diagnostic purposes. Antibodies, including agglutinins, appear in the serum of infected persons after about seven days from the onset of the disease, and progressively increase in amount.

Three methods are described below for carrying out the diagnostic agglutination test with patient's serum. One is a microscopic technique which has been much used for the purpose and has proved very satisfactory. The second is a macroscopic technique which is preferred by some bacteriologists to the microscopic method. The third is a procedure which has been adopted as a standard microscopic method in many laboratories in Great Britain.

(1) *Microscopic Agglutination Test.*—Young living cultures in a fluid serum medium (e.g. Fletcher's, Stuart's or Korthof's, p. 196 *et seq.*), grown for four days at 30° C. and then for three days at room temperature, may be used for the test. Alternatively, a formalised culture has been used, but only pure formalin (analytical reagent) should be added,

and its concentration must not exceed 0·5 per cent. Otherwise, formic acid is liberated in the preparation and causes acid-agglutination. The formolised culture can be stored, and if kept in bottles covered with black paper, and in the dark, may remain satisfactory for some time.

A series of doubling dilutions of serum is prepared, e.g. from 1 in 5 to 1 in 640 or higher (p. 245); an equal volume of the culture is added to each, and the mixtures are incubated for three hours at 37° C. A loopful is then taken from each and placed on a slide, and the drops are examined (for agglutination of the spirochaetes) by dark-ground illumination with a dry dark-ground condenser and first a $\frac{2}{3}$ -in. and then a $\frac{1}{4}$ -in. objective. When young living cultures are used for the test, and if the serum reacts positively, agglutination is observed in the lower dilutions and lysis in the higher; with formolised cultures lysis is absent.

With living cultures a titre of 1 in 30 has been considered significant, but the titre of the reaction may rise to 1 in 1000 or over. A control must be included to ensure that any agglutination observed is not occurring independently of serum.

As there may be some degree of serological difference among strains of the leptospira, it has been recommended by some workers that a polyvalent agglutinable antigen (prepared from multiple strains) should be used.

(2) *Macroscopic Agglutination Test*.—A four to seven days' culture (as above) is used as the spirochaetal suspension. This culture may contain a certain amount of sedimented material and therefore the supernatant fluid should be pipetted off, taking care not to disturb the sediment on removal of the culture bottle from the incubator. To obtain satisfactory results the culture should be of such density as to show, by dark-ground illumination with a $\frac{1}{4}$ -in. lens, not less than 40 leptospirae per microscopic field. The general technique of the test is similar to that of other agglutination tests (p. 243). Doubling dilutions of the serum are prepared ranging from 1 in 2 to 1 in 64 or higher, and to 0·2 ml. of each of these is added 0·6 ml. of the culture, the final dilutions ranging from 1 in 8 to 1 in 256 or higher. The mixtures are incubated for two to four hours at 37° C. when readings are made, and the tubes are also allowed to stand overnight for later readings. Visible agglutination in a 1 in 32 dilution may be significant at an early stage of the illness; but on repeated testing a rising titre is observed reaching even 1 in 1000 or over, and this provides conclusive evidence of infection. (It has been pointed out that when formolised cultures are used for this test, low-titre reactions, e.g. under 1 in 400, must be regarded as of only doubtful significance.)

(3) *Microscopic Agglutination Test* (Broom).—Cultures are grown in Korthof's medium, a large inoculum, at least $\frac{1}{10}$ volume, being used, and incubated at 25°–30° C. preferably 26° C. for 5–7 days. Add pure formalin to give a final concentration of 0·2 per cent. Some workers use living cultures, but with these lysis may occur as well as agglutina-

tion. Formolised cultures are preferable as being more convenient, equally sensitive and showing agglutination only. If kept in bottles covered with black paper, and in the dark, they remain satisfactory for several months.

The technique of the test, with the procedure for making dilutions by the dropping method (p. 247), 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 (formolised)	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

Keep tubes in the refrigerator (4° C.) overnight for interaction to take place. Then place a drop from each tube on a large slide and examine with a 16 mm. (10 \times) objective, using a black centre-stop and an "intense" source of light (p. 69) to give dark-ground illumination. Agglutination of the leptospirae is easily observed.

The titre for confirmation of the diagnosis is 1/1000, but 1/300 may be significant. A rising titre is highly significant.

A control must be included as in method (1).

Examination of Rats for Leptospiral Infection.—The most satisfactory method is to transmit the disease to guinea-pigs by injecting intraperitoneally an emulsion of the ground-up tissue of both kidneys, but for success it is essential that fresh material should be used. Carcasses of rats that have been dead for some time are unsatisfactory. Scrapings from the kidneys can also be examined microscopically by dark-ground illumination, and indirect evidence of infection can be obtained by the agglutination test with serum, titres of 1 in 100 being considered significant.

Examination of Water for Leptospirae.—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.

OTHER PATHOGENIC LEPTOSPIRAE

Various leptospiral infections, other than the typical infectious jaundice due to *Lept. icterohaemorrhagiae*, have now been described and defined, and a group of leptospires can be recognised, the individual members of which differ in pathogenicity to man and animals, serological characters, their natural hosts, etc. It must be noted that though differences among these organisms can be demonstrated by agglutination and agglutinin-absorption tests, they are related in antigenic composition; and it has been pointed out that the leptospires comprise a "mosaic" of antigens distributed in various combinations, so giving origin to the many serological types. The following exemplify the various types or species which have been recognised in addition to *Lept. icterohaemorrhagiae*.

Leptospira hebdomadis.—An organism described as the cause of "Seven-day fever" of the East. It is morphologically identical with *Lept. icterohaemorrhagiae*. In some cases it has been demonstrated in the peripheral blood, and at a later stage of the illness appears in the urine. It is pathogenic for young guinea-pigs, producing a febrile illness rarely associated with jaundice, and with less tendency to haemorrhages than other leptospiral infections. These animals may also recover from the infection. It is not virulent to rats or mice. This organism differs serologically from the other pathogenic leptospires. It is harboured by a field-mouse (*Microtus montebelloi*), and excreted in the urine, as in the case of rats infected by *Lept. icterohaemorrhagiae*. The mode of infection is probably similar to that in infectious jaundice.

Leptospira autumnalis.—This organism has been found associated with a disease in Japan called Akiyami, or harvest sickness, and clinically resembling a mild infectious jaundice. Cases may be confused with "seven-day fever," but *Lept. autumnalis* can be distinguished from *Lept. hebdomadis* by its high infectivity to guinea-pigs, in which it produces typical haemorrhagic jaundice. It can be differentiated from *Lept. icterohaemorrhagiae* by serological reactions. Field rats are reservoirs of this infection. The organism has also been found in the field-mouse, *Apodemus speciosus*.

Leptospira grippo-typhosa.—Has been described in "Swamp fever" of Eastern and Southern Europe, and certain parts of Asia. It can be differentiated serologically from *Lept. icterohaemorrhagiae* and is only weakly pathogenic to guinea-pigs, though infective to mice. The main animal reservoir is a field vole.

Leptospira canicola.—This organism, serologically distinct from *Lept. icterohaemorrhagiae*, has been described in infectious jaundice of dogs, and has also been found in human infections, transmitted from dogs. It does not occur in rats. It is of lesser pathogenicity to guinea-pigs than *Lept. icterohaemorrhagiae*, but is virulent to young hamsters (*Cricetus auratus*). Canine and human infections occur in Great Britain. The infection in man is comparatively benign and jaundice is only

manifest in a minority of cases. Symptoms of meningitis are common and nephritis is not infrequent.

Leptospira pyrogenes.—Has been reported in a febrile disease occurring in Sumatra, associated with some degree of jaundice and albuminuria. This organism is pathogenic to guinea-pigs, but its effects are irregular. It has been isolated by cultivation from the blood. *Lept. pyrogenes* has been found to be serologically distinct from other races of leptospira. Certain species of rats are reservoirs of the infection.

Leptospira australis A and *B*.—These organisms have been described as the causal agents of "Coastal fever" in North Queensland. (The latter is probably the same species as *Lept. pyrogenes*, *vide supra*.) The condition is of some severity, but jaundice only rarely occurs. Lymphadenitis is a common feature. The animal reservoirs are *Rattus culmorum* and *Rattus rattus*. The infection is readily transmitted to guinea-pigs by inoculation.

Leptospira pomona (which appears to be the same species as *Lept. australis A*) and *Leptospira mitis* have also been reported in Australia, where they produce a seven-day fever among farm workers who become infected from pigs and cattle. *Lept. pomona* likewise produces infections among pigs in certain parts of Europe, e.g. Switzerland, Italy, and this infection may be conveyed to persons working with these animals.

Leptospira sejroe was first observed in human infection in the island of Sejroe (Denmark). The disease is relatively mild. It has also been recorded in other parts of Europe. Certain rodents are carriers of the organism.

SPIRILLUM MINUS (SPIROCHAETA MORSUS MURIS)

A causative organism of Rat-bite fever. Though originally 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 $2\text{--}5\mu$ in length and relatively broad, with regular short coils numbering one for each micron of the length of the organism. Longer forms up to 10μ 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.

Successful cultivation has been claimed by certain workers using Smith-Noguchi or Shmamine medium. The latter is prepared by adding 0.5 gm. sodium nucleate to 160 ml. of sterile horse serum; carbon dioxide is passed through the mixture for a few minutes until the medium becomes transparent; it is then heated on three suc-

cessive days at 60° C., and on the fourth day at 65° C. for ten minutes, when it becomes semi-coagulated.

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 easily by dark-ground illumination. Guinea-pigs develop a progressive disease and die of the infection ; in mice the organisms gradually disappear from the blood without producing, as a rule, a lethal effect. 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 *Actinomyces muris* (*Streptobacillus moniliformis*), p. 532. 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. The infection is very amenable to treatment with organic arsenicals. Infection by *Actinomyces muris* 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.

Antibiotic therapy.—*Spirillum minus* infections respond to treatment with aureomycin, and this is the antibiotic of choice in the treatment of the infection, but penicillin has also been used successfully.

"Pseudospirochaetes" ↗

When dark-ground illumination is used for examining blood specimens for spirochaetes, certain filamentous structures may be seen which, unless critically studied, can readily be mistaken for spirochaetes, and particularly leptospirae—hence the term "pseudospirochaetes." They are found mostly in blood specimens which have been kept for some time and in blood *post mortem*. They also appear in large numbers in blood which has been heated at 37° C. or higher temperatures. These filaments appear to be derived from erythrocytes and may be seen attached to or projecting from these cells. In wet preparations they may show a waving movement which may be mistaken for

biological motility. They vary in size and thickness, some being very fine and slender, others thicker, and the latter type often presents a "beaded" appearance by dark-ground illumination which simulates that of a leptospira. Curvature in these filaments may also suggest the spirality of the spirochaetes and some may even exhibit recurvature at their ends like that of a leptospira.

The absence of true biological movement and axial rotation differentiate them from spirochaetes in the active state, and when films of the blood are dried, fixed and stained by a silver impregnation method they are either not demonstrable or if stained they fail to show the true and characteristic features of spirochaetes. (See Noguchi, H., *J. Amer. Med. Assoc.*, 1926, **86**, 1327.)

CHAPTER XXI

RICKETTSIA GROUP; BARTONELLA

THE generic name *Rickettsia* is applied to a group of organisms whose biological relationships are still undetermined. They are sometimes classified with the viruses and have also been regarded as intermediate between bacteria and viruses. These organisms flourish in the alimentary tract of certain blood-sucking arthropods (e.g. lice, fleas, mites, ticks and bugs), and several species have now been described. Some have no relationship to human or animal disease, but certain are pathogenic to the mammalian host of the ectoparasite in which they are found, and which thus acts as the vector of the infection. Thus, *Rickettsia prowazekii* constitutes the specific agent of classical typhus fever and is transmitted by lice which have fed on typhus cases.

While investigating the cause of typhus fever, Ricketts and Wilder found in the alimentary tract of infected lice minute diplococcal or rod-shaped structures, usually less than 1μ in their long diameter, which stained reddish or purple by Giemsa's method. These small bodies ("Rickettsia bodies") were also found in infected arthropods transmitting other diseases such as Trench fever and Rocky Mountain Spotted fever, and were regarded as the aetiological agents of these diseases. Similar bodies have been found in non-infective ectoparasites, but in relatively small numbers and infrequently. Such rickettsiae, e.g. *Rickettsia da rocha-limae* of lice, represent non-pathogenic species. In addition, similar organisms have been found almost constantly in the intestinal tract of the bed bug (*Cimex lectularius*) and sheep ked (*Melophagus ovinus*). The pathogenic rickettsiae, though they may be grown in tissue cultures and in the embryonated egg like the filterable viruses (p. 577), have not yet been cultivated on artificial media.

RICKETTSIA PROWAZEKII

This is the causative agent of the classical louse-borne Typhus fever of Europe and Asia. The disease is transmitted by *Pediculus humanus* (*corporis* and *capitis* varieties, mainly the former). A similar infection can be produced in monkeys by inoculation with blood from human cases. Infection can also be communicated to guinea-pigs by the intraperitoneal injection of patient's blood taken during the height of the illness. After an incubation period of about nine or ten days,

the animal's temperature rises to 105°–106° F. and remains above normal for four to eight days. The guinea-pig does not succumb to infection, and on recovery does not react to a second inoculation. If the animal is killed on the first or second day of the fever, the blood is infective for other guinea-pigs. At autopsy, the spleen is found to be enlarged and the peritoneum congested. The skin on histological examination shows the presence of an exanthematous reaction.

The infective agent is demonstrable (by experimental inoculation) in the blood, spleen, brain and other organs. It is easily destroyed by heat and antiseptics, but is resistant to drying. It is not filterable.

Rickettsia prowazekii can be demonstrated in the alimentary tract of lice which have fed on the blood of typhus patients. It appears in stained films as a small diplococcal or rod-shaped body, usually $0\cdot3\text{--}1\mu$, but is pleomorphic and may attain a length of $4\text{--}20\mu$. Motility is absent. It stains reddish purple with Giemsa's stain, but very feebly with ordinary stains, and is Gram-negative. It may be demonstrated, often in large numbers, in film preparations, stained by Giemsa's or Castaneda's method (pp. 108, 117), from the gut contents of lice seven to ten days after infection. The organisms multiply in the epithelial cells, which become distended with them and finally rupture. They are present in the excreta of the louse and infect the human subject through the bite-wounds or through scratches and other abrasions. The experimental disease referred to above has been produced by inoculation with the gut contents of infective lice. Rickettsiae are also demonstrable microscopically in the vascular endothelium of the skin, brain, etc., in the human disease and in the experimental infection. When infective material is inoculated into the anterior chamber of the eye of the rabbit or guinea-pig, the organisms produce an acute inflammatory condition, and are found in large numbers in the cells of Descemet's membrane. The organism is essentially an intracellular parasite.

Up to the present *R. prowazekii* has not been cultivated on artificial media, but multiplies in endothelial cells of a tissue culture and in the chorio-allantoic membrane of the embryo-chick (p. 577). Good growth has also been obtained in the yolk sac of the embryonated egg, suspensions from which being many hundred times more infective than other cultures.

It has also been found that when virulent rickettsiae from the guinea-pig are inoculated into the nose of mice and cotton-rats a

pneumonic condition is produced, the organisms multiplying in large numbers in the lungs.

The serum of typhus patients contains O agglutinins for types of *Proteus* designated "X2" and "X19," originally isolated from the urine of cases, but the reaction is much stronger with the latter organism. This serological feature of the disease is a fairly constant one, and the agglutination reaction with *Proteus* X19 (Weil-Felix reaction) has therefore been utilised for diagnosis. The reaction is not present in conditions other than typhus fever and closely related infections (*vide infra*), with the exception of *Brucella* infections in which it may occur. The immunological significance of the phenomenon has not yet been elucidated (although it has been suggested that a specific polysaccharide substance is common to *Proteus* X types and *R. prowazekii*), but the Weil-Felix reaction is not to be interpreted as indicating any aetiological relationship of *Proteus* to the disease.

DIAGNOSIS OF CLASSICAL TYPHUS FEVER

Reference has been made above to the recognition of *R. prowazekii* in lice and in tissues, and to the results of animal inoculation.

The Weil-Felix reaction constitutes a routine diagnostic test and is performed in exactly the same manner as the Widal test (p. 243), except that an O-agglutinable suspension of *Proteus* X19 is used instead of the enteric organisms. The tubes are incubated at 37° C. for two hours and then allowed to stand at room temperature overnight, when final readings are made.

As the agglutinins are of the O type, an alcoholised suspension (p. 249) made from an O culture is used for the test (*Proteus* O-X19). As this may revert to the H form, it should be grown on dry agar and, if necessary, subcultures are made from non-spreading separate colonies (Felix).

(A standard suspension can be obtained from the Central Public Health Laboratory, London.)

The minimum titre acceptable for diagnostic purposes is 1 in 100, but agglutination frequently occurs in much higher dilutions. The reaction appears about the sixth or seventh day of the disease, and a rising titre can be demonstrated on repeated testing. In persons who have received typhus

vaccine within three months, marked agglutination in a dilution of 1 in 200 or over is considered suggestive of infection.

Agglutination test with *Rickettsia*—*vide infra*.

RICKETTSIA MOOSERI

This species is responsible for the so-called murine type of typhus fever. The condition was first recognised in the United States and in Mexico where it is designated "Tabardillo." When infective material is inoculated into the peritoneum of the male guinea-pig, an inflammatory reaction develops in the scrotal sac (Neill-Mooser reaction), with rickettsiae flourishing in the endothelial cells. This is not observed when inoculations are made from European typhus. The infection can be transmitted to rats in which the resistance has been lowered by X-radiation. Wild rats constitute a reservoir of the infection, and the rat flea (*Xenopsylla cheopis*) and rat louse (*Polyplax spinulosus*) serve as vectors among rats, and the rat flea is the vector from rats to man. Once established in the human subject the infection is transmitted by lice. The serum of cases (as in European typhus) agglutinates *Proteus* X19. This form of typhus fever occurs also in Europe (e.g. Fièvre nautique of Toulon), Middle East and Malaya ("Urban typhus"), and other parts of the world.

It may be noted that in South Africa three rickettsial infections have been reported: louse-borne typhus, murine typhus and a tick-borne typhus-like condition (p. 564).

Agglutination Test with Rickettsiae

In the Middle East during the war, agglutination tests with suspensions of rickettsiae derived respectively from classical and murine typhus were successfully used for diagnostic purposes (van Rooyen and Bearcroft¹), and it has been possible in this way to distinguish between these two types of the disease, whereas both yield similar results in agglutination tests with *Proteus* X strains. Concentrated and purified rickettsial suspensions from egg yolk sac cultures (*vide supra*) have been found suitable for the tests, after dilution with a phosphate buffer mixture of pH 7.2. The opacity of the suspension should approximate to that of Brown's standard No. 1. Serum dilutions ranging from 1 in 100 to 1 in 6400 are made up in physiological saline in the usual way, and an equal volume of the rickettsial suspension is added to each. The test includes also a suspension-control without serum. Dreyer's conical agglutination tubes are very suitable for the tests. The tubes are incubated at 42° C. for four hours and then placed in the refrigerator at 0°–4° C. overnight, after which readings are made. The agglutinated rickettsiae form very fine granular floccules, and to make readings and observe accurately the

¹ *Edin. Med. Jour.*, 1943, 50, 257.

end-point, the tubes are examined with a bright artificial illuminant against a dark background. Sharp rotation of the tubes assists in the recognition of the clumps. If there is any difficulty, films are made from the deposit after removal of the supernatant fluid, and stained by a method suitable for demonstrating rickettsiae (p. 560). The clumps are seen as small regular masses in the film.

Normal serum or serum from other diseases rarely agglutinates rickettsiae beyond a titre of 1 in 100. The reaction in typhus appears about the sixth or seventh day of the illness when the titre may be 1 in 200; by the eighth or ninth day the titre may rise to 1 in 400, while on the tenth to fourteenth day it reaches 1 in 800 to 1 in 6400. The test with the two types of rickettsiae in parallel, especially after strong reactions have developed by the tenth to fifteenth day, allows a differential diagnosis to be made by virtue of the definitely higher titre for the infecting type of rickettsia. Thus, serum from a case of classical typhus should give a titre for the homologous type three to four times higher than that for the murine type.

IMMUNITY TO TYPHUS FEVER

Recovery from typhus is followed by a lasting immunity, and sera of convalescent persons have been used prophylactically in the incubation period. Some success has been achieved in prophylactic immunisation with killed suspensions of rickettsiae, obtained by emulsifying in phenol-saline the intestines of artificially infected lice, e.g. Weigl's vaccine. A type of vaccine, which has now been extensively used, is produced from suspensions of rickettsiae grown in the yolk sac of the embryonated egg, and killed with formalin and phenol (Cox and Bell).

OTHER INFECTIONS DUE TO RICKETTSIAE

In India and the Far East, typhus-like conditions occur which are frequently of a milder type than European typhus—e.g. *Japanese Flood Fever* (*Tsutsugamushi*) transmitted by a mite (e.g. *Trombicula akamushi*) and due to a rickettsia designated *R. tsutsugamushi*. Mites become infected in the larval stage, the infection continues into the adult and is transmitted to the eggs, the larvae from these conveying the disease to the human subject. The so-called "rural" or "scrub" typhus of Malaya is a similar infection. A peculiarity of this disease is the fact that while the Weil-Felix reaction with *Proteus X19* is absent, a variant of this organism (the Kingsbury type—XK) is agglutinated by the serum. Rats and probably other rodents are reservoirs of the infection.

