

The English Language Book Society publishes low-priced, unabridged editions of important British textbooks. Below is a list of some other medical books available under the ELBS imprint.

Anderson
Muir's Textbook of Pathology
Edward Arnold

Baron
A Short Textbook of Chemical Pathology
Hodder & Stoughton

Cheesbrough and McArthur
A Laboratory Manual for Rural Tropical Hospitals
Churchill Livingstone

Freeman and Bracegirdle
An Atlas of Histology
Heinemann Educational

King
A Medical Laboratory for Developing Countries
Oxford University Press

Mason and Swash
Hutchison's Clinical Methods
Bailli  re Tindall

Morley
Paediatric Priorities in the Developing World
Butterworths

Ogilvie
Chamberlain's Symptoms and Signs in Clinical Medicine
John Wright

Roitt
Essential Immunology
Blackwell Scientific

Thompson
A Short Textbook of Haematology
Pitman Medical

Introduction to Medical Laboratory Technology

Fifth Edition

F. J. BAKER F.I.M.L.S., F.I.S.T.

**R. E. SILVERTON F.I.M.L.S.,
L.I.Biol.**

With the collaboration of

**D. CANNING, F.I.M.L.S.
(Haematology and Blood Transfusion)**

**D. KILSHAW, F.I.M.L.S.
(Clinical Chemistry)**

**J. LAW, F.I.M.L.S.
(Histology)**

**R. SHANNON, M.Phil., F.I.M.L.S.
(Microbiology)**



English Language Book Society/Butterworths

**Butterworth & Co (Publishers) Ltd
88 Kingsway, London WC23 6AB**

© Butterworth & Co (Publishers) Ltd 1976

All rights reserved. No part of this publication may be reproduced or transmitted in any form or by any means, including photocopying and recording, without the written permission of the copyright holder, application for which should be addressed to the Publishers. Such written permission must also be obtained before any part of this publication is stored in a retrieval system of any nature.

First published 1954

Second edition 1957

Revised 1960

Reprinted 1961

Third edition 1962

Reprinted 1964, 1965

Fourth edition 1966

**Reprinted 1968, 1969, 1970, 1971, 1972,
1974, January 1975, August 1975**

Fifth edition 1976

Reprinted 1978, 1980, 1982

ELBS edition first published 1978

Reprinted 1980, 1982, 1985

ISBN 0 407 00154 9

Cataloging in Publication Data

Baker, Francis Joseph.

Introduction to medical laboratory technology.

Bibliography: p.

Includes index

I. Medical laboratories—Technique. I. Silverton, R. E., joint author. II.

Title. III. Title: Medical laboratory technology.

RB37.B28 1976 616.07'5

ISBN 0-407-00154-9

**Printed in Great Britain by
Butler & Tanner Ltd, Frome and London**

Preface

During the twenty-two years which have elapsed since this work was first published, laboratory medicine has undergone many changes. These changes relate not only to the more specialized and sophisticated techniques now used, but also to fundamental changes within the profession itself. Automation, data processing and other technical advances have all helped to bring about not only procedural change but changes in outlook. In the United Kingdom, the name of the professional body has been changed from the Institute of Medical Laboratory Technology to the Institute of Medical Laboratory Sciences (IMLS). This in itself reflects the progress which has been made. The examination structure for medical laboratory scientists has similarly been changed, and currently a National Certificate pathway is the common route to Associateship of the IMLS and State Registration. Graduates are entering the IMLS and the profession in increasing numbers, and the formation of a College of Pathologists, subsequently the Royal College of Pathologists, are added signs of the need for an understanding of the increasing complexities of modern medical laboratory sciences.

These many changes have been borne in mind in preparing the 5th Edition of this work. The contents have been enlarged, updated and in many instances have been completely re-written.

Despite the changes in the layout, contents of the book, and the profession to which it applies, the original title *Introduction to Medical Laboratory Technology* by which it is universally known, has been purposely retained.

A further change that has taken place in recent years is the introduction, particularly into the National Health Service, of SI Units. Système International d'Unités (SI Units) were approved internationally in 1960 and are becoming generally accepted in science and medicine in Europe and some other parts of the world.

A table of SI units, showing the changes in nomenclature and the abbreviations used, will be found at the end of the book (p. 681).

It will be seen that we have used SI units throughout the text,

PREFACE

with the exception of pounds per square inch (psi). This measurement has been retained as most autoclave gauges are calibrated this way and it seemed pointless, at this moment in time, to adopt the equivalent SI unit, and thereby produce unnecessary complications. The more common term *ml* has also been retained instead of *cm³* for measurement of volume.

Because of the increase in specialization, we have again sought the advice of many colleagues in various disciplines throughout the UK but in particular we would like to record our appreciation of the following, who have acted as collaborators in their various specialities:

Mr D. Canning, FIMLS
Mr D. Kilshaw, FIMLS
Mr J. Law, FIMLS
Mr R. Shannon, MPhil, FIMLS.

We are hopeful that the 5th Edition of *Introduction to Medical Laboratory Technology* will be as valuable to the many types of readers as the earlier editions proved to be.

Introduction

Medical Laboratory Science is a complex subject embracing a number of different disciplines. The post-war years brought a dramatic increase in the use of the laboratory whose role is to assist in the diagnosis, treatment and control of disease. The increased demands on the laboratory inevitably resulted in the introduction of more specialized and sophisticated procedures including mechanization, automation and data processing. To keep abreast with this modern development, a more academic background has become necessary for students entering the profession.

Entrants into the profession are now required to undergo formal training at a recognized college of further education or to possess a relevant university degree.

These examinations or qualifications coupled with satisfactory experience in a recognized laboratory lead to State Registration by the Council for Professions Supplementary to Medicine.

The professional body—the Institute of Medical Laboratory Sciences—is responsible for providing the opportunities to qualify by examinations. At the present moment the National Certificate pathway is the main route to qualification. The IMLS have members on the Joint Committees (who are responsible for the certificates in Medical Laboratory Subjects/Sciences) and the IMLS appoint the Assessors for these various certificates.

Newcomers to the profession are reminded that it is not a profession to be taken up lightly. In order to pass the qualifying examinations, many leisure hours will have to be devoted to study, and, even when qualified, the medical laboratory scientist will still have to keep abreast with modern developments and trends by the regular reading of the appropriate journals. The late arrival of a specimen in the laboratory, the occurrence of which is by no means infrequent, may also necessitate working after hours and sacrificing private arrangements. Considerable satisfaction, however, will be derived not only from the interesting nature of the work, but also from the knowledge that the duties undertaken during each working day are for the benefit of the community. The importance of his work and his obligations to the patient must

therefore be remembered at all times and placed before any personal consideration.

The Medical Laboratory Technician's Board of the Professions Supplementary to Medicine issued a statement to all registered practitioners of medical laboratory sciences which is reproduced here with their approval.

No registered medical laboratory technician should:

1. Hold himself out as a person, who by training and experience, is professionally qualified to diagnose or treat disease in man or animal.
2. Knowingly accept, obtain, assist in obtaining or report on any specimen for the purpose of the diagnosis and/or treatment of disease, or make any investigation for those purposes unless the diagnosis and/or treatment are to be performed by a registered medical, dental, or veterinary practitioner.
3. Knowingly disclose to any patient or to any other unauthorised person the result of any investigations or any other information of a personal or confidential nature gained in the course of practice of his profession.
4. Advertise, whether directly or indirectly, or associate himself in any way with advertisement for the purpose of obtaining specimens for laboratory investigations.
5. Knowingly falsify or suppress a report of any laboratory investigation with which he may be concerned.

1

General Laboratory Glassware and Apparatus

GLASSWARE

Glassware is widely used in medical laboratories, and it is essential to become thoroughly familiar with the common varieties. This chapter is concerned only with some of the more general types, and specialized equipment is not considered.

Composition of glass

Laboratory glassware is usually manufactured from borosilicate glass, a material developed to conform to certain well-defined characteristics. It is resistant to the action of chemicals with the exception of hydrofluoric and phosphoric acid and is made to withstand mechanical breakage and a sudden change of temperature. Resistance to thermal shock necessitates a low coefficient of thermal expansion. Glassware produced from the soda-lime type of glass does not meet this requirement and is easily broken by the mechanical stress produced by a sudden change of temperature.

Hardened glass, such as Pyrex, has a low soda content and is manufactured especially to resist thermal shock. The walls of the vessels are generally thicker than those made from soda-lime glass and the low soda content increases the chemical durability of the glass. With the less expensive soda-lime glassware, however, free soda is present on the walls, and must be neutralized before use. The main ingredients of borosilicate glass are as follows:

	<i>Per cent</i>
Silica (SiO_2)	80.6
Sodium oxide (Na_2O)	4.15
Boric oxide (B_2O_3)	12.6
Aluminium oxide (Al_2O_3)	2.2

CARE OF GLASSWARE

All glassware must be handled carefully. Breakages can sometimes be dangerous, and they may result in the loss of valuable and irreplaceable material. Certain precautions must be observed.

1. Flasks and beakers should be placed on a gauze mat when they are heated over a bunsen flame.
2. Test-tubes exposed to a naked flame should be made of heat-resistant glass (such as Pyrex).
3. If liquids are to be heated in a bath of boiling water, the glass containers used should be heat-resistant. It is safer to immerse the containers in warm water, which is then brought to the boil, than to plunge them directly into boiling water. Similarly, sudden cooling of hot glass should be avoided, unless it is specifically required.
4. When diluting concentrated acids, thin-walled glassware should be used. The heat evolved by the procedure often cracks thick glass.
5. Heat expansion is liable to crack bottles if their caps are screwed on tightly. If heat is to be applied, flasks held in retort stands should not be tightly clamped.
6. Containers and their corresponding ground-glass stoppers should be numbered, to ensure correct matching when stoppers are replaced. When these bottles or flasks are being used, the stoppers should be laid on clean filter paper, to avoid scratching them.

Cleaning of glassware**GENERAL GLASSWARE**

The cleaning of all glassware is simplified by rinsing in tap water immediately after use. Contaminated material, however, must always be sterilized before cleaning is commenced.

New glassware may be cleaned by washing in a detergent such as Pyroneg and then rinsed thoroughly in tap water. Soda-lime glassware should have the free alkali neutralized by standing the glass in a 5 per cent solution of hydrochloric acid. This is followed by several rinses in tap water and in distilled water. If it is desired simply to neutralize free alkali given off by new glassware, it may be steeped in 1 per cent hydrochloric acid for several hours. This is followed by thorough rinsing. The glass is then dried in a hot-air oven. To test that the free alkali has been neutralized, autoclave the glassware in neutral distilled water, and when cool, check the

pH of the water. If excess alkali has been given off (the pH is high, see p. 131) re-steep the glassware in the hydrochloric acid. If free alkali still persists after several treatments the glassware should be discarded.

Slides required for blood films must be absolutely grease-free. They are soaked overnight in dichromate cleaning fluid (*see below*) or in nitric acid. After thorough rinsing, they may be stored in methylated spirit until required. For general purposes, new slides may be stored directly in spirit. It is safer not to reclaim slides used for films of tuberculous material.

BIOCHEMICAL GLASSWARE

Chemical cleaning is necessary for the following reasons.

1. Traces of reagents left in tubes and containers may interfere with later chemical investigations, for example the *o*-toluidine test for occult blood may be positive if the glassware contains even minute traces of dried blood.
2. Air bubbles may be trapped between greasy surfaces and contained liquid, resulting in inaccurate volumetric readings.

Procedure for rendering glassware chemically clean

1. Preparation of cleaning fluid:

Potassium dichromate	10 g
Concentrated sulphuric acid	25 ml
Distilled water	75 ml

Grind the dichromate crystals in a pestle and mortar, and add the powder to the distilled water in a heat-resistant flask. Pour in the acid very slowly. The heat evolved hastens the dissolving of the potassium dichromate.

Note—This fluid should be handled with caution, rubber gloves and apron being worn to protect the hands and clothes and an eyeshield to protect the eyes. If clothes or skin are splashed with the fluid, they should immediately be washed in water, and any residual acid neutralized with a weak alkali. This, in turn, is washed off with tap water.

After repeated use, the colour of the fluid may darken. When this occurs, fresh fluid should be prepared.

2. Steep the glassware in the cleaning mixture for several hours.
3. Remove the glassware, and wash it thoroughly in tap water, to remove all traces of acid and preferably leave in fresh water overnight.

4. Rinse twice in distilled water.
5. After allowing surplus water to drain off, dry the glassware in a hot-air oven.

Procedure for cleaning glassware with detergents

The use of detergents for cleaning glassware is becoming increasingly popular in medical laboratories. These detergents, which are available in either liquid or powder form, owe their cleansing action to the manner in which they reduce the interfacial tension of water with that of oily or greasy substances.

Detergents possess the following advantages over ordinary soaps:

1. Their cleansing action is unaffected by the temperature of the water.
2. They are equally efficient in water which is either slightly alkaline or slightly acid.
3. They have no coagulative action on proteins.

One serious disadvantage of some detergents is their haemolytic action on red blood cells, the slightest trace of detergent being capable of producing haemolysis. This point must always be borne in mind, particularly with glassware destined to be used for haematology or blood transfusion work. The following procedure should be adopted when using detergents for cleaning glassware:

1. Rinse the glassware thoroughly in cold tap water.
2. Place in the detergent solution and brush thoroughly.
3. Wash thoroughly in running tap water.
4. Rinse three times in distilled water, using fresh distilled water for each rinse.
5. Drain off excess water and dry in the hot-air oven. Glassware dried in the hot-air oven should be packed, mouth downwards, in metal baskets, the bottoms of which are lined with thick blotting paper.

Recommended detergents are Decon,* Decon 90 and RBS 25†. These are phosphate-free, surface-active agents suitable for cleaning all glassware and plastics including those contaminated with radioactive material.

It has been stated that accurately calibrated volumetric glassware should never be heated in the oven as the expansion and con-

* Obtainable from Decon Laboratories Ltd, Ellen Street, Portslade, Brighton, Sussex.

† Obtainable from Chemical Concentrates Ltd, 41 Webb's Road, London SW11.

traction of glass that occurs may render the graduations inaccurate. Some workers, however, have shown that this is not the case and that Grade A glassware may be sterilized in a hot-air oven.

Cleaning of pipettes

1. Steep the pipettes overnight in cleaning fluid.
2. The following morning, wash them thoroughly in tap water, preferably leave overnight in fresh water, and rinse in distilled water.

To facilitate washing, connect the pipette to a water pump, using rubber tubing of suitable bore, and suck tap water through for several seconds. Follow this with two or three rinses of hot distilled water.
3. Dry the pipette with two or three brief rinses of acetone. Drying is best effected by sucking through small volumes of acetone and air successively. Repeat this procedure until the internal surface is quite dry. Alternatively an electrically heated pipette dryer may be used.
4. Wipe the outside of the pipette.
5. To avoid breakage, store the pipettes in drawers lined with lint. It is convenient to fit the drawers with separate compartments for each size and type of pipette.

Note—Immediately after use, pipettes should be rinsed in tap water, especially when they have held proteinous fluid, for example blood. Should the pipette be heavily contaminated with such material, it may be cleaned by standing it in a strong solution of caustic soda. This treatment should not be prolonged, as the alkali dissolves glass and may cause an alteration in contained volume. A pipette which has been used for measuring stain can often be cleaned rapidly by rinsing it through with hydrochloric acid.

The cleaning procedures described above do not apply to pasteur pipettes. After use with infected material, these are placed in a disinfectant solution.

Standardized glassware

Apparatus used for the measurement of liquid volume, for example, pipettes, burettes, volumetric flasks and cylinders, are divided into three grades, depending on the accuracy of calibration.

The limits of these grades are defined by the British Standards Institution, and the manufacturers mark each piece of standardized glassware with the appropriate symbol. The maker's assurance, however, is the only guarantee that the product

conforms with the BSI criteria. For example, tolerances laid down for bulb-type pipettes are given in Table 1.1.

Table 1.1 TABLE OF TOLERANCES FOR DELIVERY PIPETTES (BULB TYPE)

Capacity in ml	2	10	20	50
Time of outflow seconds, grade A	7-15	18-25	20-35	25-40
Tolerance \pm ml grade A	0.01	0.02	0.02	0.04
Time of outflow seconds, grade B	7-20	15-40	20-50	25-60
Tolerance \pm ml grade B	0.02	0.04	0.05	0.08

The most accurately calibrated glassware available in Britain carries certificates from the National Physical Laboratory. Each such piece of apparatus is etched with the letters NPL. This glassware is necessary only for the highest standards of accuracy.

Example of the markings on an NPL Class A pipette are:

NPLA	NPL Class A
5 ml	5 ml volume
D20 °C	Delivery pipette: volume correct at 20 °C
10 + 15	10 s to deliver: 15 s to drain
32867	Certificate number

By international agreement, pipettes and vessels 'to deliver' are marked with the letters 'Ex', while those designed 'to contain' are marked with the letters 'In'. The delivery time is no longer given and indeed it is recommended that the tip of the jet be kept in contact with the inside of the receiving vessel for approximately 3 s after movement of the meniscus has appeared to cease.

General glassware

BEAKERS

These have capacities of from 5 to 5000 ml. They are usually made of heat-resistant glass, and are available in different shapes. The type most commonly used is the *squat form*, which is cylindrical and has a spout. There is also a *tall form*, usually without a spout. Conical and flask-shaped types are available, but these are not widely used. Beakers are often supplied in sets or *nests* of assorted sizes. Some may be graduated, while others may be made of polystyrene.

BOTTLES

These are made in many shapes. Some of the more general types are described.

Reagent bottles are supplied in 25–1000 ml capacities. They are cylindrical, have narrow necks, and are fitted with ground-glass or polythene stoppers.

Screw-capped bottles are supplied in 5–1000 ml capacities, and may be round or flat (the 5 ml size is often called a 'bijou' bottle). The caps may be made of metal or plastic. These bottles are used for holding specimens, solutions and media. Metal caps should never be used on bottles containing mercuric chloride, as this substance will attack the metal.

Winchester quart bottles are of 2000 ml capacity, and are available in white or brown glass. They may be fitted with glass stoppers, corks or rubber bungs. They are useful for storing stock solutions and reagents, and for specimens, for example, samples of urine collected over 24 h.

Drop bottles are of about 50 ml capacity, and are made in white or brown glass, with a narrow neck and a slotted glass stopper. They are designed for delivery of drops of solutions, such as stains. After use the stoppers should be turned, so that the contents are not open to the air.

Polythene bottles, although made of plastic material, should be mentioned here. They are of various sizes and shapes, and some are fitted with a nozzle, for use as 'wash bottles'.

BURETTES

These are used for measuring variable quantities of liquid, and are made in capacities of 1–100 ml. They are long graduated tubes of uniform bore and are closed at the lower end by means of a glass stopcock, which should be lightly greased for smooth rotation.

Technique of using burettes

1. Before use, half-fill the burette with distilled water, and allow it to be discharged through the tap. Droplets adhering to the glass indicate a greasy surface. If this is seen, chemical cleaning is necessary.
2. Rinse the burette two or three times with small volumes of the solution to be measured, discharging the washings through the tap.
3. With the tap closed, clamp the burette in a vertical position,

and pour in the liquid to be measured through a funnel, until the meniscus rises above the zero mark of the burette. Remove the funnel.

4. Open the stopcock tap until the meniscus of the liquid exactly coincides with the zero mark. Make sure that the tap is completely free of air-bubbles, and that the tip has no droplets adhering to it. The burette is now ready for use.
5. When work with the burette is complete, the fluid is drained out. The burette is rinsed through several times with tap water, and then with distilled water.
6. For storage, the burette is clamped in an inverted position.

CENTRIFUGE TUBES

These are made of hardened glass, nylon or plastic material that can withstand the centrifugal strain. The bottom of the tubes may be round or conical. The latter type is preferable, because the deposit is concentrated into a smaller volume. Some centrifuge tubes are calibrated up to 10 ml. These markings may be useful, provided the graduations have been checked for accuracy.

DESICCATORS

It may be necessary to dehydrate substances, or to keep them in an anhydrous state. This may be effected by storing them in a desiccator over a water-absorbent chemical, such as anhydrous calcium chloride or phosphorous pentoxide. Evacuation of air increases the rate of dehydration, and some desiccators are therefore made of glass strong enough to withstand a vacuum. Others are not designed for this purpose, and have no tap in the lid. The tap, when present, and the opposed surfaces of jar and lid are of ground-glass.

Technique of using vacuum desiccators

1. Place the substance to be stored in an open container, and rest this on the zinc gauze sheet, which forms a platform above the desiccating chemical. Lubricate the tap and the opposing surfaces of jar and lid with Vaseline, petroleum jelly or stopcock grease, to ensure that all junctions are airtight.
2. Slide the cover onto the jar, and rotate it into position.
3. Open the tap, and connect the outlet to a vacuum pump. If this is of the water type, always insert a trap-bottle between the pump and desiccator to prevent any backflow

of water into the desiccator from a sudden fall in water pressure (p. 19).

4. When the jar has been sufficiently evacuated, close the tap, turn off the pump, and disconnect the desiccator.
5. To open the vessel, release the vacuum. Turn the tap gradually, allowing air to be sucked in. Slide the lid off the jar. If the desiccator has been kept in a refrigerator, allow the temperature of the glass to rise to that of the room, before opening the tap. This prevents condensation of moisture on the inside of the cold glass.

EVAPORATING BASINS

These are shallow vessels made of porcelain, silica, or heat-resistant glass.

FUNNELS

Buchner funnels (see p. 490).

Filter funnels are used for pouring liquids into narrow-mouthed containers, and for supporting filter papers during filtration. Some funnels have a fluted inner surface.

Separating funnels are for separating immiscible liquids of different densities, for example, ether and water.

Technique of using separating funnels

1. Close the tap, and add the mixture of liquids until the bulb is about half full.
2. Insert the stopper, and holding both tap and stopper firmly in position shake to facilitate extraction.
3. Release the pressure from time to time by inverting the funnel and gently opening the tap.
4. Stand the funnel upright and allow the liquids to separate.
5. Remove the stopper and open the tap carefully, allowing the lower layer to run out slowly.
6. Close the tap just before the last drop of the lower layer has escaped.

FLASKS

Flasks have capacities of 25–6000 ml.

Conical flasks (Erlenmeyer) are useful for titrations, and also for boiling solutions when it is necessary to keep evaporation to a minimum. Some have a side arm, suitable for attachment to a vacuum pump. Buchner or Seitz filters can be inserted into rubber bungs, and used in conjunction with these flasks.

Flat-bottomed round flasks are convenient containers in which to heat liquids. A gauze mat should be interposed between flask and flame. These flasks are widely used in the preparation of bacteriological culture media.

Round-bottomed flasks can withstand higher temperatures than the flat-bottomed type. They may be heated in a naked flame, or in an electrothermal mantle.

Volumetric flasks are flat-bottomed, pear-shaped vessels with long narrow necks, and are fitted with ground-glass or plastic stoppers. Most flasks are graduated to contain a certain volume, and these are marked with the letters 'C' or 'In'. Those designed to deliver a given volume are marked with the letters 'D' or 'Ex'. A horizontal line etched round the neck denotes the stated volume of water at a given temperature, for example, 20 °C. The neck is narrow, so that slight errors in reading the meniscus result in relatively small volumetric differences.

Technique of using volumetric flasks

To prepare an accurate solution of known concentration, proceed as follows.

1. Transfer the accurately weighed substance to the flask, using a funnel.
2. Using a little of the solvent, wash any residual traces of the weighed substance from the watchglass and funnel into the flask. Half-fill the flask with further solvent.
3. Stopper the flask, and shake it until the weighed substance is completely dissolved.
4. Add solvent until the lower margin of the meniscus reaches the etched line on the flask neck. Invert to mix.

Variations of temperature cause changes in volume. If the solution is warm, it should be allowed to cool to room temperature, or ideally the temperature at which the glass was calibrated, before the volume is made up to the line.

MEASURING CYLINDERS

These are supplied in 10–2000 ml capacities. Some are made of heat-resistant glass, and some are fitted with ground-glass or plastic stoppers. Measurement of liquids can be made quickly with these vessels, but a high degree of accuracy is impossible because of their wide bore.

PESTLE AND MORTAR

These are used for grinding solids, for example calculi and large crystals of chemicals. Those of unglazed porcelain have a porous surface, and those of heavy glass are made with roughened surfaces. Some are of agate, and these are uniformly smooth. After use, always clean the pestle and mortar thoroughly, for chemicals may be driven into the unglazed surfaces during grinding, resulting in contamination when the apparatus is next used.

PETRI DISHES

Petri dishes are flat glass or plastic containers which have a number of uses in the medical laboratory. They are used predominantly for the cultivation of organisms on solid media. They are made with diameters of 5–14 cm. For further details reference should be made to the chapter on The Use of Culture Media (Ch. 23).