A typhus-like condition of America, *Rocky Mountain Spotted Fever*, has been shown to be due to a species designated *Rickettsia rickettsii*. The organism is demonstrable in certain ticks (e.g. *Dermacentor andersoni* and *variabilis*) which transmit the disease. It is similar in appearance to *R. prowazeki*. This infection is passed from one generation

of ticks to another. No animal reservoir of infection has yet been defined. Rickettsia bodies have been observed also in endothelial cells in the human disease. The agglutination reaction with *Proteus* X19, X2 and XK occurs in this infection, but is usually weak and undifferentiated.

Similar infections such as "Weigl's disease" (*R. weigl*) and recurrent fevers in Poland, Russia, Japan and in Africa have been described.

Sao Paulo Typhus of Brazil is transmitted by ticks. In this condition the serum is stated to agglutinate strongly *Proteus* X19, but this is questioned as the disease seems to be related to, if not identical with Rocky Mountain spotted fever. The causative organism has been designated *R. brasiliensis*.

Fievre boutonneuse of the Mediterranean is transmitted to man from dogs by a tick. The serum gives a weak reaction with *Proteus* X19 and X2. The infecting organism is *R. conori* which is closely related to *R. rickettsii*.

Trench Fever was prevalent among troops during the war of 1914-18 and like typhus was louse-borne. A rickettsia (*R. quintana*) was found in lice fed on patients, and has been regarded as the cause of the disease. It has been stated that unlike *R. prowazekii* it does not invade the alimentary epithelium of the insect and is less pleomorphic.

The causal organism of a previously unknown rickettsial disease in New York has been isolated by Armstrong and his associates. Mice are the reservoir of infection, and the organism (*Rickettsia akari*) is transmitted by mouse mites.

The *Q Fever* of Australia (*R. burneti*) is a tick-borne disease for which the bandicoot is the likely animal reservoir. Atypical pneumonia may occur in severe cases. There is also hereditary transmission in the tick. In this disease the serum does not agglutinate the X strains of *Proteus*. A similar disease, *X Fever* of Montana, has been described as being due to a rickettsia (*R. diaporica*). Both *R. burneti* and *R. diaporica* are stated to be filterable.

During the war of 1939-45, cases of Q fever were recognised among troops in the Mediterranean, and in some atypical pneumonia occurred.

Recent investigations have shown that *R. burneti* infections are widely distributed in the world and cases have been found in England. The infection occurs in farmers, dairy workers and those whose occupation brings them into contact with cattle, sheep and goats. It is apparent also that persons may become infected by inhalation of dust containing dried tick excreta from cattle hides. Consumption of raw milk may also cause the disease, cows being infected and carrying the organism for long periods in their udders. It seems likely that ticks constitute a reservoir of the infection, that from them the organism is transmitted to domestic animals and from the latter occasionally to the human subject. In man the infection may take the form of an indefinite febrile illness, but sometimes is clinically an "atypical pneumonia." Diagnosis can be made by the complement-fixation test with serum and an antigen consisting of the cultivated rickettsia.

Heart-Water of cattle in South Africa is a tick-borne disease, and a rickettsia (*R. ruminantium*) has been demonstrated in the vascular endothelium of infected animals and in the arthropod vector.

Tick-borne fever of sheep in Great Britain has been regarded as a rickettsial disease. In blood films stained by Giemsa solution inclusion bodies are observed in the cytoplasm of the granular leucocytes and monocytes, and these bear resemblances to the Rickettsiae. A similar infection has been found in cattle.

The agglutination reactions of the various rickettsial infections may be summarised as follows (after Felix).

		<i>Proteus</i>		
		X19	X2	XK
Classical typhus . . .	Louse-borne	+++	+	-
Tabardillo . . .	Murine type	+++	?	-
Fièvre nautique . . .	of typhus	+++	?	-
Urban typhus of Malaya . . .		+++	+	-
Tsutsugamushi (of Japan and Sumatra) . . .	Mite-borne	-	-	+++
Scrub typhus of Malaya . . .		-	-	+++
Rocky Mountain spotted fever . . .	Tick-borne	+	+	+
Fièvre boutonneuse . . .		+	+	-
South African tick fever . . .		+	+	+

+ marks indicate the relative strengths of the reactions.

Note.—Occasionally in the tick-borne fevers (e.g. in America and South Africa) X2 is moderately or strongly agglutinated, but not X19.

Antibiotic therapy.—In general Rickettsiae are sensitive to aureomycin, terramycin and chloromycetin, and these antibiotics may be used in the treatment of rickettsial diseases.

BARTONELLA BACILLIFORMIS (OROYA FEVER)

Oroya fever is a disease occurring in Peru, with a high degree of mortality, characterised by intermittent pyrexia and anaemia.

The infective agent—*Bartonella bacilliformis*—invades red blood corpuscles and is found as minute rod-shaped forms $0\cdot3\text{--}2\cdot5\mu$ in length, while coccal forms also occur. It is actively motile. By Giemsa's method it stains reddish violet in colour; the extremities appear thickened and darker in colour, and the outlines hazy. The organism is Gram-negative. Cultures are obtained on solid or semi-solid blood media, but not in ordinary broth or agar. The optimum temperature is $25^\circ\text{--}28^\circ\text{ C.}$, and the organism can survive in blood or in cultures for many weeks.

The disease can be reproduced by the intravenous inoculation of monkeys, except that anaemia does not occur. Subcutaneous inocula-

tion into the eyebrows of a monkey also produces nodules in which numerous organisms are present.

In *Verruga peruana*, a disease characterised by a nodular eruption, an organism closely resembling *Bartonella bacilliformis* has been isolated. Noguchi's work proved that these organisms are serologically identical, and that Oroya fever and Verruga peruana have the same aetiological origin. Both diseases are transmitted by sandflies.

It seems possible that the organism described as *Anaplasma* (p. 644) in an infection of cattle and found also in red corpuscles is related biologically to *Bartonella*.

Bartonella muris.—It has been observed that after splenectomy in rats, organisms of the same type as *Bartonella bacilliformis* frequently appear in the red cells, and this infection is associated with marked anaemia and often produces a fatal result. The organism has been named *Bartonella muris*. Presumably, latent infection is prevalent in rats associated with a certain degree of natural immunity, which is broken down by the removal of the spleen. The infection is louse-borne. A similar organism has also been noted in mice, following splenectomy.

CHAPTER XXII

THE VIRUSES¹

AND SPECIAL METHODS APPLICABLE TO THE STUDY OF VIRUSES

A STEADILY increasing volume of knowledge has been obtained in recent years regarding the group of infective agents producing disease in man, animals and plants, which, on account of their minute size, can pass through the pores of earthenware, collodion, and other filters capable of arresting the bacteria.

The question has been much debated whether these filterable pathogenic agents are living organisms, and they have been designated "filterable viruses" or simply "viruses." All the evidence at present available, microscopical and experimental, indicates that such viruses, as they affect man and animals, are entities comparable with, though very much smaller than the ordinary microbes, and alternative explanations are difficult of acceptance. The smallest viruses of animals, however, approximate in size to that of the molecules of the more complex proteins, and it has been found that the infective agents of certain virus diseases of plants, e.g. tobacco mosaic disease, can be isolated in the form of crystalline proteins of high molecular weight. Such agents are nevertheless self-reproducing like true organisms, and transmissible from one individual host to another like the pathogenic microbes, setting up in each case, as a rule, a characteristic disease. Whatever the true biological nature of the filterable viruses may be, they behave like parasitic microbes and their effects are studied by methods similar to those applicable in bacterial diseases.

Three characteristics have been cited in respect of which viruses differ from bacteria : (a) "ultramicroscopic" size, (b) filterability and (c) non-cultivability on inanimate substrates. The terms ultramicroscopic and filterable, however, are not applicable to the whole series of viruses. Thus, some viruses are sufficiently large to be demonstrated by ordinary microscopic methods, while smaller viruses can be photo-

¹ For further information regarding the viruses of human disease reference may be made to *Textbook of Virology*, by A. J. Rhodes and C. E. van Rooyen, 1953, Baltimore.

graphed by ultra-violet light and by the electron microscope (*vide infra*). Moreover, organisms which cannot be classed with the viruses may pass earthenware filters. In the present state of our knowledge and technique, the non-cultivability of the viruses on inanimate food materials constitutes the most distinct difference between them and the bacteria. For their cultivation in the laboratory, a substrate consisting of living tissue cells, preferably embryonic cells, is required. Thus, many viruses have now been cultivated along with surviving tissue cells, in tissue cultures or in the chick embryo. In fact, as will be shown later, the viruses may be regarded as intracellular parasites.

As explained on p. 62, the limit of microscopic resolution for individual particles is about 0.25μ when direct illumination by transmitted light is used, and is governed by the wave-length of the light employed. By dark-ground illumination, however (*q.v.*), a very minute object may be rendered easily visible owing to its refraction of light, and is seen as a luminous spot. No resolution is obtained ; that is, no details of its form or structure are revealed, but its presence merely is noted. The shorter the wave-length of the light used, the smaller the object that can be resolved. Thus, if short-wave ultra-violet light be used, objects smaller than 0.25μ can be resolved, but as the eye is insensitive to such short waves, photographic methods must be employed. For this work a special microscope, having an optical system constructed entirely of quartz (since glass absorbs ultra-violet rays), must be employed. Under these circumstances, Barnard succeeded in resolving and photographing objects 0.075μ in diameter. In addition, while a virus in the natural state may be below the limit of visibility or resolution, after staining it may be of sufficient size to be seen and resolved as a result of the deposition of stain on the surface of the particle.

Recently, by means of the electron microscope (p. 81) the difficulty of resolution has been overcome, and photographs of the smallest viruses can now be obtained at exceedingly high magnifications.

As already pointed out, the viruses of animal diseases appear to be living entities comparable with the known micro-organisms, but smaller in size. They propagate, infect tissues and incite immunity like micro-parasites, and there is no reason why the range of micro-organisms in nature should be limited to the size of the smallest microbes visible with the ordinary microscope. Just as bacteria range in size

from that of the anthrax bacillus to the pleuro-pneumonia organism, so also viruses vary from the size of the smallest bacterium to that almost of a protein macro-molecule.

Moreover, they seem to form a continuous series of varying size, as determined by filtration through graded collodion membranes, by the rate of deposition when centrifuged at high speed, and by electron microscopy (p. 571).

It has been suggested that the viruses are the end-result of a retrograde evolution from ordinary microbial forms to an extreme state of parasitism, in which most of the functions of the independent living organism have been lost, along with the corresponding cell substance, and only a vestige remains. Such entities on the basis of this assumption are unable to synthesise substances essential for cell growth and can only function as living organisms by obtaining these from the cells of their hosts. In this way also is explained their intra-cellular habitat in the tissues. It is thought that, while the larger viruses may still retain some of the enzyme systems necessary for vital activity, in the descending scale of size of these infective agents there is loss of more and more of such functions, so that the smallest viruses are devoid of all the enzyme systems necessary for the synthetic activities characteristic of a living organism, and may constitute merely protein macro-molecules; these, however, still represent the elements necessary for reproduction and maintenance of species. In the appropriate cell, however, the virus obtains the substances necessary for its growth and becomes to all intents and purposes a living organism.

When viruses incite immunity in the animal body the immune serum shows specific inactivating properties towards the virus concerned, while the antibodies can be absorbed by suitable virus material. Comparable with the serological types of bacteria, such as the pneumococcus, are the different antigenic types of the foot-and-mouth disease virus, and the types of the influenza virus. It is of further significance that the viruses when transmitted by animal passage, in the same or even different species, "breed true to type" and reproduce the same disease.

The bacteriophages, which will be dealt with later, present a close analogy to the viruses and may reasonably be regarded as bearing a parasitic relationship to bacteria like that of the viruses to the tissues of animals and plants.

The position of the *Rickettsia* group, which has been described in the previous chapter, and its relationship to the

viruses require consideration. Certain writers actually include these organisms in the group of viruses, and the relationship between them and some of the larger viruses is apparently a close one. The Rickettsiae have mostly proved non-filterable through filters which arrest bacteria, and though smaller than bacteria are in general larger than the viruses. They are easily demonstrable also by ordinary microscopic methods. The Rickettsiae, however, resemble the viruses in their inability to grow on an inanimate substrate while they can be cultivated readily with living cells, *e.g.* in the embryonated egg. They are also intracellular parasites. Until the biology of all these infective agents is better known, it is perhaps expedient to classify them separately.

SIZES OF THE VIRUS BODIES

Viruses were first graded in size by filtration through thin collodion membranes of standard porosities (p. 134).

Their sizes can also be estimated by centrifuging at very high speeds (60,000 r.p.m.) and determining the rate of sedimentation, the virus being retained by a disk of thick filter-paper at the bottom of the container. The sedimentation rate follows a fixed law (Stokes' law), depending on the diameter of the particle, its density, the viscosity and density of the medium, the height of the liquid column, the distance of the filter-paper from the axis of rotation, and the speed in r.p.m. The values of the factors enumerated above can be determined and the size of the particles calculated. By this method, which entails high technical skill and elaborate apparatus, the sizes of many of the different virus bodies have been computed, and they agree closely with the sizes estimated by filtration experiments.

Measurements recently made by electron microscopy have added further to our knowledge of the sizes of a number of viruses.

The following table indicates the approximate or average sizes of various filterable viruses. Owing to their minuteness the unit $m\mu$ (millimicron) is used, *i.e.* one-thousandth part of a μ , or 0.000001 mm. Many of the figures are based on collective findings by different observers and some also on the results obtained by different methods, preference being given to measurements made by electron microscopy.

<i>Virus, etc.</i>	<i>Approx. Size in μ</i>
Staphylococcus (for comparison)	1000
<i>Serratia marcescens</i> (<i>Bacillus prodigiosus</i>) (for comparison)	750
<i>Rickettsia</i> (for comparison)	475
Psittacosis	450
Lymphogranuloma inguinale	440
Fowl-pox	330×265
Canary-pox	310×260
Molluscum contagiosum	300×225
Infectious ectromelia of mice	300×210
Vaccinia	260×210
Pleuro-pneumonia organism — granular form (for comparison)	150
Rabbit fibroma (Shope)	150
Herpes simplex	150
Pseudo-rabies	150
Rabies (fixed virus)	125
Influenza	125-100
Borna disease of horses	100
Bacteriophages	from 100 to 10
Vesicular stomatitis of horses	100
Fowl plague	90
Rous sarcoma of fowls	70
Lymphocytic chorio-meningitis	50
Rabbit papilloma	44
Rift Valley fever	30
St. Louis encephalitis	25
Poliomyelitis	25-10
Yellow fever	22
Louping-ill	19
Foot-and-mouth disease	10
Protein molecules (for comparison)—	
— egg albumin	10×2·5
— serum globulin	6-7
— serum albumin	5-6

FILTRATION OF THE VIRUSES

As the viruses are distinguished by their capacity to pass through filters which are impervious to ordinary bacteria, it is essential to understand the types and limitations of the various filters used. The term "filterable" was originally applied in respect to rather coarse earthenware filters of the Berkefeld type, which would just hold back the smallest bacteria, e.g. *Serratia marcescens*,¹ but must now be considered more in relation to the porosity of the filter used.

Apart from the viruses, two types of filterable organisms have been recognised :

(1) Filterable bacteria ; e.g. *Dialister pneumosintes*, and similar anaerobic bacteria of the respiratory passages, which pass coarse filters ; the pleuro-pneumonia organism, and similar bacteria (p. 535) ; and the reported filterable forms of some other bacteria.

(2) Certain spirochaetes and vibrios which, owing to their extremely slender form, their flexibility or motility, are able to pass through the coarser filters, especially if the time of filtration is prolonged.

It is important to note that the mere passage of an organism through an earthenware filter candle does not justify the term "filterable virus" being applied to it. When describing a virus or organism as filterable it is necessary therefore to indicate (1) the type of filter ; (2) the porosity of the filter ; (3) the pressure employed during filtration ; (4) the time of filtration ; (5) the nature and the pH of the fluid in which the material is suspended, and (6) the controlling organism with which the filter has been tested.

Filtration is not the mere mechanical passage of particles of a certain size through slightly larger pores, but depends on physico-chemical factors. Thus, the carthenware filter consists mainly of magnesium and calcium silicates, and according to the electric charge on the virus, so the particles will be adsorbed or be able to pass through. The more acid the solution, the more liable are the particles to become adsorbed. In addition, the adsorption of a virus by protein or tissue, the amount of the latter substances present, the temperature and pressure of filtration, affect very profoundly

¹ *Serratia marcescens* (*B. prodigiosus*) is a minute Gram-negative saprophytic bacillus (Table, p. 571) which, growing on culture medium, produces a red pigment.

the results obtained. Filtration, therefore, depends on many variable and uncontrollable factors, and inability to pass an earthenware filter is no measure of size.

It must also be emphasised that usually the presence of virus in the filtrate can be determined only by animal experiment or by cultivation in the embryo-chick (p. 577). As certain viruses are very susceptible to changes in hydrogen-ion concentration, it is essential that the reaction of the virus-material be carefully adjusted, otherwise the virus may be inactivated and so adjudged non-filterable.

The various types of filters and the technique of filtration are dealt with on p. 132 *et seq.*

When working with filterable viruses, controls must always be made of the filtered material. It must be cultivated both aerobically and anaerobically to show that no bacteria are present. Inoculation of animals susceptible to the virus should be carried out, and the filtrate should give rise to the train of symptoms characteristic of the disease.

HUMAN DISEASES REGARDED AS DUE TO FILTERABLE VIRUSES¹

Epidemic poliomyelitis.

Encephalitis lethargica and other forms of encephalitis.

Ascending myelitis due to virus "B."

Rabies (common also to dogs and certain other animals).

Lymphocytic chorio-meningitis and Pseudo-lymphocytic-chorio-meningitis.

Bornholm disease (epidemic myalgia or pleurodynia).

Herpes simplex.

Herpes zoster.

Varicella (chicken-pox).

Smallpox (variola), alastrim and vaccinia.

Measles.

German measles (rubella).

Molluscum contagiosum.

Common wart.

Foot-and-mouth disease (transmissible from cattle).

Trachoma.

Inclusion conjunctivitis, urethritis, and cervicitis.

Conjunctivitis due to the virus of Newcastle disease of poultry.

¹ In many of the diseases listed there is indubitable evidence of virus causation, but in others, experimental proof is incomplete.

Epidemic keratoconjunctivitis.
 Influenza.
 Common cold.
 Primary atypical pneumonia.
 Psittacosis and Ornithosis (common also to parrots and certain other birds).
 Mumps.
 Infective mononucleosis (glandular fever).
 Lymphogranuloma inguinale (or climatic bubo).
 Yellow fever.
 Infective hepatitis.
 Rift Valley fever (common also to sheep and cattle—enzootic hepatitis).
 Phlebotomus fever.
 Dengue.

Viruses have been classified according to their apparent affinities for particular tissues : those having an affinity for nervous tissues are referred to as *neurotropic* ; for the skin, *dermotropic* ; for internal organs, e.g. liver, *viscerotropic* ; for various tissues and organs, *pantropic*. Such classification and nomenclature are unsatisfactory, since some viruses are both dermatropic and neurotropic, and tropism may be modified by passage in animals ; for example, a viscerotropic virus may become neurotropic by adaptation.

Representative Diseases of Mammals, Birds and Insects generally accepted as due to Filterable Viruses :—

Canine distemper.
 Hard pad disease of dogs.
 Rabies (*vide supra*).
 Feline distemper.
 Feline pneumonitis.
 Feline infectious enteritis.
 Foot-and-mouth disease of cattle (and certain other animals).
 Cow-pox (*vaccinia*—*vide supra*).
 Pseudo-rabies, or mad itch, of cattle and other animals.
 Cattle plague (*rinderpest*).
 Malignant catarrh of cattle.
 Ephemeral fever of cattle.
 Vesicular stomatitis of horses.
 African horse sickness.
 Borna disease of horses.
 Equine encephalomyelitis (transmissible to man).
 Infectious anaemia of horses.
 Periodic ophthalmia of horses.

Epizootic abortion of mares.
Louping-ill (encephalomyelitis) of sheep (transmissible to man).
Scrapie of sheep.
Sheep-pox.
Blue-tongue of sheep and goats.
Nairobi sheep disease.
Contagious pustular dermatitis of sheep.
Swine fever (or hog-cholera).
Swine influenza.
Vesicular exanthem of swine.
Encephalitis of foxes.
Infectious myxomatosis of rabbits.
Infectious fibromatosis of rabbits.
Infectious papillomatosis of rabbits.
Rabbit-pox.
Virus III infection of rabbits.
Encephalitis of rabbits.
Salivary-gland disease of guinea-pigs.
Infectious ectromelia of mice.
Filterable tumours of fowls, *e.g.* Rous sarcoma.
Fowl plague.
Newcastle disease of fowls.
Infectious laryngo-tracheitis of fowls.
Infectious bronchitis of chickens.
Fowl-pox, including roup.
Infectious encephalomyelitis of chickens.
Leukaemia of fowls.
Fowl paralysis.
Blue comb of chickens.
Pigeon-pox.
Canary-pox.
Psittacosis and Ornithosis.
Pacheco's parrot disease.
Silkworm jaundice.
Sac brood of bees.

GENERAL CHARACTERS OF THE FILTERABLE VIRUSES

Certain characteristic features are common to many of the viruses :—

1. Great infectiousness of the associated disease.
2. The production of a solid immunity when recovery from the disease takes place ; and such immunity may be of long duration.
3. Filterability.
4. Invisibility by ordinary microscopic methods.

5. Non-cultivability *in vitro* by ordinary methods applicable to bacteria.

6. Resistance to glycerol.

7. The presence of intracellular "inclusion bodies" in lesions.

1. GREAT INFECTIOUSNESS OF THE ASSOCIATED DISEASE.— This is seen in smallpox epidemics, where, in spite of careful quarantine, cases continue to occur. Foot-and-mouth disease is another example; the difficulty of control is due to the amazing rapidity of spread, not only from animal to animal, but from one locality to another.

As a group, the viruses are highly pathogenic even in minute doses. Thus, the virus of yellow fever is capable of causing infection of the monkey in a quantity as small as 0.000001 ml. of a 10 per cent. emulsion of brain tissue from an infected animal.

2. THE PRODUCTION OF SOLID AND DURABLE IMMUNITY.— In individuals surviving infection there is a high degree of immunity which often lasts for a considerable period of time. Advantage is taken of this in immunisation against smallpox and yellow fever by inoculation with an attenuated virus. In addition, specific antisera may be prepared, as in the case of swine fever and rinderpest, which are highly protective against the respective viruses. On the other hand, the immunity following some virus infections may be of only short duration.

<i>Prolonged Immunity</i>		<i>Short Immunity</i>
Smallpox.	Measles.	Herpes simplex.
Vaccinia.	Yellow fever.	Influenza.
Poliomyelitis.	Rift Valley fever.	Common cold.
Chicken-pox.	Mumps.	Dengue.
		Trachoma.

It was at one time supposed that immunity could not be stimulated by injecting "dead" virus into susceptible animals as is the case with killed-culture vaccines of bacteria, and it has been thought that "living" virus is absolutely essential for an immunity response. It has been shown, however, that formolised or phenolised virus can be used to produce active immunity against louping-ill, rabies, canine distemper, etc.

3 and 4. FILTERABILITY and INVISIBILITY of the viruses have already been discussed.

5. CULTIVATION.—The filterable viruses are not cultivable

on ordinary media, and their presence has been generally recognised by their action on susceptible animals. Many viruses can be grown, however, in association with the proliferating cells of tissue cultures, in embryonic tissues as in the fertile egg, and even along with surviving adult cells *in vitro*. Thus, vaccinia virus can be cultivated by using fresh hen or rabbit kidney minced and mixed with fresh serum and Tyrode's solution.¹ The conditions necessary for virus growth are not yet fully understood, and the procedures are still somewhat empirical. It would appear that the obligatory intracellular parasitism of many of the viruses precludes their cultivation like the bacteria on inanimate substrates.

Different conditions of growth are necessary, and these must be provided, just as for the bacteria whose oxygen and temperature requirements, nutrient materials, growth factors, optimum H-ion concentration, etc., must be studied. Thus the viruses of herpes simplex and vaccinia multiply in surviving tissue, and proliferating cells are not essential, while the psittacosis and influenza viruses require actively growing cells. Certain viruses grow in tissue cultures irrespective of the nature of the tissue cells, but the virus of foot-and-mouth disease requires particular tissues from certain species—e.g. the pads, lips, tongue or hairy skin of the embryo guinea-pig, or epidermis of bovine tongue.

Many viruses—e.g. of vaccinia, fowl-pox, influenza, psittacosis, etc.—can be cultivated in the chorio-allantoic membrane of the developing chick. This provides a comparatively simple and convenient "medium." Lesions are often produced in the membrane in the form of opacities; later, greyish-yellow plaques are observed. Massive lesions and death of the embryo may result. Some viruses, however, may be propagated serially without producing any visible lesions. Certain viruses grow better in other tissues of the developing chick, e.g. lymphogranuloma inguinale virus in the yolk sac, influenza virus in the allantoic sac.

Various forms of egg-cultivation, and the methods of inoculation, etc., are detailed on p. 619 *et seq.* For a full description of the technique, reference should be made to the Medical Research Council's Special Report Series, 1946, No. 256, by W. I. B. Beveridge and F. M. Burnet.

6. RESISTANCE TO GLYCEROL.—Ordinary non-sporing bacteria are killed by 50 per cent. glycerol in a comparatively short time. Many of the filterable viruses, on the contrary, retain their viability much longer in this material than in

¹ *Tyrode's solution.*—NaCl, 8·0 gm., KCl, 0·2 gm., CaCl₂, 0·2 gm., MgCl₂, 0·1 gm., NaHCO₃, 1·0 gm., glucose, 1·0 gm., NaH₂PO₄, 0·05 gm., distilled water, 1 litre. The solution is adjusted to pH 7·4 and sterilised by filtration through a Seitz filter.

any other fluid when kept at 4°C. The preservation of the vaccinia virus used prophylactically against smallpox is accomplished by means of glycerol. Other viruses which keep for prolonged periods in glycerol at low temperatures are : poliomyelitis virus, the virus of herpes simplex, and the rabies virus. On the other hand, some viruses, e.g. rinderpest virus and virus III of rabbits, will survive for less time in glycerol than certain bacteria. It is essential that the glycerol should be pure and free from mineral acids, esters and sulphates.

It may be noted here that the filterable viruses infecting man and animals are very readily destroyed by heat and by most of the generally used disinfectants, but may survive outside the body for considerable periods of time, and in many instances can also resist natural desiccation.

7. INCLUSION BODIES.—When lesions occurring in virus diseases are examined histologically, there are frequently found within the cytoplasm or the cell nucleus abnormal structures which may be of two types :—

- (1) "Elementary bodies"
- (2) "Inclusion bodies."

Elementary bodies appear to represent the actual virus, and are exemplified by the "Paschen bodies" which are seen in smallpox and vaccinia lesions in the exudate as well as in the cells. When suitably stained they appear as small round coccus-like bodies, about 0.2μ on size. Similar bodies have been found in varicella and herpes zoster, while they are demonstrable in the lesions of psittacosis, molluscum contagiosum, ectromelia, fowl-pox, etc. They can be demonstrated by Paschen's or Castaneda's stain (p. 117) and by prolonged staining in Giemsa's solution, when they appear red or purplish.

Recently, in many virus diseases, elementary bodies representing apparently the infective units of these diseases, have been more clearly defined by electron microscopy ; and virus bodies not previously demonstrated have now been photographed in this way.

Inclusion bodies may be :—

(a) Cytoplasmic : exemplified by the Negri body found in the nerve cells of the central nervous system in rabies, the Guarnieri body of smallpox, the Henderson-Paterson body or "molluscum body" of molluscum contagiosum, and the Bollinger body of fowl-pox. These inclusion bodies are acidophilic in nature, and with Mann's methyl-blue-eosin (p. 116)

or Giemsa's stain are coloured pink by the eosin. The Negri bodies are round or oval, and vary considerably in size (p. 584). Guarnieri bodies are found in the cytoplasm of epithelial cells of the skin lesions of smallpox and vaccinia. They stain with acid dyes and are often seen lying in clear spaces in the protoplasm. They may vary in size from 1μ to $15-20\mu$, and may be as large as the cell nucleus. Similarly, Bollinger bodies are acidophilic cytoplasmic inclusions, round or oval in shape, and varying considerably in size. In certain virus diseases the cytoplasmic inclusions are basophilic in their staining reactions, e.g. psittacosis, lymphogranuloma. Further details of the characters of inclusion bodies are given later under various virus diseases.

(b) Intranuclear: exemplified in the following diseases, being demonstrated by Giemsa's stain, polychrome methylene blue and eosin, or Mann's stain :

Herpes simplex Chicken-pox Herpes zoster Pseudo-rabies Borna disease Yellow fever Rift Valley fever Pacheco's parrot disease	} in the epidermis. } in the brain. } in the liver.
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Virus III infection of rabbits—in the cornea, skin and testis.
Guinea-pig salivary gland infection—in the ducts.

These intranuclear inclusions are acidophilic in nature, and vary in size.

It has been shown that a single isolated Bollinger body (of fowl-pox) is infective for the fowl and therefore contains the virus. When suitably stained and examined microscopically the Bollinger bodies are seen to consist of minute particles—termed Borrel bodies—in an amorphous matrix. This has been confirmed by electron microscopy. These Borrel bodies, which are similar to the elementary bodies of variola and vaccinia, may be regarded as the virus. In fowl-pox it was thus shown that the inclusion bodies consist of aggregates or colonies of the virus which is multiplying as an intracellular parasite.

By microdissection it has been possible to remove a molluscum body from the epithelial cell, and on opening this inclusion it was found to contain a large number of elementary bodies surrounded by amorphous material (van Rooyen).

Likewise the inclusion body of smallpox and vaccinia has been shown by electron microscopy to consist of an aggregate of elementary virus bodies.

The inclusion bodies were formerly supposed to consist of a mantle

or sheath around the infective agent, and were known as "*chlamydozoa*," and while some inclusion bodies may represent degenerative or reactionary changes in the cell due to the action of the virus, recent evidence all points to the fact that they are aggregates or collections of virus units.

POLIOMYELITIS

Poliomyelitis is an acute infection, occurring mainly in children, which affects the central nervous system with usually a special localisation in the anterior cornu of the spinal cord.

The disease can be communicated to susceptible animals (*vide infra*) by the inoculation of filtrates of infective tissue.

The virus passes through Berkefeld V and N filters. Its size is about 10–25 m μ . It is easily destroyed by heat (55° C. for thirty minutes), but can withstand the action of weak phenol for some days. In brain tissue, preserved in 50 per cent. glycerol at 4° C., it remains viable for years.

The disease can be transmitted to monkeys (e.g. *Macaca mulatta*) by the intracerebral or intravenous inoculation of human brain or spinal cord material. *Cynomolgus* and various other monkeys are also susceptible, and the former can be infected by feeding. The animals develop the typical disease, and show in the central nervous system histological lesions similar to those of human cases. The disease can also be transmitted from monkey to monkey. Chimpanzees inoculated orally in most cases develop an inapparent infection and carrier state, the virus being present in their faeces.

Certain strains of the virus have been transmitted (after passage in monkeys) to the white mouse and cotton-rat.

Laboratory diagnosis can be established by inoculation of monkeys.

It was at one time thought that the source of infection was secretion droplets from the nose and throat of persons suffering from the disease or, more frequently, healthy persons acting as carriers of the virus; it was also considered that the nose was the avenue of infection, and that the virus reached the central nervous system *via* the olfactory nerves, but this has not been established. Recent work has shown that the virus is present in the faeces of patients and also of healthy carriers, and that it can be recovered from sewage (p. 626). The possibility has therefore to be considered of the infection spreading from the intestinal tract to the central nervous system through the abdominal sympathetic nerves. The virus is found also in the tonsils and oro-pharynx of

infected persons, and may invade the central nervous system *via* afferent cranial nerves. The occurrence of viraemia in experimental animals has further suggested the possibility of transport of the virus by the blood stream. Excretal contamination of food may be a factor in the spread of infection in the community. It has also been shown that the virus can be carried by flies, but the epidemiological importance of this has not yet been assessed.

It has proved difficult to obtain laboratory cultures of the virus. Certain strains of virus, however, have now been cultivated in cultures of human embryonic tissues and of monkey testis. The virus exhibits a marked "cytopathogenic" effect on the cells of the tissue culture.

Recent serological studies have indicated that there are three antigenic groups of the poliomyelitis virus.

The serum of convalescents contains neutralising antibodies, but its value in the treatment of the pre-paralytic or early paralytic stages of the disease is doubtful, as once the virus has entered the cell, serum has no effect on it. *Gamma* globulin from pooled normal adult serum is at present under investigation as a prophylactic.

Acute Ascending Myelitis due to Virus "B."—This virus was isolated from the central nervous system of a case of ascending myelitis subsequent to the bite of a monkey. From the available evidence it is assumed that the virus occurs in a latent form in monkeys. Its neurotropism has been demonstrated by experimental inoculation of rabbits.

Bornholm Disease (Epidemic Myalgia) and the Coxsackie Virus.—The "Coxsackie" virus, as it has been generally named in the literature, was described by Dalldorf and associates who isolated it from the faeces of children with clinical symptoms similar to those of poliomyelitis. It was found that the virus could be transmitted to suckling mice, more mature animals being insusceptible. When young mice were injected intracerebrally with virus-containing material they developed muscular paralysis but without lesions in the central nervous system; on the other hand pronounced hyaline degeneration occurred in skeletal muscles. The virus has likewise been found in the throats and stools of infants suffering from supposed non-paralytic poliomyelitis with pains in the limbs, and it has been demonstrated in the faeces of cases along with the true poliomyelitis virus.

The Coxsackie virus has now been isolated from cases of Bornholm disease (or epidemic myalgia) in America and Europe, including Great Britain, and possibly some cases of supposed infantile poliomyelitis in which it has been found have been actually of the same pathological nature as Bornholm disease. The virus seems to have a wide geographical distribution. In Great Britain a laboratory infection with an American strain has been recorded: the symptoms corresponded

to those of Bornholm disease and infection with the virus was indicated by the presence of specific complement-fixing antibodies in the serum. A volunteer was inoculated intranasally with the same strain and developed pleurodynia. Sera from cases with histories of a condition corresponding to Bornholm disease have been found to give a complement-fixation reaction with the Coxsackie virus.

The Coxsackie virus has also been isolated in the United States from the condition of "herpangina," characterised by sore throat, faecal ulcers and abdominal pain.

Serological differences have emerged among strains of the virus, and multiple types have been recorded. It is estimated that the size of the virus is about 10–15 m μ .

ENCEPHALITIS

The following different types are recognised : (1) Encephalitis lethargica, (2) St. Louis encephalitis, (3) Australian encephalitis, (4) Japanese encephalitis, (5) West Nile encephalitis, (6) Russian spring-summer encephalitis, (7) Equine encephalomyelitis, transmissible to man.

Encephalitis Lethargica.—Little is known of the virus of this disease except that claims have been made of its occasional transmission to rabbits and monkeys. It is probable, however, that the virus of herpes simplex was responsible for the infection in these animals (p. 585). Much confusion has arisen in experimental work on this disease when rabbits have been used, because these animals suffer naturally from a spontaneous meningo-encephalitis, and this condition has been mistaken for the experimental disease. The precise aetiology of encephalitis lethargica is still obscure.

St. Louis Encephalitis.—This form of encephalitis was first defined by the isolation of the causative virus in an epidemic at St. Louis, U.S.A. The virus is transmissible to monkeys and mice by intracerebral inoculation. In monkeys the experimental disease is of a mild type. The virus is about 25 m μ in size and passes Berkefeld V and N and Seitz filters. It has been grown in the chorio-allantoic membrane of the embryo chick and in certain types of tissue culture. The disease, which is transmitted by mosquitoes, has no relationship to encephalitis lethargica. There is evidence that wild and domestic birds (*e.g.* poultry) may constitute reservoirs of the infection, the virus being transmitted in these birds by mites.

Australian Encephalitis.—In 1917, 1918 and 1925 epidemics of encephalitis occurred in South-eastern Australia from which a virus, pathogenic for monkeys and sheep, was isolated. The strains were lost before other similar viruses were avail-

able for comparison, but a relationship to Louping-ill and Japanese encephalitis B viruses (*vide infra*) was suggested on grounds of the type of disease produced in experimental animals and the histology of the lesions. In 1951 a similar outbreak occurred which has been described as Murray Valley Encephalitis. The virus isolated from this outbreak shows some serological relation to viruses of the Japanese B type but none to Louping-ill virus.

Japanese Encephalitis.—Two types of encephalitis have been described in Japan, encephalitis lethargica (type A), and "summer" encephalitis (type B) which is mosquito-borne. The virus of the latter condition is probably about $18\text{--}20\text{ m}\mu$ in size, and passes Berkefeld W and Seitz EK filters. The virus grows in a tissue culture of chick-embryo brain. It is destroyed at 55° C. in thirty minutes but remains virulent in glycerol for many months. The condition can be transmitted to mice and monkeys, producing a severe meningo-encephalitis in the latter animal (*cf.* St. Louis encephalitis).

West Nile Encephalitis.—The virus has some antigenic relationship to that of Japanese encephalitis type B, and the St. Louis encephalitis virus.

* **Russian Spring-Summer Encephalitis** is related to louping-ill (p. 614) and is transmitted by ticks, e.g. *Ixodes persulcatus*. It is transmissible experimentally to sheep, mice and monkeys. Domestic animals and wild rodents may be reservoirs of the infection.

Equine Encephalomyelitis is referred to on p. 616.

In addition to the viruses above described certain others which infect the human subject and are neurotropic in animals have been reported from tropical Africa and South America.

RABIES

This disease is communicated to the human subject by the bite of a rabid dog or other animal, the infective virus being present in the saliva of the animal. A paralytic form of rabies has been reported, *e.g.* in Trinidad, in which the infection is transmitted by the bite of the vampire bat.

The virus is localised in the nervous tissues, and in animals also in salivary glands, and is transmitted through the body only along nerve tracts. The incubation period in man is usually from thirty to sixty days, but may extend to several months in some cases.

The size of the virus is about $125\text{ m}\mu$ and it is filterable through Berkefeld and Chamberland L_1 and L_2 filters.

Elementary bodies have not been definitely identified. Characteristic inclusion bodies—"Negri bodies"—occur in the cytoplasm of the nerve cells, particularly in the hippocampus of the brain and in the cerebellum. They consist of round, oval or angular bodies varying in size from $0\cdot5\mu$ to 20μ , staining pink (with a slight purple tint) by Giemsa's, Mann's or similar stain. The larger Negri bodies may contain one or more dark-coloured granules. The nature of these structures has not yet been elucidated, although their occurrence is a specific feature of rabies. The virus has been cultivated in tissue-cultures and in the chick embryo. It is easily destroyed by heat (55° C. for fifteen minutes) and chemicals, e.g. formalin, mercuric chloride, etc., but is resistant to glycerol. Phenol (0·5 per cent.) inactivates the virus, and phenolised suspensions of infected rabbit's or sheep's brain are used for prophylactic immunisation.

Rabies can be transmitted experimentally to the dog, rabbit, guinea-pig, mouse and other animals. In rabbits, intracerebral inoculation produces paresis in seven to twenty-one days, and death occurs in fifteen to twenty-eight days. Infection can usually be produced through all the usual parenteral routes—intracerebral, intraneurral, intravenous, subcutaneous and intramuscular. *Post mortem* the characteristic Negri bodies are found in stained smears or sections of the brain. The virus becomes enhanced in virulence after repeated passage in the rabbit, until it produces rabies regularly in six to seven days and then becomes the "fixed" virus (as designated by Pasteur).

When a person is bitten by a dog suffering from rabies, prophylactic immunisation by the Pasteur method, or a modification, is carried out without delay, and when there is any doubt as to the condition of the dog, an accurate diagnosis is essential.

If available, the animal is kept under observation in strict isolation; survival for ten days would usually exclude a diagnosis of rabies, since rabid dogs invariably die within this period. If unmistakable symptoms of the disease are observed, the animal is killed and the diagnosis confirmed by laboratory examination. If the laboratory is at some distance, the head is removed and forwarded in 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. 108) or by Mann's or Lépine's stain (p. 116). For sections, the tissue is fixed in Bouin's or Zenker's fluid (pp. 121, 122) and stained as above. 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 the rabbit with brain emulsion is also carried out as described on page 284, and if the virus is present, paresis and death occur as described above.

Pasteur's method of immunisation of persons bitten by a rabid dog was to inject suspensions of spinal cords from rabbits infected with "fixed" virus, the virulence or pathogenicity of which had been reduced to varying degrees by drying. The first injection was a suspension of cord dried for twelve days, and subsequent daily injections of progressively more virulent nervous tissue were given. This attenuated virus vaccine has been largely superseded by the phenolised virus referred to above.

Pseudo-rabies (Aujeszky's Disease—Mad Itch)

This is a disease affecting the central nervous system and occurring in dogs, cattle, horses, pigs, sheep, rats and certain other animals. 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 blood and organs. It is not found in the saliva. Antigenically it is distinct from the virus of rabies and has no connection whatsoever with the latter. It has been cultivated by tissue-culture methods. The size of the virus is about 150 m μ . 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.

HERPES SIMPLEX

If the serous fluid from a herpes vesicle be inoculated on the scarified cornea of a rabbit, there follows a severe keratitis which can be propagated indefinitely through a series of animals. Some of the rabbits show cerebral symptoms and later die of encephalitis. Herpes virus is present in the brain unassociated with other organisms. It is very

infective for rabbits. Recovered animals show a marked immunity.

The virus is filterable through Berkefeld and coarse Chamberland filters. Its size is about $150\text{ m}\mu$. It can be cultivated easily in minced rabbit testis with rabbit plasma and Tyrode's solution, and in the chorio-allantoic membrane of the developing chick. Visible focal lesions are produced in the membrane. Even after prolonged culture the virus retains its neurotropic properties. In the corneal epithelium and nerve cells of infected rabbits, nuclear inclusions ("Lipschütz bodies") may be demonstrated. These are acidophilic intranuclear bodies, at first small, but later they coalesce and occupy the greater portion of the nucleus. Typical inclusions can also be demonstrated in the cells of the culture. Elementary bodies can be demonstrated by ordinary microscopy after suitable staining, as in the case of the virus bodies of vaccinia (p. 117).

This virus also causes the condition of *aphthous stomatitis* in young subjects. It seems probable that it is often present in an inactive phase in the mouths of normal persons, but becomes active as a result of some lowering of resistance, e.g. during a febrile illness—hence the name *herpes febrilis*. Specific neutralising antibodies have been demonstrated in the blood serum of normal persons.

Herpes genitalis is due to a virus similar to that of herpes simplex.

HERPES ZOSTER

This disease is characterised by an erythematous eruption of the skin, later becoming vesicular, its location corresponding to the cutaneous distribution of a sensory nerve. The trunk is most frequently affected, on one side only, the distribution of the eruption being that of intercostal nerves (shingles). Other areas affected are the head and genito-urinary region. As compared with varicella, to which it is related epidemiologically, herpes zoster is more frequent in adults and not so common in children.