PIPETTES

These are used to measure liquid volumes of up to 50 ml. There are several types, each having its own advantages and limitations.

AUTOMATIC PIPETTES AND DISPENSERS

These are designed to measure and deliver variable volumes of fluids. There are many types on the market (*Figures 1.1, 1.2 and 1.3*) which will deliver micro and macro volumes. With many of them, disposable tips are available.

Another type of automatic pipette is that shown in *Figure 1.4*. These pipettes are compact, versatile units, designed for rapid repetitive work. Their working principle is that pressure from the reservoir moves the piston contained within the graduated barrel. The position at which the piston is set can be adjusted by means of a micro-adjustor, thereby allowing the volume of the reagent delivered to be changed. Turning the stopcock key to the left, drives the piston along from the right end of the barrel until it reaches the stop, forcing the volume of liquid before it out of the delivery jet. Turning the stopcock key in the opposite direction reverses the procedure, thereby producing a rapid and continuous delivery of the reagent.

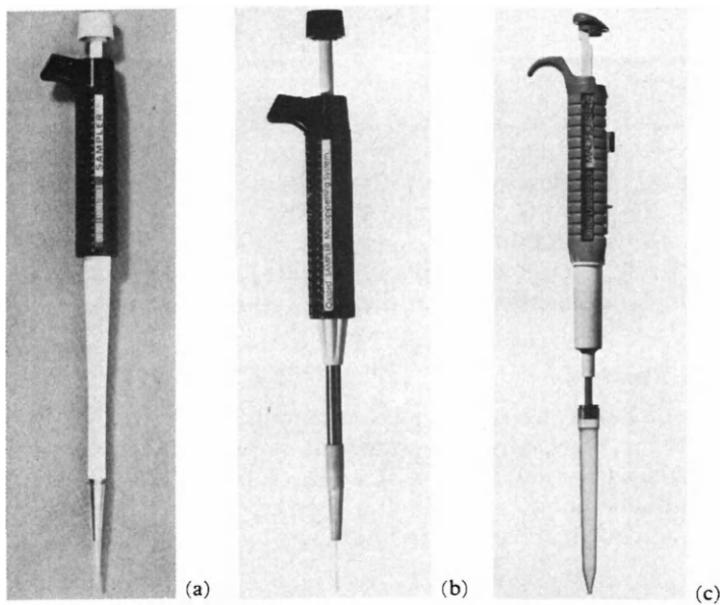
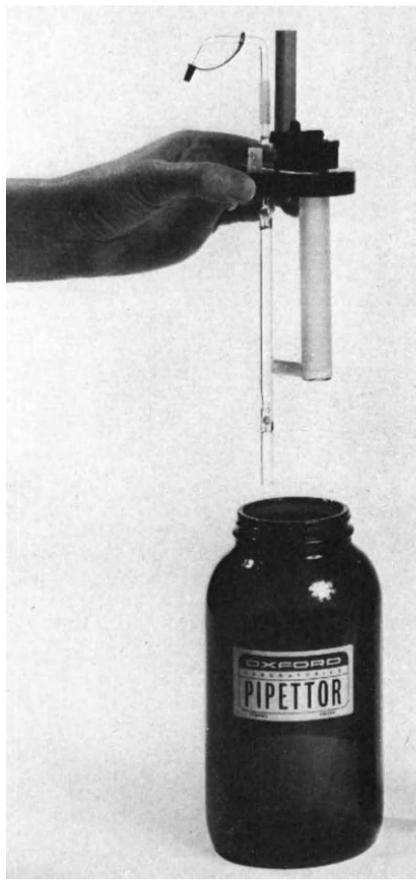


Figure 1.1. (a) Ultra-micro sampler. (b) Sampler. (c) Macro set. (Reproduced by courtesy of The Boehringer Corporation (London) Ltd)



(a)



(b)

Figure 1.2. (a) Model S Dispensor. (b) Pipettor. (Reproduced by courtesy of The Boehringer Corporation (London) Ltd)

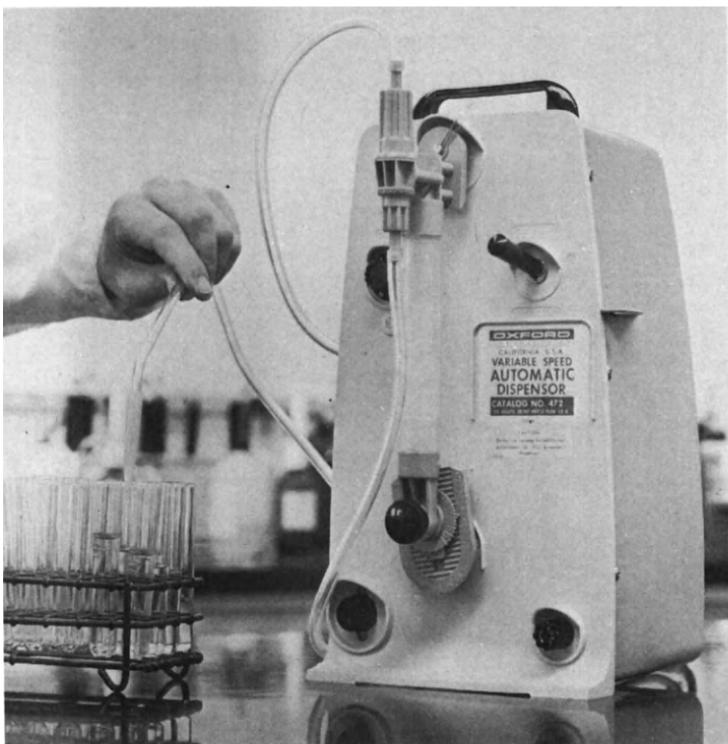


Figure 1.3. Automatic Dispensor. (Reproduced by courtesy of The Boehringer Corporation (London) Ltd)

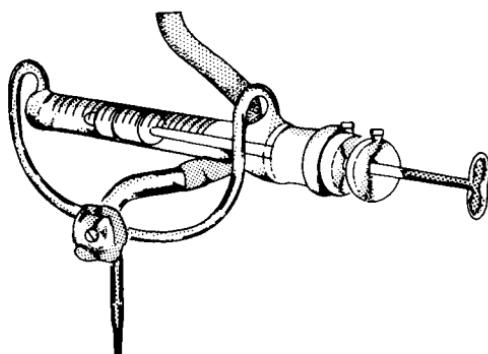


Figure 1.4. Exelo double action automatic pipette

Delivery pipettes

Delivery pipettes are made in 1–50 ml capacities. They are calibrated to *deliver* a constant volume of liquid under certain specified conditions. These pipettes are marked with the letter 'D', the temperature at which the pipette was calibrated, that is, 20 °C unless otherwise stated, and a letter denoting the grade. Pipettes are calibrated using water, and consequently their use for fluids of different viscosity results in inaccurate measurements. Such liquids, for example glycerin and ether, are most accurately measured with pipettes designed to be rinsed out.

There are two types of delivery pipette: the volumetric and the graduated types.

The *volumetric (bulb) type* is the most accurate type of pipette in everyday use. The outflow and drainage times are usually marked on the bulb.

Technique of using volumetric pipettes

1. Rinse out the pipette with the fluid to be measured.
2. Fill it by suction, until the liquid rises above the graduation mark. Retain the liquid at this level by placing the dry forefinger over the mouthpiece of the pipette.
3. Reduce the pressure of the finger, allowing the liquid to run slowly down to the mark.
4. Read the meniscus at eye-level, to avoid any error due to parallax.
5. Wipe the outside of the stem with a clean cloth, and remove any drops on the tip by touching it against a glass vessel.
6. Remove the finger, and the pipette will deliver its contents. Hold the tip of the pipette against the inside of the receiving vessel, until the liquid has run out.
7. When the free flow has ceased, hold the pipette against the side of the receiver for a further 15 s, allowing it to drain. The entire volume will now have been delivered.
8. The residual drop in the tip is discarded.

The graduated type

These pipettes are satisfactory for most routine purposes, if a high degree of accuracy is not essential. They are used in the same way as the volumetric pipette, but the graduations along the stem enable variable amounts of liquid to be delivered.

The least accurate sector of the graduated stem is the tapering

point. Some pipettes are made with graduations that do not extend down to the tip.

Pipettes 'to contain'

Some pipettes are not designed for delivery of a given volume of liquid, but are made to *contain* it. A pipette of this type holds a stated volume, and in order to transfer its contents completely, one must rinse out the pipette after it has drained. There are two types of pipette made *to contain*: the bulb type and the graduated type.

Bulb type pipettes are made to contain a specified volume. They are available in 0.2–10 ml volumes.

Many micro-methods of blood analysis, for example blood sugar and urea methods, require accurate measurement of 0.2 ml of blood and a 'wash-out' pipette should be used for this purpose.

Technique of using bulb type wash-out pipette

1. Fill the pipette with liquid and allow the meniscus to fall to the mark 'O' and wipe the outside with a cloth.
2. Run the liquid slowly into the receiving vessel, until the pipette is empty but for a residual drop at the tip.
3. Remove the last traces of solution in the pipette by rinsing it out two or three times, and add the rinsings to the delivered volume in the receiving vessel.

Graduated pipettes

Graduated pipettes are made for measuring volumes of 0.2–25 ml. They are used in the same way as the bulb type (Ostwald) pipette.

Haemacytometer pipettes are of the wash-out type. For further details and the method for checking the calibration see pp. 571 and 576–7.

'TRAP-BOTTLES'

These are used as a safeguard against the back-flow of water when water-vacuum pumps are being used.

Technique of using the trap-bottle

1. Using rubber tubing, connect the side arm of the flask to the vacuum pump.
2. Connect the apparatus to be evacuated to a short piece of glass tubing projecting from the bung of the trap-bottle.

WATER-VACUUM PUMP (water Venturi pump)

This indispensable piece of laboratory equipment may be made of metal or of glass. The principle of its action is illustrated in *Figure 1.5.*

If the vacuum pump fails during use, negative pressure in the

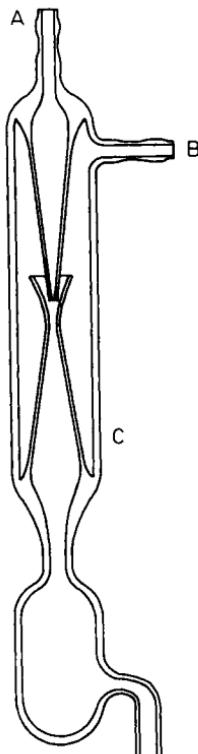


Figure 1.5. The principle of the Venturi pump. Inlet A is connected to the water-tap. When the tap is turned on fully, water flows rapidly into tube C which has a constriction near its upper end. Air is sucked into the rapidly flowing jet of water, and the negative pressure created inside the jacket of the pump causes air to be sucked in through inlet B

system will cause water to be sucked back. The interposed trap-bottle will receive the water, and prevent it from flowing back into the vessel being evacuated. A glass Venturi pump is available incorporating an all glass non-return valve, making a trap-bottle unnecessary.

APPARATUS

Autoclaves

The autoclave is used for sterilization by steam under pressure (*see Chapter 22*).

Centrifuges

Centrifuges are used to hasten the deposition of substances suspended in liquids. The suspended matter is deposited in order of weight, the heaviest element being the first to settle.

There are many types of centrifuge, but the basic principle is the same, that is, the use of centrifugal force. When this exceeds the force of gravity, heavier elements are thrown to the bottom of the tube. The earlier centrifuges were operated by hand or water pressure but most modern laboratories are equipped with electrically driven models.

For the purpose of comparison, centrifugal force is referred to as RCF (Relative Centrifugal Force). The RCF is taken as a guide to the separating capacity of a centrifuge and the RCF value for any centrifuge may be calculated from the following equation,

$$\text{RCF (in g)} = 1.118 \times R \times N^2 \times 10^5$$

where R = The radius in cm from the centre of the centrifuge shaft to external tip of centrifuge tube,

N = The number of revolutions per minute (rpm) of the centrifuge head.

Small models are designed to centrifuge a series of 15 ml amounts in conical tubes or 30 ml amounts in thick-walled round-bottom tubes at speeds of 3000–5000 rpm. Larger free-standing models will centrifuge total volumes of up to 2000 ml at 3000–5000 rpm. Laboratory centrifuges with speeds up to 20000 rpm are not uncommon and much higher speeds still can be reached in ultra centrifuges as with the refrigerated type used in virus studies.

The large type of modern centrifuge has a built-in revolution counter, by means of which a constant check may be kept of the number of revolutions per minute which the head rotates. The small clinical centrifuges are not so equipped and their speed must be assessed by the use of a tachometer. A hole is provided in the centre of the centrifuge lid, through which the spindle of the tachometer is inserted. The tachometer must be held firmly in position during use in order to ensure that its spindle remains firmly engaged with that of the centrifuge.

Centrifuges are used with two types of head—‘swing out’ and ‘angle’—based on the principles of horizontal or angle sedimentation.

In angle sedimentation particles only move a short distance through the liquid before reaching the outer wall of the container where they join the stream of particles travelling towards the bottom of the tube. In horizontal sedimentation particles have to travel a longer path through the liquid but the deposit is evenly compacted at the bottom of the tube whereas in angle sedimentation the wedge-shaped sediment spreading up the side of the container may be easily disturbed when centrifuging stops and the tube is removed.

The angle head centrifuge is capable of higher speeds than the swing-out models, owing to less air resistance, but the speed of the ‘swing-out’ head can be increased in certain machines by the attachment of a wind shield.

Technique for centrifugation

1. Remove all the metal cups or buckets from the centrifuge head.
2. Ensure rubber cushions are present in all metal cups.
3. Insert the tubes or bottles to be centrifuged into buckets, and place one on each pan of a crude balance.
4. With a pipette, add a little water to the lighter *bucket*, not to the tube or bottle, until the weight of the two buckets and contents are balanced.
5. Place the buckets containing the tubes in diametrically opposite positions in the centrifuge head, and close the lid. If using the ‘swing out’ head, position the trunnions (or bucket carriers) carefully before inserting the buckets.
6. Start the motor, and *gradually* increase the speed until the required number of revolutions per minute is reached.
7. When the tubes have been centrifuged sufficiently, switch off the motor, and allow the centrifuge to stop.

Points on care and maintenance

The siting of the centrifuge is important, bench models being positioned on a firm base.

Notes—Always balance the buckets. Failure to do this will cause excessive wear on the driving bushes and breakage of tubes due to vibration.

Do not increase the speed of revolution too rapidly, and never slow the revolving head manually. Wait until it has stopped before attempting to remove the buckets or tubes.

The interior bowl of the centrifuge should be frequently wiped with a disinfectant.

The carbon brushes need replacing periodically and a supply of these should always be available.

Lubrication on the modern centrifuge is seldom necessary, the manufacturers having fitted in sealed grease units.

In certain procedures it is necessary to prevent aerosols from being blown out into the laboratory. It is now possible to use capped centrifuge tubes and buckets. Alternatively, the centrifuge should be housed in an exhaust inoculating cabinet. Buckets should be frequently examined for any flaws.

The production of chemically pure water

As tap water contains many dissolved salts and gases, it is unsuitable for most laboratory work. The water must therefore be purified by one of two methods, distillation or the use of ion exchange resins.

DISTILLATION

Using a still, the water is boiled and the resultant steam condensed onto a cold surface. The condensed steam is then collected as 'distilled water'. The condenser of a still should preferably be made of pure tin or fused quartz, as pure water readily absorbs ions from glass. A knife point of potassium permanganate and a few pellets of sodium hydroxide added to the tap water before commencing distillation will oxidize steam volatile organic compounds which might otherwise be carried over into the distilled water receiver. It is also a good plan to discard the first and last portions of the distillate. If really pure water is desired, it may be distilled three times (triple distilled). If 'pyrogen-free' distilled water is required (*see p. 650*), the still must be equipped with a suitable anti-splash device which allows only pure steam to pass through and prevents any droplets from passing into the condensate.

In the simplest form of 'still', tap water is heated in a flask and the steam given off is conveyed by glass tubing to a 'Liebig' condenser. This consists of a central tube into which the steam is passed. An outer glass jacket provides for circulation of cold tap water around the inner tube. The fall in temperature causes condensation of the steam into distilled water which is collected into the receiving flask.

The rate of distillation with this apparatus may not be adequate to supply the routine needs of a large laboratory. There are several commercial stills available which deliver distilled water at rates

ranging from 2 to 200 l per h. These may be heated by gas or electricity, and incorporate an automatic water feed which maintains the volume of boiling water.

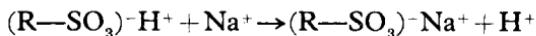
For some purposes, water must be glass-distilled, that is, at no stage must the steam or distillate come into contact with any surface other than glass.

DE-IONIZATION BY ION EXCHANGE RESINS

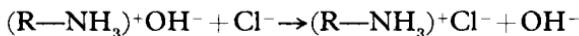
Although this technique, which is used routinely in many laboratories produces water free from ions, not all non-electrolyte contaminants may be removed, that is the water is not pyrogen-free. There may also be some extraction of organic impurities from the resins, but under normal circumstances the water obtained by this method is purer than that obtained by distillation. Water purified by ion exchange resins is sometimes called 'conductivity water' as it has such a low electrical conductivity that it is suitable for use in such measurements. Although the theoretical pH of pure water is 7.0, in practice, pure water rapidly becomes acidic when exposed to air, due to the absorption of carbon dioxide and the formation of carbonic acid.

Ion exchange resins are of two types: (a) cation exchange resins ($R-SO_3^-$) H^+ which are insoluble acids, and (b) anion exchange resins ($R-NH_3^+$) OH^- which are insoluble bases. R represents a polystyrene resin.

The mode of action of ion exchange resins may be illustrated by the following. If, for instance, water containing sodium chloride is passed through a column of cation exchange resin, the Na^+ cations replace the H^+ cations of the resin



The emerging water now contains H^+ ions (obtained from the resin) together with the original Cl^- anions. If this water is now passed through the anion exchange resin, the Cl^- replaces the OH^- anion of the resin



The water now contains H^+ and OH^- ions which combine to form H_2O . In this way the water is made ion-free.

In practice, the two resins are usually mixed together in one column, as a 'mixed-bed' de-ionizer. To obtain pure water, simply pour the water to be purified on to the mixed polystyrene resins,

and the water emerging after passing through this column is pure water.

Ion exchange resins may be regenerated by passing HCl through the cation resin, followed by washing well with water, and by passing NaOH through the anion resin, and washing with water. If the resins are of the 'mixed-bed' type, it is necessary to separate the two resins first by passing an upward flow of water through the mixture. The two resins, being of unequal density, will separate out.

2

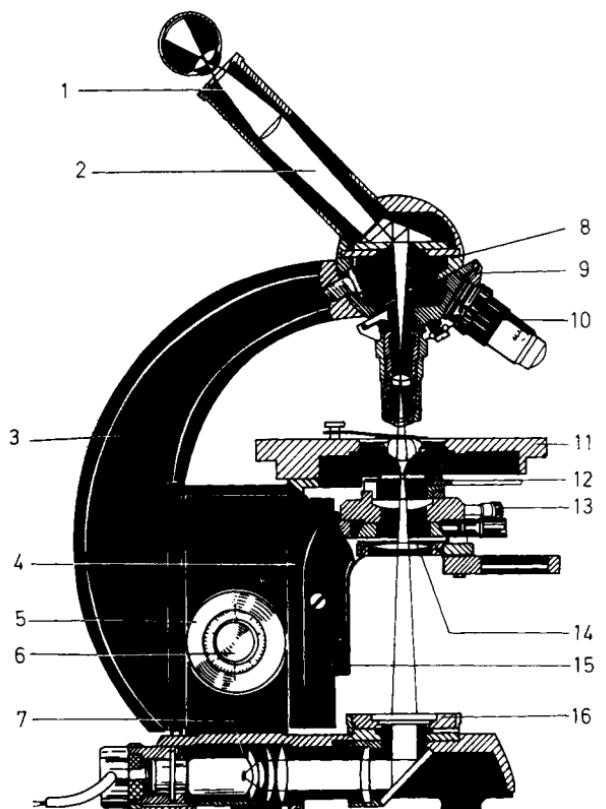
Elementary Microscopy

The modern compound microscope is an indispensable piece of apparatus in all medical laboratories, and a theoretical knowledge of its working principles is essential. It is a precision instrument, and its efficient use requires some measure of skill and training. The magnification and clarity of the image depend upon the quality of its lenses, but definition is readily lost if the instrument is improperly used.

Time spent in the systematic setting up of the microscope and lamp is amply repaid by the results obtained. Microscopes are made in two forms, monocular and binocular. For general purposes monocular instruments are satisfactory, but for prolonged use the binocular types are less fatiguing and today most microscopes used in medical laboratories are of this design. In essence, a microscope consists of an objective lens and eyepiece, with the mechanism necessary for focusing them. A bright light is passed through the object under examination and into the objective lens, which is the main magnifying agent. The rays of light emitted at the upper end of the objective form an image which is viewed through the eyepiece.

Many advances have been made in the design of microscopes in recent years. The limb or tube of the modern monocular microscope is now often fixed rigidly to the base, which is constructed to contain a built-in illuminant. The eyepiece is held in an inclined tube which is attached to a tube head into which the objectives are screwed. Focusing is accomplished by raising or lowering the stage, the coarse and fine adjustment being situated in the stage support together with the mechanism for supporting and focusing the sub-stage condenser (*Figure 2.1*).

In the older type of monocular microscope, many of which are still in use, the construction is different. The base or foot is sufficiently solid to hold the instrument stable, even when tilted in use. The limb is pivoted to the foot and at its lower end carries the stage, sub-stage condenser and reversible mirror with plane and concave surfaces. The body is attached to the upper end of



- | | |
|--------------------------------------|--|
| 1 = Eyepiece | 9 = Revolving nosepiece |
| 2 = Inclined tube | 10 = Objective |
| 3 = Tube support | 11 = Stage |
| 4 = Stage support | 12 = Condenser (aperture) diaphragm |
| 5 = Coarse adjustment knob | 13 = Knob for swinging condenser front lens in and out |
| 6 = Fine adjustment knob | 14 = Auxiliary condenser lens |
| 7 = Illuminating tube with collector | 15 = Condenser carrier |
| 8 = Tube head | 16 = Diaphragm insert |

Figure 2.1. Diagram illustrating a modern monocular microscope

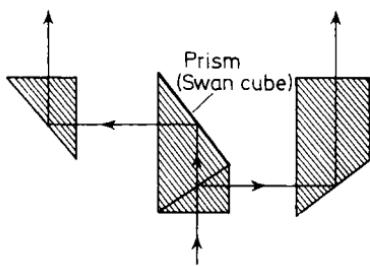
the limb and contains coarse and fine adjustments by which the body-tube is raised or lowered.

The body-tube houses the draw-tube, in which the eyepiece rests. An objective mount or nosepiece is attached to the lower end of the body-tube and objectives of varying focal length may be screwed into this mount.

In the modern binocular microscope (*Figure 2.2a*), the rays



(a)



(b)

Figure 2.2. (a) A modern binocular microscope. (b) This diagram illustrates the principle of the binocular microscope

reflected from the object are equally divided between the two eyepieces. This is achieved by the use of a prism, known as a Swan cube. All models are based on the principle illustrated in *Figure 2.2b*.

A sliding adjustment is provided enabling the operator to set the eyepieces at a comfortable interpupillary distance.

With modern instruments, both oculars can be focused. This allows the interpupillary distance to be read from a scale between the eyepieces and adjustments made for variations in tube length by setting the eyepiece tube scale at the same reading. Should the observer's vision be at fault the image is first focused with the emmetropic eye and the necessary adjustment made for the ametropic eye. If the instrument has only one focusing collar, it is set up as for the monocular microscope using the fixed eyepiece. The adjustment on the other is used to correct any discrepancy between the observer's eyes.

Before dealing with the components of a microscope, it is necessary to explain certain terms.

Refraction

Refraction is the change in direction of light passing obliquely from one medium to another of different optical density. *Figure 2.3a* shows the path of a ray of light passing from air into a glass plate and out into the air again. At B, the point of entry of the ray into the glass, a line XY, called the 'normal', is perpendicular to the surface of separation of the media. The ray AB is refracted towards the normal along BC in the glass, and away from the normal along CD in the air.

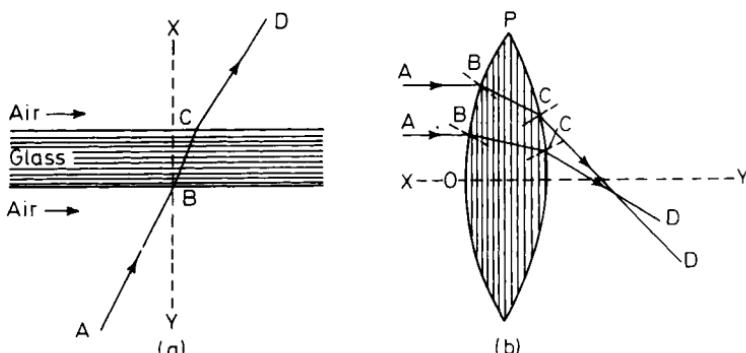


Figure 2.3. Diagrams to illustrate refraction of light rays passing through (a) a glass plate, and (b) a bi-convex lens

In general, a ray of light passing from a rarer to a denser medium is refracted *towards* the normal, but when passing from a denser to a rarer medium is refracted *away* from the normal.

Refractive index

In *Figure 2.3a* the ray AB is termed the incident ray, and the ray BC is the refracted ray. The angle ABY is therefore termed the angle of incidence, and CBX the angle of refraction. The sine of the angle of incidence divided by the sine of the angle of refraction is a constant quantity for any two given media and is called the Refractive Index.

In *Figure 2.3a* the sine of the angle ABY divided by the sine of angle CBX determines the refractive index of the glass.

SPHERICAL ABERRATION

Spherical aberration is the indistinct or fuzzy appearance of the outer part of the field of view of a lens, which is caused by the non-convergence of rays to a common focus.

Figure 2.3b shows rays of light ABCD entering and leaving a

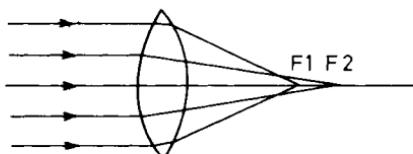


Figure 2.4. Diagram to illustrate spherical aberration by a bi-convex lens. The marginal rays intercept the axis at a point closer to the lens (F1) than the more central rays (F2)

bi-convex lens. Because of the curvature of the lens, 'normals' at points along the surface are not parallel to one another. The direction which rays of light will take when refracted by the lens will therefore vary according to their place of entry. It will be seen that rays entering the lens near the centre O are refracted less than those entering the lens at the more peripheral part, towards P. Rays from an object therefore tend not to be brought to a common focus, and the result is a distorted image (*Figure 2.4*).

CHROMATIC ABERRATION

When white light is passed through a prism it is split into a spectrum of colours ranging from red through orange, yellow, green, blue, indigo and violet. These colours when combined reproduce white light. A bi-convex lens also splits white light into its com-

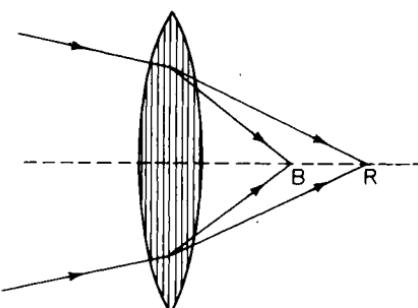


Figure 2.5. Diagram to illustrate chromatic aberration in a bi-convex lens. R - Red component of light coming to a common focus. B - Blue component of light coming to a common focus

ponent colours, the blue light being refracted more than the red so that it comes to a focus nearer to the lens (Figure 2.5.). This non-convergence of the coloured components of white light to a common focus is termed chromatic aberration. This term is used to describe the coloured fringes sometimes seen round the edge of an object viewed through a lens.

PRINCIPAL FOCI OF A CONVERGING LENS

A bi-convex lens has two spherical surfaces which curve outwards (Figure 2.6). It is called a *converging lens* as rays of light passing through the lens converge to a focal point. The centre of the lens surfaces are called the *centres of curvature*. A straight line between these two centres is the *principal axis*. A line, at right angles to

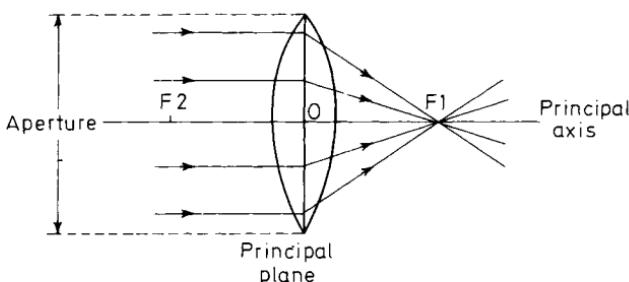


Figure 2.6. Diagram illustrating the principal axis and plane of a bi-convex lens. F₁ and F₂ are the principal foci. O is the optical centre and the distance O-F₁ the focal length

this axis, which passes through the centre of the lens is termed the *principal plane*. The diameter or width of the lens is called its *aperture*.

Rays of light entering a converging lens parallel to the principal axis are refracted towards and across this axis. The point at which they cross is called the *principal focus*. A bi-convex lens has two principal foci, one on either side.

Optical centre

A ray of light that enters one side of a lens or lens system and emerges parallel to the entering ray, will pass through the *optical*

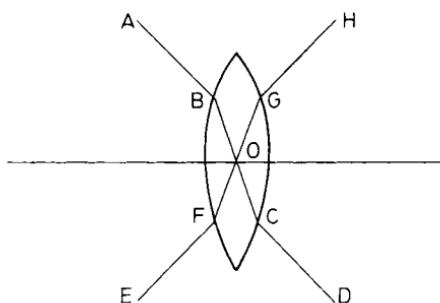


Figure 2.7. Diagram showing optical centre of a converging lens. AB is parallel to CD: EF is parallel to GH: O = optical centre

centre. A ray acting similarly when entering the opposite side of the lens, will also pass through the optical centre. The point at which these two rays cross will therefore be the optical centre (Figure 2.7).

FOCAL LENGTH

The distance between the optical centre and the principal focus (the focal point) is the *focal length* of that lens. This must not be confused with *working distance* which is the distance between the surface of the lens and the focal point.

PRINCIPAL FOCUS OF A DIVERGING LENS

A bi-concave lens has two surfaces which curve inwards (Figure 2.8) and is called a diverging lens. Rays passing parallel to the principal axis of a diverging lens are refracted away from the principal axis as though originating from the principal focus.

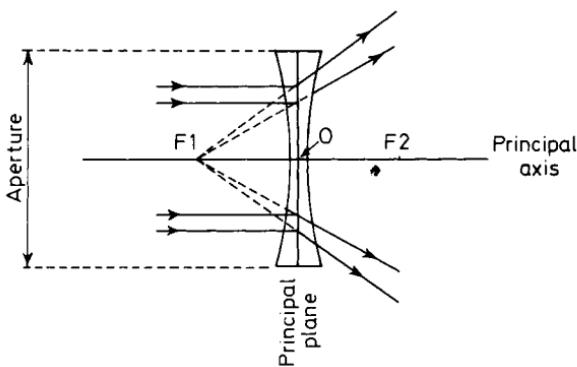


Figure 2.8. Diagram illustrating the manner in which rays are refracted away from the principal axis by a diverging lens. The principal focus of the lens is shown by the use of broken construction lines

Image formation

REAL IMAGE

A converging lens can produce either a *real image* or a *virtual image*. A real image is an inverted image, which can be projected onto a screen, and is formed when the object is placed outside

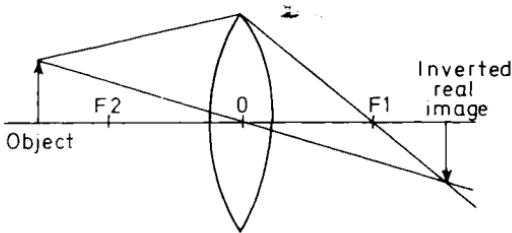


Figure 2.9. Diagram showing the formation of a real image

the focal length of the lens or lens system (Figure 2.9). The size of the image produced depends on the distance between the lens and the object.

For example,

if u = distance between object and lens

$2f$ = twice the focal length of the lens.

Then when

u is greater than $2f$ a diminished image is produced

u is equal to $2f$ an image of the same size is produced

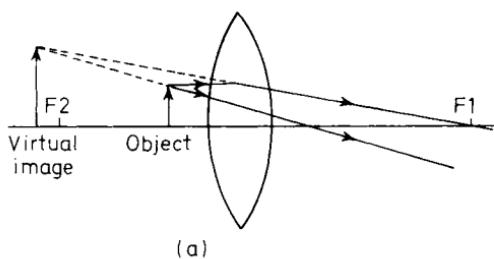
u is less than $2f$ (but more than f) a magnified image is produced.

VIRTUAL IMAGE

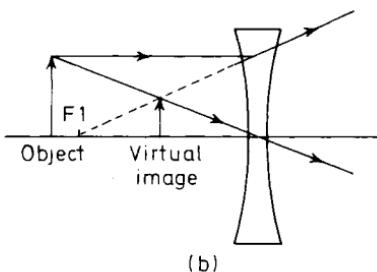
If the object is placed within the focal length of the converging lens, then a *virtual image* is formed which has no physical existence, and cannot be projected onto a screen (*Figure 2.10a*).

A diverging lens will always produce a virtual image which is erect (not inverted) but which is diminished (*Figure 2.10b*).

In the compound microscope two sets of lens systems produce



(a)



(b)

Figure 2.10. (a) Diagram illustrating (by the use of broken construction lines) formation of a magnified virtual image by a converging lens when the object is within the focal length of the lens. (b) Diagram illustrating (by the use of broken construction lines) diminished virtual image by a diverging lens

the magnified image, namely the objective and the ocular. The objective produces the primary image, which is brought to focus in the plane of the eyepiece diaphragm by the field lens, and is then viewed with the eye lens. The primary image is a real, magnified image of the object, which must therefore be at a greater distance from the objective than the focal length ($u < 2f$ produces magnified image, *see above*). The primary image when viewed by

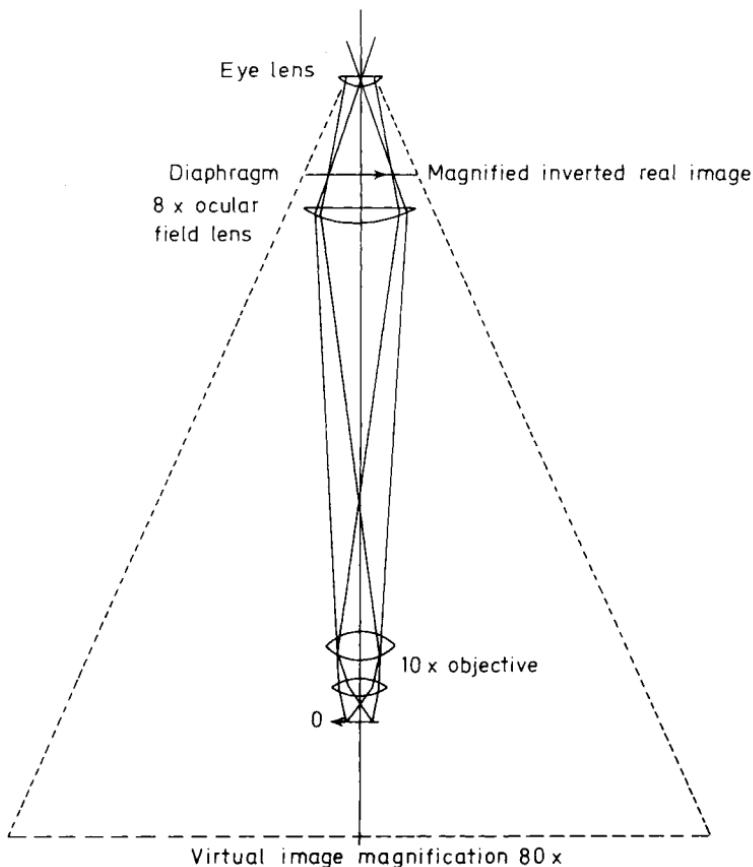


Figure 2.11. Rays from the object O are brought to a focus by the field lens in the plane of the eyepiece diaphragm as an inverted, magnified real image. The image is within the focal length of the eye lens, resulting in the production of an inverted, magnified virtual image

the eye lens is within the focal length of the lens and a magnified virtual image is produced (Figure 2.11).

COMPONENTS OF THE MICROSCOPE

Optical components

The optics of the compound microscope can be most easily understood by considering the components in stages. The optical system consists essentially of a condenser, an objective and an eyepiece (Figure 2.12).

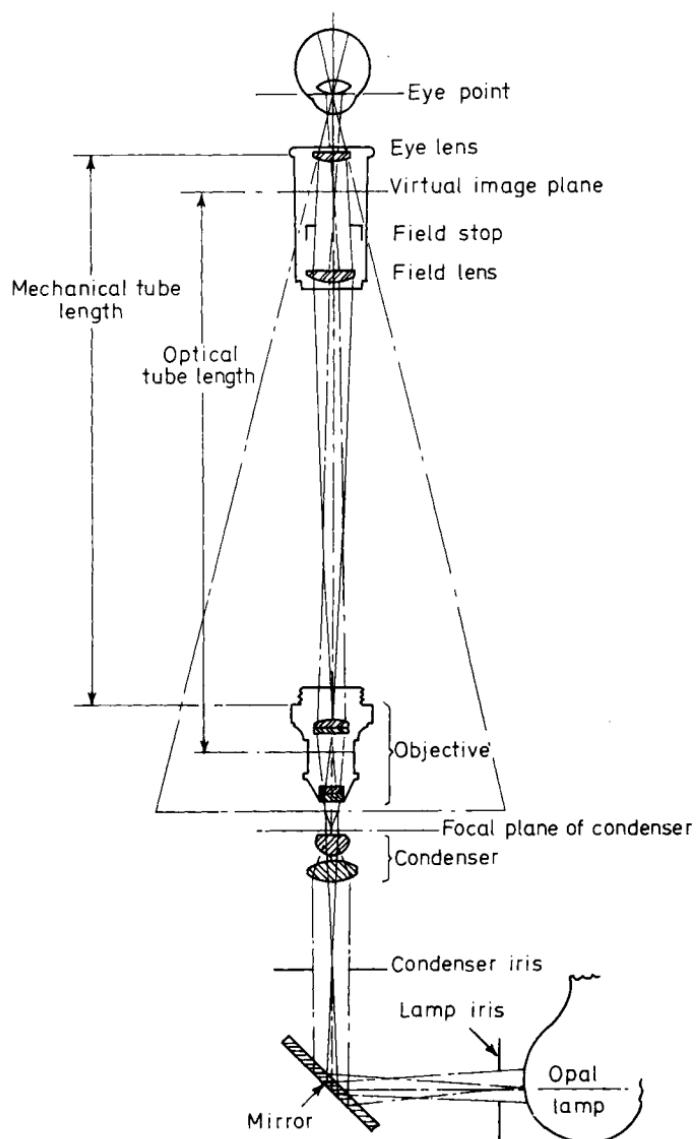


Figure 2.12. Diagram illustrating the optical system of a microscope

Rays from a light source are directed into the sub-stage condenser, which brings them to a common focus on the object, for example blood film. Having illuminated the object, light rays pass through the objective, and produce the primary image in the plane of the eyepiece diaphragm. The eye lens magnifies the image and brings it into focus on the retina of the eye as a virtual image.

The retina forms the inner coat of the eyeball and acts as a screen. It is composed of small, highly specialized cells of which two types, the rods and cones, are sensitive to light. Under normal conditions and when the eye is at rest, distant objects are registered as being in focus. Magnification is achieved by the simple process of reducing the distance between the eye and the object, thereby increasing the visual angle and spreading the image over a larger area of the retina (*Figure 2.13*).

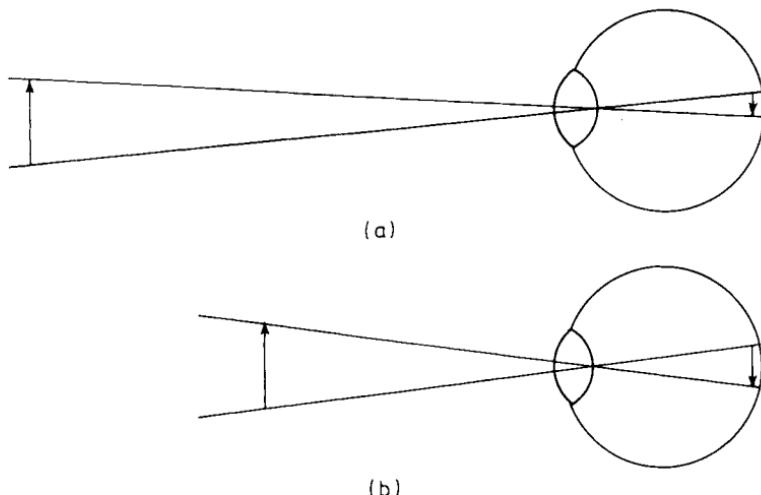


Figure 2.13. Diagram illustrating the manner in which the retinal image is increased as the object is brought closer to the eye, thereby increasing the visual angle

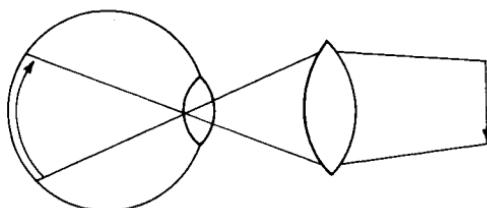


Figure 2.14. Diagram illustrating the manner in which a convex lens interposed between the object and the eye increases the visual angle and size of the retinal image

The eye has a minimum focusing distance of 25 cm, however, and objects brought closer than this appear indistinct. Further magnification can only be achieved by placing a lens, or system of lenses such as a microscope, between the object under examination and the eye (*Figure 2.14*).

OBJECTIVES

The aberrations mentioned previously are corrected in greater or lesser degree, in the modern microscope, by using combinations of lenses of different shape and types of glass. The average objective (achromatic) brings to a common focus the rays of red and blue light, that is, it is corrected for two spectral colours. In addition, spherical aberration is corrected for light of one colour.

For highly critical work, apochromatic objectives are necessary. These employ internal positive lenses (convex) of fluorite, and negative lenses (concave) of barium flint. Such lenses bring rays of three different colours to a common focus, and are said to be corrected for three spectral colours. In addition, they correct the spherical aberration for two spectral colours, that is, spherical aberration is minimized provided the light used consists only of these two colours. They necessitate the use of a compensating eyepiece. Apochromatic lenses are so costly that semi-apochromatic ones are often used instead. Apochromatic lenses are usually reserved for oil immersion or high-powered objectives, preferably in conjunction with a compensating eyepiece. Of recent development are planachromatic objectives which are designed to provide the flattest possible field. *Figure 2.15* illustrates the lens arrangement in the common objectives.

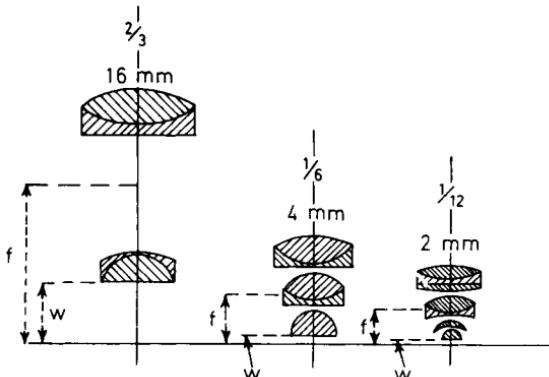


Figure 2.15. Lens arrangement of the common objectives showing the relative focal lengths and working distances. f : focal length; w : working distance

The focal length of a lens is the distance between its centre, or in the case of a system of lenses their optical centre, and the point where a parallel beam of light is brought to a focus. The focal length of a high-power objective is shorter than that of a low-power (*Figure 2.15*). The focal length should not be confused with the working distance, which is the distance between the front lens of an objective and the object on which it is sharply focused (*Figure 2.15*). The working distance is relative to the numerical aperture; the higher the numerical aperture of the objective the shorter the working distance.

Objectives of focal length over 3 mm have air between the front lens of the objective and the object under examination (dry objective).

Objectives with focal length under 3 mm use fluid between the front lens and the object under examination. This fluid should have the same refractive index as glass. Special cedar-wood oil is generally used, unless otherwise stated on the objective, for example water immersion. The chromatic aberration produced by this procedure is corrected in the lens system.

RESOLVING POWER

The power of a lens to reveal detail is referred to as the resolving power or resolution of the lens. It may be defined as the ability to reveal closely adjacent structural details as being actually separate and distinct.

The resolving power of a microscope is largely dependent upon

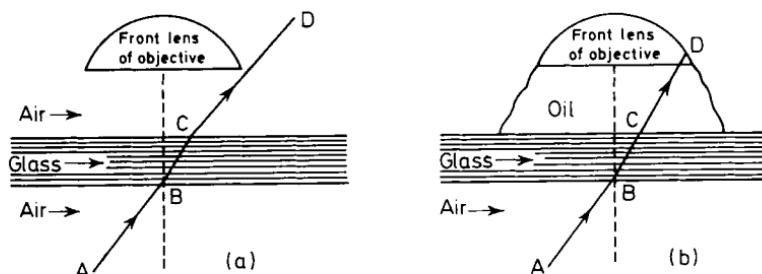


Figure 2.16. Diagram to illustrate angle of light entering objective

the angle of light entering the objective. It will be seen from *Figure 2.16* that the presence of oil between objective and slide conserves many of the light rays, which would otherwise be lost by refraction. In *Figure 2.16a*, ABCD is the path of a ray of light through a glass slide. It is refracted towards the normal on entering the glass, BC,

and away from the normal, CD, on entering the rarer medium, air; this ray of light would not enter the objective. In *Figure 2.16b* a similar ray of light, ABCD, behaves exactly the same on entering the glass, BC. It is not refracted when leaving the slide, however, as oil has the same refractive index as the glass. The ray CD will, therefore, pass into the objective.

Numerical aperture

The resolution, or resolving power, of an objective is partly dependent upon the cone of light collected by the front lens. The Numerical Aperture (NA), an optical constant, is defined as the product of the refractive index of the medium outside the lens (n), and the sine of half the angle of the cone of light absorbed by the front lens of the objective (U), that is, $NA = n \times \sin U$ (*Figure 2.17*).

The wider the cone of light, the greater the NA. Thus, if two objectives of the same focal length have lenses of different diameters, one will admit a greater angle of light, and therefore have a higher NA. In *Figure 2.17a*, ACB is the angle of the cone of light entering the front lens of the objective. AB is the diameter of the front lens of the objective. C is the object being viewed.

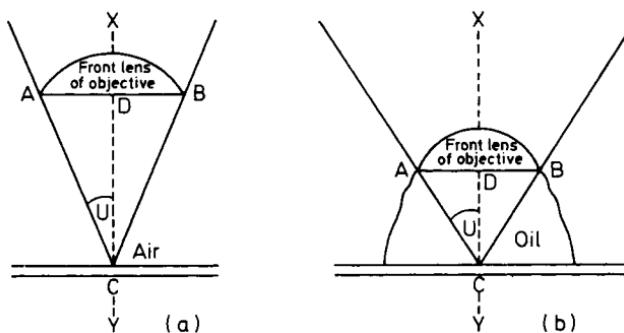


Figure 2.17. Refractive index of objectives (a) without oil, (b) with oil

The NA of that objective is therefore: $n \times \sin U$.

As the external medium is air, the value of n is unity.

$$\text{Therefore } NA = \sin U = \frac{AD}{AC}$$

In *Figure 2.17b*, as the external medium is oil with a refractive

index of 1.5, the NA of that objective is

$$1.5 \times \frac{AD}{AC}$$

The NA for dry lenses may be calculated by measuring the angle U with an apertometer. As the external medium is air, the value for n is unity. The NA is normally marked on the objective.

With oil-immersion objectives, somewhat higher values are obtained for NA, owing to the higher figure for n . Most oils used have a refractive index of about 1.5.

EYEPieces

The eyepiece most commonly used is of the Huygenian pattern. This is composed of two plano-convex lenses which are arranged

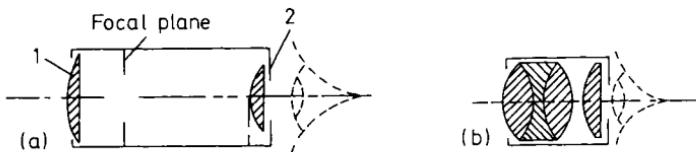


Figure 2.18. Eyepieces; (a) Huygenian type (1: field lens; 2: eye lens); (b) compensating eyepiece

with their convex surfaces facing the objective. The two lenses are of different sizes, the front (or field) lens having a focal length twice or three times that of the eye lens. A diaphragm is situated within the eyepiece at the focal plane of the eye lens (*Figure 2.18a*).

Compensating eyepiece

This is primarily designed for use with apochromatic objectives. The apochromatic objectives are under-corrected for the magnification of light of various colours, and compensating (over-corrected) eyepieces are designed to rectify this (*Figure 2.18b*). The over-correction can be seen by looking through the eyepiece at a distant light source. A red fringe will usually be seen at the edge of the diaphragm, in contrast to the blue fringe produced with an ordinary Huygenian ocular.