The condition is aetiologically distinct from herpes simplex, and the causal agent is identical with or very closely allied to the virus of varicella. Elementary bodies somewhat similar to those of vaccinia can be demonstrated microscopically in specially stained films of the vesicle fluid. These virus bodies are also demonstrable by electron microscopy and resemble in size and shape those of varicella and the pox

diseases (*q.v.*). Intranuclear acidophilic inclusions have been observed in epithelial cells. Inoculation of the skin in the human subject with fluid from the vesicles may produce the typical vesicular lesions, and immunity to re-inoculation results. The virus does not grow in the chorio-allantoic membrane of the developing chick.

In addition to its dermatropic properties the virus is neurotropic, as lesions are also found in the posterior root ganglia.

VARICELLA (CHICKEN-POX)

Varicella shows close epidemiological association with herpes zoster and, like the latter, is due to a filterable virus. Thus, cases of herpes zoster have been followed by outbreaks of chicken-pox. In the lesions of varicella, elementary bodies similar to those of the other pox diseases can be demonstrated; by electron microscopy they appear to be brick-shaped and measure about 242 by 210 m μ . Intranuclear inclusions, like those in herpes zoster, have been observed in the epidermal cells. Serological experiments have shown a pronounced cross-immunity between the varicella and herpes zoster viruses, which are now regarded as being identical, or very closely allied.

Material from herpes zoster cases may produce typical varicella when inoculated into susceptible persons. This is not the case in children who have had varicella, while children inoculated with zoster material are subsequently immune to inoculation with varicella.

LYMPHOCYTIC CHORIO-MENINGITIS

In this condition the patient becomes acutely ill with symptoms of meningitis, but recovers in a short time. Fatal cases are very rare. The pathological condition is an acute leptomeningitis affecting particularly the basal meninges and producing an exudate of lymphocytes throughout the entire ventricular system.

The cerebro-spinal fluid shows an increase in lymphocytes, usually about 200 per c.mm. but rising sometimes to 1500. The spinal fluid is sterile by ordinary methods of cultivation.

The disease is due to a virus, about 50 m μ in size, which passes Berkefeld, Seitz, and Chamberland L₁, L₂ and L₃ filters. It is present in the blood in the early stage of the disease and later in the spinal fluid when meningitic signs appear. Inclusion bodies, acidophilic and intranuclear, have been described in the brains of infected animals.

The virus can be cultivated in the chorio-allantoic membrane of the chick, but no recognisable lesions develop. The virus is easily destroyed by heat (at 55° C. for twenty minutes), but resists 50 per cent. glycerol for many months.

The disease can be transmitted by intracerebral inoculation in guinea-pigs, mice and monkeys. If mice are inoculated subcutaneously there is no apparent infection, but intracerebral injection is fatal in seven to nine days. Guinea-pigs are killed by several strains of the virus following subcutaneous, intraperitoneal or intracerebral inoculation. The infection occurs naturally in mice, and the virus is present in the urine and nasal secretion of these animals. The source of infection in man is still obscure. Mice act as a reservoir, and the infection may be conveyed by the excreta and by dust, although in America it has been suggested that the disease is insect-borne.

Neutralising antibodies may be found in the serum of recovered persons and also of contacts. Similar antibodies occur in infected animals. The serum may be tested by mixing it with the virus and, after incubation for four hours, injecting the mixture intracerebrally into mice. All the strains isolated are serologically identical.

In diagnosing the condition the blood and cerebro-spinal fluid should be examined to exclude cultivable organisms, and cell counts of the cerebro-spinal fluid are carried out. The fluid does not coagulate on standing (*cf.* tuberculous meningitis). Mice (from virus-free stocks) are inoculated intracerebrally with 0·02 ml. spinal fluid, and guinea-pigs are inoculated intraperitoneally or intracerebrally with whole blood and cerebrospinal fluid. If the virus is present, the animal becomes ill in about six days with ruffled fur and convulsive movements, dying about the seventh to ninth day after injection.

The serum of recovered cases gives a complement-fixation reaction with infected guinea-pig spleen as the antigen. Complement-fixing antibodies appear much earlier than neutralising antibodies which may not be demonstrable till several months after recovery and may sometimes remain completely absent.

Pseudo-lymphocytic-chorio-meningitis has been described as due to a virus which is 150–225 m μ in size, in contrast to the smaller dimensions of the virus of true lymphocytic chorio-meningitis. The two diseases also differ immunologically.

Swineherd's disease (pseudo-typoid meningitis) occurring in certain parts of Europe is a disease characterised by lymphocytic meningitis which may be preceded by intestinal symptoms. The infective agent is present in the blood, urine and, in some cases, spinal fluid. The infection is experimentally transmissible to pigs, mice, etc., and may occur naturally in pigs from which it is passed to the human subject. Some observers have regarded the infection as due to a virus, but it

should be noted that a leptospiral infection transmitted from pigs occurs in swineherds (p. 556) and the aetiology of "Swineherd's disease" is still somewhat indefinite.

VARIOLA (SMALLPOX) AND VACCINIA

Variola or Smallpox is due to a virus, but filtration experiments have not always been successful owing to the adsorption of the virus by the filter candle and protein material in the suspension. In the skin lesions, inclusion bodies, "Guarnieri bodies," may be seen and also minute granules, "elementary bodies" or "Paschen bodies." Similar inclusion and elementary bodies are found in vaccinia (*vide infra*).

The virus from cases of smallpox, when inoculated by scarification into various species of monkeys, produces typical localised "pocks," but only occasionally a general eruption with fever and illness. The virus from the human disease or the experimental infection in monkeys, when transmitted serially in rabbits or calves by skin inoculation, may become modified or mutated to vaccinia virus (*vide infra*), but successful adaptation, for unknown reasons, is rare.

A solid immunity is produced by an attack of the disease, or by "vaccination" with vaccinia virus.

The virus of *spontaneous vaccinia* or *cow-pox* (p. 37) is very closely related to that of smallpox, and, as shown above, the smallpox virus can be so modified by passage in calves as to assume the features of the vaccinia virus. In fact most laboratory strains of vaccinia virus have originated in this way. Thus the term "vaccinia virus" now refers to a virus with certain features irrespective of its origin. This virus, like that derived from smallpox, is not readily filterable. Localised pocks are readily produced in calves and rabbits by inoculation of the scarified skin. "Vaccination" in human beings is the introduction of vaccinia virus into a small area of skin, with subsequent development of a pustule or pock. Artificial immunisation against smallpox by this method depends on the close antigenic similarity between the smallpox and vaccinia viruses.

Downie¹ has shown by cross-absorption of immune sera with elementary body suspensions that there is an antigenic difference between the virus of *spontaneous cow-pox* and laboratory strains of vaccinia virus. The results of agglutination, complement-fixation and neutralisation tests with absorbed sera suggest that although the antigens

¹ Downie, A. W., *Brit. J. Exp. Path.*, 1939, **20**, 158.

of cow-pox and vaccinia viruses are very much alike, there are qualitative differences in the heat-labile component (p. 593). These, and certain differences in the lesions produced experimentally, would indicate that the virus of *spontaneous* cow-pox is not completely identical with the strains of vaccinia virus examined.

The inoculation of rabbits or calves with vaccinia virus, after scarification of the skin, is followed by an inflammatory reaction on the third day, which becomes papular. By the fifth day the papules have developed into vesicles filled with clear fluid which soon becomes purulent. About eight to ten days after inoculation scabbing occurs. Such reactions are obtained even with a high dilution of vesicular fluid, e.g. from 1 : 1000 up to even 1 : 100,000. If the virus be injected intravenously in large amounts into the rabbit, generalised lesions occur not only in the skin but in the internal organs. In the epithelial cells, after cutaneous inoculation of the rabbit, cytoplasmic inclusion bodies (Guarnieri bodies) may be seen, and in the early stages large numbers of elementary bodies (Paschen bodies) about $0\cdot2\mu$ in size.

These elementary bodies, which appear to be the infective units of the disease, were first noted and described by Buist, of Edinburgh, in 1886.

They have been found to consist of protein, carbohydrate, and lipoid with small amounts of copper, riboflavin and biotin. This is illustrative of the constitution of the larger viruses. As observed by the electron microscope the virus bodies of vaccinia present a brick-shaped appearance, as contrasted with the rounded bodies of certain other viruses, e.g. herpes simplex, and are about $260\times210\text{ m}\mu$ in size.

Elementary bodies have been separated and purified by high-speed centrifuging, and they can be agglutinated by a specific antiserum from recovered animals, while complement-fixation tests can be carried out with the separated bodies as antigen.

Cultivation.—The virus of vaccinia has been successfully cultivated, first in a tissue culture and later by a simplified method introduced by H. B. and M. C. Maitland. The medium consists of minced fresh adult kidney, either from a hen or a rabbit, Tyrode's solution and fresh serum. Bacteria-free vaccinia virus is mixed with 0.3 ml. minced kidney tissue, 3 ml. fresh rabbit serum are added, and the whole is diluted to 10 ml. with Tyrode's solution. The mixture is distributed in 2 ml. amounts in Carrel flasks and incubated at 37°C . The kidney cells do not proliferate but remain alive for five to six days, and, in association with them, multiplication of the virus takes place. The virus likewise has been cultivated in a simple medium of chick embryo and Tyrode's solution. The virus also grows well in the chorio-allantoic membrane of the developing chick, producing characteristic lesions or plaques. Elementary bodies can be demonstrated in cultures.

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 out and collected 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 the lymph must be kept at a temperature below 0° C. if its potency is to be maintained for any length of time.

A modification adopted at the Lister Institute (McClean¹) 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 48 hours; this generally lowers the bacterial count to the requirement of the Therapeutic Substances Regulations though if necessary the treatment may be prolonged for a further 24 hours; after this two parts of glycerol are added and the lymph is stored at -10° C.

Vaccination has usually been carried out by application of calf-lymph to a superficially scarified area of the skin. In infants and young children three $\frac{1}{4}$ inch scratches (made with a Hagedorn needle) may be inoculated; in older children and adults vaccinated for the first time it has been recommended that only one such inoculation should be made since in such cases post-vaccinal encephalitis may occur, though this is extremely uncommon (p. 594). In a person lacking immunity a papule forms at the site of inoculation in 3-4 days and this becomes vesicular in 5-6 days; in 8-10 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 21st day, leaving a

¹ *Lancet*, 1949, 2, 476.

depressed scar. In persons who have been recently vaccinated and possess a satisfactory immunity there may be no reaction or a papule appears more rapidly than in the non-immune subject and resolves without the development of a 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, a "vaccinoid" reaction is noted : a papule appears quickly, becoming also vesicular and pustular more quickly than in the completely non-immune person.

Multiple Pressure Method.—This is now tending to supersede the scarification method. A drop of lymph is 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. In this way the inoculum is forced into the deeper layers of the epidermis. The area inoculated may be only one-eighth 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 and less severe reactions.¹

It has been suggested that instead of virus derived directly from animals, cultivated virus (from the developing chick or chick-embryo medium) should be used for smallpox immunisation, the material being injected intracutaneously. Further evidence from mass vaccinations is still needed to prove that the resultant immunity is as durable as that following vaccination with "calf-lymph."

Serological Diagnosis.—Craigie and Tulloch's modification of Gordon's precipitation test has been used for distinguishing smallpox from chicken-pox. The crusts from the suspected case are dried in a desiccator overnight and ground in an agate mortar. The powder is weighed, triturated with saline, allowed to stand for an hour, and the mixture is centrifuged. Dilutions of the crusts (1 in 100 to 1 in 800) are made, and to these are added varying dilutions (1 in 20 to 1 in 80) of immune sera obtained by injecting rabbits with saline extracts of vaccinia crusts from a vaccinated rabbit. On incubation, if the case is one of smallpox, flocculation will occur in some of the tubes, there being optimum dilutions of crusts and serum which give the maximum flocculation. Chicken-pox crusts yield no flocculation. A similar experiment with vaccinia crusts from the rabbit is set up at the same time to serve as a positive control.

Complement-fixation tests are also positive with smallpox or vaccinia crusts and a known immune rabbit serum. This test has been strongly recommended by Craigie and Wishart, since not only is it 8-10 times

¹ See *Bacterial and Virus Diseases*, H. J. Parish, Edinburgh, 1951.

more sensitive than the flocculation test, but it is more easily carried out. The crusts of 6-8 lesions (0.008 gram) are sufficient, and are prepared as described for the flocculation test. The test is carried out like the Wassermann reaction. A disadvantage is that occasionally crusts may show marked anticomplementary properties.¹

It may be noted here that the antigen of the vaccinia virus has been shown to be composite, and two components, heat-labile and heat-stable (at 70° C.) respectively, have been differentiated. Corresponding antibodies have been demonstrated in immune sera.

Paul's Test.—This consists in light scarification of a rabbit's cornea and application of material from the skin lesion to the eye. After forty-eight hours, if the lesion is variolous, small elevated spots are seen on the cornea, and can best be observed if the eye is enucleated and placed for a minute in a solution of 2 parts saturated aqueous mercuric chloride and 1 part absolute alcohol; under these conditions the lesions appear white and opaque.

Chorio-allantoic Cultivation (p. 620).—This has been regarded as the most delicate diagnostic test. Typical plaques appear in seventy-two hours. (Growth in the chorio-allantoic membrane also differentiates variola and vaccinia; the plaques of the latter appear in forty-eight hours, and are larger and often accompanied by haemorrhage and death of the embryo.)

Microscopic Diagnosis.—van Rooyen and Illingworth² have shown how early smallpox can be diagnosed and differentiated from chickenpox by the recognition of the characteristic elementary bodies in stained films from the skin lesions. The method is as follows: after cleansing with ether, the central parts of several lesions are scraped with a sharp-pointed scalpel, and films are prepared; these, after drying, are treated with normal saline for a few minutes and again dried; they are then treated with a mixture of equal parts of alcohol and ether for three minutes and dried; they are stained by Paschen's method (p. 117). Recently electron microscopy has been suggested for diagnosis.

Summary of Procedure in the Laboratory Diagnosis of Smallpox.—If a prodromal eruption is present or a papular eruption, Paul's test and examination for elementary bodies are carried out.

If the eruption is vesicular and the vesiculation early, the microscopic test for elementary bodies is still practicable.

If the vesicles are well developed, Paul's test and the complement-fixation test are carried out with vesicle fluid.

If pustules are present, their contents or crusts are used for Paul's test and the flocculation or complement-fixation reaction.

The cultivation test in the chorio-allantoic membrane, as mentioned above, constitutes a most delicate means of diagnosis, but is only applicable in special laboratories in which nine to twelve days chick embryos are available for the immediate diagnostic test.

¹ For full details, see Craigie, J., and Wishart, F. O., *Canad. Publ. Hlth. J.*, 1936, 27, 371.

² *Brit. Med. J.*, 1944, 2, 526.

For details of collection of specimens in the laboratory diagnosis of smallpox, see F. O. MacCallum, *J. Roy. San. Inst.*, 1952, 72, 112.

Inhibition of vaccinia virus haemagglutination by human serum. The vaccinia virus agglutinates the red corpuscles of certain species, like the influenza virus (p. 597), and the reaction is inhibited specifically by immune serum. This inhibition by serum from suspected cases of smallpox has recently been studied as a diagnostic procedure.

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. This is noted particularly in the white matter of the cerebrum, mid-brain and pons, and also in the cord. Congestion and infiltration with mononuclear cells are also present.

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 is an entirely different disease, due possibly to a separate virus or toxic agent, but activated by the antecedent disease. 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.

MEASLES

Knowledge of the virus of measles is still somewhat limited. The disease is not communicable to ordinary laboratory animals, but it has been claimed that, under favourable circumstances, a modified form of measles may be produced in the monkey by inoculation with blood taken at the height of the disease. A similar result has been obtained by intratracheal inoculation of filtrates of naso-pharyngeal washings from early cases. The experimental condition has been transmitted through a series of animals and the infective agent shown to be filterable.

Measles has also been transmitted experimentally to the human subject by inoculation with blood and secretions from the respiratory passages.

The virus can be grown in the chorio-allantoic membrane of the developing chick, infective blood or filtrates of throat washings being used as the inoculum. The cultivation can

be carried out serially, and a measles-like disease has been reproduced in young monkeys from the culture. Cultivated virus has also reproduced the disease under experimental conditions in the human subject.

Such cultivated virus, after numerous passages in eggs, becomes attenuated, and has been used by some investigators as a vaccine for active immunisation of children.

Serum from convalescent patients has marked protective powers, and if injected in amounts of 5-10 ml., before the fifth day after exposure to infection, will usually prevent the disease in young children. If given after this time, the disease is modified and the risk of complications considerably reduced.

If convalescent serum is not available, serum from young adults ("Adult measles serum") who have had measles in childhood is also efficacious in attenuating an attack of the disease, although a much larger dose must be given.

The doses of Adult serum are :—Under 3 years of age, 10 ml.; 4 years of age, 15 ml.; 5 years of age, 20 ml. It is not recommended that Adult measles serum should be given to children over 5 years of age owing to the large quantities of serum required. If prophylaxis or attenuation of the disease is desired in such children, convalescent measles serum, 15-20 ml., should be used.

A preparation made from human placenta termed "Placental globulin" is also used in measles prophylaxis. The dose is 2 ml. given intramuscularly.

Gamma (γ) globulin from human adult serum has likewise been employed with success in the prevention of measles. It has been prepared by precipitation with alcohol, and also recently by ether precipitation.

RUBELLA (OR GERMAN MEASLES)

Knowledge of the virus of this disease is still incomplete. It has been shown that monkeys are susceptible to experimental transmission by inoculation with blood or nasal washings from cases, and the illness produced appears to bear a resemblance to the human disease. It is of special interest and importance that congenital lesions affecting the ear, eye, heart and brain may result in children born from mothers who have suffered from rubella in the first three months of pregnancy. Deaf-mutism is a special example. Apparently the virus infects the foetus *in utero* and attacks particular tissues.

THE COMMON COLD (INFECTIOUS CORYZA)

The causative agent of the common cold is a filterable virus, and can be transmitted experimentally to man by the inoculation of filtrates of nasal secretion from cases into the

upper respiratory tract. It has also been claimed that this virus is transmissible to chimpanzees, producing a condition similar to the human disease, and that it can be cultivated in tissue cultures ; but this has not been fully confirmed. Some workers, however, have obtained laboratory cultures of the virus in the embryonated egg. Certain findings have also suggested the possibility of different types of virus being concerned in the aetiology of infectious coryza. All recent studies have fully confirmed the virus aetiology of the disease and its experimental transmission to the human subject, but the characters of the virus and the factors involved in the causation of the disease still await further elucidation.

INFLUENZA

In 1933 Wilson Smith, Andrewes and Laidlaw showed that influenza is due to a filterable virus which is transmissible to ferrets. Filtrates from nasal washings of influenza patients, when instilled intranasally into these animals, produce a characteristic train of symptoms. After forty-eight hours the temperature rises, the animal shows signs of general illness, and on the third day catarrhal symptoms appear. The eyes are watery, and there is a variable amount of watery discharge from the nose. The disease lasts for a few days only and is not fatal. It can be transmitted serially in ferrets either by contact or by intranasal instillation of virus material. No other method of inoculation induces the disease. Immunity follows recovery from the infection and persists for about three months. The virus is found only in the nasal mucous membrane of the animal and not in blood, lymph glands or spleen. The infection can be transmitted to mice after adaptation in ferrets. In mice, lung lesions occur, varying from small haemorrhages to complete consolidation. The infection has also been transmitted experimentally to hedgehogs. It has been estimated that the size of the virus is about $100\text{--}125\text{ }\mu$. By electron microscopy the virus bodies usually appear as rounded or oval structures but filamentous forms have also been demonstrated.

Strains of the influenza virus have been adapted for growth in the chorio-allantoic membrane of the developing chick. At first the lesions associated with growth of the virus are trivial, but the virulence increases by passage. The virus multiplies rapidly in the allantoic sac, and this is the main source of virus used in experimental work. Rapid growth also occurs in the amniotic cavity, and direct intra-

amniotic inoculation of throat washings—either filtered or treated with penicillin—is now used as a standard method of isolation of the virus.

The disease can be induced in ferrets only with material from acute influenza cases, and not from normal persons or those with common colds. Serum from recovered animals neutralises the virus, while human serum from influenza convalescents also neutralises the virus. Even serum from normal persons may have a neutralising effect, probably due to antibodies resulting from previous infection.

All recent work has indicated that there are several varieties of the virus differing in antigenic characters. The original virus, described above, has been designated "influenza A virus," and virus strains subsequently isolated with similar antigenic characters are given the same designation. An "influenza B virus" was later defined and has been found in outbreaks in various parts of the world; it is completely distinct serologically from the A virus, and is more difficult to transmit to ferrets and mice. As in the case of virus A influenza, sera from patients convalescent from virus B infection show specific neutralising antibodies. Within each of these two serological types, multiple subtypes have now been recognised by further serological analysis. A "C" type of influenza virus quite distinct serologically from A and B has recently been described.

Vaccination of the human subject against influenza, with formolised concentrated virus grown in the allantoic sac, has been shown to be of value in man if given shortly before exposure to infection. The vaccine contains several strains of the A and B viruses. The immunity to influenza virus, either natural or acquired by vaccines, is not of long duration.

By immunisation of horses an antiserum has been produced which, in mouse experiments, is capable of neutralising or inactivating the virus.

In 1941, Hirst described the phenomenon of *agglutination of fowl's red cells* by preparations of *influenza virus* (p. 622). (The red cells of various birds, reptiles, amphibia, man and certain rodents are likewise agglutinated.) He infected chick embryos with the influenza virus and showed that the egg fluid contained an agglutinating substance. He further showed¹ that serum containing neutralising antibodies for the influenza virus (as determined by the protection of mice from the pathogenic effect of the virus) inhibited the agglutination of fowl's red cells, and that such inhibition was specific for the particular type of virus con-

¹ Hirst, G. K., *J. Exp. Med.*, 1942, 75, 49.

cerned. Normal sera also inhibit the agglutination reaction, but in a much lower dilution. The inhibition of agglutination demonstrates *in vitro* the increase of influenza virus antibodies in human serum after an attack of influenza as readily as the protection test in mice, for which it is a simple substitute. (It may be noted here that certain other viruses, e.g. vaccinia and mumps, also produce this haemagglutination reaction.)

It has been shown that there is a *virus-inactivating agent* present in *human nasal secretion* which is capable of neutralising all types of influenza virus, and also other viruses such as those of herpes, louping-ill, etc.¹ It has no action on the viruses of vaccinia, ectromelia, fowl-pox, psittacosis, or pseudo-rabies. There is a slight but significant action on the virus of poliomyelitis. Inactivation proceeds at 37° C. but not at 0° C., while activity is destroyed by boiling for ten minutes. There is no correlation with the lytic agent, lysozyme, found in tears, etc.

The *complement-fixation reaction* can also be utilised for the serological identification of the types and subtypes of the influenza virus with specific immune sera, and for determining the presence of antibodies in human serum with known cultivated strains of the virus.

Two antigenic factors have been demonstrated in egg cultures : one represented by the virus bodies and the other called "soluble" represented by a much smaller particle. The latter is non-infective, non-haemagglutinating but still capable of acting as antigen in the complement-fixation test.

Influenza virus is closely related to that of *swine influenza*, and, although they are different antigenically, there is a common antigenic factor. It should be noted that in swine influenza two separate agents are operative—namely, the virus, and *Haemophilus suis*. The virus alone produces a mild, almost unrecognisable disease, while *H. suis* by itself is harmless. In the natural disease the two agents act together, the virus being the essential factor. When the virus of swine influenza is inoculated in the ferret, however, a febrile catarrh is produced and the disease is not modified by simultaneous inoculation with *H. suis*. Swine influenza virus can be established in the mouse, producing pulmonary consolidation.

"Influenza Pneumonia" of Calves.—In recent studies of this condition a virus has been demonstrated and transmitted to mice. Enteritis—"scours"—may be associated with the pneumonic condition.

Influenza-like infections are also observed in other animals and are probably due to viruses : thus, a virus has been demonstrated in *equine influenza*.

Diagnosis of Influenza

The methods at present available may be summarised as follows :

(a) nasal inoculation of ferrets with nasal washings or sputum from

¹ Burnet, F. M., Lush, D., and Jackson, A. V., *Brit. J. Exp. Path.*, 1939, 20, 377.

suspected cases ; (b) a week after the commencement of the illness or in convalescence, the demonstration in the serum of specific antibodies by the neutralisation test in animals, the inhibition of the haemagglutination reaction and the complement-fixation test : for this purpose, known strains of the A and B viruses are used ; the results should if possible be compared quantitatively with those obtained by testing similarly the sera of cases at the commencement of the illness ; a "rising titre" demonstrated in this way is conclusive evidence of infection ; (c) amniotic inoculation and egg-cultivation, the cultivated virus being identified serologically with specific immune sera as referred to above.¹

YELLOW FEVER

The infection occurs naturally in various species of monkeys and probably other mammals in tropical Africa and South America, and is spread by forest mosquitoes, e.g. *Aedes simpsoni*, *Aedes africanus*, *Aedes leucocelaenus* and *Haemagogus capricorni* (in Brazil). In these areas human infection may occur by mosquito transmission from monkeys—the so-called "Jungle Yellow Fever"—but the typical epidemic spread in man is normally by *Aedes aegypti*, the well-known "domestic" mosquito. In the Sudan epidemic of 1940, however, the vectors were the "non-domestic" species *Aedes vittatus* and *Aedes furcifer (taylori)*. The disease can be transmitted experimentally to various monkeys, but the most susceptible, and hence the animal of choice for experimental work, is *Macaca mulatta*. Infection is brought about either by the bite of infected mosquitoes or by the inoculation of patients' blood during the early days (three to four) of the disease. The virus can pass fine filters, its size being about 22 m. μ . The pathological changes in the livers of fatal human or monkey cases consist of a characteristic coagulative hyaline necrosis (the "Councilman lesion") scattered diffusely throughout the liver, but often more marked in the mid zone of the lobules, and preceded or accompanied by fatty degeneration and cloudy swelling. Specific intranuclear inclusions ("Torres bodies") are constantly present in the livers of monkeys, but in only about 25 per cent. of human cases, and in the latter are therefore not a diagnostic criterion. The serum of convalescent cases has strong protective properties.

The virus can be transmitted to mice by intracerebral inoculation and produces an encephalitis; after twenty or more passages it becomes "fixed" (with neurotropic properties).

¹ Burnet, F. M., *Brit. J. Exp. Path.*, 1940, 21, 147.

This virus produces encephalitis in monkeys and some rodents, such as guinea-pigs, field voles, squirrels, etc. The neurotropic virus leads to the production of immune bodies which protect against the viscerotropic strain.

The virus has been cultivated in the chorio-allantoic membrane of the developing chick, in a chick-embryo medium, and in the carcinoma cells of tumour-bearing mice.

A considerable amount of work has been carried out on prophylactic immunisation against yellow fever. Several forms of vaccine have been adopted ; but that in general use is prepared from ten to eleven days chick embryos infected with the attenuated strain of the virus ("17 D"). The infected embryos are ground up, and frozen and dried *in vacuo*. The vaccine is standardised in terms of lethal doses for the mouse ; the usual dose for immunisation being 500–1000 mouse doses. For administration the dried material is made up in 0·5 ml. sterile water or saline and this must be injected within 30 minutes. Immunity is produced after ten to fifteen days and full protection is maintained for at least four years, and probably longer periods.

In surveys of yellow fever districts, tests of immunity to the disease are made by the "mouse protection test." The mouse is injected intraperitoneally with the serum to be tested and "fixed" virus (infected mouse brain), while a small quantity of starch solution is injected intracerebrally. If the serum contains no antibodies the mouse dies of encephalitis within ten days.

Diagnosis of Yellow Fever

The diagnosis of yellow fever has assumed great importance with increasing air travel, and one or more of the following methods is employed :

(a) *Histological Examination*.—In fatal cases a piece of liver is removed either at *post-mortem* examination or by the viscerotome, and examined histologically for the characteristic changes (*vide supra*).

(b) *Isolation of the Virus*.—During the first three or four days of the illness the virus is present in the blood, as mentioned above, and the serum is injected intracerebrally into mice. When signs of encephalitis appear, the mouse is killed and an emulsion of the brain is injected subcutaneously into a *Macaca mulatta* monkey, in which animal further investigations are carried out.

(c) *Mouse Protection Test*.—The serum is taken early in the illness and again during convalescence (ten to fourteen days), and the mouse protection test is carried out. A change from a negative to a positive reaction is definite proof that the illness was yellow fever.

It should be noted that these methods of diagnosis demand considerable experience and special laboratory resources.

Psittacosis and Ornithosis

Psittacosis is an epizootic disease of parrots and parakeets, which may affect man, often with fatal results. Recent work suggests that other species of birds may suffer from the disease under natural conditions; its occurrence in fulmar petrels has been established and shown to be the source of human cases in the Faroe Islands and Iceland. The disease can be transmitted experimentally to birds of the parrot family and many species of finch, and to mice, monkeys and guinea-pigs. The virus is present in the organs of the infected host and is found in greatest quantity in the spleen and liver, and also in the lungs when these organs are involved, as is often the case in human psittacosis. It passes through the coarser filters, such as the Berkefeld V and Chamberland L_{1a}. The average size of the virus is estimated to be about 450 m μ but smaller forms also occur (*vide infra*) and it is these which render it filterable. Its resistance to glycerol, though greater than that of the non-sporing bacteria, is not so great as that of most viruses. Psittacosis virus is present in the tissues in the form of visible bodies which stain readily with Giemsa's solution and by rickettsial staining methods, such as Castaneda's. In film preparations of virulent material stained by Castaneda's method, round or slightly oval elementary bodies are seen, the clear blue colour of which contrasts with the pink of the cells and background; these bodies are found lying free or inside reticulo-endothelial cells which are sometimes filled to bursting point. The elementary bodies can be freed from cellular material by submitting a suspension of virulent material to fractional centrifugation. The washed particles so obtained are agglutinated specifically by an anti-psittacosis serum and fix complement with it. Like all other viruses, psittacosis virus requires living cells for its multiplication, and it appears to grow within the cells. In the laboratory it can be readily cultivated in the embryonated egg, e.g. in the yolk-sac. Bedson and Bland showed that this virus, when multiplying, passes through a regular sequence of morphological changes. The elementary body or filterable phase on entering a cell changes into a larger form about 1 μ in size, which appears to multiply by division. As multiplication proceeds there is a progressive diminution in size of the virus bodies, and in forty-eight to seventy-two hours after infection of the cell they are all once more of the minute elementary type. The multiplying virus also forms a more or less compact colony in the cytoplasm of the cell, consisting of virus bodies embedded in a homogeneous matrix of basophilic material. (A similar developmental cycle has been demonstrated in the case of the viruses of lymphogranuloma inguinale and inclusion conjunctivitis.)

Complement-fixation tests with an antigen of infected mouse spleen emulsion or cultures of the virus in the yolk sac of the developing chick are employed for the diagnosis of psittacosis in the human subject and for detecting the disease in imported parrots (p. 623).

The psittacosis virus is susceptible to chloromycetin and aureomycin and these antibiotics have proved of value in the treatment of cases.

The relationship of psittacosis virus to other viruses of similar morphology.—Recent work has shown that in addition to birds of the parrot family and fulmar petrels, pigeons and the domestic fowl may suffer from psittacosis. Since the strains of virus from these different sources, though similar in their antigenic structure, differ in their host-range and pathogenicity, it would be more correct to speak of viruses of the "psittacosis group" rather than of psittacosis virus; while the term "ornithosis" is suggested for general application to these infections, psittacosis being retained for infections in or from the parrot family. It has been recognised for some time that there is a close resemblance in morphology and staining characters of the viruses of psittacosis, lymphogranuloma inguinale (p. 604), trachoma (p. 605) and inclusion conjunctivitis (p. 606). They all stain by Castaneda's method and might be termed the "Castaneda-positive group." Included in this group are the mouse pneumonia virus of Nigg¹ which occurs in a latent infection among many mouse stocks and can be activated by various means, and the viruses of meningo-pneumonitis of Francis and Magill,² and of atypical pneumonia (p. 611) of Eaton, Beck and Pearson.³ The virus of meningo-pneumonitis came from a ferret which had been inoculated with naso-pharyngeal washings from a case of influenza; it behaves like a strain of psittacosis virus of pigeon origin. All these viruses are antigenically related. This has an important practical bearing on the serological diagnosis of infections caused by them. For example, the complement-fixation test for psittacosis or lymphogranuloma inguinale cannot be considered specific; the sera from cases of trachoma and inclusion conjunctivitis may fix complement with a psittacosis antigen, and the Frei test (p. 604) may be positive not only in lymphogranuloma inguinale but also in infections due to the psittacosis group. The interpretation of these laboratory investigations has to be made in the light of the clinical and epidemiological findings.

Pacheco's disease of parrots, though producing a similar clinical picture in the bird, differs from psittacosis in many respects. It is not transmissible to other species of birds and does not infect man. The virus has not been demonstrated microscopically but produces nuclear inclusions in affected cells.

Diagnosis of Psittacosis

(1) *Suspected human case of the disease.*—(a) *Acute.*—Emulsify sputum (which contains the virus) in saline or phosphate buffer solution (pH 7·4), and centrifuge to deposit cells and the majority of the bacteria. Inoculate four to six mice intraperitoneally each with 0·5 ml. of the supernatant fluid. If the case is one of psittacosis, some or all of the mice will die in seven to ten days showing signs of experimental

¹ *Science*, 1942, **85**, 49.

² *J. Exp. Med.*, 1938, **68**, 147.

³ *J. Exp. Med.*, 1941, **73**, 641.

psittacosis, namely a glairy peritoneal exudate, and enlargement of the spleen and liver. Films from the exudate and spleen, when stained with Castaneda's or Giemsa's stain, show typical virus elementary bodies. It is recommended for confirmation also to inoculate an emulsion of the infected mouse spleen into two further mice, which should die in seven to ten days with similar symptoms, the virus bodies being demonstrated microscopically in the peritoneal exudate and spleen.

If the mice inoculated with the human material survive for ten days, they are then killed, the spleen is examined microscopically for virus bodies and an emulsion of the spleen is inoculated intraperitoneally into four to six mice. If none of these die within ten days, the case is presumed negative. If any of the mice show signs of disease or die, they are examined as above, and further mice are inoculated with spleen emulsion to confirm the diagnosis.

(b) If the case is *past the acute stage or convalescent*, a complement-fixation test should be carried out as described on p. 623.

(2) *Examination of birds.*—If a dead bird is received, the spleen is examined with the naked eye for enlargement. In a budgerigar the spleen may be so small that only a microscopical examination can be made and the liver is then used for inoculating mice. In the case of a parrot the spleen is large enough for both film preparations and animal inoculation. A suspension of the spleen or liver is inoculated into four to six mice which are observed for ten days. Animals dying within that period, if positive, show a glairy peritoneal exudate and enlarged spleen, in which the virus can be seen microscopically after staining by Castaneda's or Giemsa's methods. Further animals are inoculated with spleen emulsion and should die within seven to ten days with macro- and micro-scoptic evidence of psittacosis. If the mice inoculated from the bird survive for ten days, they are then killed, the spleens are examined microscopically and spleen emulsion is injected into further mice. If these are not dead within ten days, the case may be regarded as negative. If the parrots have recovered, blood may be obtained from the superficial ulnar vein under ether anaesthesia, and a complement-fixation test carried out.

Strict personal precautions should be taken by laboratory workers in handling materials from birds or experimental animals suffering from psittacosis, as these are highly infective to the human subject.

Mumps

The infection is transmitted by the salivary secretion and may be spread by secretion droplets. Injection of the saliva from early cases of mumps into the parotid duct in monkeys produces, after an incubation period of six to eight days, a rise in temperature, with swelling and oedema of the gland. The disease can be transmitted in series by inoculation with infective gland tissue. These animals can be inoculated intracerebrally and by injection of virus directly into the parotid gland. Cats have also been infected experimentally. The

infection in the monkey has been transmitted to human volunteers. The virus is filterable through Berkefeld filters, resists desiccation and is preserved by 50 per cent. glycerol. It is neutralised by serum from recovered cases of mumps. Elementary bodies have been observed by electron microscopy ; their size is about 200 m μ .

The virus may be grown in the allantoic, amniotic or yolk sacs. The cultivated virus agglutinates fowl and guinea-pig red cells like the influenza virus and immune serum inhibits this reaction specifically. These reactions can be utilised for the recognition of the virus in inoculated eggs and for demonstrating specific antibodies in human serum (*cf.* influenza). The complement-fixation reaction can likewise be used for antigen-antibody tests. Antigens for complement-fixation or allergic skin tests can be prepared from infected monkey parotid gland or chick embryo fluids or tissues. Formolised preparations of infected monkey parotid gland and chick embryo fluids have been investigated for efficacy as vaccines.

**Lymphogranuloma Inguinale (Lymphogranuloma venereum
or Climatic Bubo)**

This disease is transmitted by venereal contact. It has increased in frequency in this country and on the Continent. The appearance for a few days of a small primary sore on the external genitals is followed in males by a swelling of the lymph nodes in the groin ; in females where the primary sore is often situated in the vagina, the inflammatory reaction may affect the peri-anal tissues, leading to stricture of the rectum and elephantiasis of the genitals. The virus inoculated into the groins of guinea-pigs, monkeys and dogs produces a bubo; inoculated intracerebrally into monkeys, mice, guinea-pigs, dogs and cats, it gives rise to meningo-encephalitis. Intra- and extra-cellular elementary bodies have been described in human and animal lesions, and also larger inclusion bodies. A life-cycle similar to that of the virus of psittacosis has been described. Apparently the elementary bodies after invading cells develop into larger forms which by successive divisions constitute compact basophilic inclusions, and these finally divide into large numbers of elementary bodies. The virus passes Berkefeld, Seitz and coarse Chamberland filters. Its average size is estimated to be about 440 m μ . The virus has been grown in tissue cultures containing embryonic guinea-pig brain tissue and in the yolk sac of the chick embryo. Yolk-sac inoculation has been found to be the most reliable method of isolation of the virus. Serum from recovered cases is capable of neutralising the virus. Pus from infected lymph nodes, diluted 1 in 5 with saline and heated to 60° C., when inoculated intradermally in persons who have suffered from climatic bubo leads to a specific allergic reaction in the skin (Frei's reaction). Brain tissue from infected mice has been used for preparing the Frei reagent, but, if used, a control test with normal mouse brain should also be carried out. The yolk-sac culture

described above is a more satisfactory antigen for the Frei reaction and is used also for a diagnostic complement-fixation test.

Phlebotomus Fever

This disease of tropical and subtropical climates is transmitted by the sand-fly, *Phlebotomus papatasii*. From experiments on the human subject the transmission of the disease by this insect has been established and evidence has been obtained that the virus (present in the blood) is filterable. It has been cultivated in the chorio-allantoic membrane of the developing chick.

Dengue

This infection is transmitted by the mosquito, *Aedes aegypti*. Experiments have been carried out in the human subject, in whom the disease has been reproduced by the injection of unfiltered and filtered blood. The virus apparently persists in the circulation for five days. It can pass fine Chamberland filters. Some strains have been adapted to produce infection in mice by intracerebral inoculation. The virus has been cultivated in the chick embryo. Immunity following an attack of dengue usually lasts for one to four years.

Molluscum Contagiosum

In the epithelial cells of the lesions, large inclusion bodies, mainly acidophilic in their staining reactions, can be observed. These have been called "molluscum bodies" or "Henderson-Paterson bodies." Material from the lesions stained by Giemsa's or Paschen's stain shows elementary bodies. These are estimated to be about $300 \times 225 \text{ m}\mu$ in size.

It has been shown by van Rooyen that the molluscum body is a pear-shaped structure, about $30\mu \times 20\mu$ in size, inside the epithelial cell. By microdissection he has been able to isolate the molluscum body, which on being opened is found to contain numerous elementary bodies suspended in an amorphous matrix. The elementary bodies are quadrangular or brick-shaped like those of vaccinia. The molluscum body apparently constitutes a phase in the life-cycle of the virus. With filtrates from the lesions, positive inoculation results have been obtained in the human subject.

Common Wart

This has been reproduced in the human subject by inoculation of the skin with Berkefeld filtrates from the lesions. The incubation period is long (one to twenty months). Inclusion bodies have been described in the cytoplasm and nucleus of the cells.

Trachoma

Trachoma has been shown to be due to a virus which can be transmitted experimentally from one person to another. Filtration experiments with Berkefeld candles have been mainly negative, but positive

results have been obtained with collodion membranes. The size of the virus is stated to be about 200 m μ . Characteristic inclusions—"Halberstaedter-Prowazek bodies"—are present in the conjunctival epithelium. Stained by Giemsa's method they may be blue, violet or red in colour and are seen to contain small elementary bodies. Films from the conjunctival secretion stained by Giemsa's method show small reddish elementary bodies surrounded by non-staining material. The virus is easily killed by heating at 55° C. for fifteen minutes and dies rapidly outside the body. The disease can be transmitted to man, but it is doubtful if animals have been successfully infected, though it has been claimed that certain monkeys have been experimentally infected. Immunity to trachoma is of short duration.

A small motile Gram-negative haemophilic bacillus, *Noguchia granulosis*, has also been found associated with trachoma.

Inclusion Conjunctivitis

This term includes three forms of follicular inflammation of the conjunctiva, (a) swimming-bath conjunctivitis, (b) conjunctivitis of the same type but independent of infection in swimming-baths, and (c) non-bacterial ophthalmia neonatorum. Each of these is caused by the same virus and characterised by inclusion bodies in the cytoplasm of the epithelial cells of the conjunctiva. Swimming-bath conjunctivitis is rarely seen in Britain but is common in the continent of Europe and in the United States. It is spread by persons with infection of the urethra ("inclusion urethritis") and cervix uteri ("inclusion cervicitis") urinating in the bath. The disease may also be acquired by contact with infected persons or infants suffering from inclusion conjunctivitis. Non-bacterial ophthalmia neonatorum is contracted during passage by the infant through the birth canal of the mother. Thus, the virus of inclusion conjunctivitis localises primarily in the genito-urinary tract, and conjunctivitis is a secondary disease. The virus resembles, but is apparently distinct from, that of trachoma.

Diagnosis is based on the finding of inclusions within the epithelial cells of the conjunctiva. The lids are everted and the conjunctival surface swabbed free from pus. A drop of adrenalin solution is applied, the conjunctiva is scraped with a scalpel without drawing blood, and films are made on slides. These are fixed in methyl alcohol and stained with dilute Giemsa's solution (1 drop of stain in 1 ml. distilled water or buffer solution of pH 7.0) for twenty-four hours.

The cellular inclusions show two phases, (1) a minute elementary body, also found extracellularly, stated to be about 250 m μ in size, and (2) an "initial" body which may be 800 m μ in size. The elementary body penetrates the epithelial cell and develops into the larger initial body which later divides up into elementary bodies, and these infect new cells. The cycle of growth takes about forty-eight hours, and is similar to that of the psittacosis virus (p. 601).