Mechanical tube length

This is the distance which separates the top lens of the eyepiece from the point where the objective screws into the revolving nose-piece (*Figure 2.12*). Most objectives are made to be used with a mechanical tube length of 160 mm. This distance can easily be checked by measuring with a ruler.

Optical tube length

This is the distance between the upper focal plane of the objective and the lower focal plane of the eyepiece (*Figure 2.12*) and is of a similar distance to that of the mechanical tube length. The magnifying power of the microscope is dependent in part on the optical tube length. In practice, however, the mechanical tube length is used for calculating the total magnifying power of the microscope, or the individual power of the eyepiece and objectives.

The use of the draw-tube

Extension of the draw-tube in older types of instruments produces magnification of the final image, but its primary function is the elimination of spherical aberration when using coverglasses of incorrect thickness. Objectives are usually corrected for the standard No. 1 coverglasses, and shortening of the tube length is desirable if very thick coverglasses are used.

COVERGLASS THICKNESS

No. of coverglass	Thickness in mm
0	0.18
1	0.145
2	0.220
3	0.300

CONDENSERS

The sub-stage condenser is the most neglected part of the optical system in a microscope. The important role which it plays is frequently overlooked, and as a general rule, while a great deal of attention is given to the optical qualities of the objectives, very little care is taken to ensure that the condenser is of a sufficiently high quality to allow maximum resolution to be achieved by the other optical components.

Only two working errors are possible with components situated above the stage; (1) the tube length may be set incorrectly, and on modern instruments this is no longer adjustable, and (2) the front lens of the objective may be racked down too far, resulting in damage to the objective and the specimen; this is also allowed for in many modern objectives which have the front component spring loaded.

In the case of the sub-stage condenser, many cardinal sins may

be committed when working with the microscope. Incorrect centring, failure to adjust it correctly for Köhler or Nelson illumination, indiscriminate use of the auxiliary lens, top lens or the iris diaphragm, each of these factors can separately or collectively reduce the resolution of which the objective may be capable.

Condensers fall into two categories, bright field and dark field.

Bright-field condensers

Condensers under this heading are used with transmitted light for routine work. The most common type is the Abbé condenser, designed by Professor Abbé in 1872. It consists of two lenses and an iris diaphragm. As no correction is made for chromatic or spherical aberration, a considerable amount of scattering occurs. This can be reduced by partly closing the iris diaphragm, or by placing immersion oil on the top lens of the condenser, under the object slide. For critical work, an achromatic condenser which consists of a series of lenses is essential.

The NA quoted for Abbé condensers is frequently as high as 1.20 but, due to the lack of correction, the iris diaphragm must be partly closed in order to produce an aplanatic cone of light. This reduces the working NA of the condenser markedly and with it the effective working NA of the objective.

For critical work a three-lens type of Abbé achromatic condenser may be used but for the best results an aplanatic condenser should be selected. The maximum working NA of the objective/condenser system is then obtained.

The working NA of the objective/condenser system is the arithmetic mean of the two. Thus an objective with NA 1.2 and a condenser with working NA of 0.4 is equal to:

$$\frac{1.2 + 0.4}{2} = 0.8$$

This means that only two-thirds of the possible NA of the objective is being utilized.

Low-power objectives necessitate the use of low-power condensers if the whole field is to be illuminated. This may be achieved by removing the top lens of the condenser. Some manufacturers produce condensers with top lenses which may be flipped in or out of the optical train as required.

Dark-field condensers

These are designed so that the object under examination is illuminated very obliquely. The light rays passing through the con-

denser are lost unless they are deflected or refracted into the objective by the object under examination. The field as seen under the microscope is therefore black, but any solid material present is clearly illuminated (*Figure 2.19*).

Most dark-ground condensers have a fixed focus and must be used with thin slides and coverglasses being usually corrected for slides 1.2 mm in thickness and No. 1 coverglass. A more expensive type of focusing condenser is available, however, which will allow slides and coverglasses of varying thickness to be used. All types

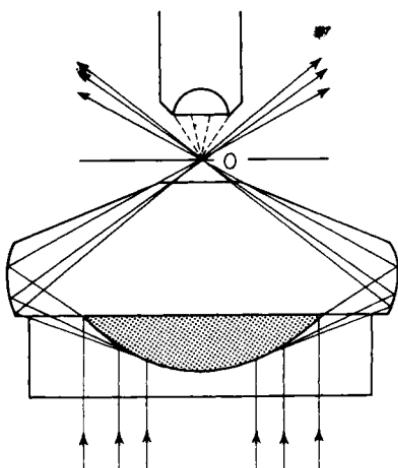


Figure 2.19. Diagram illustrating the way in which light rays are reflected from the central spherical surface and outer cardioid reflecting surface in the cardioid darkfield condenser. The rays of light strike the object O at an oblique angle

should be used as immersion condensers, cedar-wood oil being the usual immersion fluid. The type of dark-ground condenser usually available in medical laboratories cannot give a perfectly black background when used in conjunction with an objective which has a numerical aperture higher than 0.90. Such objectives should have their aperture decreased by the insertion of a funnel stop—a small metal tube. Some high-power objectives incorporate an iris diaphragm for this purpose (Davis diaphragm).

An intense source of light is necessary for dark-ground illumination. Objects examined by dark-ground illumination appear larger than they actually are: this is due to their light scattering properties.

Filters

A variety of light filters is available for use with the microscope. Light filters may be used to (a) increase resolution and contrast; (b) decrease light intensity and glare; and (c) absorb excess heat.

Neutral filters

These are used to decrease the brilliance of the illuminant without reducing the operating voltage of the bulb. They are manufactured in a number of densities, two or more of which may be used together to obtain the effect of their combined strength.

Coloured filters

These have a number of different functions. 'Daylight blue' is the most commonly used filter in medical laboratories. By absorbing those light rays of longer wavelengths and only transmitting the shorter, resolution is increased. Green filters similarly increase resolution but also decrease glare. Increased contrast can be obtained with coloured filters by selecting one that is complementary to the colour of the object under examination.

Colour correction filters

These cover a very wide range. They are used mainly in colour photomicrography in order to correct the colour temperature of the light source to agree with that of the film.

Heat-absorbing filters

These are used in conjunction with medium and high-intensity lamps. Their purpose is to absorb the heat rays.

Exciter filters

These are used in fluorescence microscopy for transmitting light of a selected wavelength.

Barrier filters (secondary filters)

These are used to protect the retina from injury by preventing the passage of ultraviolet light. They must, therefore, only transmit light of a longer wavelength than the exciter filter. In addition, they serve to increase the brilliance of the fluorescent image by producing a dark background.

Source of illumination

The resolution achieved by the optical components of the microscope is dependent in part upon the intensity and adjustment of

the light source. Correct alignment is therefore essential when the microscope is set up.

Daylight is a poor illuminant for microscopy as it imposes strict limitations on the use of the instrument and makes standardization impossible. It should only be used when no alternative light source is available and then only with low-power objectives. Most microscope lamps use electricity as the source of energy and good lamps possess the following features.

The base is of sufficient size and weight to ensure perfect stability. The lamphouse has good ventilation to prevent overheating, but is light-tight, except for the working orifice, and is supported on a stand which permits smooth adjustment of the height. Facilities are provided which permit adjustment in both a vertical and horizontal direction, and provision is made for coloured filters to be interposed in the light path. The better lamps are fitted with a condenser and an iris diaphragm and have a focusing mechanism for providing Köhler illumination. A rheostat, or variable voltmeter is also incorporated to give control over the intensity.

Electric light bulbs of 25–60 watts are used in the simplest form of student lamps; the bulb should be of an opal type rather than a frosted glass one, as the latter do not give even illumination and the filament, which can be seen, is in focus when the microscope is adjusted for critical illumination. Most microscopes in medical laboratories have a coil filament bulb as the illuminant. This is of low voltage, usually 6–12 volts, and must be used in conjunction with a transformer and variable voltmeter. Lamps of this nature have a small but intense light source which is very often of uneven brilliance. It is therefore essential to use Köhler illumination with this form of illuminant (*see p. 52*). Microscopes manufactured with built-in illumination are invariably provided with this type of light source, which is pre-centred. To comply with the conditions necessary for Köhler illumination a condenser is provided and an iris diaphragm is built into the foot of the instrument. If the condenser is not adjustable provision is made for varying the position of the bulb in relation to the condenser (*Figure 2.20*).

A widely used type of illuminant in microscopy is the quartz-iodine vapour lamp. It is a 12-volt, 100-watt illuminant which is used in conjunction with a variable voltmeter. The tungsten-iodine filament is enclosed within a quartz envelope. The lamp is rich in light at the 400 nm level of the spectrum and is therefore useful for blue light fluorescence.

The most efficient source of intense illumination is undoubtedly

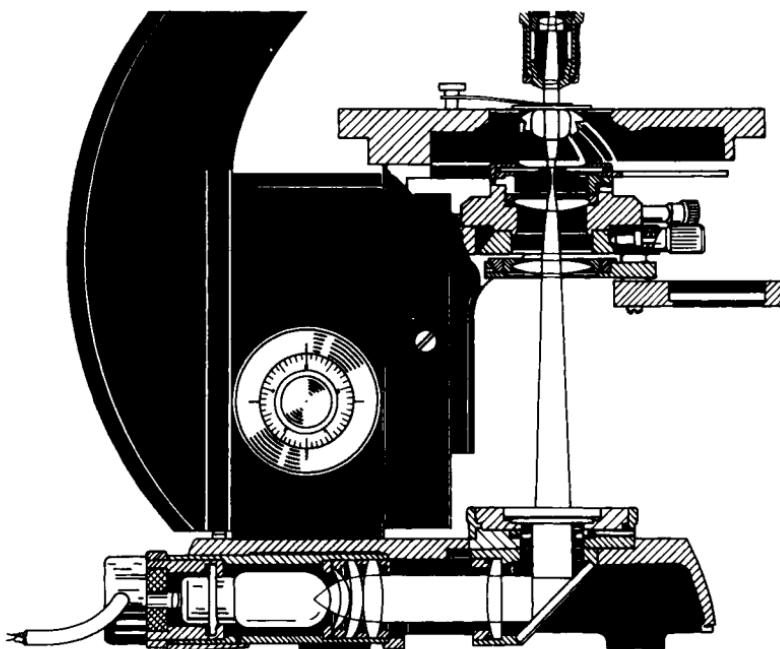


Figure 2.20. Built-in illumination

the high-intensity mercury vapour lamp. The main application of this lamp in medical laboratory work is in ultraviolet microscopy, particularly for studying immunofluorescence reactions.

Mechanical components

To complete the microscope, certain mechanical details must be considered.

The coarse adjustment is a rack and pinion utilized in older models for connecting the body-tube to the body. A similar device is used for attaching the sub-stage condenser to the base of the limb. The fine adjustment, allowing precision in focusing, may be of several different designs. Modern microscopes are different in construction. The body-tube is permanently fixed to the limb, and focusing is performed by racking the stage up or down.

The fixed stage is a basic component of the microscope, but only allows manual movement of the object slide. This disadvantage can be overcome by the mechanical stage.

Mechanical stages are fitted with two scales and Vernier plates running at right angles to each other, for the purpose of recording

a particular field in the specimen under examination. For this reason, the habit should be adopted of always placing the slide on the stage with the label at the same end. Some workers also use these scales for making approximate measurements of relatively large objects, for which purpose the eyepiece should be fitted with cross wires or a marker.

The Vernier system consists of a main scale, which is divided into millimetres, and a Vernier plate which has a scale 9 mm in

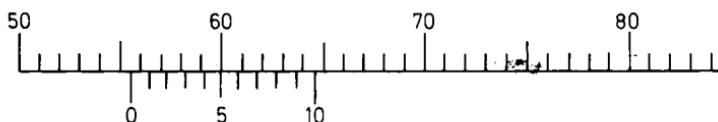


Figure 2.21. Diagram illustrating Vernier system. The main scale shows a reading of 55 and the Vernier scale a reading of 5. The reading is recorded as 55.5

length but divided into ten equal divisions. The zero mark on the Vernier plate is used as the reference point for recording. When this falls between two divisions on the main scale, the lower one should be recorded, and the Vernier plate scale examined to see which of its divisional lines coincides with a reading on the main scale. The reading from the Vernier plate is then recorded as a decimal reading. Thus, if the zero on the Vernier scale falls between 55 and 56 on the main scale and the Vernier plate reading is 5, the reading recorded is 55.5. Readings from both Vernier scales should be taken in order to re-locate a particular field in a slide (*Figure 2.21*).

MICROMETRY

Vernier scales are not suitable for making accurate measurements with the microscope and, when this is necessary, specialized equipment is required. The most widely used type in medical laboratories is the stage micrometer and micrometer eyepiece. The actual measuring is done with the micrometer eyepiece but it must be emphasized that the scale used is a transfer scale to be compared against a known standard with which it must first be calibrated.

Micrometer eyepiece

This may be one of several types of eyepieces (Ramsden, Huygenian, compensating) containing an engraved micrometer scale at the level of the diaphragm. The eye lens is provided with a

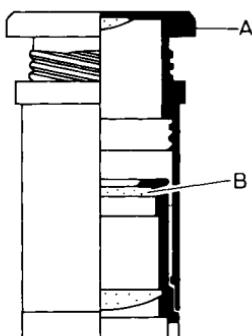


Figure 2.22. Eyepiece micrometer. A Eye lens which can be focused. B Graticule with scale

means of adjustment in order to bring the scale into sharp focus (*Figure 2.22*).

Stage micrometer

This consists of a 3×1 slide, in the centre of which is an engraved scale with finely divided divisions mounted beneath a coverglass. The scale is usually 2 mm in length and the divisions are 0.1 mm and 0.01 mm in width (100 μm and 10 μm respectively).

METHOD OF USE

1. Focus the eye lens sharply on the engraved scale by pointing the eyepiece towards an illuminated surface and adjusting the eye lens.
2. Place the eyepiece in the draw-tube and ensure that the tube is set to the correct working distance.
3. Place the stage micrometer in position and focus on the scale.
4. Turn the eyepiece until both scales are parallel.
5. Study the two scales carefully and record the number of larger divisions on the eyepiece scale that corresponds to a whole number on the stage micrometer. When necessary the draw-tube can be adjusted to ensure that an accurate reading is obtained (*Figure 2.23*).
6. From the readings obtained, calculate the ratio of the two scales.
7. Having calculated the size of the division on the eyepiece micrometer, place the specimen to be examined on the stage and make the necessary reading. The eyepiece micrometer should be calibrated for each objective used.

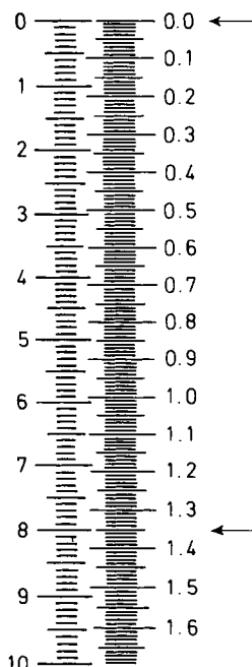


Figure 2.23. Diagram illustrating the manner in which the eyepiece micrometer is calibrated with the stage-micrometer. In this example, 80 divisions correspond to 1350 μm , each division therefore being 16.9 μm , in length

For example:

From *Figure 2.23* it will be seen that 80 small eyepiece divisions are equal to 135 small stage-divisions.

As each small stage division is 10 μm in width,

$$80 \text{ eyepiece division}$$

$$= 1350 \mu\text{m}$$

$$\therefore 1 \text{ eyepiece division}$$

$$= \frac{1350}{80} \mu\text{m}$$

$$= 16.9 \mu\text{m}$$

MAGNIFICATION

At the normal optical tube length of 160 mm, the total magnifying power of the compound microscope is the product of the magnification of the objective and eyepiece; for example, with objective

$\times 40$ and eyepiece $\times 10$, the magnification would be $40 \times 10 = 400$. If the tube length is varied, the final magnification is

$$\left(\frac{\text{Magnification of objective}}{} \right) \times \left(\frac{\text{Magnification of eyepiece}}{} \right) \times \left(\frac{\text{Working tube length}}{\text{Normal optical tube length}} \right)$$

For example, with objective $\times 40$, eyepiece $\times 10$ and working tube length 180 mm, total magnification is

$$40 \times 10 \times \frac{180}{160} = 450$$

If objectives are marked with focal length only, total magnification is

$$\left(\frac{\text{Magnification of eyepiece}}{} \right) \times \left(\frac{\text{Working tube length}}{\text{Focal length of objective}} \right)$$

For example, with eyepiece $\times 10$, objective of focal length 4 mm and working tube length of 160 mm total magnification is

$$\frac{10 \times 160}{4} = 400$$

Empty magnification

This is the term used to describe magnification which produces an increase in the apparent size of an object without revealing any new detail. In other words empty magnification is magnification without resolution.

SETTING UP THE MICROSCOPE

If the best results are to be obtained it is of the utmost importance that the microscope is set up correctly. Two methods of adjusting the illumination, the Nelson and Köhler methods, are in universal use. Of these methods, the latter is rapidly becoming the more widely used and research microscopes equipped with built-in illumination are designed to be adjusted for Köhler illumination.

The term 'critical illumination' was coined during the latter part of the last century by E. M. Nelson, the famous British critical microscopist. The method was devised in order to obtain maximum resolution and to achieve this even illumination of the whole field was sacrificed. An image of the light source is focused in the

object plane, resulting in the object under examination being illuminated by a solid cone of light. Maximum resolution is therefore obtained in the centre of the field, the size of which is controlled by means of the sub-stage condenser (*Figure 2.24*).

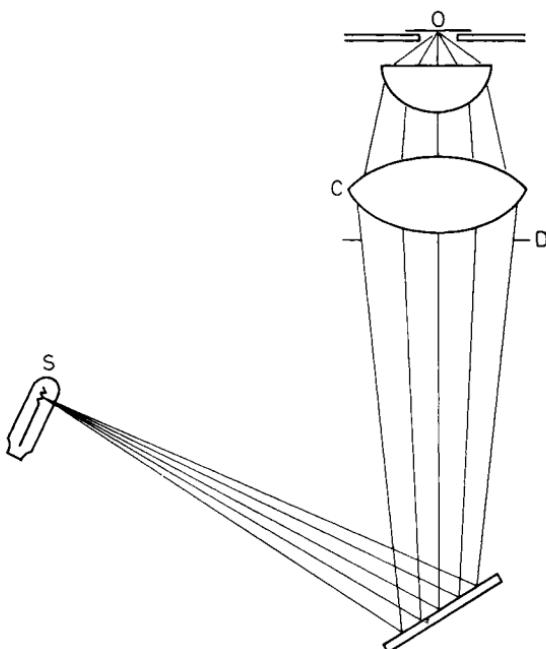
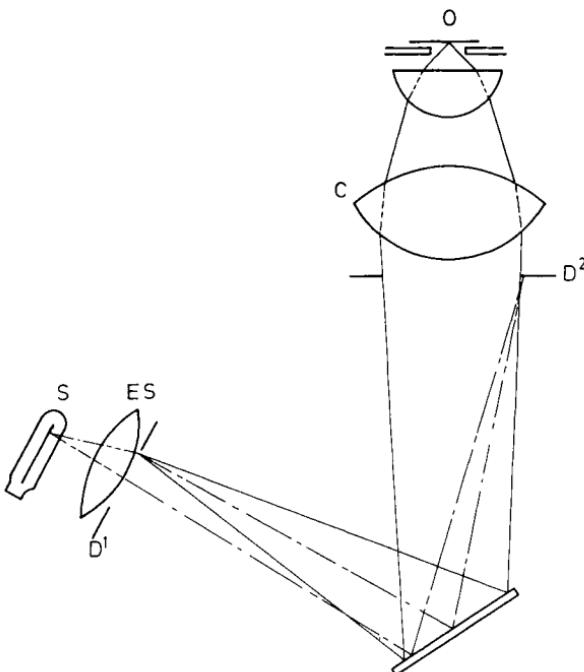


Figure 2.24. Diagram illustrating critical illumination (Nelson). The light source S is imaged in the plane of the object O by the sub-stage condenser C. The diaphragm D in the sub-stage condenser serves as a field diaphragm

Köhler illumination is named after the German scientist August Köhler, who was famous for his photomicrography and who introduced the method in 1892. With Köhler illumination the whole field is illuminated evenly, a condition which is essential for photomicrography. In order to obtain Köhler illumination it is necessary for the lamp to be fitted with a condenser and an iris diaphragm. The lamp condenser is used to project an enlarged image of the lamp filament, which is brought to focus on the iris diaphragm of the sub-stage condenser. When so focused, the effective source is imaged in the object plane, the whole field being flooded with parallel light. The lamp diaphragm, which should have a large aperture and be mounted as near as possible to the lamp con-

denser, serves as a field diaphragm; the sub-stage iris diaphragm is used as an aperture diaphragm (*Figure 2.25*).

A field diaphragm controls the area illuminated but does not



*Figure 2.25. Diagram illustrating Köhler illumination. An image of the actual light source *S* is formed in the plane of the sub-stage iris diaphragm. *D*¹ serves as a field diaphragm, the sub-stage iris diaphragm *D*² being the aperture diaphragm*

change the brilliance of the illumination or affect the working numerical aperture of the system.

An aperture diaphragm controls the brilliance of the illumination and the working numerical aperture of the system, but does not affect the size of the area illuminated.

Köhler illumination

1. The microscope bench should be firm, of suitable height and free from vibration.
2. Place the microscope about 10 in in front of the lamp diaphragm and direct the beam on to the centre of the plane surface of the mirror.
3. Place a 16 mm objective and a low-power eyepiece in the

optical train, insert a neutral filter in the light path and adjust the mirror to obtain maximum illumination.

4. Place a stained slide on the stage and focus on the specimen. Close the sub-stage iris diaphragm, remove the eyepiece and by means of the centring screws ensure that the sub-stage condenser is correctly aligned.
5. Rack up the sub-stage condenser, replace the eyepiece and adjust the lamp condenser until an enlarged image of the light source is focused on the sub-stage diaphragm. This can be observed by using a small hand-mirror.
6. Open the sub-stage diaphragm, close the lamp diaphragm and focus the sub-stage condenser to obtain a sharp image of the lamp diaphragm in the object plane.
7. Adjust the mirror to bring the centre of the lamp diaphragm image into the centre of the field.
8. Open the lamp diaphragm until the field is just filled with light, remove the eyepiece and adjust the sub-stage diaphragm until the back lens of the objective is just filled with light.

Note—If the microscope is equipped with built-in lighting designed to work on the Köhler principal steps 2 and 7 of the above procedure may be omitted.

SETTING UP THE MICROSCOPE FOR DARK-FIELD ILLUMINATION

1. Switch on the electric current, place a piece of lens paper over the eyepiece and adjust the illuminant and tail-piece mirror until the maximum illumination is obtained. Remove the bright-field condenser.
2. Fix the dark-field condenser into position and swing the $10\times$ objective into the optical train.
3. Place a drop of oil on the top lens of the condenser and lower surface of the slide to be examined and carefully place the slide in position, taking care to avoid the formation of air bubbles between the oiled surfaces.
4. Examine the specimen and adjust the condenser until a small, but intense area of illumination is obtained, centring the condenser if necessary.
5. Swing the $40\times$ objective into the optical train and focus. Correct for maximum illumination by closing the lamp

diaphragm and re-focusing the light spot with the condenser. Re-centre the condenser if necessary.

6. Open the lamp diaphragm until the minimum working field is illuminated and examine the specimen.
7. Rack the body-tube up slightly, apply a drop of oil to the area of the slide to be examined and swing the immersion objective into the optical train. Carefully lower the body-tube until the front lens of the objective is just less than the working distance from the slide. Complete the focusing with the fine adjustment by *raising* the body tube. When the specimen is in focus, make any final adjustment necessary to the condenser.

FLUORESCENCE MICROSCOPY

The term 'fluorescence' was first coined by George Gabriel Stokes in 1852 to describe the reaction of fluorspar when illuminated with ultraviolet light. Basically, fluorescence is the absorption and re-emission properties possessed by certain substances, whereby short-wave radiation is absorbed and re-emitted as light of a longer wavelength, resulting in the object acquiring a luminous appearance.

The development in recent years of intense light sources rich in short-wave radiation, has given a tremendous impetus to the development of the fluorescence microscope as a diagnostic and research instrument. As a result, many companies now market microscopes especially designed for this purpose. The essential requirements for fluorescence microscopy are the following:

1. a suitable light source,
2. a heat-absorbing filter,
3. exciter filters,
4. condenser,
5. objective,
6. barrier filter,
7. eyepiece.

The light source

A variety of light sources is available, the final selection being dependent upon the work to be undertaken. At the present time the two most widely used lamps are the Osram HBO 200 mercury vapour lamp and the quartz-iodine lamp. Of these the latter, which

is relatively cheap, is quite adequate for a great deal of work in the medical laboratory, such as screening for malignant cells and identifying mycobacteria, for which purpose blue light fluorescence is used. For more detailed research work, however, particularly in the field of immunology where ultraviolet light of a shorter wavelength is desirable, the mercury vapour lamp is said to be preferable.

Heat-absorbing filter

This must be interposed between the lamp and the exciter filters. It may be either liquid or glass, the latter being more convenient. Liquid filters consist of a cuvette with a solution of copper sulphate and ammonium sulphate and in addition to serving as heat-absorbing filters may also serve as an exciter filter by cutting out the red light. The glass type are manufactured commercially and when using the mercury vapour lamp should be at least 4 mm in thickness to provide adequate protection. Examples of glass filters are Chance-Pilkington OX 2 and Schott KG 2.

Exciter filters

The correct selection of the exciter filters is perhaps the most important single factor in fluorescence microscopy. These also may be either liquid or glass, but the advantages of the latter are such that they are rapidly becoming the automatic choice. The filter,

Table 2.1 EQUIVALENT FILTER COMBINATIONS USED IN FLUORESCENCE MICROSCOPY

		Chance	Ilford	Leitz	Reichert	Zeiss
<i>Bright field</i>	Exciter	OX 1A +	828 +	UG 1 +	E 1	UG 1 +
		OB 10	502	BG 38		BG 12
	Barrier	OY 13	102	uv Abs.	SP 1	41
						UG 1
<i>Dark field</i>	Exciter	OX 1	828	UG 1	E 2	+ UG 5
	Barrier	OY 10	805	uv Abs.	SP 2	41-65
<i>Blue uv</i>	Exciter		502 OB 10 +	BG 12 +	E 3	BG 12 +
			804	BG 38		BG 12
	Barrier	OY 13 +	109 +	Blue Abs.	SP 3	50
		OY 3	104			

or combination of exciter filters used, depends upon a number of factors, including the light source, the specimen under examination, the fluorochrome used as the staining reagent and the barrier filter. Instructions for the use of exciter filter combination are supplied by the manufacturers and the student should follow their directions. In addition, the original papers in which the staining procedure in use was first described should be consulted, in order to establish the filter combination originally recommended. A table of equivalent filters is given above (*see* Table 2.1).

Condenser

Special quartz condensers are manufactured for use in fluorescence microscopy but are only necessary for specific purposes. In general, condensers made from crown glass are adequate but it is important that the condenser does not contain too many components which are cemented together. In many instances, a cardiod dark-ground condenser is to be preferred, as this provides greater contrast.

Objectives

These should be of simple construction. When available, planachromatic objectives should be used to provide a flatter field. If immersion objectives are used, the immersion oil must be 'fluorescence-free'. Special immersion oil is manufactured for this purpose but oxidation can cause it to fluoresce after the bottle has been opened a few months.

Barrier filters (secondary filters)

A barrier filter is an essential for fluorescence microscopy and specimens should not be examined by ultraviolet light without a barrier filter being positioned in the optical train. Microscopes manufactured especially for fluorescence microscopy usually have a turret or slide in the body, in which the filters are housed. Instruments adapted for fluorescence normally have the barrier filter located in the eyepiece either as a clip-on attachment or resting on the diaphragm. The latter is in direct focus of the eye lens and must therefore be free from specks of dust and fingerprints.

The barrier filter is complementary to the exciter filter and selection should therefore be made with care. The facilities provided in research microscopes for having a series of barrier filters is one of many advantages of purchasing specially designed equipment.

Eyepiece

For fluorescence microscopy low-power oculars are to be preferred. The construction should be as simple as possible, the most widely used being the Huygenian pattern.

Fluorochromes

Mention was made earlier of the use of fluorochromes as staining reagents in fluorescence microscopy. These are organic dyes which fluoresce when subjected to short-wave radiation, and which have an affinity for certain substances. Fluorescence produced by the

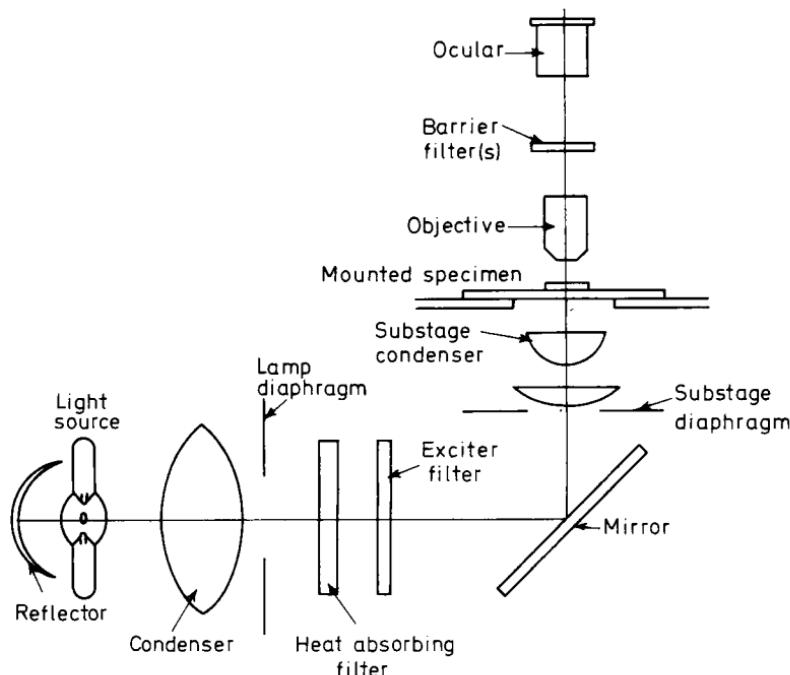


Figure 2.26

use of fluorochromes is termed 'secondary fluorescence'. A knowledge of the wavelength at which fluorochromes absorb the exciting radiation and re-emit the absorbed energy is of great importance when selecting the exciter and barrier filter combination. Ideally, the transmission of the exciter filter should match the emission peak of the fluorochrome being used.

Using the fluorescence microscope

There is no special technique for using the fluorescence microscope other than applying the general rules for good microscopy and adjusting it for Köhler illumination and ensuring that the correct filters are in position prior to switching on the lamp. The art in obtaining high-quality results lies more in a thorough knowledge of the fluorochromes and filters in use. When using a mercury vapour lamp a record should be kept of the number of hours for which the lamp is used.

SOME DO'S AND DON'TS OF MICROSCOPY

DO cover the microscope when not in use.

DO remove all immersion oil from the objective immediately after use. This may easily be done by wiping with lens paper or a soft cloth. Xylene may be used sparingly.

DO clean the optical parts with lens paper before use.

DO support the microscope carefully, when moving it.

DON'T rack the objective downwards to bring the object into focus whilst looking down the microscope.

DON'T attempt to dismantle objectives.

DON'T use a high-power objective when a low-power one is sufficient.

DON'T lubricate the microscope with any oil other than that provided for the purpose.

DON'T place wet preparations on the stage without wiping the undersurface of the slide.

3

Safety Precautions

The Health and Safety at Work Act, April 1975, requires employers to provide adequate safety precautions and regulations. Workers in medical laboratories are exposed to many dangers, not only from infected material but also from the dangerous compounds and apparatus which they use as a daily routine. These precautions must be observed by all members of the staff, not only for the good of themselves as individuals, but for the safety of all concerned. While the application of safety precautions is only a matter of using a little common sense, unnecessary accidents frequently occur in most laboratories. The following rules are given here for guidance but all laboratory staff should be familiar with the publication *Safety in the Pathology Laboratory* issued by the Department of Health and Social Security and Welfare Office.

All accidents which occur in the laboratory must be reported immediately to the person in charge. Protective clothing must be worn when in the laboratory, but should always be removed before leaving the premises. Personal clothing should be suited to the nature of the work. The wearing of jewellery should be limited to a minimum and care should be taken to ensure that rings and other ornaments, when worn, are not attacked by acids or other chemicals.

Examine all glassware before use, paying particular attention to the mouthpiece of pipettes. When pipetting always use a teat, never the mouth. Glassware with damaged edges should not be used. Examine carefully for any defects on all flasks, beakers and other containers in which acids or alkalis are to be dissolved, and reject those with the slightest flaw. Discard all broken glass into a bucket reserved for the purpose and not into the waste paper basket. Do not clutter up the sink with dirty glassware; in the event of an accident you may wish to use the sink quickly.

After using strong acids and alkalis be sure to wipe the neck of the bottle before returning it to the shelf. Always replace reagent bottles on the shelves and never leave bottles of acids and alkalis where they may be overturned. Neutralize immediately all acids

and alkalis that are spilt. All bottles must be clearly labelled to show their contents.

Always use a fume-cupboard when working with chemicals which evolve a toxic or irritant vapour. Do not use wash bottles belonging to other people; this practice is both unhygienic and unreliable. Put away immediately all microtome knives and other sharp-edged instruments as soon as they are no longer required. Keep the working space on the bench clear so that if an accident occurs the minimum number of articles will be involved.

PRECAUTIONS AGAINST INFECTION

Prior to appointment all staff should undergo a medical examination, which should include screening for *M. tuberculosis* (Heaf or Mantoux Test) and Hepatitis B Surface Antigen (Australia Antigen). If the Heaf or Mantoux test is negative, it may be necessary for them to have a course of BCG vaccine. Periodic X-ray (1 or 2 per annum) should be carried out if working in close contact with this organism. Prophylactic vaccine should be administered to those members of staff working with certain types of organisms, for example *S. typhi*.

Spillage from specimens should be wiped up immediately with absorbent cotton wool dipped in disinfectant. Suitable disinfectants are 1 per cent Hycolin, 2 per cent Clearsol, 1 per cent Sudol and 10 per cent Chloros. All vessels that have contained contagious specimens must be sterilized before being washed up. Sterilization may be accomplished by autoclaving at 15 lb pressure per in² for 15 min*. Disposable sputum containers should be incinerated after sterilization. Laboratory coats, post-mortem gowns and infected towels must always be sterilized before being sent to the laundry. Soiled swabs should be discarded into an infected material bin or a jar of disinfectant, and not into the waste paper basket.

All glassware and containers used to examine wet specimens, for example slides and trays, must be placed into a disinfectant immediately the investigation is completed. Instruments sterilized by flaming, for example platinum loops, should be held at arm's length while being flamed in order to eliminate the danger of infection from spattering.

When no longer required discard cultures into an appropriate

* Pressure is not given in S.I. units as autoclave pressure gauges are marked in lb per in².

receptacle for sterilizing. Never remove the cultures once discarded until they have been sterilized.

Always wash your hands with soap and water after handling cultures and specimens before leaving the laboratory. Smoking should not be permitted in the laboratory.

The inoculating cabinet

All work involving the handling of infective specimens should be performed under cover of an inoculating cabinet. Further details are given in Chapter 27.

PRECAUTIONS AGAINST FIRE

When working with highly flammable chemicals in the laboratory the danger of fire should always be kept in mind and adequate precautions taken. Flammable chemicals include benzene, xylene, toluene, acetone, ether and alcohol. It should be remembered that the greater fire hazard is from the vapour given off from the chemical. To guard against this all flammable chemicals must be securely stoppered when not in use. When working with flammable chemicals all naked flames in the near vicinity should be extinguished. A waste bottle should be kept in all laboratories for the purpose of discarding flammable chemicals. When full, the contents of the waste bottle may be disposed of in a safe place. On no account smoke when handling flammable chemicals.

Fire emergency measures

In the event of fire the following steps should be taken:

1. Sound the alarm.
2. Evacuate from the immediate vicinity of the fire.
3. Close all the doors and windows, turn off all gas and electrical appliances. Attack the fire if possible with the appliances available but without taking personal risk.

The following equipment for dealing with all minor fires and controlling larger ones should be available in all laboratories. All members of the staff should also be familiar with the location of the fire apparatus adjacent to their own laboratory in cases of emergency.

HOSES

These must be checked regularly to ensure that they are in good working order.

WATER AND SAND BUCKETS

These must be kept filled and covered, checked and refilled periodically.

FIRE BLANKETS

These must be fixed in easily accessible positions. They are effective in smothering and preventing the spreading of fires. They are particularly useful for extinguishing fires involving clothing or cotton wool.

FIRE EXTINGUISHERS*Foam type*

Used on fires started by solvents immiscible with water, for example xylene. They should *not* be used where live electrical circuits are exposed.

Soda-acid type

Used on fires involving solid material such as paper and wood. They may also be used on water-miscible solvents but *not* where live electrical circuits are exposed.

Carbon dioxide type

Used on small fires and where live electrical circuits are exposed. It should not be used on liquid fires.

STORAGE OF CHEMICALS

Acids must be kept in glass-stoppered bottles preferably in a drip tray. Winchester quart bottles should be stored at floor level.

Alcohol. Duty-free alcohol must be kept locked, and all details of its use recorded.

Customs and Excise officials periodically inspect the stock.

Ammonia must be kept tightly stoppered and away from heat and other chemicals.

Bromine ampoules must be stored in absorbent material.

Cyanide and all other poisonous chemicals must be clearly marked 'poison' in red letters, and kept locked in a poisons cupboard; details of all poisons issued should be entered in a poison record book.

Deliquescent and hygroscopic chemicals must be stored in airtight containers. Such chemicals include potassium and sodium

hydroxide, sodium carbonate, phenol and phosphorus pentoxide.

Ether must be kept in a glass bottle, stoppered with a tinfoil-covered cork, or a waxed-lined bakelite screw top. Never use a rubbing bung, as ether attacks rubber, and never store in a refrigerator.

Hydrogen fluoride attacks glass and must be stored in a gutta-percha or polythene bottle.

Hydrogen peroxide must be kept in a brown glass bottle in a refrigerator. Exposure to warmth and light causes oxygen to be evolved, and a pressure sufficient to cause the bottle to explode may be built up.

Flammable liquids. Keep well stoppered in a metal container clearly marked 'flammable'. Stocks of such fluids should be kept in a store used solely for this purpose. This store room should have a sunken floor so that in the event of breakages no liquid will flow from the room. Keep the bottles as cool as possible; never use the liquids near a naked flame.

Iodine must be kept in a brown glass bottle with a glass stopper. Never use a rubber bung, as iodine attacks rubber.

Potassium hydroxide solution should be stored in bottles waxed on the inside, as it attacks glass, forming sodium silicate. Glass stoppers must never be used, as the CO₂ in the air combines with the NaOH, forming Na₂CO₃ which acts as a cement, firmly fixing the stopper into position. The solution in daily use should be stored in an aspirator. A soda-lime guard tube will absorb and prevent any CO₂ from entering the aspirator.

Potassium permanganate must be stored in a dark glass-stoppered bottle, as it decomposes when exposed to light.

Sodium hydroxide solution (*see potassium hydroxide*).

Sodium must never be allowed to come into contact with water, as spontaneous combustion will result. Keep completely covered with ligroin, naphtha or xylene.

Silver nitrate solution must be kept in a dark, glass-stoppered bottle. Exposure to light decomposes the silver nitrate to silver oxide.

Sodium nitroprusside must be stored in a dark, glass-stoppered bottle, as it decomposes upon exposure to light.

IMMEDIATE TREATMENT OF ACCIDENTS

First-aid treatment

All accidents which occur in the laboratory *must* be reported and entered in the accident book. It is emphasized that the following procedures are only emergency measures and must be followed immediately by adequate treatment.

GENERAL TREATMENT OF SUPERFICIAL WOUNDS

If a limb is involved, raise it to reduce the bleeding. To arrest the flow of blood apply digital pressure with a clean dressing to the wound. Fragments of glass should be left unless they can be easily removed with a sterile dressing. Blood clots which form should not be disturbed. Place a dry sterile dressing over the wound and, if the bleeding is profuse, back the dressing with cotton wool. Bandage firmly into position and obtain further treatment in the casualty or out-patients department.

GENERAL TREATMENT OF BURNS AND SCALDS

Dry burns and scalds

Pain produced by burns may be reduced by immersing in cold water as soon as possible or applying a cold compress. Burn dressings, such as tannic acid, should only be applied under a doctor's supervision and blisters must not be punctured.

Chemical burns

Dab away as much of the chemical as possible and bathe with water. If the corrosive chemical is an acid, sprinkle powdered sodium bicarbonate over the site of injury. If acid splashes into the eye, wash with water from a wash bottle and bathe with 5 per cent sodium bicarbonate in an eyebath. If the corrosive chemical is an alkali, sprinkle powdered boracic acid over the affected area. In the event of an alkali entering the eye, immediately wash the eye with water or 1 per cent acetic acid. Phenolic burns should be washed with surgical spirit.

All cases of burns must be referred to a doctor as quickly as possible.

Poisoning

Corrosive poisoning

This is caused by swallowing strong acid or alkali. Treatment consists of rinsing the mouth immediately, followed by copious

draughts of water. If acid has been swallowed, Milk of Magnesia should be given. For alkali poisoning, very dilute acetic acid should be administered. Vomiting should *not* be encouraged.

Contamination by infected material

In the event of an accident involving infected material occurring, for example cutting a hand, medical treatment must be obtained immediately.

Electric shock

Switch off the power at source. If the patient is unconscious send for medical assistance immediately and if necessary apply artificial respiration.

4

Collection and Reporting of Specimens

As many different types of specimen are received daily in a routine pathology department, it is necessary to observe certain details to ensure an accurate report with the minimum of delay.

1. The specimen should be clearly labelled with the patient's name, hospital number, ward, date and time of collection.
2. A fully completed 'request form' should accompany each specimen with the details mentioned above, together with the nature and origin of the specimen, the provisional diagnosis, the investigation required, and any other relevant information that can aid the laboratory in setting up the correct test.

In all routine laboratories the quality of the specimen has an effect on the tests that are performed and their results. For example, a sputum for bacteriological examination would be of little value if the specimen was mainly saliva; a urine for culture whose delivery to the laboratory had been delayed for a considerable time would again be of little value as any organisms present would be multiplying within that urine, giving a false picture of possible infection; a clotted blood sample would be of little value for a white cell count etc., therefore for a laboratory to give an adequate service, there must be two-way communication. It is essential that physicians know what specimens to send for a particular investigation, what container should be used and how quickly it should be delivered to the laboratory.

On the other hand, it is equally important that the laboratory help the physician in these matters. One way of achieving this, is to issue to the medical staff a list similar to that in Table 4.1, which covers the majority of tests performed in laboratories. Not all of these tests, however, are included in this book.

Receipt of specimens

Hospitals vary with the way in which specimens are collected from

Table 4.1 EXAMINATION, TYPE AND BLOOD VOLUME REQUIRED

<i>Examination required</i>	<i>Type of blood</i>	<i>Volume required (ml)</i>
<i>Bacteriology</i>		
Culture	Into special blood culture bottles (see p. 523)	10
Virus agglutination	C	10
Widal		
VD and other serology		
<i>Biochemistry</i>		
Acid phosphatase	C	2.5
Alcohol	C	2.5
ADP/AMP	C	5
Alcohol ¹	C or H	2.5
Aldolase	C	2.5
Alkaline phosphatase	C	2.5
Alkali reserve	C	2.5
Amino acids	H	10
Amylase	C	2.5
Ascorbic acid ²	C	10
Bicarbonate total	H	2.5
Bilirubin	C or H	2.5
Bromide	H or C	2.5
Calcium	C	2.5
Chloride	C or H	2.5
Cholesterol	C or H	2.5
Cholinesterase	H or C	2.5
Congo red	C	10
Creatinine	C or H	2.5
Creatine	C or H	6
Fibrinogen	O	2.5
Flocculation tests	C	10
Folate	C	5
Glucose	C, H or O	2.5
Iron	C	10
Iron-binding capacity		
Lipase	H or C	5
Liver function tests	C	10
Methaemoglobin	H or C	2.5
Methaemalbumin	H or C	2.5
Non-protein nitrogen	H or C	6
Urea nitrogen	H or C	2.5
Phosphorus	H or C	2.5
Proteins/albumin/globulin	H or C	6
Pyruvate	10% trichloracetic acid	2.5
Salicylate	H or C	2.5
Sodium	H or C	2.5
Sulphaemoglobin	H or C	2.5

Table 4.1 (cont.)

<i>Examination required</i>	<i>Type of blood</i>	<i>Volume required (ml)</i>
Transaminases	C	5
Urea	H or C	2.5
Uric Acid	H or C	2.5
Vitamin A	H or C	10
Vitamin B ₁₂	C	5
<i>Other biochemical tests</i>		
All these tests require 5–10 ml of clotted blood		
MDH (malate dehydrogenase)	GT	
Non-esterified fatty acids	HBDH	
Phospholipids	CPK	
Pyruvate kinase	Isoenzymes	
SD4 (sorbitol dehydrogenase)	Lactate	
Total lipids	ICDH	
Triglycerides (neutral fat)	G-6 P.DH	
LDH	Galactose	
<i>Haematology</i>		
Absolute values	{ E	Volume indicated on EDTA bottle. Generally 2.5 ml
Differential cell count		
Erythrocyte sedimentation rate		
Haemoglobin		
Red cell count		
White cell count		
Blood grouping	{ C	5–10 ml
Cold agglutinin		
Compatibility tests		
Paul Bunnell		
Rose Waaler		
Clotting studies	P or S	Volume indicated on bottles. Generally 2.5 ml
Red cell fragility	H	5–10 ml

C = clotted; E = EDTA (Sequestrene); H = heparinized; O = oxalated; P = Heller and Paul (Wintrobe); S = Sodium citrate

¹ Preferably collected under paraffin or in tube filled to stopper.

² Specimen should be sent to laboratory as quickly as possible.

Table 4.2 OTHER SPECIMENS, EXAMINATIONS AND CONTAINERS REQUIRED

<i>Specimen and examination required</i>	<i>Specimen container</i>
FAECES	
<i>Bacteriology</i>	
Organisms	Sterile wide-mouth screw-capped bottle.
Amoeba	Fresh specimen sent immediately.
<i>Biochemistry</i>	
Faecal fat	In a waxed carton.
Occult blood	
GASTRIC CONTENTS	
<i>Bacteriology</i>	
Tuberculosis	Universal containing 5 ml of trisodium phosphate.
<i>For other investigations the pathologist should be consulted</i>	
HAIR	
<i>Bacteriology</i>	
Fungi	Fold the hairs into clean white paper.
PLEURAL, PERITONEAL AND OTHER EFFUSIONS	
<i>Bacteriology</i>	
Organisms	20 ml into sterile bottle containing 1 ml of 20 per cent sodium citrate.
<i>Histology</i>	Bottle large enough to accommodate the whole specimen. No sodium citrate.
PUS	
<i>Bacteriology</i>	
Organisms	Sterile 1 oz universal bottle or swab.
SALIVA	Sterile 1 oz universal bottle.
SPUTUM	
<i>Bacteriology</i>	
Organisms	Sterile 1 oz universal bottle.
Tuberculosis	Wax carton.
<i>Histology</i>	
Tumour cells	Sterile 1 oz universal bottle.
URINE	
<i>Bacteriology</i>	
Organisms	Sterile 1 oz universal bottle.
Tuberculosis	24 h specimen in a Winchester quart bottle or Early morning specimen in universal bottle.
<i>Biochemistry</i>	
Routine examination	Sterile 1 oz universal bottle.
Electrolytes	24 h specimen in a Winchester quart bottle.
Pregnancy test	Early morning specimen collected in 200 ml bottle. At least 12 days after last expected MP.

the wards. On receipt of the specimen, many hospital laboratories have a central area where it is given a laboratory number, the information on the request form accompanying the specimen is checked, and the specimen and form despatched to the appropriate laboratory.

Reporting

After the specimen has been processed in the laboratory, it is essential that the information obtained be conveyed to the physician. The 3 Rs of reporting are reliability, rapidity and relevance. Reliability of course speaks for itself. The results must be reliable and they must be transmitted as rapidly as possible. Relevance is of vital importance. Although many tests may be performed on a specimen, it is advisable that only those relevant to the request should be sent. For example, if several antibiotic susceptibility tests are performed on an organism, it is not always advisable to give every result. Only those relevant and consistent with the antibiotic policy of the hospital should be mentioned. The need for information to be given as rapidly as possible often means telephoning results to the ward. This can be a highly dangerous procedure. There are many examples of misinterpretation of results due to either the speaker not being precise, or the recipient misunderstanding. A classic example of this was when the specimen was received from the theatre for a rapid result. The result was phoned through as an adenocarcinoma which was interpreted as 'had no carcinoma'. If results are telephoned, it is essential for the person to read back what has been said so that no mistakes can occur. A written report should be sent as soon as possible.