The virus does not resist drying and is quickly killed by heat at 58° C. Experimental infection can be produced in several species of

monkey, including *Macaca mulatta* and the baboon, which develop a disease comparable with a mild human infection. The typical inclusion bodies are present in the animal lesions. These animals have also been infected experimentally with material from persons with "inclusion urethritis" and "cervicitis." Filtrates of virus-containing material are infective to animals. Experimental infections have been produced in the human subject. In monkeys the virus dies out after several passages. Intracerebral inoculation in the mouse and rabbit fails to produce encephalitis, while the chorio-allantoic membrane of the chick cannot be infected. No general immunity is conferred by an attack. Neither viricidal antibodies nor agglutinins have been demonstrated in the blood of convalescents. Complement-fixation tests with psittacosis antigen and convalescent serum have been negative.

Epidemic Keratoconjunctivitis

This condition has recently been studied in Europe and the United States, and investigators have obtained evidence of a virus causation. Thus, intracerebral inoculation of mice with infective material produced a convulsive condition and death. The estimated size of the virus is approximately $75\text{ m}\mu$. From the experimentally inoculated mouse the infection can apparently be re-transmitted to the human subject, a mild form of the disease resulting on conjunctival inoculation.

Infective Hepatitis

This disease, which has an incubation period of approximately twenty to forty days, is caused by a virus, though this aetiological agent has not been definitely isolated. It has been transmitted to volunteers by the oral administration of unfiltered faeces and Seitz filtrates of faeces collected in the acute stage and up to the eighth day of jaundice. Virus has likewise been demonstrated in blood and serum. Successful transmission with naso-pharyngeal washings from cases of the naturally occurring disease has not yet been reported.

From biopsy material obtained by liver-aspiration it has been possible to study the pathological process, and it has been shown that the jaundice is due to a hepatitis with liver cell necrosis. The lesions may be diffuse, zonal or mixed. The hepatitis usually resolves completely and rapidly. When the disease runs a longer course, some residual fibrosis in the portal tract may still be present after apparent recovery.

In recent years a clinically similar condition has occurred sixty to one hundred and thirty days after the injection of certain batches of measles convalescent serum, yellow fever vaccine (containing human plasma), pooled adult serum and mumps convalescent plasma. It has not been possible to reproduce the disease in any laboratory animal by the inoculation of these icterogenic blood products. Experiments in human volunteers have shown that the causative agent is present in the blood in the pre-icteric and icteric stages, and can be transmitted

by various parenteral routes. Findlay and Martin¹ have reported from West Africa successful transmission by intranasal inoculation of naso-pharyngeal washings collected in the pre-icteric and early icteric stages. According to MacCallum² it has not been possible to transmit the disease by oral administration of faeces from cases in which the condition was induced by injection of icterogenic blood products. The causative agent can pass through a Seitz filter and survive heating at 56° C. for an hour. It is inactivated by two and a half hours' exposure to ultra-violet radiation consisting of 2650 Å for one hour and 2537 Å for one and a half hours. It has been suggested that the agent is the same as that causing infective hepatitis, but the differences in the transmission experiments, as well as the apparent absence of cross-immunity in tests on volunteers, point to the existence of different causative agents.²

The same pathological picture is seen in both the above types of hepatitis.

Hepatitis following the injection of arsenical drugs may be caused by either agent, but is usually due to that of serum hepatitis. Apparently it results from infection conveyed from one person to another by imperfectly sterilised syringes and needles.

Infective Mononucleosis (Glandular Fever)

The evidence, though incomplete, suggests that the disease is due to a virus. Thus, it has been shown that blood from cases injected into monkeys may produce a definite mononucleosis after an incubation period of two to three weeks, and in some experiments the infection has apparently been transmitted serially in monkeys, while the virus has also been found to be filterable through a Seitz disk.

In a few cases presenting the clinical features of infective mononucleosis, *Listeria monocytogenes* has been isolated, and the aetiology of the disease requires further careful investigation.

A laboratory method of diagnosis is the *Paul-Bunnell reaction* in which the serum of an infected or recovered person agglutinates sheep red cells. The test is performed as follows. Heat the serum at 55° C. for twenty minutes. Make a series of doubling dilutions of the serum with saline in 0.5 ml. amounts in 3-in. × $\frac{1}{2}$ -in. tubes, ranging from 1 in 16 to 1 in 1024, as described on p. 245 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 hours. 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

¹ Findlay, G. M., and Martin, N. H., *Lancet*, 1943, 2, 678.

² MacCallum, F. O., *Proc. Roy. Soc. Med.*, 1946, 39, 655.

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 hours. This avoids fallacious results from "cold agglutination" (p. 611) 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 (Forsmann'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. 40).

It has been pointed out by certain authors that the type of antibody present in infective mononucleosis differs in certain respects from the Forsmann antibody, and also from that found in normal serum, and that this difference can be determined by agglutinin-absorption tests as follows :—

<i>Antibody</i>	<i>Treated with emulsion of guinea-pig kidney</i>	<i>Treated with ox red cells</i>
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.

Reagents.

1. *Patient's serum.*—1·0 ml. is required. Heat the serum in a water-bath at 56° C. for thirty minutes.

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

¹ Barrett, A. M., *J. Hygiene*, 1941, 41, 330.

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 hour. 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 hour 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 hours 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 minutes before re-suspension of the cells is attempted.

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 suspensions, but unaffected by guinea-pig kidney-emulsion.

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.

Using the absorption technique, as detailed above, a titre of 1 in 28 in rows (a) and (b) is significant.

Primary Atypical Pneumonia

This condition, also referred to as "acute pneumonitis," "acute interstitial pneumonitis," or "virus pneumonia," is caused by a variety of viruses, some of which have been identified. Of these, the viruses of the psittacosis group (p. 602) have first claim for consideration, since their aetiological connection with atypical pneumonia is the most firmly established. The available evidence, which is mainly serological, suggests that they are responsible for about 5-15 per cent. of cases. There is also some evidence that the virus of lymphocytic chorio-meningitis may occasionally give rise to pneumonia of this type, and the same is probably true of the virus of lymphogranuloma inguinale. Accepting the two latter viruses as occasional causes of atypical pneumonia, this would only account for, at the most, one-fifth of all cases, and the precise aetiology of the remainder is as yet unknown. It should be remembered that the influenza virus and *Rickettsia burneti*¹ (Q fever, p. 564) may also produce clinical conditions diagnosed as "atypical pneumonia." Other viruses worthy of consideration in the aetiology of the disease are: the virus of Weir and Horsfall,² which, under experimental conditions, produced pneumonia in the mongoose but failed to infect other experimental animals, including the ferret, mouse and monkey; the virus isolated by Eaton and his associates³ by the inoculation of cotton-rats; the feline pneumonia viruses of Blake, Howard and Tatlock,³ and of Baker⁴; the meningo-pneumonitis virus of Francis and Magill⁵ which was isolated from ferrets inoculated from persons with a respiratory-tract infection resembling influenza; and the pneumonia virus of Horsfall and Hahn⁶ recovered from persons with pneumonia and also from normal mice. In the main these viruses seem to be related to the psittacosis group. The aetiological problem of "atypical pneumonia" still requires further elucidation.

"Cold auto-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 washed group O human red cells. The mixtures are placed in a refrigerator

¹ *J. Exp. Med.*, 1940, **72**, 595.

² *Science*, 1942, **96**, 518.

³ *Yale J. Biol. Med.*, 1942, **15**, 139.

⁴ *Science*, 1942, **96**, 475.

⁵ *J. Exp. Med.*, 1938, **68**, 147.

⁶ *J. Exp. Med.*, 1940, **71**, 391.

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. (See Meiklejohn, G., *Proc. Soc. Exp. Biol. and Med.*, 1943, 54, 181.)

Agglutination test with Streptococcus "MG."—This organism, frequently isolated from cases of primary atypical pneumonia, is a non-haemolytic streptococcus with apparently distinctive serological characters. Convalescents may show specific agglutinins for it in their serum, and the agglutination test is of some value in retrospective diagnosis. For details of the organism and the serological test reference should be made to *Diagnostic Procedures for Virus and Rickettsial Diseases*, American Public Health Association, New York, 1948, p. 131.

Foot-and-Mouth Disease

Foot-and-mouth disease is an extremely infectious epizootic condition of cattle, pigs, sheep, goats and deer, characterised by the occurrence of vesicles on the feet and in the mouth, along with constitutional symptoms of infection. It may also occur naturally in the hedgehog. The infection is occasionally communicated to man. The disease can be transmitted experimentally by the inoculation of fluid from the vesicles, even after filtration through the finest earthenware filters. A suspension of vesicular epithelium is likewise infective. No visible or cultivable organisms have been found aetiologically associated with the disease, and the infection is due to a filterable virus. The virus is present also in the blood, saliva, milk and excreta of infected animals, and it has been thought that recovered animals may carry the infection for considerable periods, but this is doubtful. The size of the virus, which is one of the smallest, has been estimated as approximately 10 m μ , but it has been suggested that the virus particles are rod-shaped and that their long diameter is greater than this. It has been cultivated in tissue culture by using the pads, lips, tongue and hairy skin of embryo guinea-pigs but is now readily grown in cultures of epidermal tissue from the bovine tongue. Under experimental conditions the disease can be transmitted to cattle, sheep and pigs by inoculating the scarified mucous membranes of the lips and mouth. It can also be produced experimentally in guinea-pigs by cutaneous inoculation in the hairless pads of the feet. Within 24 to 48 hours pyrexia results, and an inflammatory condition of the foot, with a vesicular eruption like that of the natural disease. Secondary vesicles occur on the other feet and in the mouth. The animal usually recovers and is thereafter immune to further infection. The virus is present in the circulating blood as well as in the vesicular lesions. Suckling mice and rats are susceptible to the disease by experimental

inoculation, and in these animals the virus multiplies in the skeletal muscles ; adults cannot be infected. If slowly dried, the virus retains its viability for considerable periods, and the disease may be transmitted by certain vehicles of infection, e.g. fodder, as well as by contact. It is highly susceptible to heat, being rapidly killed at 55° C., and to changes in pH, e.g. a pH greater than 10 or less than 6 rapidly inactivates the virus. Three main immunological types of the virus have been recognised, namely, O, A and C and three further types, S.A.T. 1, 2 and 3, have been described. Animals recovered from one type are not immune against another type. Considerable differences may occur in the antigenic behaviour of different strains of any one type. Immunity has been produced by the injection of inactivated virus, but the protection afforded lasts only 4 to 6 months and vaccination is only of assistance in controlling the disease in countries where it is enzootic. Immunity following recovery is apparently due to a neutralising antibody present in the blood serum.

Vesicular exanthem of swine is a virus disease which occurs in California. It is of interest that though the clinical condition is indistinguishable from foot and mouth disease, the virus is antigenically distinct from that of the latter infection.

Vesicular stomatitis of horses resembles foot-and-mouth disease in some features and may be transmitted naturally to cattle. The virus is, however, antigenically distinct and differs also in being larger (about 100 m μ) and in being cultivable in the chorio-allantoic membrane of the chick.

(The virus of foot-and-mouth disease is not infective to the horse.)

Contagious pustular dermatitis of sheep.—The manifestations of this disease are pustules on the lips and round the mouth and on the mucosa of the mouth, the cornea and in other parts of the body. The infection is transmissible experimentally in lambs by inoculation of the skin with material from the pustules, and filtrates of infective material are also capable of causing lesions : but little is yet known about the characters and relationships of the virus (p. 618). The disease has been controlled under field conditions by means of a vaccine representing a modified strain of the virus. Human infections with the virus of contagious pustular dermatitis of sheep may sometimes occur through contact with diseased animals.

Rift Valley Fever

This disease, also known as enzootic hepatitis, was first described by Daubney and Hudson in 1931 in Kenya where it caused an extremely fatal epizootic amongst lambs. The ewes also were affected, but not so fatally. The native shepherds and Europeans engaged in the epizootic developed a dengue-like fever with severe back pains, while laboratory workers also became infected. The virus of Rift Valley fever passes fine Chamberland filters, and by means of collodion membranes its size has been determined as about 30 m μ . It has been cultivated in tissue cultures of chick embryo in Tyrode's

solution. In addition to being infective for sheep and man, the disease can be transmitted by blood, liver or spleen tissue to cattle, monkeys and rodents such as rats, mice, field voles, dormice and squirrels. It is not infective for birds, reptiles, rabbits or guinea-pigs. In the natural disease in the sheep there is marked necrosis of the liver, with haemorrhages into other organs. Inclusion bodies are numerous in the liver. The serum of recovered animals neutralises the virus. The virus is resistant to phenol, and in defibrinated blood containing 0·5 per cent. phenol will retain its activity for many months in the cold. Mice can be immunised with virus inactivated by formalin. There is evidence to suggest that the virus is transmitted by a mosquito ; it has also been suggested that it enters the body by the respiratory tract. It is of interest that Findlay and Howard have demonstrated the virus in larvae of *Taenia crassicollis* from the livers of mice which had been infected with the virus, and they have put forward the suggestion that under natural conditions the Rift Valley fever virus might infect the larvae of *Taenia solium* in East Africa, which could thus transmit the disease to man.

Louping-Ill

This disease is an encephalomyelitis of sheep characterised by cerebellar ataxia and disorder of nervous functions. It is common in certain parts of Scotland and England. The same condition may also occur in cattle. The virus is readily filterable through Berkefeld and coarse Chamberland filters. Its size has been estimated as about 19 m μ . It can be cultivated in the chorio-allantoic membrane of the chick embryo and in a medium of chick embryo tissue *in vitro*. The virus can be transmitted by intracerebral injection of emulsions of infected brain and spinal cord to sheep, pigs and mice, and in the latter animal also by intranasal instillation, the virus entering the olfactory bulbs and from there spreading throughout the nervous system. The evidence shows that the tick, *Ixodes ricinus*, is the vector of the disease, which is found only in those parts of Great Britain where this tick is present. *Ixodes ricinus* may transmit the infection in both the nymphal and adult stages. Another infection of sheep, "Tick-bite fever" (p. 565), probably due to a rickettsial organism demonstrable in the leucocytes, is regarded as predisposing to the louping-ill infection.

Successful prophylaxis has been achieved with a vaccine of formolised sheep brain and spinal cord tissue from an animal inoculated with the virus.

Cases have been recorded of transmission of this virus to laboratory workers and others handling infective material. Russian spring-summer encephalitis is due to a related virus, p. 583.

Scrapie

This is a disease of sheep which occurs in Great Britain and in Europe. The incubation period is said to be protracted but is not definitely

known ; under experimental conditions it is about 4-5 months. The nervous system is affected and pruritus is a marked symptom; there may also be incoordination of gait. It has been found possible to transmit the disease experimentally in sheep by intracerebral inoculation of brain and spinal cord from affected animals. Experimental infection has also been produced by other routes of inoculation. The size of the virus is not definitely known but it will pass a gradocol membrane of A.P.D. 0.41μ . It remains viable in the dried state for periods of over 2 years and is resistant to concentrations of formalin which inactivate other viruses.

Canine Distemper

Distemper is a specific infectious disease of great prevalence in young dogs and other animals, such as ferrets, silver foxes and fitches. The common manifestations are pyrexia and coryza, followed by secondary inflammation of the respiratory or alimentary system. Experimental evidence shows that the infective agent is a filterable virus. The disease can be transmitted experimentally to dogs and ferrets by subcutaneous inoculation with discharges from infected animals, and also with blood, serum and certain tissues, e.g. spleen. The incubation period in the ferret is 9-11 days. Filtrates of infective material are also virulent. Cytoplasmic and nuclear inclusions are seen in the epithelial cells of the respiratory passages and the urinary bladder, and their occurrence has been regarded by certain observers as of diagnostic significance. The virus has been cultivated in the chorio-allantoic membrane of the chick.

Active immunisation of dogs as a prophylactic against distemper can be achieved by the injection of either (1) 5 ml. of "vaccine" followed fourteen days later by 1 ml. "virus"; or (2) 1 ml. "virus" followed one and a half to two hours later by 10 ml. hyperimmune serum. The "vaccine" consists of tissues from infected dogs, mesenteric glands and spleen, which are ground, emulsified and treated with formalin. The "virus" is derived from the spleen and mesenteric glands of infected ferrets. A 20 per cent. emulsion is made and centrifuged. The supernatant fluid is filled into ampoules, dried, and sealed under nitrogen. The hyperimmune serum is obtained from dogs immunised with distemper virus.

Haemophilus bronchisepticus and other bacteria, at one time described as the causal agents of distemper, are secondary invaders.

Hard pad disease affects dogs of all ages and is characterised by hyperkeratosis of the pads of the feet and a high death rate. The condition is due to a virus which is possibly related to the distemper virus, being infective to ferrets after an incubation period of 23 days (*cf.* distemper).

Cat distemper is characterised by marked catarrh of the respiratory passages and a long period of illness. It is due to a different virus from that of canine distemper; this virus seems to have some relationship to the psittacosis and the mouse pneumonia viruses.

Feline infectious enteritis is an acute disease of cats characterised by a short period of illness and a high death rate. The virus is different from that of cat distemper. Formolised infected spleen appears to provide a vaccine of some value.

Borna Disease of Horses

This is an infectious meningo-encephalomyelitis of horses in Europe, characterised by lesions in the central and peripheral nervous systems. After general systemic disturbances the animal becomes paralytic and usually dies, the mortality being at least 75-80 per cent. The virus is essentially neurotropic. It may also infect cattle and sheep under natural conditions. It can be transmitted experimentally by intracerebral inoculation of these animals, and also rabbits, guinea-pigs, rats, mice, fowls and monkeys. The virus can pass Berkefeld and the coarse and medium grades of Chamberland filter. Its size is computed as 100 (85-125) $m\mu$. The microscopic lesions in the brain are perivascular and meningeal infiltrations with mononuclear cells, with also some diffuse infiltration. Similar lesions occur in the cord. The nuclei of the nerve cells contain characteristic inclusion bodies known as "Joest-Degen corpuscles," and are more numerous in the cells of the spinal ganglia. They vary in size from 3 or 4μ down to the limit of resolution, and by Mann's methyl-blue eosin stain are coloured red. The larger bodies are similar to the Negri bodies of rabies. The virus material treated with phenol and glycerol possesses immunising properties.

Equine Encephalomyelitis

Equine encephalomyelitis is a disease of horses which in recent years has been prevalent in the North American continent. It is due to a virus of which there are two distinct types—a "Western" and "Eastern." It is about 25 $m\mu$ in size and can be cultivated in the chorio-allantoic membrane of the chick. The infection is readily transmitted experimentally to white mice. The disease can be transmitted by various species of mosquitoes, and human infections occur. It seems likely that certain domestic mammals and birds and also wild species of these are reservoirs of infection. There is some evidence that bird mites may carry the infection. Since the introduction of formolised chick-embryo culture vaccines (1939) preventive inoculation of horses seems to have greatly reduced the incidence of the disease. A third antigenic type has been isolated in Venezuela, which is more pathogenic to mice than the types found in the United States.

Rinderpest

Rinderpest or cattle plague is characterised by elevation of temperature, glairy discharge from eyes and nose, ulceration of the lips and pads and severe diarrhoea, followed by weakness and death, usually in four to seven days. *Post mortem* there is extensive submucous haemorrhage in the intestine, and ulceration is usually present along

the whole alimentary canal. The virus passes Berkefeld and coarse Chamberland filters. It is not free in the blood stream, but is present in the leucocytes. It is rapidly destroyed by drying, by heat, and by chemicals, and if kept at room temperature. It survives for some months if infected blood is frozen solid, but is rapidly destroyed by glycerol. Regarding the special characters of the virus little is known. It has been cultivated in the chick embryo, but has not yet been measured or photographed. The virus is present in the discharges, all of which are infective, particularly that from the nose ; infection is normally through a mucous surface and not by an insect carrier. Recovery results in a solid and lasting immunity. Formolised spleen pulp from infected animals has been found to confer protection when used as a prophylactic vaccine. Injection of serum together with virulent blood has also been used to produce immunity in herds. At present the most effective vaccines are prepared from living virus adapted to other animals, e.g. goat, rabbit, and consequently less virulent for cattle. Virus cultivated in the fertile egg has also proved an effective immunising agent.

Swine Fever

This disease was at one time thought to be due to an organism of the *Salmonella* group, *S. cholerae-suis*, but a filterable virus is the causal agent. In the acute disease there is a febrile generalised infection with haemorrhagic inflammation of the intestinal mucous membrane. The incubation period is about six days. In chronic cases there is a necrotic condition of the mucosa in which *S. cholerae-suis* is found. Secondary infection of the lungs by a *Pasteurella* organism may also complicate the disease.

The natural mode of infection is by ingestion, and exceedingly minute doses of virus will cause the disease. The virus is present in the blood plasma and not in the corpuscles.

The virus passes fine filters. It is fairly resistant and survives moderate heat and freezing. It can also survive in cured hams and pickled pork, while desiccation does not destroy it. The virus is resistant to disinfectants and glycerol, but is soon destroyed by putrefaction.

The immunity following an attack is lasting, and immune serum together with infected blood has been used for immunisation. The use of *crystal violet vaccine* is effective. This vaccine is prepared by mixing defibrinated blood from infected pigs with crystal violet and ethylene glycol and incubating for fourteen days at 37° C. The vaccine contains the virus in an "inactivated" form ; it is incapable of setting up the disease and confers protection against the natural infection for at least twelve months.

Animal Pox Diseases

Various domestic animals are susceptible to pock-like eruptive skin lesions, namely, horses, sheep, cattle, goats and swine. Fowls suffer from an analogous disease.