Reporting systems vary from hospital to hospital and no universal reporting system would necessarily be accepted by everyone. Cumulative reporting, if carried out correctly, is probably the most helpful way both to laboratory and physician. This necessitates each patient having a master card for each discipline and the results are entered onto this card and photocopied, the photocopy being delivered to the ward. By this method the physician can see at a glance any changing pattern of results and this also gives the laboratory a check on their results when compared with the previous ones. In larger hospitals, reporting is performed utilizing computers, results being transmitted direct to the ward. Whichever system is used, an adequate check must be made that

1. the correct details of the patient's name, ward, hospital number etc. have been filled in,

2. the correct result has been entered and the report delivered to the right place.

Ward etiquette

The collection of blood or cytology specimens may necessitate visits to the wards or out-patients departments. As this may well represent the only direct professional contact with the patient (the public) it is desirable that a good impression be created.

Before entering the ward it is both courteous and essential to obtain permission from the sister or nurse in charge, thereby preventing visiting the patient at inconvenient times. It must be remembered that the cooperation of the nursing staff can often facilitate the collection of specimens.

It is important to gain the confidence of the patient and appearance can go a long way towards achieving this. A clean coat reserved for visiting patients, has the dual role of reducing the risk of cross-infection and creating a good impression.

The patient should be put at ease, and submitted to the least possible discomfort. The operator therefore should be certain that all apparatus and reagents necessary for the collection of the specimens are to hand.

To avoid errors, it must be emphasized that specimens should be labelled, correctly and immediately, in the ward and not left until returning to the laboratory.

Postal specimens

The sending of specimens by post is governed by regulations laid down by the Postmaster General. These rules must be strictly adhered to at all times.

1. The specimen must be sent by letter post only and must be labelled clearly 'FRAGILE WITH CARE' and 'PATHOLOGICAL SPECIMEN'.
2. The specimen must be in a sealed container.
3. The sealed container must be packed in a wood or metal box which contains sufficient absorbent material such as sawdust or cotton wool to prevent any possible leakage should the container be damaged en route.

The full regulations may be obtained from the Post Office and if any doubt about a type of box or container in general use exists, the person in charge should submit it to the Secretary of the General Post Office for confirmation of its suitability. Failure to do so may lead to the loss of the specimen and prosecution of the person sending it.

PREPARATION OF SPECIMEN CONTAINERS

It is invariably the duty of the Pathology Department to prepare and issue the many varied containers used for the collection of specimens. Most laboratories in the UK use commercially prepared anticoagulant bottles (disposable), which saves valuable preparation time. For those who have to prepare their own bottles the method of preparation is listed below.

STERILE UNIVERSAL BOTTLES

These are 1 oz glass or plastic bottles, with screw caps. After suitable cleaning and drying the glass bottles are capped and autoclaved at 15 lb for 20 min.

The plastic bottles are purchased sterilized.

SEQUESTRENE (EDTA) BOTTLES

Dry salt

Sequestrene (disodium salt of ethylenediamine)

Ethylenediamine tetra-acetic acid	10 g
Distilled water	100 ml

Dissolve the salt in the distilled water. Deliver 0.05 ml of solution into small bottles or tubes marked at the 2.5 ml level. Allow the water content of the solution to evaporate at room temperature. Fix screw caps or corks to tubes and label as containers for 2.5 ml of blood.

HELLER AND PAUL OXALATE MIXTURE (WINTROBE)

Ammonium oxalate	1.2 g
Potassium oxalate	0.8 g
Distilled water	to 100 ml

Dissolve the salts in the distilled water. Deliver 0.25 ml of the solution into small bottles or tubes marked at the 2.5 ml level. Place the bottles or tubes in an oven at 60 °C to evaporate the water content from the oxalate solution. Fix screw caps to bottles or corks to tubes and label as ready for use with 2.5 ml of blood.

HEPARIN BOTTLES

Disposable bottles obtainable commercially.

Heparin is a physiological anticoagulant and is used in a concentration of 0.1–0.2 mg per ml of blood.

Lithium heparin is to be preferred for electrolyte studies.

SODIUM FLUORIDE-POTASSIUM OXALATE

Sodium fluoride	1.2 g
Potassium oxalate, neutral	6.0 g

Grind the two salts to a fine powder and dissolve in 100 ml of distilled water. Distribute in screw-capped bottles in volumes of 0.05 ml for each ml of blood. Evaporate to dryness in an oven which does not exceed 60 °C.

Notes—

1. Ammonium oxalate should not be used as an anticoagulant for blood which is to be examined biochemically, as it prevents the determination of nitrogen and ruins any analysis which requires the use of Nessler's reagent.
2. Sodium fluoride prevents glycolysis. It should not be used as a preservative, however, when the blood is to be examined for urea by a urease method.

SODIUM CITRATE

Sodium citrate	3.8 g
Distilled water	100 ml

Dissolve the salt in the distilled water. Distribute in small screw-cap bottles in 5 ml or 10 ml amounts and sterilize by autoclaving. Label according to the volume.

SWABS**Throat, eye, vaginal swabs**

A swab for one of these areas consists of a plastic applicator around which a small whisp of absorbent wool is wound to give a small pledget approximately 12 mm in length and 2–3 mm in width.

Ready-made and sterilized disposable swabs are available commercially.

Laryngeal swabs

Laryngeal swabs are made from approximately 22.5 cm of brass wire which is slightly bent 5 cm from one end. Absorbent cotton wool is wrapped round this end which is then inserted into a large test-tube, plugged with non-absorbent cotton wool and sterilized by dry heat. Alternatively, calcium alginate wool can be used in place of absorbent cotton wool. This wool will dissolve in a sodium

salt solution, and has proved very effective for laryngeal swabs for mycobacteria. The whole swab is investigated, and there is no loss of material by immersion into acid and alkali. Sterilize by autoclaving.

Pernasal swabs

These consist of a small piece of absorbent cotton wool mounted on a fine wire. The wire used should be polished nicrome SWG 22 roughened at one end to prevent the wool from slipping. The wire must be thin and flexible to ensure easy passage into the nares. Place in a 150 × 15 mm tube, plug with non-absorbent wool and sterilize in the hot-air oven.

Pharyngeal (post-nasal) swabs

These consist of a piece of absorbent cotton wool attached to a 20 cm length of flexible wire. The wire used should be copper SWG 18 prepared as for pernasal swabs. The cotton wool end should be bent like a hockey stick for easy passage into the pharynx. The whole swab is then enclosed in a piece of curved glass tubing and plugged at both ends with non-absorbent wool. Sterilize in the hot-air oven.

COLLECTION OF AUTOPSY SPECIMENS

Histology

Small pieces of tissue: In bottle containing a suitable fixative.

Whole organs: Preserved in Wentworth's solution or 10 per cent formol saline (p. 325).

Bacteriology

Small pieces of tissue: In sterile 1 oz universal bottle.

COLLECTION OF BIOPSY SPECIMENS

Histology

In bottle containing a suitable fixative.

Bacteriology

In sterile 1 oz universal bottle.

5

Some Fundamentals of Chemistry

The student should have previous knowledge of the following—elements, atoms, isotopes and formulas; chemical units such as atomic weight, molecular weight, gram-atom and gram-molecule. Familiarity with the four fundamental laws of chemistry is also assumed but they have been included because of their importance.

1. Law of Conservation of Mass

Mass can neither be created nor destroyed. This means that in a chemical reaction, there are the same number of atoms of each element in the products as there were in the reactants.

2. Law of Constant Composition

A given chemical compound always contains by weight the same elements in the same proportions.

Note—Many compounds do not obey this law, e.g. ferrous sulphide has variable composition and rarely has the precise formula FeS. Such compounds are called non-stoichiometric or Berthollide compounds.

3. Law of Multiple Proportions

When two elements, A and B, combine to form more than one compound, the masses of element B, which combine with a fixed mass of element A, are in simple numerical proportion to one another.

4. Law of Reciprocal Proportions (or equivalent proportions)

The masses of two elements A and B which combine separately with a fixed mass of element C are in a simple ratio to the mass ratio in which A and B combine with each other.

Equivalents

The law of reciprocal proportions means that to every element may be assigned a number representing the mass of element which will combine with a fixed mass of the substance of reference. This number is called the equivalent weight. The equivalent weight expressed in grams is called the gram-equivalent.

THE GAS LAWS

Although an ideal gas is purely hypothetical, the usefulness of the laws is that they are obeyed very closely by gases under normal conditions.

1. Boyle's Law

The volume of a given mass of gas at constant temperature is inversely proportional to its pressure, i.e.

$$pv = k$$

where p is the pressure and v is the volume of a fixed quantity of gas at constant temperature; k is the constant.

2. Charles' Law

At constant pressure the volume of a given mass of any gas is proportional to its temperature on the absolute scale.

If t is the temperature on the centigrade scale, then the absolute temperature T (K) is equal to $t + 273$.

Therefore Charles' Law can be expressed as:

$$\begin{aligned} v &= k(t + 273) \\ v &= kT \end{aligned}$$

Boyle's and Charles' Law can now be combined to give the following relationship:

$$pv = kT \quad \text{or} \quad \frac{pv}{T} = k$$

The absolute temperature (kelvin) scale is, as mentioned above, obtained by adding 273 to the temperature in degrees celsius.

For example

$$\begin{aligned} 20^\circ\text{C} &= (20 + 273) = 293\text{K} \\ -10^\circ\text{C} &= (-10 + 273) = 263\text{K} \end{aligned}$$

Boyle's and Charles' Law can be further elucidated by the following calculation:

A gas occupies 250 ml at 30°C and at a pressure of 730 mmHg. Calculate the volume of gas at 760 mm and 0°C .

$$\frac{p_1v_1}{T_1} = \frac{p_2v_2}{T_2}$$

$$\begin{array}{lll} p_1 = 730 \text{ mm} & v_1 = 250 \text{ ml} & T_1 = (273 + 30) = 303\text{K} \\ p_2 = 760 \text{ mm} & T_2 = 273 & \end{array}$$

$$v_2 = \frac{730 \times 250 \times 273}{303 \times 760} = 216 \text{ ml}$$

3. Avogadro's Law

Equal volumes of all gases at the same temperature and pressure contain equal numbers of molecules. One gram mole of any gas at a given temperature and pressure will therefore occupy a definite volume, which will be the same for all gases. Therefore the value k in the equation $pv = kT$ will also be the same for all gases. This standard value k is usually indicated by a special symbol R , the gas constant.

General gas equation

The three gas laws combine to give

$$pv = nRT$$

where p = pressure of gas

v = volume of the gas

n = moles of gas molecules

R = gas constant

T = absolute temperature

The value of R depends upon the units of p and v

$$R = \frac{pv}{nT}$$

Numerical value of the gas constant

Experimentally, the volume of one mole of a perfect or ideal gas occupies 22.4 litres at 0 °C and at 1 atmosphere (standard temperature and pressure, STP).

The value R is then:

$$R = \frac{pv}{nT} = 1 \times 22.4 = 0.082 \text{ litres/atmos/degree/mole}$$

Find the volume of 3.2 g of oxygen at 0 °C and 100 atmospheres:

$$pv = nRT$$

One mole of oxygen weighs 32 g

$$\text{Therefore, moles of oxygen} = \frac{3.2}{32} = 0.10$$

$$v = \frac{nRT}{P} \quad \text{or} \quad v = \frac{0.10 \times 0.082 \times 273}{100}$$

$$v = 0.0224 \text{ litres or } 22.4 \text{ ml}$$

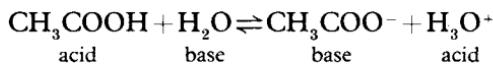
ACIDS AND BASES

There are several definitions of acids and bases, but the one which is particularly helpful is that of Bronstead-Lowry, who defined acids and bases in terms of proton exchange.

An acid is a substance existing as molecules or ions which can donate a proton, i.e. a proton donor; a base is a molecule or ion which can accept a proton, i.e. a proton acceptor.

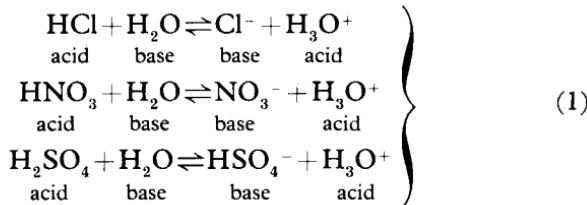


where \rightleftharpoons is the reversible reaction sign. This can be considered further by the interaction of acetic acid and water



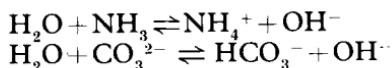
Acetic acid is the proton donor and water the proton acceptor; therefore water is a base. However, the hydroxonium ion (H_3O^+) is also a proton donor and the proton acceptor is the acetate ion.

Other examples are:



The essential constituent of acidic solution in water is the hydroxonium ion (H_3O^+). It is responsible for the typical acidic properties namely: (a) sharp taste; (b) ability to change the colour of blue litmus to red; (c) ability to evolve carbon dioxide when added to carbonates.

HCl , HNO_3 , H_2SO_4 are termed *strong acids* because they are ionized almost completely in dilute solutions so that there are virtually no molecules of the acid present. Examples of weak acids are lactic and acetic because they do not completely dissociate even in dilute solutions. In the presence of bases stronger than itself, water can function as an acid. This is illustrated by the donation of protons to such bases as ammonia and the carbonate ion (Na_2CO_3)



The essential constituent of bases (alkaline solutions) is the hydroxyl ion (OH^-). It is responsible for the typical properties of alkaline solutions, namely: (a) soapy feeling; (b) ability to change the colour of red litmus to blue; (c) the ability to neutralize acids.

NaOH , KOH are termed *strong bases* because they completely dissociate in water liberating the hydroxyl ion.

In the equations (1) the bases Cl^- , NO_3^- and HSO_4^- are termed weak bases because their power of accepting a proton is low.

The trends in acid strength and basic strength are shown in Table 5.1 which illustrates the important principle: the stronger the acid the weaker the base.

Table 5.1 ACID-BASE CHART

Acid	Formula	Base	Formula
Water	H_2O	Hydroxide ion	OH^-
Bicarbonate ion	HCO_3^-	Carbonate ion	CO_3^{2-}
Ammonium ion	NH_4^+	Ammonia	NH_3
Acetic acid	CH_3COOH	Acetate ion	CH_3COO^-
Phosphoric acid	H_3PO_4	Dihydrogen phosphate ion	H_2PO_4^-
Hydroxonium ion	H_3O^+	Water	H_2O
Nitric acid	HNO_3	Nitrate ion	NO_3^-
Hydrochloric acid	HCl	Chloride ion	Cl^-
Sulphuric acid	H_2SO_4	Bisulphate ion	HSO_4^-

THE DISSOCIATION OF WATER

Pure water is a bad conductor of electricity. The presence of small amounts of dissolved substances, however, increases the conductance considerably. Distilled water rapidly acquires impurities from the air and from the walls of the containing vessels. Water used as a solvent has to be specially prepared so that its own conductance will be as small as possible. For example, water obtained by demineralizing ordinary water by ion exchange resins is described as *conductivity water*.

Pure water is very slightly ionized due to the slight dissociation into H_3O^+ and OH^- ions.

For simplicity it is usually written



Applying the law of mass action

$$K = \frac{(\text{H}^+)(\text{OH}^-)}{(\text{H}_2\text{O})} \quad (3)$$

Since the degree of dissociation is very small (H_2O) can be considered to be constant, so therefore we have a constant K_w which is the ionic product of water, expressed as:

$$K_w = (\text{H}^+)(\text{OH}^-) \quad (4)$$

It can be calculated from conductivity experiments that at 25°C , 1 litre of water contains approximately 1×10^{-7} moles of both hydrogen and hydroxyl ions.

Hence from (4)

$$K_w = 10^{-7} \times 10^{-7} = 10^{-14}$$

K_w increases with rise in temperature, but as 25°C is commonly used for carrying out experiments, the value at this temperature is the one most frequently used. As the K_w for all aqueous solutions at 25°C will always equal 10^{-14} , if an acid is added to water the H_3O^+ ion concentration will rise but there will be a proportionate decrease in OH^- . Likewise, if alkali is added, there is a rise in OH^- concentration but a proportionate decrease in H_3O^+ .

HYDROGEN ION CONCENTRATION AND pH

As hydrogen ion concentration of solutions are a matter of great practical importance, expressing values in terms of 10^{-7} moles per litre is not very convenient.

Sørensen devised the convenient units of pH (puissance d'hydrogen), strength of hydrogen, or pOH (puissance d'hydroxyl) to overcome this difficulty. pH is therefore defined as the logarithm to the base 10 of the reciprocal of the hydrogen ion concentration or the negative value of the logarithm to the base 10 of the hydrogen ion concentration.

$$\text{pH} = \log \frac{1}{(\text{H}^+)} = -\log (\text{H}^+)$$

$$\text{pOH} = \log \frac{1}{(\text{OH}^-)} = -\log (\text{OH}^-)$$

But we have said that $(\text{H}^+)(\text{OH}^-) = K_w$

Therefore $(\text{pH})(\text{pOH}) = -\log K_w$

Now if $(\text{H}^+) = 10^{-7}$

$$\text{pH} = \log 10^{-7} = \text{pH } 7.0$$

$$\text{or } (\text{H}^+) = 10^{-2}$$

$$\text{pH} = \log 10^{-2} = \text{pH } 2.0$$

It follows therefore that:

A neutral solution has a pH of 7.0

An acid solution has a pH less than 7.0

An alkaline solution has a pH greater than 7.0

Now if we consider 0.1N(M) HCl:

This acid completely dissociates and the (H^+) concentration is 10^{-1} moles per litre.

Therefore pH = 1.0.

Likewise 0.1N(M) NaOH completely dissociates and the (OH^-) concentration is 10^{-1} moles per litre.

Since

$$(H^+)(OH^-) = 10^{-14}$$

$$(H^+) = 10^{-13}$$

Therefore pH = 13.0.

In the case of 0.15N(M) HCl, its (H^+) concentration is 1.5×10^{-1} moles per litre

$$\log(H^+) 0.15 = -1.1761$$

$$-1 + 0.1761 = -0.8239$$

$$pH = 0.8239$$

As weak acids and bases are not completely dissociated, the concentration of ions and hence 'acidity' and 'alkalinity' are less.

For example:

the pH of 0.1M or 0.1N acetic acid = pH 2.9

pH of 0.1M or 0.1N ammonia = pH 11.1

Find the pH of 0.1M(N) acetic acid, when the degree of dissociation is 2.0 per cent.

$$(H^+) = 0.020 \times 10^{-1} = 0.0020 \text{ g moles per litre}$$

Now if the acid was completely dissociated, the ionic concentration would be 10^{-1} g moles per litre.

$$(H^+) = 0.002 = 2.0 \times 10^{-3}$$

$$pH = \log \frac{1}{2.0 \times 10^{-3}}$$

$$= \log \frac{1}{0.301 \times 10^{-3}} - (-3 + 0.301)$$

$$pH = 2.699 \text{ (2.70)}$$

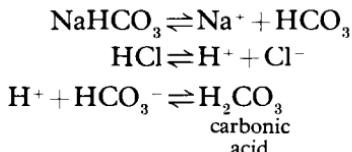
Measurement of pH

See Instrumentation, Chapter 6.

BUFFER SOLUTIONS

It is possible to prepare solutions of known pH by making up solutions of strong acid or alkali of known concentration. For example, 0.0001N HCl has a pH of 4.0 while 0.0001N NaOH has a pH of 10.0. However, these solutions do not retain a constant hydrogen ion concentration for long, as they accept impurities from the air and the walls of the containers. This can best be illustrated by the following examples.

When small amounts of a strong acid or base are added to water, there is a considerable increase in (H^+) ion and (OH^-) ion concentration resulting in a lower and higher pH respectively. On the other hand, when an equal amount of *acid* is added to a solution of a salt of a weak acid such as sodium hydrogen carbonate ($NaHCO_3$), the pH is lowered only slightly. This is because the salt of the weak acid is freely dissociated in solution and the anion (HCO_3^-) is a strong base which readily takes up (H^+) ions.



The result is the replacement of a strong acid freely dissociated by a weak acid only slightly dissociated.

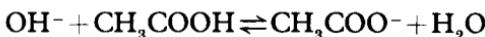
The solution of the salt of a weak acid which absorbs hydrogen ions in this way is a *buffer*. A buffer solution can therefore be described as a mixture of substances in solution which controls the (H^+) ion concentration of that solution and maintains it on the addition of reasonable amounts of acid or alkali. It usually contains a mixture of a weak acid and a salt of a strong base, or a weak base and its salt with a strong acid, for example a mixture of acetic acid and sodium acetate.

Mode of action of acetic acid and sodium acetate

The above-mentioned buffer contains both acetate ions and acetic acid molecules. On the addition of acid to the buffer, the acetate ions combine with the (H^+) ions to form acetic acid.



While upon the addition of base, the (OH^-) ions combine with the (H^+) ions from acetic acid to form water and an acetate ion.



In this way the additional (H^+) ions and (OH^-) ions are removed from the solution. Other examples of buffers are

1. hydrochloric acid and potassium hydrogen phthalate
2. hydrochloric acid and potassium chloride
3. potassium dihydrogen phosphate and disodium hydrogen phosphate.

Buffering capacity and range of buffer action

Different buffers have a different 'buffering capacity', that is they vary in their resistance to the addition of (H^+) and (OH^-) ions. By varying the proportions of the constituents in a buffer system solutions of different pH may be prepared. For example, in the appendix it can be seen that if 50 ml 0.2M potassium hydrogen phthalate is added to 46.60 ml 0.2M HCl and diluted to 200 ml with distilled water a pH of 2.2 is obtained. On the other hand pH 8.3 is obtained when the same amount of phthalate is added to 2.65 ml of HCl and diluted to 200 ml. However, the range of buffer action of a particular system is limited because the buffering capacity reaches a maximum at a certain pH and falls off on either side of its maximum point.

SOLUTIONS

Solute and solvent

In a solution of one substance in another, the dissolved substance is called the solute and the substance in which the solute is dissolved is called the solvent.

Saturated solutions

If a few grams of sodium chloride are added to 100 ml of water the salt will dissolve, but if further quantities are added a stage is reached when solid sodium chloride remains and no more dissolves. Such a solution is said to be saturated. A saturated solution, therefore, is one which contains as much solute (dissolved substance) as it can dissolve, in the presence of the solid solute. This last proviso is very important, because it is possible to prepare, sometimes quite easily, supersaturated solutions containing more solute than the saturation value. However, supersaturated

solutions are unstable, and the addition of solute to them causes precipitation of excess dissolved solute until the concentration falls to the saturation value; that is a supersaturated solution cannot exist in the presence of solid solute. Therefore, when making a saturated solution always make sure that some solute remains undissolved.

Solubility

The concentration of a saturated solution at a particular temperature is called the solubility of the solute in the particular solvent.

Solubilities are usually expressed as the number of grams of solute dissolved in 100 ml of distilled water (*see Appendix*).

Temperature effects

Usually solubility rises with temperature. For example, the solubility of sodium hydroxide at 100 °C is eight times higher than it is at 0 °C. The temperature effect is very variable, however, the solubility of sodium chloride increases hardly at all between 0 °C and 100 °C, and for a few compounds, such as lithium carbonate the solubility decreases with rise in temperature.

Concentrations of solutions

The concentration of a solution is the amount of solute in a given amount of solution. There are several ways of expressing concentrations.

1. Percentage solutions (*w/v*) contain x g of solute in 100 ml of solution, for example, 20 per cent Na_2CO_3 is made by dissolving 20 g of solid sodium carbonate in distilled water and making the final volume up to 100 ml with distilled water.
2. Weight per cent solutions (*w/w*) contain x g of solute in 100 g of solution.
3. A mole (mol) is the molecular weight of a substance expressed in grams.

When studying chemical changes it is often desirable to consider the *numbers* of reacting atoms, ions and molecules rather than their masses. Numbers are measured in *moles* and one mole is the number of carbon atoms in 12 g of neutral atoms of carbon 12. This number = 6.023×10^{23} , that is a mole of O_2 means 6.023×10^{23} oxygen molecules; of Na: 6.023×10^{23} sodium atoms; of NO_3^- : 6.023×10^{23} nitrate ions.

The mass of a mole of a substance is obtained from the fact

that a mole of atoms of carbon 12 (the basis of the atomic weight scale) has a mass of 12 g. Hence, there is one mole of atoms in 1.008 g H; 16 g O; 32.06 g S; 23 g Na; 35.45 g Cl. Similarly, there is 1 mole of molecules in 32 g O₂; 98.08 g H₂SO₄; 18.015 g H₂O, and 1 mole of sodium ions and 1 mole of chlorine ions in 58.443 g NaCl.