Cow-pox has already been referred to (p. 589) and may affect man, e.g. milkers, under natural conditions, but some confusion still exists in the literature concerning the relation of this disease to the other animal poxes. This is largely due to the erroneous assumption that because the clinical syndromes and histological features are similar or identical, the viruses producing such lesions are also closely related. The viruses proved to be related to the variola-vaccinia group of man are those of *cow-pox*, *rabbit-pox* and *horse-pox*. It may be noted that the virus of infectious ectromelia (*vide infra*) of mice also shows certain relationships to the variola-vaccinia group. Horse-pox causes the condition known as "grease" or contagious equine pustular stomatitis, but some consider that this clinical syndrome is not an independent disease and is merely cow-pox infecting the horse.

Sheep-pox is strictly specific for its host and the virus is quite unrelated to any other of the pox group. It is an extremely serious disease when it occurs in epizootics, often causing high mortality. The statement that flocks can be immunised against sheep-pox with cow-pox (vaccinia) is incorrect, but some claim good results by using a mixture of sheep-pox virus and immune serum from a recovered animal.

The clinical syndromes known as *Goat-pox* and *Contagious pustular dermatitis* (or *stomatitis*) affecting goats and sheep are due to a group of viruses immunologically related (p. 613). There is no relationship between this group and either sheep-pox or the vaccinia group.¹

Swine-pox is clinically similar to cow-pox and sheep-pox but is due to a specific virus unrelated to these viruses. It is also a serious disease, and on the Continent may have a mortality rate of 20 per cent. or more. In Britain, however, the disease is usually very mild, the lesions are not definitely pock-like, while the infection is often confined to very young pigs. It is probable that the severe outbreaks are due to true swine-pox, and the mild infections are caused by vaccinia.

Fowl-pox and Roup.—These diseases are both caused by the same virus, the relationship of which to the other pox viruses is doubtful. In the infected bird the virus gives rise to inclusion bodies (Bollinger bodies), and elementary bodies (Borrel bodies) are also demonstrable (p. 579). It can be cultivated in the chorio-allantoic membrane of the developing chick. Fowls can be immunised by the inoculation of pigeon-pox virus, which has a low pathogenicity for the fowl, but is probably a modified form of the fowl-pox virus.

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 viscera. In the epithelial cells of the skin and intestine large acido-

¹ Bennett, S. C. J., Horgan, E. S., and Haseeb, M. A., *J. Comp. Path.*, 1944, 54, 131.

philic cytoplasmic inclusions are found, and elementary bodies about 100–150 m μ 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 this virus and the variola-vaccinia viruses.

CERTAIN SPECIAL METHODS APPLICABLE TO THE STUDY OF VIRUSES

Cultivation of Viruses in the Embryonated Egg

Fertile hens' eggs of not less than 53 grams are used, preferably with light-coloured shells, e.g. from white Leghorn birds. The shells should be perfectly clean. Incubation of the eggs must commence not later than 10 days, and if possible within 5 days, after being

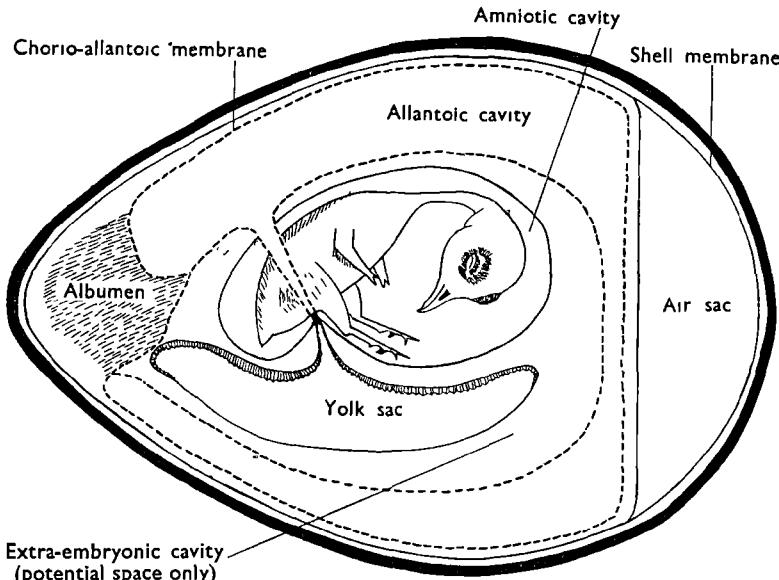


Diagram of embryonated hen's egg after 11 days' incubation, to illustrate the various sites of inoculation with viruses (R.H.A.S.).

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 35° to 37·8° C. (according to the virus dealt with), and the eggs are turned twice daily. Before inoculation, e.g. after 7–12 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 engine. A vulcanite carborundum disk (*e.g.* vulcarbo No. 8) can be recommended. Special care must be taken to avoid damage to the underlying membranes and the consequent bleeding. Mounted dissecting needles, forceps, scissors, syringes and capillary pipettes are required for the manipulations involved in inoculating the egg. Inoculation must, of course, 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° - 36° C. They must be kept stationary, resting on special stands or trays.

Inoculation of the Chorio-Allantoic Membrane.

The eggs are incubated usually for 12 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 both shell and 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.* over the chorio-allantoic membrane. The opening is finally sealed with a piece of cellophane (which has been sterilised in boiling water) dipped in sterile 10 per cent. egg albumin containing chloroform as a preservative; a convenient and efficient substitute is commercial transparent adhesive tape ("Scotch tape") 1 in. wide. The inoculated egg is incubated for 2 to 4 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 for further investigation.

Allantoic Inoculation.

For this purpose eggs are incubated for 10 to 11 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. Melted paraffin wax is then applied to the groove and the surrounding area. A small drop of the inoculum is placed over the groove with a pipette and a needle is thrust through this drop, the paraffin wax, the groove and the underlying membranes to a depth of a few millimetres. When the needle is withdrawn the drop of fluid is automatically aspirated ; and then the remainder of the inoculum, the total bulk of which is 0.05 ml., is similarly deposited and aspirated. The opening is sealed with melted paraffin. Incubation is carried out for 2 days and the egg is then refrigerated for 4 to 18 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 13 to 14 days' incubation. On candling, the densest part of the embryo is marked and an oval area (2×1.5 cm.) in the long axis of the egg is drilled at this point. Two further cuts of 1 cm. are made within the oval to form a triangle with the periphery of the oval, 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 oval 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 capillary pipette 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. The egg is incubated for 3 to 5 days at 35°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 5 to 6 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 20- or 21-gauge needle, 3 to 3·5 cm. long, the needle being 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. 0·5 to 1·0 ml. of inoculum is introduced. The opening is sealed with melted paraffin. Incubation is then carried out and the egg is candled daily. The yolk 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 decanted into a Petri dish, but it is an advantage first to extract the embryo by cutting the membranes and umbilical stalk and then withdrawing the yolk-sac by traction on the umbilical stalk. The sac is drained and its contents are collected; in some cases where the sac membrane itself contains a large amount of virus, this is retained for further investigation.

Haemagglutination Tests with the Influenza Virus

Blood Suspension.—Red blood corpuscles are obtained by drawing 10 ml. of fowl's blood from a wing vein into 2 ml. 5 per cent. sodium citrate. The citrated blood is passed through muslin to remove small clots and then centrifuged; the corpuscles are washed three times with several volumes of normal saline solution (p. 257). From the corpuscular sediment a 10 per cent. suspension in normal saline is prepared and this may be kept at 4° C. for a week. This stock suspension is diluted to 0·5 or 0·25 per cent. for the actual test.

Virus Preparation.—Partially purified virus should be used. The virus is cultivated in the allantoic sac (p. 621) and after 2 days' incubation the shell over the air-sac is removed and both the chorio-allantois and amnion and their blood-vessels are torn. The egg is rotated to mix the blood with the fluids, and the mixture is then withdrawn into a large centrifuge tube or bottle and immediately placed at 4°–6° C. The red corpuscles agglutinate and are separated by centrifuging for 3 minutes at 500 to 1000 r.p.m. The supernatant fluid is discarded and the compact and coherent sediment is rinsed with normal saline at 4° C.; this is done by adding slowly 2–3 ml. saline (cooled to 4° C.) without disturbing the sediment and then pipetting it off, the rinsing being repeated three times; finally, saline equal to one-tenth of the volume of the original material is added, the temperature is raised to 37° C. and kept at that level for 2½ hours. The agglutinated cells now separate and disperse, and the virus dissociates. The mixture is then centrifuged and the supernatant fluid constitutes the virus material to be used for testing. This preparation retains its haemagglutinating potency in the refrigerator for 3 weeks.

Titration of Virus.—Two series of 0.5 ml. amounts of doubling dilutions of the virus material are prepared in small test-tubes (7.5×1.0 cm.), one series from 1 in 20 to 1 in 5120 and the other from 1 in 30 to 7680. To each tube 0.5 ml. of 0.5 per cent. blood suspension is added; a control tube is included in the test, containing blood suspension and saline only. The tubes are shaken and then allowed to stand at room temperature for 1½–2 hours, when the results can be read. In the absence of agglutination the cells sediment to form a small compact disk of deposit; complete agglutination is denoted by the spread of the cells in the form of a film over the foot of the tube. The titre is stated as the reciprocal of the highest dilution which yields complete agglutination, the results of the duplicate titrations being averaged.

Titration of Antibodies in Serum.—The virus is made up in a dilution representing quarter-titre. To each of a series of 10 small test-tubes 0.5 ml. of blood suspension is added, the first tube receiving 0.5 per cent., the others 0.25 per cent. suspension. To the first tube 0.5 ml. of a 1 in 8 dilution of serum is added and mixed with the blood; then 0.5 ml. of this mixture is transferred to the second tube and so on throughout the series. To each tube 0.5 ml. of the quarter-titre virus is added and mixed with the other contents of the tube. The final range of dilutions of the serum will be 1 in 32 to 1 in 16,384. The following controls are included in the test: (1) blood suspension *plus* 0.5 ml. of the virus material—complete agglutination should occur in this tube; (2) blood suspension *plus* 0.5 ml. of a 1 in 8 dilution of normal serum—this serves for comparison with the tubes containing the higher concentrations of the serum tested; (3) blood suspension *plus* 0.5 ml. saline to serve for comparison with tubes containing the higher dilutions of the serum tested. The tubes are shaken and allowed to stand at room temperature for 1½–2 hours. The reciprocal of the highest dilution of serum which completely prevents agglutination is stated as the titre of the serum.

In testing human sera it may be noted that normal sera may give low-titre reactions—below 128; convalescent sera give titres from 256 to 2048. If tests are done at the beginning of the illness, a four-fold rise in titre after convalescence is highly significant. (See Francis and Salk, *Science*, 1942, **96**, 499; Salk, *J. Immun.*, 1944, **49**, 87.)

Complement Fixation Tests in Virus Diseases¹

Psittacosis

The general technique of the test is similar to that of the Wassermann test described on p. 263, 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

¹ Bedson, S. P., and Bland, J. O. W., *Brit. J. Expt. Path.*, 1949, **10**, 393.

inoculating with psittacosis virus the yolk-sacs of 6 to 8 days' chick embryos (p. 622). 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.

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 30 minutes.

Test Proper.—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 2½ hours at room temperature, after which 0·2 ml. of the red cell suspension is added. Readings are taken after 30 minutes' 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.

Influenza

The general principles of the test are similar to those described for psittacosis.¹

¹ Hoyle, L., *Month. Bull. Min. of Hlth. and P.H.L.S.*, 1948, 7, 114.

Antigen.—A stock antigen was at one time obtained from portions of consolidated lung tissue from mice killed or dead on the third or fourth day after intranasal inoculation with virulent virus. The tissue was desiccated *in vacuo* over calcium chloride and remained stable for a period of years. Liquid antigen was prepared by grinding a weighed amount of the dry lung tissue with an abrasive (*e.g.* sintered glass powder—fine grade) in a mortar to make a fine and uniform suspension. It was then diluted with saline to make a 1 per cent. suspension and clarified by centrifuging at 5000 r.p.m. for 15 minutes. To the supernatant fluid a few drops of chloroform were added, and it was allowed to stand overnight in the refrigerator. A fine precipitate resulted and this was removed by centrifuging next morning. The antigen could be kept for many weeks in the refrigerator.

Satisfactory antigens may now be prepared from allantoic fluid of infected chick embryos. Fluids with high haemagglutinin titres or concentrations prepared by Salk's method (pp. 622, 623) can be used.

The antigen is tested in doubling dilutions *e.g.*, 1/2 to 1/16 with known positive sera to ascertain the highest active dilution; a quarter of this dilution is used in tests with unknown sera.

The "soluble" form of antigen (p. 598) has recently been recommended for the complement-fixation test. (For details of preparation etc., see Hoyle, L., *Brit. J. Exp. Path.*, 1948, 29, 390.)

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. Complement preserved by Richardson's technique (p. 266) is satisfactory.

Sera.—Inactivated at 56° C. for 30 minutes.

Test Proper.—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 2½ 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 1 hour 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 30 minutes. Readings are made after the cells have settled, and 50 per cent. haemolysis or more indicates absence of complement-fixation in the particular tube.

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. 592), 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.

Preparation of Specimens of Faeces for Inoculation into Animals in demonstrating the Poliomyelitis and Coxsackie Viruses

A large sample of faeces is collected, e.g. 15-25 grams. It should be transported to the laboratory in a container packed in ice, and kept refrigerated until the following treatment is commenced :—

Grind at least 5 grams in a mortar with normal saline to make a 20 per cent. W/V suspension.

Allow this to stand overnight in the refrigerator.

Centrifuge at 2000 r.p.m. for 30 minutes and remove the supernatant fluid.

Add 20 per cent. ether to this fluid, and keep overnight in the refrigerator.

Centrifuge it at 2000 r.p.m. for 30 minutes.

Place the mixture in a sterile beaker and evaporate the ether with a vacuum pump.

Test for bacteriological sterility.

Penicillin and streptomycin may be added, 500 units and 500 µg respectively per ml.; but this is often unnecessary as the material may be found to be already sterile.

The above procedure suffices for inoculation of the material into suckling mice as in the isolation of the Coxsackie virus; no toxic effects result as a rule when doses of 0.05 ml. intraperitoneally and 0.01 ml. intracerebrally are injected.

When intracerebral inoculation of monkeys is contemplated in the case of the poliomyelitis virus, 10 ml. of the final supernatant fluid (as above) are centrifuged at 4500 r.p.m. in an angle centrifuge, and the resultant supernatant fluid after a sterility test is used for inoculation.

(For fuller details of these methods, reference should be made to *Diagnostic Procedures for Virus and Rickettsial Diseases*, cited above.)

THE BACTERIOPHAGE, AND TRANSMISSIBLE LYSIS

The phenomenon of transmissible lysis of bacteria was discovered 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 the phenomenon of transmissible lysis is

due to a minute organism (*Bacteriophage*¹) which is parasitic and destructive to bacteria, propagating or multiplying at the expense of the bacterial cells. According to this view it belongs to the same category as the filterable viruses, and undoubtedly a bacteriophage exhibits a close analogy to the viruses. If a filtrate containing the lytic agent or phage¹ is added to a young broth culture and this is used for inoculating a plate of medium, the resulting growth shows clear circular plaques where the phage is acting; these have been spoken of as "colonies of bacteriophage." This indicates the particulate nature of a phage, which has also been established by the same methods as those applied to the viruses (p. 570). Phages vary, however, in size from about 100 m μ to 10 m μ . Recently, electron microscopy has clearly revealed phages as corpuscular structures; some of them (e.g. *Esch. coli* phage) have a tail-like process which gives them a tadpole-like shape. Others seem to be rounded or cubical in shape. Phage particles introduced into a bacterial culture are first adsorbed on the surface of bacterial cells and then apparently penetrate the cells, quickly thereafter increasing in quantity or number; later the cell becomes disrupted and the increased number of phage particles are liberated.

Phages have considerable resistance to physical and chemical agents; they may withstand heating at 75° C. for half an hour and resist drying for long periods. They can be propagated only in living and growing cultures. As in the case of viruses, no final statement can be made as to whether the phenomenon of bacteriophagy is due to a living organism and the processes of growth and multiplication of phages are still obscure. The question of the unity or multiplicity of phages has been debated: a phage which is active for one organism may lyse related bacteria and may apparently be "adapted" to entirely different organisms. In general, phages are specific for particular species or types of bacteria, and phage specificity is dependent on the antigenic composition of the bacteria. Different types of a bacterial species may be identified by means of specific phages.

It is usually observed that, when a culture is lysed by phage, some individual organisms prove resistant and in the course of time yield a "secondary growth" which represents a strain resistant to the phage. This strain, though insusceptible to phage-lysis, may carry the phage and is designated "lysogenic" in view of the fact that culture-filtrates from it may lyse a susceptible strain. Resistant strains derived from a culture acted on by phage often constitute variants of the parent organism, and it is now recognised that phage is a powerful agency in bringing about bacterial variation.

With regard to the distribution of phages, it may be said in general that a phage active towards a particular species is commonly found associated with the organism or is present in the same environment, but exceptions to this rule may be observed.

Bacteriophage has an antigenic individuality apart from the associ-

¹ The term "bacteriophage" is generally abbreviated to "phage."

ated organism, and immunisation with phage-containing filtrates yields an antiserum which inhibits the action of the phage.

References to the practical applications of bacteriophages have been made in earlier chapters (pp. 334, 436).

Isolation of Bacteriophage

To illustrate the procedure for the demonstration and isolation of a bacteriophage, the methods applicable for obtaining phages for typhoid-paratyphoid and dysentery bacilli from faeces, sewage and water may be taken.

Faeces and Sewage.—A relatively large amount of the material (e.g. about 5 grams 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, so that conditions are sufficiently aerobic. The broth is incubated for twelve to twenty-four hours at 37° C. and then filtered through a Seitz disk (pp. 133, 134). It is preferable, however, to carry out "phage-enrichment" by adding to the broth a few drops of a young broth culture of the type of organism for which a phage is sought. The flask is incubated and the contents are filtered as above.

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.

A phage having 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; and the phage can be propagated serially in the same way by adding each filtrate to a young broth culture of the organism.

In demonstrating a phage towards the particular organism, some information may also be obtained regarding its effect on allied species or types by stroking broth cultures of these on the plate in parallel with the test organism and superimposing drops of the filtrate on them; a single plate may suffice for testing simultaneously phage action on several organisms.

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 filtrate 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 (p. 210) so that most of the surface of the medium is inoculated uniformly. (A confluent growth over the surface of the plate

is aimed at.) After incubation of the plate, small, discrete, well-separated "plaques" will be observed in the case of certain dilutions of filtrate. (When the phage is in too great a concentration the plaques are not sufficiently discrete.) A plate representing a dilution with good separation of plaques is selected, and with a small inoculating loop single plaques are subinoculated into tubes of broth inoculated with the test organism; after incubation these cultures are filtered. To ensure absolute purity it may be necessary to repeat the isolation procedure several times, preparing decimal dilutions, plating, and subinoculating discrete plaques.

Water.—200 ml. of water are added to a flask containing 100 ml. triple-strength broth. This is inoculated with 2 ml. of a young broth culture of the organism for which the phage is sought, and the procedure is thereafter the same as that described above. Larger quantities of water can be examined by multiplying the number of flasks and 200 ml. volumes of water tested.

CHAPTER XXIII

THE PATHOGENIC FUNGI

INFECTIONS produced by the fungi (p. 4) are usually designated "mycoses."¹

FUNGI IMPERFECTI

The more important of the mycoses are due to organisms of the group above-named, *i.e.* fungi whose complete life-cycle is still imperfectly known, though it is possible that many of them are asexual stages of *Ascomycetes* (p. 633). The group includes *Microsporon*, *Trichophyton*, *Achorion*, etc.

Microsporon.—This organism is the common cause of scalp ringworm in children. It rarely attacks the body. It is apparently unable to infect the scalp after puberty. The mycelium develops in the horny layer of the scalp epidermis, and in the hair medulla from which hyphae pass through the cortex, and produce a covering of small spherical "spores" (approximately 5μ in diameter) arranged in the form of a mosaic on the outside of the hair. Grown artificially, *Microsporon* develops large circular colonies with a raised centre, and radiating folds. The surface of the colonies is velvety, due to the projecting aerial hyphae. Though the surface remains white or buff-coloured, the substance of the colony may develop a brown pigmentation. Various species have been recognised, e.g. *Microsporon audouini* (the common type in ringworm of the scalp among children), *Microsporon canis* (dermatomycosis of dogs), *Microsporon felinum* (of cats), *Microsporon equinum* (of horses). Species of animal origin, e.g. *Microsporon felinum*, may infect the human subject.

Trichophyton.—This organism may occur in ringworm of the scalp, nails and glabrous skin. It may also be found in beard ringworm. The fungus mycelium, as it develops in the hair medulla or epidermis, produces characteristic chains or "rosaries" of oval or rectangular "spores," which are larger (5μ to 8μ) than those of *Microsporon*. In artificial

¹ For detailed information on this subject, reference may be made to *Medical Mycology*, by Dodge, London, 1936; *Elements of Medical Mycology*, by Swartz, London, 1944; and *Manual of Clinical Mycology (Military Medical Manuals, National Research Council)*, by Conant, Martin, Smith, Baker and Callaway, Philadelphia and London, 1946.

culture the different species vary in detailed characters, but present the same general type of growth as *Microsporon* though the colonies are more dense and opaque. A common species in Great Britain occurring in scalp ringworm is *Trichophyton crateriforme*, which produces raised colonies with crater-like depressions. Under this designation, however, several different types are included. *Trichophyton acuminatum* is a common species in scalp ringworm in Europe. It is characterised by a conical peak in the centre of the colony which has a creamy-white colour and powdery surface. Some species are markedly pigmented, e.g. *Trichophyton rosaceum*, *Trichophyton violaceum*, *Trichophyton sulphureum*.