Molar solutions (mol/l; M) contain one mole of the solute dissolved in and made up to 1000 ml with solvent. For example:

M Na₂CO₃ contains 105.988 g/l
 M NaCl contains 58.44 g/l

5. Normal solutions (N) contain 1 g equivalent per 1000 ml of solution. For example:

N Na₂CO₃ contains 53.00 g/l
 N NaCl contains 58.44 g/l

Note—since the equivalent weight of a substance is a variable quantity, the expression of a solution strength in terms of normality can be ambiguous unless the reaction the substance is to undergo is known.

6. Millimoles per litre (mmol/l) expresses the strength in terms of the molecular weight in milligrams per litre of solution. For example:

A solution containing 1 mmol/l of NaCl contains 58.44 mg/l
 A solution containing 1 mmol/l of Na₂CO₃ contains 53.00 mg/l

Note—‘normal saline’ must not be confused with a normal solution of sodium chloride. ‘Normal saline’ usually refers to physiological saline (0.85 per cent), which is isotonic with body fluids. Normal sodium chloride solution is 5.85 g per cent.

SIMPLE QUALITATIVE ANALYSIS

Simple tests to identify certain cations and anions.

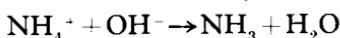
Cations

Flame test—moisten the substance with concentrated hydrochloric acid, dip a platinum wire or porcelain rod into the mixture and apply it to the base of a non-luminous bunsen flame.

Table 5.2

<i>Flame coloration</i>	<i>Colour seen through blue glass</i>	<i>Inference</i>
Persistent yellow	Invisible	Sodium
Violet	Crimson	Potassium
Brick-red	Light green	Calcium
Green flashes	Bluish green	Barium
Green (blue zone)	Bluish green	Copper

Ammonium NH_4^+ —boil with 5N sodium hydroxide solution. Ammonia gas, which turns moistened red litmus paper from red to blue, and has a characteristic smell, is evolved.



Calcium Ca^{2+} —to a concentrated solution of the substance made alkaline with ammonium hydroxide add a 3 per cent solution of ammonium oxalate. A white precipitate of calcium oxalate, $\text{CaC}_2\text{O}_4 \cdot \text{H}_2\text{O}$, is formed.

Iron—when freshly prepared, all ferrous solutions are pale green in colour; on standing in air they are oxidized to the yellowish-red ferric state.

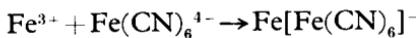
Fe^{2+} —a solution of a ferrous salt gives a dirty-green gelatinous precipitate of ferrous hydroxide when made alkaline with sodium hydroxide solution.

Fe^{3+} —a ferric salt solution gives a reddish-brown gelatinous precipitate of ferric hydroxide with sodium hydroxide.

Fe^{3+} —intense blue precipitate (Prussian blue)



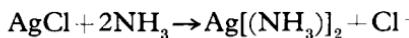
formed with a 5 per cent solution of potassium ferrocyanide $\text{K}_4[\text{Fe}(\text{CN})_6] \cdot 3\text{H}_2\text{O}$



Anions

Halides—to a solution in distilled water, add dilute nitric acid, followed by silver nitrate solution.

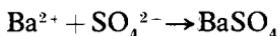
Chloride—white curdy precipitate of silver chloride, which is readily soluble in ammonia solution.



Bromide—whitish-yellow precipitate of silver bromide, not readily soluble in ammonia solution.

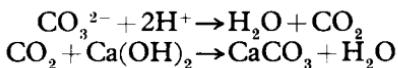
Iodide—yellow precipitate of silver iodide, insoluble in ammonia solution.

Sulphate—to a solution in water, add dilute hydrochloric acid, followed by a 6 per cent solution of barium chloride. White precipitate of barium sulphate.



Nitrate—brown ring test: to a cold solution add an equal volume of a freshly prepared saturated solution of ferrous sulphate. Pour a few ml of concentrated sulphuric acid into a boiling tube, and then very carefully, so as to avoid mixing, pour the prepared solution down the side of the tube to form a layer on top of the sulphuric acid. A brown ring forms at the junction of the two liquids, due to the formation of the unstable compound $\text{Fe}(\text{NO})\text{SO}_4$. On shaking and warming, nitric oxide, NO , is evolved, and the brown colour disappears.

Carbonate—to the solid add dilute nitric acid. Effervescence occurs, and carbon dioxide, which is liberated, can be detected by passing the gas into 'limewater' (saturated solution of calcium hydroxide), when a white precipitate of calcium carbonate is formed.



QUANTITATIVE ANALYSIS

Volumetric (titrimetric) analysis

In volumetric analysis, the volume of a solution of accurately known concentration is allowed to react quantitatively with a solution of the substance being titrated. The solution of known concentration is the standard solution containing a definite number of gram-equivalents or moles per litre. The unknown substance is titrated by adding standard solutions until the reaction is just complete. This 'end-point' is shown by a colour change, due either to the standard solution or a colour change given by an indicator, or the end-point may be revealed by the deposition of a precipitate. The complete process is called a *titration*. There are three main categories of titration: (1) neutralization reactions; (2) oxidation-reduction reactions; and (3) precipitation reactions.

In volumetric analysis there are always two types of standard solutions, primary and secondary.

PRIMARY STANDARDS

To obtain a reference for volumetric analysis, certain primary standards are used which should satisfy the following requirements:

1. It must be stable, easy to obtain, to dry and to preserve in a pure state.
2. It should have a large equivalent weight, to lessen the effect of errors in weighing.
3. It must not be altered in air during weighing (i.e. absorb moisture during weighing).
4. It must be readily soluble in the solvent.
5. The titration reaction with the standard solution should be stoichiometric (theoretical end-point) and practically instantaneous.
6. It should not give rise to any product likely to interfere with the titration.

Ideal primary standards are difficult to obtain and hence a substance between the ideal requirements is usually made.

<i>Primary standards</i>	<i>For titration with</i>	<i>Indicator</i>
Anhydrous sodium carbonate (Na_2CO_3)	Hydrochloric acid (HCl)	Methyl orange
Sodium chloride (NaCl)	Silver nitrate (AgNO_3)	Potassium chromate
Sodium oxalate $(\text{Na}_2\text{C}_2\text{O}_4)$	Potassium permanganate (KMnO_4)	Potassium permanganate
Silver nitrate (AgNO_3)	Potassium thiocyanate (KCNS)	Ferric alum
Potassium hydrogen phthalate $(\text{KHC}_8\text{H}_4\text{O}_4)$	Bases	Phenolphthalein
Disodium tetraborate (borax) $(\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O})$	Hydrochloric acid (HCl)	Methyl red

SECONDARY STANDARDS

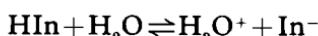
Unlike primary standards which can be accurately weighed, some substances cannot be made directly into solutions of known concentrations. They must first be prepared to an approximate concentration and then titrated against a primary standard to obtain the precise concentrations. Such substances are known as 'secondary standards'. Various acid solutions, for example hydrochloric (not constant boiling point mixture), nitric, sulphuric and acetic acid, can be diluted to a given approximate concentration, and by suitable titration against the base, a secondary standard of precise concentration can be prepared. On the other hand, sodium and potassium hydroxide for example, although in solid form, cannot be weighed accurately, as the material deliquesces and combines with the carbon dioxide in the air. Solutions of greater concentration than those finally required are prepared, titrated against a suitable standard solution and then diluted to give the required standard solution. The concentration is again checked by repeating the titration procedure. This is rather a cumbersome way of standardization and it may be more convenient to work out a factor for the secondary standard (see p. 95). Sodium and potassium hydroxide solutions are not stable unless kept in a suitable container (polythene) and if required for daily use, the vessel must be fitted with a soda-lime guard tube to prevent CO_2 from entering the solution.

NEUTRALIZATION INDICATORS

Neutralization indicators are substances which dissociate in solution into two (or more) differently coloured forms, the nature of the form present being governed by the pH of the solution. An indicator may be used for determining the pH of a solution or for determining the end-point of an acid-base titration.

Theory of indicators

Indicators are either very weak acids or very weak bases. Let HIn represent the weak acid indicator. In water this will be ionized as follows:



where HIn and In^- have different colours.

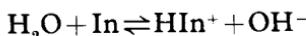
The equilibrium constant is

$$\frac{(\text{H}_3\text{O}^+)(\text{In}^-)}{(\text{H}_2\text{O})(\text{HIn})} = K$$

If an acid is added to the indicator solution, the effect of the added H_3O^+ ions will be to decrease the concentration of In^- and increase the concentration of HIn . That is, the indicator will be almost completely in the HIn coloured form.

On the other hand, if an alkali is added the acidic indicator HIn itself is neutralized and will almost entirely exist as the In^- coloured form.

Let In represent the weak base indicator. In water this will be ionized as follows:



where In and HIn^+ have different colours.

The equilibrium constant is

$$\frac{(\text{HIn}^+)(\text{OH}^-)}{(\text{H}_2\text{O})(\text{In})} = K$$

If an alkali is added, the OH^- ions will be almost completely in the In coloured form and, if acid is added, the basic indicator is neutralized and will almost entirely exist in the HIn^+ coloured form.

Each indicator has a pH range over which a visible colour change occurs called the 'colour change interval'. Some of the properties of the more common indicators are given below.

Table 5.3 QUANTITATIVE ANALYSIS

Indicator	Colour in 'acid' solution	Colour in 'alkaline' solution	Colour change interval
Methyl orange	Red	Orange-yellow	3.1–4.6
Methyl red	Red	Yellow	4.2–6.3
Bromothymol blue	Yellow	Blue	6.0–7.6
Phenol red	Yellow	Red	6.8–8.4
Phenolphthalein	Colourless	Red	8.0–9.8

Acid-base titrations

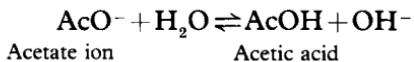
At the equivalence point, the ions in solution are the same as if the pure salt of the base and acid had been dissolved, and the resultant pH depends on the extent to which the salt is hydrolysed.

STRONG ACID: STRONG BASE (for example, sodium hydroxide and hydrochloric acid)

The pH at the equivalence point is 7 because the salt, for example sodium chloride, is not hydrolysed. Also, a very small amount of titrant at the equivalence point causes a large change in pH from about 3 to 10, so it is not necessary to employ an indicator having a colour change interval covering pH = 7. In practice, any indicator between methyl orange and phenolphthalein is satisfactory. This assumes that carbon dioxide from the air is not dissolved in the alkali because dissolved carbon dioxide acts as an acid to phenolphthalein. If the alkali is not carbon dioxide-free then methyl orange is a suitable indicator. In the titration of sodium carbonate with hydrochloric acid the solution is saturated with carbon dioxide at the equivalence point causing a pH of about 3.5, and again methyl orange is the indicator that should be used.

WEAK ACID: STRONG BASE (for example, acetic acid and sodium hydroxide)

The pH at the equivalence point is > 7 , that is, alkaline because the anion of the weak acid, for example acetate ion, combines with water to form un-ionized acid and hydroxyl ions.

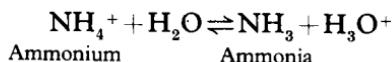


Because the relationship $(\text{H}_3\text{O}^+)(\text{OH}^-) = 10^{-14}$ always holds, the formation of OH^- ions by the hydrolysis is accompanied by a decrease in H_3O^+ concentration, and hence a pH > 7 .

Apart from the fact that the equivalence point occurs on the alkaline side, the change in pH for a small addition of titrant at the equivalence point varies from about 6 to 11. Methyl orange is quite valueless for this titration, since it completes its colour change before the equivalence point is reached. Phenolphthalein should be used for this class of titration.

STRONG ACID: WEAK BASE (for example, hydrochloric acid and ammonium hydroxide)

The pH at the equivalence point is < 7 , that is, acidic, because the cation of the weak base, for example ammonium, combines with water to form un-ionized base and hydroxonium ions.



The addition of a small quantity of titrant at the equivalence point causes a pH change from about 4 to 8. Phenolphthalein is not satisfactory and methyl red or methyl orange should be used.

WEAK ACID : WEAK BASE (for example, acetic acid and ammonium hydroxide)

The pH at the equivalence point is 7, but the change in pH is so gradual that no indicator gives a sharp colour change. For this reason such titrations are not practicable.

PREPARATION OF VOLUMETRIC SOLUTIONS

Volumetric analysis, like many other types of analysis, has changed over the past few years, the change being mainly in the introduction of molar instead of normal solutions.

A normal solution (N) contains 1 g equivalent per 1000 ml of solution.

1. The equivalent weight of an acid is the weight which contains 1 g replaceable hydrogen.

e.g.

HCl mol wt 36.5	equivalent weight 36.5
H ₂ SO ₄ mol wt 98	equivalent weight 49

2. The equivalent weight of a base, is the weight which contains one replaceable hydroxyl group.

e.g.

NaOH mol wt 40	equivalent weight 40
Ba(OH) ₂ mol wt 171.36	equivalent weight 171.36 ÷ 2 = 85.68

3. The equivalent weight of an oxidizing or reducing agent is the weight which contains or reacts with 1 g available hydrogen, or 8 g available oxygen. But the equivalent weight of certain substances can vary, depending upon the type of reaction in which they are involved. The equivalent weight of potassium permanganate in acid solution is as follows:



i.e. 2 moles of KMnO₄ give up 5 moles of oxygen in acid solution, therefore the EW is

$$\frac{2\text{KMnO}_4}{10} \quad \text{or} \quad \frac{\text{MW}}{5}$$

A molar solution contains 1 g mole of the solute dissolved in and made up to 1000 ml with solvent.

e.g.	1M HCl contains 36.5 g
	1M H_2SO_4 contains 98.0 g
	1M NaOH contains 40.0 g
	1M $\text{Ba}(\text{OH})_2$ contains 171.36 g

25 ml 0.1N HCl will neutralize 25 ml 0.1N NaOH, but the same cannot be said for molar solutions except when they are monovalent.

25 ml 0.1M HCl will neutralize 25 ml 0.1M NaOH and
 25 ml 0.1N HCl will neutralize 25 ml 0.1N Na_2CO_3 but
 25 ml 0.1M HCl will only neutralize 12.5 ml 0.1M Na_2CO_3 .
 25 ml 0.1N H_2SO_4 will neutralize 25 ml 0.1N Na_2CO_3 and
 25 ml 0.1M H_2SO_4 will neutralize 25 ml 0.1M Na_2CO_3 but
 25 ml 0.1M H_2SO_4 will neutralize 50 ml M NaOH.

i.e. normal solutions are equivalent to each other, as are monovalent molar solutions, but when a molar solution is divalent it is twice as strong as a monovalent molar solution.

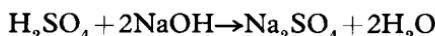
The use of factors in volumetric analysis

Factors are very important in volumetric analysis because they save all the problems of preparing exactly standardized volumetric solutions, i.e. diluting down a solution of sodium hydroxide after the initial titration until it is exactly 0.1 molar, a step which is very difficult to carry out satisfactorily.

Golden rules in volumetric analysis

FINDING A FACTOR (F) METHOD 1

If we have in our laboratory 0.02M NaOH with a factor value of 1.032 and we require to standardize a solution of approximately 0.02M sulphuric acid we then have to find the factor for the sulphuric acid.



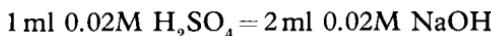
In the above equation

$$1\text{M H}_2\text{SO}_4 = 2\text{M NaOH}$$

Therefore 1000 ml M H_2SO_4 = 2000 ml M NaOH.

If 20 ml aliquot of approximately 0.02M H_2SO_4 required

38.4 ml 0.02M NaOH of factor 1.032, then from the equation we know that



Therefore

20 ml approximately 0.02M $\text{H}_2\text{SO}_4 = 38.4 \times 0.02\text{M NaOH}$ (factor 1.032)

Then

$$1 \text{ ml } 0.02\text{M NaOH} = \frac{38.4 \times 1.032}{2^* \times 20} = 0.9907 \text{ ml of exactly } 0.02\text{M H}_2\text{SO}_4$$

or F for 0.02M H_2SO_4 is 0.9907.

FINDING A FACTOR (F) METHOD 2

By standardization of a substance, e.g. NaOH, using a pure solid primary standard. First of all calculate the weight of primary standard to be weighed out.

$$1 \text{ mole of primary standard} = 1000 \text{ ml M NaOH}$$

$$\text{Then } \frac{\text{MW}}{1000} \approx 1 \text{ ml M NaOH}$$

As the titres are usually around 20 ml we then need to weigh out $(\text{MW}/1000) \times 20$ of primary standard to give a reasonable titration figure.

Example using potassium hydrogen phthalate (PHT) ($\text{C}_6\text{H}_4\text{COOHCOOK}$):

$$1000 \text{ ml M PHT contains } 204.22 \text{ g}$$

Therefore 1000 ml 0.1M PHT contains 20.422 g

Then 20 ml 0.1M PHT will contain

$$\frac{20.422}{1000} \times 20 = 0.40844 \text{ g}$$

If 0.405 g of PHT were weighed out accurately, transferred to a conical flask, dissolved in distilled water and titrated with approximately 0.1M NaOH and a titre of 19.5 ml was obtained,

$$\text{then } F \text{ for NaOH} = \frac{0.4050}{0.4084 \times (19.5/20)} = 1.017$$

*2 is included in the calculation because of the difference in equivalence.

Therefore to find the F for a substance using a solid primary standard, the following calculation can be used.

$$F = \frac{\text{weight of primary standard actually used}}{\text{calculated weight should have used} \times (\text{titre}/\text{calculated titre})}$$

Primary standards can easily be weighed out because they conform to the criteria laid down on p. 90, but in the case of secondary standards such as strong acids, a known volume of acid is usually diluted to a given volume to give an approximate solution. The volume of acid required can be readily calculated from the specific gravity and the percentage composition.

The specific gravity of concentrated hydrochloric acid is 1.18; the percentage composition is 35.4 per cent (*w/w*), and the molecular weight is 36.5, therefore the volume of acid required to make a litre of molar (normal) solution is as follows:

$$\begin{aligned}\text{Sp. gr. of concentrated HCl} &= 1.18 \quad \text{i.e. } 1 \text{ ml HCl weighs } 1.18 \text{ g} \\ \text{percentage composition} &= 35.4\end{aligned}$$

Then number of ml concentrated HCl equivalent to 36.5 g is

$$36.5 \times \frac{100}{1.18} \times \frac{100}{35.4} = 87.2 \text{ ml}$$

Thus if 87.2 ml of concentrated HCl are diluted to 1 litre, an approximately molar (normal) solution is obtained.

The specific gravity of concentrated sulphuric acid is 1.83; the percentage composition is 96.0 per cent (*w/w*) and the equivalent weight is 49. Therefore the volume of acid required to be diluted to a litre to prepare an approximately normal solution is as follows:

$$49 \times \frac{1.0}{1.83} \times \frac{100}{98} = 27.3 \text{ ml}$$

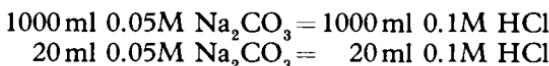
Preparation of approximately 0.1M (0.1N) hydrochloric acid

Measure out from a burette 9 ml pure concentrated hydrochloric acid into a litre volumetric flask containing about 500 ml of distilled water and dilute to 1 litre. Mix well and this will give an approximately 0.1M hydrochloric acid solution.

Standardization against pure anhydrous sodium carbonate



From the above equation 1 mole of Na_2CO_3 is equivalent to 2 moles of HCl, therefore



Then $\frac{0.05 \text{ MW}}{1000} \times 20 \text{ g of } \text{Na}_2\text{CO}_3 = 20 \text{ ml } 0.1\text{M HCl}$

or $\frac{0.05 \times 106}{1000} \times 20 = 0.106 \text{ g } \text{Na}_2\text{CO}_3 = 20 \text{ ml } 0.1\text{M HCl}$

Therefore if 0.106 g Na_2CO_3 were weighed out, dissolved in water and then titrated with the HCl solution, a reasonable titre should be obtained. This is a far better technique than making up an accurate 0.05M solution of Na_2CO_3 , because one does not need to weigh out exactly 0.106 g of solid; any weight around this figure can be used, providing it is accurately weighed (*see p. 100*).

PROCEDURE

1. AR Grade of anhydrous sodium carbonate is 99.9 per cent pure, but it does contain a little moisture and must be dehydrated by heating at 260–270 °C for 1 h and then allowed to cool in a desiccator before use.
2. Dry 5 to 6 g of anhydrous sodium carbonate as above.
3. Prepare three 250 ml conical flasks (A, B, C) with a funnel in the neck of each.
4. Weigh out accurately from a weighing bottle about 0.110 g of the pure sodium carbonate and transfer quantitatively with washing to the conical flask. Rinse the funnel thoroughly with distilled water, allowing washings to run into the flask. Add a total of about 50 ml of distilled water to dissolve completely.
5. Repeat the same procedure for flasks B and C.
6. Add a few drops of methyl orange indicator to each flask, or preferably methyl orange indigo carmine indicator.*
7. Rinse out a 25 ml burette with the approximately 0.1M acid solution several times; fill the burette to a point 2–3 cm above the zero mark and open stopcock until the jet is completely filled with liquid. Refill if necessary to bring the liquid above the zero mark; then slowly run out the excess

* Preparation of indicators:

1. Methyl orange—0.1 g methyl orange per 100 ml water.
2. Methyl orange-indigo carmine—0.1 g methyl orange and 0.25 g indigo carmine per 100 ml water. The colour change on passing from alkaline to acid solution is from green to magenta with a neutral grey colour at pH of about 4.0.

acid until the liquid meniscus is at the 0 mark. Read the position of the meniscus to 0.01 ml.

8. Place a white tile beneath the flask in order to see the colour changes more readily. Run the acid slowly into flask A from the burette. During the addition of the acid, the flask must be constantly rotated with one hand while the other hand controls the stopcock. When the orange colour lightens to a yellow tint or the green colour of the mixed indicator becomes paler the end-point is near. Rinse the walls of the flask with a little distilled water, and continue the titration drop by drop, until the colour becomes orange or a faint pink or in the case of the mixed indicator grey. This marks the end-point of the titration and the burette reading is noted.
9. Repeat the titration, using flasks B and C, and note the titration readings. Calculate the molarity from each titre. Average the values.
10. *Calculation of the molarity of HCl solution*

$$\text{molarity} = \frac{w \times t \times M}{W \times V}$$

where w = weight of primary standard used

t = theoretical titre

W = calculated weight

V = actual volume used

M = assumed molarity

Example 0.125 g of Na_2CO_3 required 19.5 ml HCl solution

$$\text{Therefore molarity} = \frac{0.125 \times 20}{0.106 \times 19.5} \times 0.1 = 0.121\text{M}$$

11. The bulk of the hydrochloric acid solution can then be diluted with distilled water to obtain a theoretically exact 0.1M solution by using the following calculation.
12. As the acid is 0.121M, it must be diluted with distilled water to obtain a concentration closer to 0.1M. The acid is 1.21 times too concentrated, therefore dilute the acid to the proportion of $1.00/1.21 = 0.826$.
13. Place 826 ml of the 0.121M HCl into a litre volumetric flask and carefully dilute to 1000 ml with distilled water and thoroughly mix.
14. If this diluted solution of acid is then titrated against

Na_2CO_3 and it is found to be 0.105M, further dilutions are unnecessary as the bottle is labelled 0.1M HCl

$$F = 1.05$$

ALTERNATIVE PROCEDURE USING NORMALITY

Weigh out accurately about 1.325 g anhydrous sodium carbonate from a weighing bottle, dissolve in water and dilute to 250 ml in a volumetric flask. Pipette 20 ml portions into 250 ml conical flasks, add a few drops of indicator and titrate as above. Note the titre. Repeat the titration until duplicates agree to within 0.05 ml of each other.

1.325 g Na_2CO_3 dissolved in 250 ml water is an exactly 0.100N solution

If 1.400 g is the exact amount weighed out, then the normality of the resulting solutions is

$$\frac{1.400 \times 0.100}{1.325} = 0.1056\text{N (0.106N)}$$

Suppose 19.5 ml of the hydrochloric acid were required to neutralize 20.0 ml of the 0.106N sodium carbonate solution, then from the formula:

$$\begin{aligned} N_1 V_1 &= N_2 V_2 \\ \text{Normality of HCl} &= \frac{0.106 \times 20.0}{19.5} = 0.1087\text{N (0.109N)} \end{aligned}$$

Preparation of 0.1M (N) sodium hydroxide

A standard solution of sodium hydroxide cannot be made from direct weighing because it is hygroscopic and contains sodium carbonate formed from atmospheric carbon dioxide. Also a solution for titration should be carbonate-free, otherwise it is not possible to obtain an exact end-point with phenolphthalein. For most purposes the AR sodium hydroxide (which contains 1.2 per cent of sodium carbonate) is sufficiently pure.