Different species associated with dermatomycoses in animals have been recognised : *Tricho. faviforme* (in cattle), *Tricho. equinum* (in horses), *Tricho. caninum* (in dogs), *Tricho. felinum* (in cats). *Trichophyton* infections in animals are not uncommon sources of human ringworm.

Achorion schönleinii.—Associated with the condition called "favus," which affects the scalp and other skin areas. The fungus forms the characteristic concave yellow disks or "scutula" centred in hair follicles. The scutulum is composed of mycelium and spores which are markedly irregular in size and arrangement. The hairs in the affected area are also invaded. The artificial growth on culture medium consists of a tough brownish layer with raised irregular folds, and a white velvety surface due to the aerial hyphae.

Achorion quinkeanum is the species of cat and mouse favus ; it is occasionally found in the human disease. A favus-like condition occurs in fowls due to *Achorion gallinae*.

Epidermophyton.—This type of organism does not attack the appendages but grows in the superficial layers of the epidermis, and skin folds are specially susceptible. The infection is exemplified by *Tinea cruris* ("Dhobi itch") ; a similar condition may occur between the toes (*Tinea interdigitalis*). A number of species of *Epidermophyton* have been recognised and differentiated mainly by the colour of growths on culture medium, e.g. *Epidermophyton inguinale* (yellow), *Epidermophyton perneti* (pink), *Epidermophyton rubrum* (deep red).

Microsporon (or *Malassezia*) *furfur* of Pityriasis versicolor, *Endodermophyton concentricum* of *Tinea imbricata* (in which the mycelial growth occurs between the rete Malpighi and stratum corneum of the epidermis), and *Endodermophyton*

castellanii of *Tinea intersepta* are further examples of "fungi imperfecti" responsible for human dermatomycoses. *Microsporon furfur* is more of the nature of an epiphyte, growing on the skin surface, than a true infecting parasite.

A fungus, designated by some writers *Microsporon minutissimum*, is associated with "erythrasma," consisting of scaly patches in the axillae and groins with some hyperaemia. The parasite infects the epidermis and grows in the form of small threads which readily break up into shorter forms resembling bacilli. It is doubtful if this organism has been cultivated, and in biological characters it is more allied to the actinomycetes than to the fungi proper.

Sporotrichon schenckii (or *beurmanni*).—Associated with subcutaneous ulcerating granulomata. The condition is designated "sporotrichosis" and occurs in man and horses. When grown artificially (at room temperature) the organism forms a typical mycelium; the hyphae are relatively narrow (2μ); oval spores are found in clusters at the ends of hyphae. In the tissues it may be difficult to demonstrate the organism by microscopic examination; the morphology is entirely different, and no definite mycelium is observed; oval or spindle-shaped forms are found; some of these are not unlike large bacilli; budding may be noted as in the yeasts.

This organism is pathogenic to mice and white rats, experimental inoculation of cultures producing an ulcerating granuloma at the site of injection.

Hemispora stellata.—The organism of hemisporosis, a granulomatous and suppurative condition occurring in the human subject and involving the skin in the form of single or multiple lesions. The infection may also occur in bones. This fungus, which appears as a mycelium with star-like conidiophores, is easily cultivated from lesions as an irregular whitish growth on the surface of the medium. It is pathogenic for the rabbit on experimental inoculation. It is not uncommon as a saprophyte and may be found in dust.

Madurella, a fungus found in mycetoma, is referred to on p. 531.

Allergic Reactions in Fungus Infections.—Deep-seated infections of the skin, especially those due to *Trichophyton*, may lead to widespread cutaneous hypersensitivity to the products of the organism. Thus, in the course of the infection, generalised skin eruptions of an allergic nature may occur, e.g. the "trichophytides." Evidence of such allergy may be elicited by injecting extracts of the organism, e.g. trichophytin. These allergic reactions are not species-specific.

(In some individuals manifestations of hypersensitivity may result from the inhalation of certain saprophytic fungi present in air or dust —p. 50.)

PHYCOMYCETES

The mycelium is made up of non-septate hyphae; asexual spores are developed in a spherical "sporangium" or spore-

case borne on the expanded end of an aerial hypha ; "zygospores" are also formed as the result of conjugation of two hyphae at their tips. These fungi are of little or no importance as pathogens, though they have been noted in auricular, nasopharyngeal and pulmonary mycoses, and in mycosis of the tongue. They are frequently contaminants of food, and of culture medium in the laboratory. The common mould *Mucor mucedo* exemplifies the group.

Rhinosporidium seeberi.—The biological relationships of this organism are doubtful. It is responsible for a polypoid disease of the nose and tumour-like lesions of the conjunctiva, uvula and mouth, occurring in India and South America, e.g. Argentine. In an early phase of its life history it appears in the tissues as a small rounded capsulated body with a single nucleus. This cell enlarges and the cytoplasm and nucleus divide into a large number of spores. The parent structure, surrounded by a thick cellulose capsule, may reach even 2-3 mm. in diameter, and with the contained spores has been regarded as analogous to a sporangium. The spores ultimately escape through a pore in the capsule and spread in the lymphatic spaces.

ASCOMYCETES

The mycelium is typically septate ; "ascospores" (usually eight in number) are developed in a spore-case ("ascus") formed at the end of a hypha. The moulds *Aspergillus* and *Penicillium* are examples of this group.

Aspergillus is a frequent contaminant of culture media, occurring as a felted, yellow, green or black mould-growth ; rows of spores or conidia develop from finger-like processes or "sterigmata" borne on an expanded aerial hypha ("conidiophore"). Pulmonary aspergillosis, due to *Aspergillus fumigatus*, may occur in birds, e.g. pigeons and penguins in captivity, and this infection has also been noted in man (among bird fanciers). *Aspergillus pictor* and also other species of fungi have been described in the skin lesions of Pinta, a disease of Central America, which is now known to be due to a spirochaete (p. 544).

Penicillium, which includes a large number of species, is one of the commonest food moulds and contaminants of culture medium. Its biology is similar in some respects to that of *Aspergillus*. In this group the conidiophore divides at its free end into a pencil of finger-like branches and on the end of each a sterigma is formed, from which the conidia are abstricted. The common green mould (*Penicillium expansum*) found on food is a representative of this group.

The important antibiotic substance, penicillin, is derived from *Penicillium notatum* (p. 22).

YEASTS AND YEAST-LIKE FUNGI

(*SACCHAROMYCETES*, *BLASTOMYCETES*)

These are often classified with the *Ascomycetes*, and the yeasts are now regarded more as a phase of other types of fungi than as a separate group. Some of them are grouped among the *Fungi imperfecti*. Their morphology and gemmation, or budding, are highly characteristic features, and though in some species hyphae occur, it is convenient to consider them separately from the moulds. An outstanding biological property of certain of these organisms is the fermentation of carbohydrates.

The pathogenic organisms of this group for convenience are classified here into three main genera : *Coccidioides*, *Monilia* (or *Candida*) and *Cryptococcus*, but the characterisation of these genera and the assigning of species to them must be regarded as provisional.

Coccidioides.—This genus shows yeast-like forms, but is also characterised by the formation of mycelium, and ascospore-like structures have been noted. The biological position of the genus is somewhat doubtful but it may conveniently be grouped among the yeast-like organisms.

Coccidioides immitis—of "Blastomycosis," an uncommon granulomatous or suppurative condition involving skin, subcutaneous tissue, lungs, etc., developing sometimes into a generalised pyaemic condition. Large cyst-like structures ($10\text{--}60\mu$) are seen in the tissues and these contain spores ($2\text{--}5\mu$). In culture septate hyphae are formed.

Monilia (or *Candida*).—This type is yeast-like in morphology and exhibits the characteristic gemmation, but hyphal filaments also occur. No ascospores have been noted. Various carbohydrates are fermented with both acid and gas production.

Monilia albicans (*Oidium albicans* or *Candida albicans*) is the species found in thrush (oral and vaginal). As seen in wet preparations from the lesions or in dried films stained by Gram's method, the organism appears as septate hyphae forming a mycelium, along with yeast-like structures which are regarded by some as buds (blastospores) from the mycelium, though they may occur as independent units, often in clusters. A similar organism occurs in bronchial and pulmonary infections.

Monilia psilosis was at one time described as the causative organism of Sprue but has no aetiological relationship to this disease.

Monilia tropicalis is a common type found in bronchial infections (broncho-moniliasis) in tropical countries.

Various other species have been described under separate designations.

Cryptococcus.—typical yeast-like forms; no hyphae occur; when grown in fermentable carbohydrates no gas is produced.

Cryptococcus gilchristi (*Blastomyces dermatitidis*)—of Blastomycetic dermatitis. In the tissues yeast-like bodies ($8\text{--}20\mu$) are observed; they have a double contour due to the thick cell wall but are not capsulate. When cultivated at 37° C . similar yeast forms develop, but at room temperature hyphae are produced. It is doubtful whether this organism should be assigned to the genus *Cryptococcus*.

Cryptococcus linguae pilosae—of "Black-tongue."

Cryptococcus farciminosus—of Epizootic Lymphangitis in horses.

Cryptococcus hominis (*Torula histolytica*).—This is a yeast-like organism which reproduces only by budding, shows no mycelial formation, and produces no endospores. The individual cells are about $5\text{--}10\mu$ in diameter and are capsulate. It has been stated to have no fermentative properties, but some strains ferment carbohydrates without gas production. It has been reported in cases of meningitis and also in pulmonary infections. Mice are susceptible to inoculation, e.g. intraperitoneally, and develop a general infection with granulomatous lesions in internal organs and muscles.

Monilia and *Cryptococcus* types have been shown to be aetiological agents of dermatitis (in circumscribed form), suppurative paronychia and deformities of the nail-plate.

Fonsecaea (or *Hormodendrum*) *pedrosoi*.—This and other closely related species are associated with the condition of chromoblastomycosis, a granulomatous disease of the skin and subcutaneous tissues usually affecting the lower extremities. No mycelium is seen in the lesions and the fungus occurs as a brown spherical sclerotic cell about 12μ in diameter with a thick outer membrane; this body may show septate subdivision. The fungus can be cultivated readily and then develops mycelium with conidiophores.

Histoplasma capsulatum.—This organism has been described in a disease characterised by splenomegaly and anaemia. It was originally thought to be a protozoon but its fungus nature has been clearly established. In the tissues yeast-like forms are seen, mostly within large mononuclear cells, and mycelium is developed in culture. The yeast forms are usually $3\text{--}5\mu$ in diameter and in stained preparations appear to be capsulate. The infection is readily transmitted to animals, e.g. guinea-pigs, rats, etc. The organism has been classified with the *Monilia* group.

DIAGNOSIS OF THE DERMATOMYCOSES

The affected hairs, or the epithelial scales from affected areas, are treated on a microscope slide with a 10 per cent. solution of caustic potash, gently warmed, and examined with a 4-mm. objective in the unstained condition under a cover-slip. The alkali clears the hairs and epidermis, and

renders the fungi easily detectable under the microscope. Artifacts that may be mistaken by inexperienced observers for fungal elements, and therefore to be remembered in diagnostic work, are oil or fat droplets, air bubbles, spaces between epithelial cells, etc. Stained preparations are unnecessary for routine diagnostic work.

In examining scales from the palms or soles in cases of suspected epidermophyton infection "ghost fungus" is frequently seen, *i.e.* rectangular spore-like structures between epithelial cells. It is now known that this appearance is not due to a fungus but to cholesterol crystals between the cells.

For artificial culture, Sabouraud's medium, adjusted to pH 5.0—5.5 (p. 195), is suitable, and it tends to restrain the growth of bacteria. The original procedure was to solidify a layer of the medium in the bottom of a conical flask. After inoculation the mouth of the flask was covered with a rubber cap over the cotton-wool plug, or sealed with paraffin (p. 213). A more convenient arrangement is to use as the container a screw-capped vial or medical flat bottle and to solidify the medium with the bottle in the horizontal position. This provides sufficient area of medium for inoculation and growth of the fungus, and the morphological characters of the growth can easily be observed. The screw-cap prevents evaporation. In making cultures from scales and hairs, immersion in 50 per cent. alcohol before "planting" them on the medium assists in the isolation of the fungus by destroying the cocci and other bacteria that may be present on the skin. The addition to the medium of 1 : 500,000 (0.0002 per cent.) gentian violet or 1 : 200,000 brilliant green assists in the isolation of the fungus by inhibiting contaminant bacteria. The cultures are incubated at 25° C. The growth of the fungus may be relatively slow, and the cultures may have to be incubated over a prolonged period to elicit characteristic appearances. It is for this reason that the containers must be sealed to prevent drying of the medium.

CHAPTER XXIV

MALARIA PLASMODIA; BABESIAE; TRYPANOSOMES; LEISHMANIAE

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.”

Three well-defined species of the malaria plasmodium are recognised :—

Plasmodium vivax—Benign tertian malaria.

Plasmodium malariae—Quartan malaria.

Plasmodium falciparum (*Laverania malariae*)—Malignant malaria.

These organisms belong to the order Haemosporidia of the Sporozoa. The plasmodium is transmitted by female anopheline mosquitoes, and goes through the sexual phase of its life-cycle in the body of the insect. Only the asexual stage 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 cell or sporozoite, containing nuclear material in the form of a chromatin granule. It was originally thought that the sporozoite directly entered a red cell, assuming an amoeboid form which gradually developed in the cell (trophozoite). Studies of bird-malaria first showed that the development in red cells is preceded by an “exo-erythrocytic” phase in reticulo-endothelial cells. In man there had been indirect evidence of such an exo-erythrocytic phase, and recently this has been definitely demonstrated. The complete sequence of events still requires further study and definition, but apparently the sporozoites invade liver cells and develop into large schizonts from which merozoites (*vide infra*) are produced and liberated, some

initiating a further exo-erythrocytic development, others now attacking red cells and becoming trophozoites in these cells. The erythrocytic trophozoite grows at the expense of the cell and accumulates altered blood pigment in the form of 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 (p. 639). 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.

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

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.

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.

DIFFERENTIATION OF THE MALARIA PLASMODIA

	PL. VIVAX (<i>Benign tertian</i>)	PL. MALARIAE (<i>Quartan</i>)	PL. FALCIPARUM (<i>Malignant</i>)
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 $\frac{1}{6}$ th or $\frac{1}{8}$ th 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 $\frac{1}{3}$ rd diameter of red cell; usually single chromatin granule	Thick round rings, about $\frac{1}{3}$ rd diameter of red cell; often in the form of equatorial bands	Small, well defined, thin; often 2 chromatin granules; often situated at the edge of red cell; about $\frac{1}{6}$ th or $\frac{1}{8}$ th diameter of red cell
Red cells (stained preparations) . . .	Swollen, pale, showing deeply stained rose to purple points, "Schuffner'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; macrogametocyte 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 (Stephens).—This is a fourth species or sub-species of malaria plasmodium, which has been reported in a few cases infected in East and West Africa. It 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*.

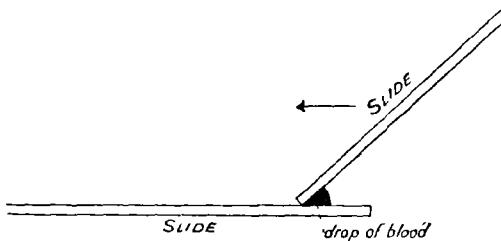
Certain other supposed sub-species of *Plasmodium* have also been described.

DIAGNOSIS OF MALARIA

THIN BLOOD FILMS.—Two or three films are made on microscope slides or $\frac{1}{4}$ -in. square No. 1 cover-slips, which have been carefully cleansed (p. 83) 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 and the area to be punctured are cleansed with 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 (p. 106). 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 $\frac{1}{2}$ -in. oil-immersion lens (p. 56) 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, and 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 is stained by Leishman's method, or Field's stain may be used in which case prior dehaemoglobinisation is not required (p. 111).

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 may be possible to determine the species or type present (Table, p. 639). 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.

It is noteworthy that 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.

Certain *cytological features of the blood* may, in the absence of actual organisms, suggest the existence of a malarial infection, e.g. a relatively high percentage of large mononuclear cells (15–20 per cent.), or the presence of leucocytes containing altered blood pigment (p. 638).

Cultivation of the Malaria Parasite.—This has sometimes been utilised for diagnostic purposes, and the following method has been recommended by Knowles. 5 ml. of blood are withdrawn by vein puncture. The interior of the syringe, before it is used, must be thoroughly washed with saline solution. The blood is placed in a sterile flask with glass beads and defibrinated. For the actual culture, stoppered sterilised tubes, 12.5 by 1.25 cm., are employed, and in each of these is placed one drop of sterilised 50 per cent. pure glucose; defibrinated blood to a depth of 2.5 cm. is then added. Partial anaerobic conditions are produced by warming the upper part of the tube and then attaching a rubber teat to the mouth. The tubes are incubated at 37° C. After twelve hours, and also after twenty-four or forty-eight hours, a small amount of the surface layer of the deposited red cells is aspirated with a capillary pipette and from this, films are made on slides and stained by the usual method. It should be noted, however, that cultivation has often proved quite unsuccessful.

BABESIAE (or PIROPLASMS)

These protozoal organisms (classified with the Sporozoa) produce disease in various domesticated 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 2μ to 4μ 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, and the eggs may also 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 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* (p. 566).

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, $12\text{--}40\mu$ long by $1\cdot5\text{--}3\mu$ 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. 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 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. 198.

TRYPANOSOMA RHODESIENSE

Associated often with a more acute form of Sleeping Sickness than that produced by *Trypanosoma gambiense*.

Glossina morsitans is the insect vector.

Morphologically it resembles *Trypanosoma 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 *Trypanosoma 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 *Trypanosoma brucei*, the organism of Nagana, but it has been shown by inoculation of man that *Trypanosoma 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*).

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, "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 or 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.

Auto-agglutination of blood cells is frequently observed in trypanosomiasis and is a useful indication for diagnostic purposes. A simple method for detecting this phenomenon is to spread a drop of blood on a slide under a cover-slip and examine microscopically. The cells instead of forming rouleaux become aggregated in compact clumps. The reaction may probably be of the same nature as the "cold agglutination" observed in primary atypical pneumonia (p. 611).

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.

They are designated as follows:

Leishmania donovani—of Kala-azar (occurring in certain Eastern countries).

Leishmania tropica—of Tropical Sore or Delhi Boil. This is generally regarded as a separate species.

“*Leishmania infantum*”—of Infantile Splenomegaly (occurring in North Africa). It is doubtful whether this form constitutes a species separate from *Leishmania donovani*.

“*Leishmania brasiliensis*”—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 (p. 198) incubated at $20^{\circ}\text{--}24^{\circ}\text{C}$. 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

(a) *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.

(b) *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 the following are therefore often substituted.

(c) *Liver Puncture*. (d) *Bone-marrow aspiration* (from the sternum or tibia).

(e) *Culture*.—Aspirated spleen or gland "juice" is cultured on N.N.N. medium.

(f) *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.

¹ Kirk, R., and Sati, M. H., *Trans. Roy. Soc. Trop. Med. and Hyg.*, 1939-1940, 33, 501.

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).¹

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 exudate of the sore (preferably at the margin) after carefully cleansing the surface and removing the surface discharge. They are stained by Leishman's stain.

LEISHMANIA INFANTUM

This organism is similar to *Leish. donovani*, and is associated with a pathological condition, similar to that of kala-azar, occurring in young children in North Africa and the Mediterranean littoral. The infection also occurs naturally in dogs, which are possibly the origin of the human disease, the organism being transmitted by an insect (probably *Phlebotomus perniciosus*). Monkeys, dogs, guinea-pigs and certain hamsters are susceptible to experimental infection.

Diagnosis is established as in kala-azar.

TOXOPLASMA

This organism, an unclassified protozoon, has been found in cases of encephalitis of young subjects (being demonstrable sometimes in the cerebrospinal fluid) and in exanthematous blood infection with pneumonitis of adults. The infection may occur in the new-born and appears to be derived from the mother *in utero*, the maternal infection being an inapparent one. The organism occurs in the form of, oval or crescentic bodies, about $6-7\mu$ by $2-4\mu$, which are found in endothelial and large mononuclear cells, but also in the free state. With a Romanowsky stain, e.g. Giemsa's, it shows a reddish nuclear structure and blue cytoplasm. It can be transmitted experimentally to various animals, e.g. guinea-pigs and other laboratory rodents. The origin of the human infection has not been defined. The organism causes natural infections in rodents.

¹ *Ind. Med. Gaz.*, 1945, 80, 896.

For diagnosis during life body fluids may be inoculated into laboratory rodents. A *neutralisation test* for demonstrating antibodies in blood serum has been used with some success in the diagnosis of chronic inapparent infection: the serum is mixed with a suspension of toxoplasma-containing material from an experimental animal and the mixture is then injected intracutaneously in the rabbit; if antibodies are present the animal is protected from infection.

Another laboratory test which has been used in the diagnosis of toxoplasmosis 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. Patient's serum can thus be tested quantitatively for the presence of antibody indicative of toxoplasma infection. The organisms used in the test are obtained from peritoneal exudate of inoculated mice. For details of the method and interpretation of results reference should be made to Sabin, A. B., and Feldman, H. A., *Science*, 1948, 108, 660; *Pediatrics*, 1949, 4, 660; and Beverley, J. K. A., and Beattie, C. P., *J. Clin. Path.*, 1952, 5, 350.

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Printed in Great Britain
at Hopetoun Street, Edinburgh,
by T. and A. CONSTABLE LTD.
Printers to the University of Edinburgh