PROCEDURE

1. The molecular weight of NaOH is 40 (equivalent weight 40), and therefore 0.1M NaOH contains 4.0 g per litre.
2. Weigh out about 5 g of dry sodium hydroxide pellets on a watch glass and transfer quantitatively to a 500 ml Pyrex beaker and dissolve in about 300 ml carbon dioxide-free dis-

tilled water. Warm if necessary. Cool and transfer quantitatively to a 1 litre volumetric flask. Wash out the beaker with more carbon dioxide-free water and transfer the washings

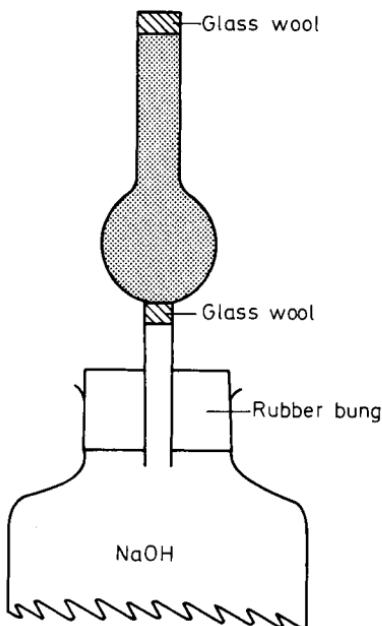


Figure 5.1. Storage container for sodium hydroxide solution

to the volumetric flask; dilute to the mark and mix well. Transfer to a reagent bottle containing a rubber stopper or, alternatively, the solution may be stored in an apparatus similar to that shown in *Figure 5.1*; a soda-lime guard tube should be used.

Standardization of sodium hydroxide solution

USING 0.1M HCl

1. Pipette 20 ml 0.1M HCl into each of four 250 ml conical flasks, add a few drops of phenolphthalein indicator. The solution should be colourless.
2. Fill a 25 ml burette in the same way as described on p. 9 and titrate until a permanent light pink coloured end-point is obtained. Note the titre.
3. Repeat the titration until duplicate determinations agree to within 0.05 ml of each other.

4. Calculation of molarity of sodium hydroxide solution.

Suppose 19.0 ml of sodium hydroxide were required to neutralize 20.0 ml 0.1M HCl of factor 1.05,

$$20.0 \text{ ml } 0.1\text{M HCl of factor } 1.05 = 21.0 \text{ ml } 0.1\text{M HCl.}$$

Therefore as 21.0 ml 0.1M HCl = 19.0 ml of approx. 0.1M NaOH.

$$\text{Molarity of NaOH} = \frac{21.0 \times 0.1}{19.0} = 0.1105\text{M}$$

0.1105M NaOH is the same as 0.1M NaOH of factor 1.105 and the reagent bottle should be labelled as such.

Also 0.1105M NaOH = 0.1105N NaOH.

USING POTASSIUM HYDROGEN PHTHALATE (PHT)



From above equation 1M NaOH is equivalent to 1M PHT, therefore 20 ml 0.1M NaOH = 20 ml 0.1M (0.1N) PHT.

$$\begin{aligned} 20 \text{ ml } 0.1\text{M NaOH} &= \frac{0.1 \text{ MW PHT}}{1000} \times 20 \text{ g of PHT} \\ &= \frac{0.1 \times 204.22}{1000} \times 20 \text{ g of PHT} \end{aligned}$$

$$20 \text{ ml } 0.1\text{M NaOH} = 0.4083 \text{ g of PHT.}$$

PROCEDURE

1. AR PHT has a purity of at least 99.9 per cent; it is almost non-hygroscopic but it should be dried at 120 °C for 2 h and allowed to cool in a desiccator.
2. Weigh out three 0.4–0.5 g accurately and transfer to 250 ml flasks in the same way as described on p. 98.
3. Add 75 ml boiled distilled water and shake gently to dissolve.
4. Titre with the sodium hydroxide solution using phenolphthalein* as indicator. Note the titre.
5. Repeat the titration with the other two weighings.
6. Calculation of molarity as for hydrochloric acid (see p. 99).
Example 0.45 g of PHT required 20.0 ml of approximately 0.1M NaOH.

$$\text{Therefore molarity} = \frac{0.45 \times 20.0}{0.4083 \times 20.0} \times 0.1 = 0.1103\text{M (0.110M)}$$

* Preparation of indicator: Phenolphthalein—0.1 g—1.0 g in 50 per cent alcohol.

7. Calculate the molarity from each weighing and average the values. Label the reagent bottle accordingly.

Preparation of 0.1M (0.1N) silver nitrate solution

AR silver nitrate has a purity of at least 99.9 per cent. Although an excellent primary standard it is still advisable to dry some finely powdered AgNO_3 at 250°C for 1–2 h, allowing to cool in a desiccator.

PROCEDURE

1. The molecular weight of AgNO_3 is 169.876 (equivalent weight 169.876) and therefore 0.1M AgNO_3 contains 16.9876 g per litre (in theory this weight should be multiplied by the purity factor for AR silver nitrate, e.g. 1/0.999).
2. Weigh out accurately 8.4938 g dissolved in distilled water and transfer quantitatively to a 500 ml volumetric flask. Dilute to the mark and mix well.

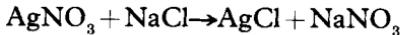
Titration of silver nitrate solution

(a) MOHR'S METHOD

Principle

In this method potassium chromate (5 per cent *w/v*) is used as an indicator and silver chromate, although only sparingly soluble, is more soluble than silver chloride. Consequently, when chloride is titrated with silver nitrate no silver chromate is precipitated until all the chloride has been precipitated. The next drop of silver nitrate after the end-point then produces a deep red precipitate of silver chromate. The solution then darkens due to the masking effect of the white chloride precipitate.

Potassium chromate should only be used in neutral solution. In acidic solution the chromate is converted into dichromate and the end-point is poor. In alkaline solution, the hydroxide ion precipitates silver oxide.



From the above equation 1M AgNO_3 is equivalent to 1M sodium chloride, therefore 20 ml 0.1M (0.1N) AgNO_3 = 20 ml 0.1M (0.1N) sodium chloride.

$$\begin{aligned} 20 \text{ ml } 0.1 \text{ M } \text{AgNO}_3 &= \frac{0.1 \text{ MW}}{1000} \times 20 \text{ g of NaCl} \\ &= \frac{0.1 \times 58.46}{1000} \times 20 \text{ g of NaCl} \end{aligned}$$

$$20 \text{ ml } 0.1 \text{ M } \text{AgNO}_3 = 0.1168 \text{ g of NaCl.}$$

Procedure

1. AR sodium chloride has a purity of 99.9–100 per cent. Although an excellent primary standard, it is slightly hygroscopic and should be dried at 250 °C for 1–2 h then allowed to cool in a desiccator.
2. Weigh out three 0.12–0.15 g accurately and transfer to 250 ml flasks in the same way as described on p. 98.
3. Dissolve in about 50 ml of distilled water and shake gently to dissolve.
4. Add 1 ml of 5 per cent potassium chromate solution.
5. Slowly add the silver nitrate solution from a burette with constant shaking until the red colour formed by the addition of each drop begins to disappear more slowly: this is an indicator that most of the chloride has been precipitated.
6. Continue the addition of the silver nitrate until a faint but distinct change in colour occurs. The faint reddish-brown colour should persist after brisk shaking. Note the titre.
7. Repeat the titration with the other two weighings.
8. Calculation of molarity as for hydrochloric acid (*see* p. 97).

Example 0.125 g of sodium chloride required 20.5 ml of 0.1M AgNO_3 .

$$\text{Therefore molarity} = \frac{0.125}{0.1168} \times \frac{20.0}{20.5} \times 0.1 = 0.1\text{M NaCl.}$$

9. Calculate the molarity from each weighing and average the values. Label the amber reagent bottle accordingly.

Alternative procedure

Weigh out accurately about 2.923 g pure dry sodium chloride from a weighing bottle, dissolve in water and dilute to 500 ml. Pipette 20 ml portions into 250 ml conical flasks, add 1 ml of 5 per cent potassium chromate and titrate as above. Note the titre. Repeat the titration until duplicate determinations agree to within 0.05 ml of each other.

Calculation

2.923 g NaCl dissolved in 500 ml water is an exactly 0.1M (N) solution. If 2.900 g is the exact amount weighed out, then the molarity of the resulting solution is

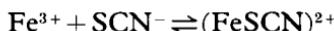
$$\frac{2.900 \times 0.100}{2.923} = 0.0993\text{M}$$

Suppose 19.7 ml of the silver nitrate solution were required to precipitate 20 ml of 0.0993M NaCl, then the molarity of the silver nitrate:

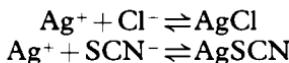
$$\frac{20.0 \times 0.0993}{19.7} = 0.1008\text{M (0.1M)}$$

(b) VOLHARD'S METHOD

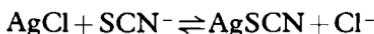
Chloride is precipitated from acidic (nitric acid) solution by excess standard silver nitrate solution, and the excess nitrate back titrated with a standard solution of ammonium or potassium thiocyanate. The indicator is ferric ammonium alum (ferric alum). At the endpoint, when all the silver ion has been precipitated, the thiocyanate ion reacts with the ferric ion to produce a reddish, brown colorization, due to the formation of a complex ferri-thiocyanate ion.



When the chloride ions have precipitated the silver ions, the excess silver ions during titration form silver thiocyanate, after which the thiocyanate may then react with the silver chloride, since silver thiocyanate is the less soluble salt.



and so



This will take place before the reaction occurs with the ferric ions and therefore there will be a considerable titration error. To prevent this, an immiscible liquid nitrobenzene is added to the reaction mixture to 'coat' the silver chloride particles and thereby protect them from the interaction with the thiocyanate.

Thiocyanates are slightly deliquescent and are unsuitable as primary standards.

Preparation of 0.1M (0.1N) ammonium thiocyanate



From equation (1) and (2) 1 mole of AgNO_3 is equivalent to 1 mole of NaCl and 1 mole of AgNO_3 is equivalent to 1 mole of NH_4SCN .

Therefore 1 mole of AgNO_3 is equivalent to 76.12 g of NH_4SCN . Then 0.1 mole of AgNO_3 is equivalent to 7.612 g of NH_4SCN .

As ammonium thiocyanate is deliquescent weigh out about 8.5 g of AR ammonium thiocyanate (or 10.5 g of AR potassium thiocyanate), dissolve it in water and dilute to 1 litre in a volumetric flask. Shake well.

Standardization against 0.1M AgNO₃

PROCEDURE

1. Pipette 20 ml of standard 0.1M AgNO₃ into a 250 ml conical flask, add 5 ml of 6M (N) HNO₃ and 1 ml ferric alum indicator*.
2. Slowly add the ammonium thiocyanate from a burette with constant shaking. At first a white precipitate is produced and as each drop of thiocyanate is added, it produces a reddish-brown colour. As the end-point approaches, the precipitate coagulates and settles, eventually one drop of thiocyanate solution produces a faint reddish-brown colour which no longer disappears on shaking. This is the end-point.
3. Note the titre and repeat the titration with two other 20 ml portions or until duplicates agree to within 0.1 ml of each other.
4. Calculation of molarity of ammonium thiocyanate. Suppose 20 ml of 0.1M AgNO₃ required 19.5 ml of ammonium thiocyanate. Then

$$\text{molarity of NH}_4\text{SCN} = \frac{20 \times 0.1}{19.5} = 0.1026\text{M (0.103)}$$

5. Transfer the solution to a brown reagent bottle and label accordingly, i.e. 0.1M NH₄SCN.

$$F = 1.03$$

Titration of chlorides (Volhard's Method)

PRINCIPLE

See above.

PROCEDURE

1. Pipette 20 ml of approximately 0.1M NaCl solution into a 250 ml conical flask.
2. Add 5 ml 6M (N) HNO₃.

* Preparation of indicator: Ferric alum indicator—40 g of ferric ammonium sulphate AR per 100 ml of distilled water to which a few drops of 6M (N) HNO₃ has been added.

3. Run into the flask from a burette 25 ml standard 0.1M AgNO_3 (sufficient to give about 5 ml excess).
4. Add 3 ml nitrobenzene AR and 1 ml ferric alum indicator and shake the flask vigorously to coagulate the precipitate.
5. Titrate the excess silver nitrate with the 0.103M ammonium thiocyanate until a permanent faint reddish-brown colour appears.
6. Note the titre and repeat the titration with two other 20 ml portions of sodium chloride solution or until duplicate titres agree to within 0.1 ml of each other.
7. Calculation of molarity of sodium chloride solution:
25 ml of standard 0.1M AgNO_3 were used in the titration. If 4.8 ml of 0.1M NH_4SCN of factor 1.03 were required to titrate the residual silver nitrate (i.e. $4.8 \times 1.03 = 4.94$ ml of 0.1M NH_4SCN). Then volume of silver nitrate equivalent to chloride

$$= (25 - 4.94) \text{ ml of } 0.1\text{M } \text{AgNO}_3$$

$$= 20.06 \text{ ml of } 0.1\text{M } \text{AgNO}_3$$

$$\text{Therefore molarity of NaCl} = \frac{20.06 \times 0.1}{20.0} = 0.1003\text{M.}$$

Titration of chlorides (adsorption indicator method)

Indicator 0.1 per cent dichlorofluorescin in 70 per cent alcohol.

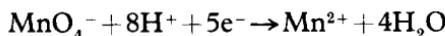
PROCEDURE

1. Pipette 20 ml of chloride solution into a 250 ml conical flask.
2. Add 5–10 drops of indicator.
3. Titrate with the silver nitrate solution in diffuse light with constant swirling. As the end-point is near, the silver chloride coagulates appreciably and the development of the pink colour upon the addition of each drop of silver nitrate solution becomes more and more pronounced.
4. Continue the titration, dropwise, until the precipitate suddenly becomes a pronounced pink or red colour. Note the titre.
5. Repeat the titration with two other 20 ml portions of chloride solution or until the individual titrations agree to within 0.1 ml of each other.

Preparation of 0.02M (0.1N) potassium permanganate solution

Potassium permanganate (KMnO_4) is a very powerful oxidizing

agent and in acid solution, the reduction can be represented by the following equation:



from which it follows that:

1M KMnO_4 is equivalent to 5.0N KMnO_4

(i.e. the equivalent weight is therefore as one-fifth of the formula weight).

Therefore

$$\frac{M}{5} = 1.0\text{N } \text{KMnO}_4$$

$$\text{or } 0.2\text{M } \text{KMnO}_4 = 1.0\text{N } \text{KMnO}_4$$

Thus

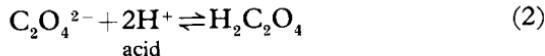
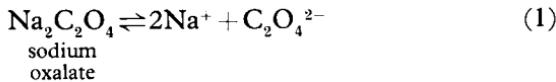
$$0.02\text{M } \text{KMnO}_4 = 0.1\text{N } \text{KMnO}_4$$

PROCEDURE

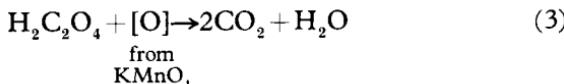
1. The molecular weight of KMnO_4 is 158.03 and therefore 0.02M KMnO_4 contains 3.1606 g per litre.
2. Weigh out 3.2–3.25 g of AR KMnO_4 and transfer to a litre beaker, add about 500 ml of distilled water and boil.
3. Allow to cool, filter the solution through a plug of purified glass wool into a clean litre volumetric flask.
4. Add further distilled water to the beaker and transfer quantitatively to the filtrate and finally dilute to 1000 ml.
5. Mix well and keep in the dark or in diffuse light until standardized.
6. Alternatively, the solution can be kept in an amber reagent bottle.

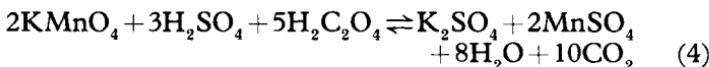
Standardization against sodium oxalate

Sodium oxalate is a primary standard whereas oxalic acid is not. An acidified solution of an oxalate is for purposes of titration with KMnO_4 solution equivalent to a solution of oxalic acid itself.



The oxidation of oxalic acid is represented as follows:

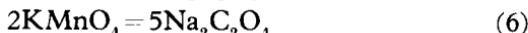




Thus



or



From the molecular equation (4), (5) and (6) it can be seen that 2 moles of potassium permanganate (KMnO_4) are equivalent to 5 moles of sodium oxalate ($\text{Na}_2\text{C}_2\text{O}_4$).

But 20 ml 0.02M KMnO_4 is equivalent to 20 ml 0.05M $\text{Na}_2\text{C}_2\text{O}_4$

Therefore

$$\frac{0.05 \times \text{MW}}{1000} \times 20 \text{ ml of } \text{Na}_2\text{C}_2\text{O}_4 = 20 \text{ ml } 0.02\text{M } \text{KMnO}_4$$

$$\text{or } \frac{0.05 \times 134}{1000} \times 20 = 0.134 \text{ g } \text{Na}_2\text{C}_2\text{O}_4 = 20 \text{ ml } 0.02\text{M } \text{KMnO}_4.$$

PROCEDURE

1. Dry about 2 g AR sodium oxalate at 105–110 °C for 2 h and allow to cool in a desiccator.
2. Weigh out accurately three 0.135–0.140 g of sodium oxalate and transfer to 250 ml conical flasks in the same way as described on p. 98.
3. Add about 50 ml recently prepared distilled water, shake well to dissolve then add about 50 ml molar (2N) H_2SO_4 and heat the mixture to about 60–70 °C.
4. Potassium permanganate does not oxidize oxalate in cold solution, a temperature of 60–70 °C is necessary to cause the reaction to begin. This temperature can be best judged by testing with the palm of the hand. When the bottom of the flask is just too hot, the temperature of the liquid is approximately correct.
5. Titrate with the KMnO_4 , heating again as the liquid cools, until a permanent pink coloration is obtained.
6. For the most exact work determine the excess KMnO_4 required to reach the end-point by adding permanganate solution to the same volume of water and dilute acid at 60–70 °C—this is usually about 0.05 ml.
7. Note titre and repeat the titration with the other two weighings.

8. *Calculation of molarity of KMnO₄*

$$\text{Actual molarity} = \frac{w \times t \times m}{W \times V}$$

w = weight of substance used

W = weight of substance calculated

t = theoretical titre

V = volume used

m = assumed molarity

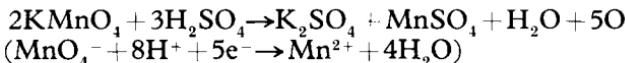
If 0.140 g of Na₂C₂O₄ require 19.5 ml of approximately 0.02M KMnO₄, and 0.134 g of Na₂C₂O₄ are equivalent to 20 ml 0.02M KMnO₄,

$$\text{Then molarity} = \frac{0.140 \times 20 \times 0.02}{0.134 \times 19.5} = 0.0214\text{M KMnO}_4$$

$$F \text{ for KMnO}_4 = \frac{0.140 \times 20}{0.134 \times 19.5} = 1.07$$

ALTERNATIVE PROCEDURE USING NORMALITY

Preparation of 0.1N potassium permanganate



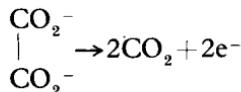
The equivalent weight is therefore a fifth of the molecular weight, 31.606. An 0.1N solution thus contains 3.1606 g per litre.

PREPARATION

See p. 107.

Standardization against sodium oxalate

Sodium oxalate is a primary standard and in acid solution the half-reaction is [see also equation (1) and (2)]



The equivalent weight is therefore half the molecular weight, 67.0. An 0.1N solution thus contains 6.70 g per litre.

PROCEDURE

1. Weigh out accurately about 1.675 g sodium oxalate which has been dried as on p. 109. Dissolve in recently prepared distilled water and dilute to 250 ml in a volumetric flask.

2. Pipette 20 ml portions (caution, poisonous) into 250 ml conical flasks, add about 50 ml molar (2N) H_2SO_4 . Heat to 60–70 °C.
3. Titrate with the KMnO_4 solution, heating again as the liquid cools until a permanent pink coloration is obtained.
4. Note the titre and repeat the titration until duplicates agree to within 0.05 ml of each other.
5. 1.67 g sodium oxalate dissolved in 250 ml distilled water is an exactly 0.100N solution. If 1.80 g is the exact amount weighed out, then the normality of the resulting liquid is

$$\frac{1.80 \times 0.100}{1.675} = 0.1075\text{N}$$

CALCULATION OF NORMALITY

Suppose 19.0 ml of KMnO_4 solution required 20 ml of the 0.1075N sodium oxalate solution for complete oxidation-reduction, then

$$\frac{0.1075 \times 20.0}{19.0} = 0.113\text{N} \quad \text{or} \quad 0.1\text{N } \text{KMnO}_4 \quad F = 1.13.$$

6

Instrumentation

ELEMENTARY COLORIMETRY AND SPECTROPHOTOMETRY

Many biochemical methods produce solutions of coloured compounds; others are involved in a chemical reaction to yield coloured substances. The measurement of the coloured solution forms the basis of a quantitative method of analysis and is used in the technique of colorimetry.

There are several methods available for measuring the concentration of coloured solutions.

1. Visual comparison

This is a procedure where solutions are matched against a set of standards using test-tubes of similar diameter. Values intermediate between a set of standards can then be approximated. This principle is used in the Lovibond comparator, but in place of liquid standards coloured glass standards are utilized.

LOVIBOND COMPARATOR (*See Figure 6.1*)

This apparatus consists of a box with compartments for tubes of the test and blank solutions, and it has a rotatable disc mounted in front of the two tubes. The central window of the box is in front of the test solution; the other window lies in front of the 'blank' solution and rotation of the disc permits the superimposition of the coloured glass standards.

Various discs are manufactured, but a standard solution of known strength should always be carried through in parallel with the test. This standard solution enables one to check the permanent standard and also ensures that no error in technique has been made.

2. Visual colorimeter (*See Figure 6.2*)

This was widely used for many years, but has now been totally superseded by photoelectric absorptiometers of one kind or another.

One type of visual colorimeter is the Dubosq, which consists essentially of two glass containers each of which contains a solid glass plunger, capable of being raised or lowered. Light from an even source of illumination concealed at the base of the instrument passes through the two solutions to be tested and through the

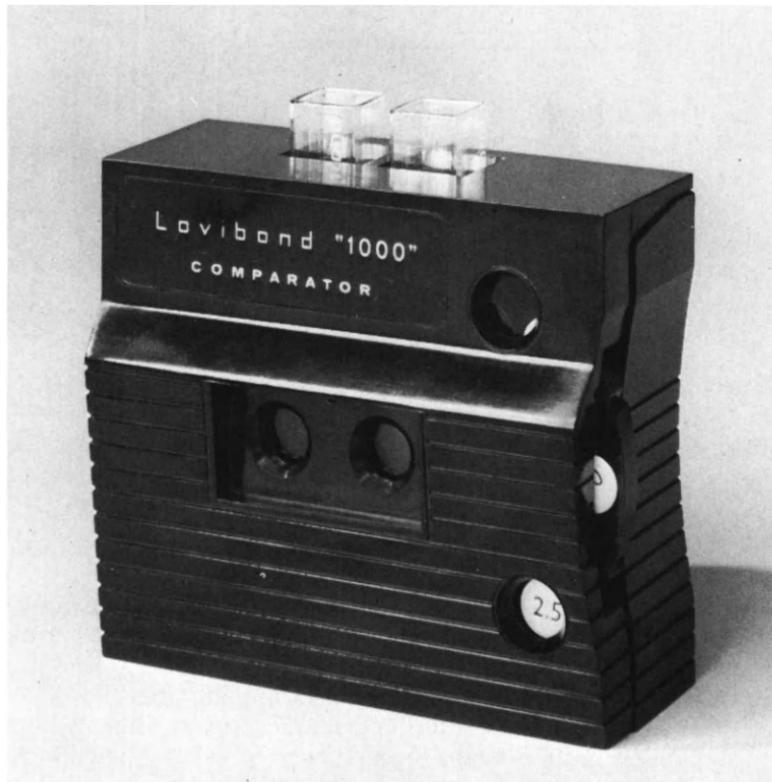


Figure 6.1. Lovibond Comparator. (Reproduced by courtesy of the Tintometer Company)

plungers. Some of the light is absorbed in passing through the solutions, the amount of absorption being dependent upon the concentration and the depth of the solution. The two beams of light are brought to a common axis by a prism system, which is focused onto an eyepiece. On looking through the eyepiece, a wide circular field is visible, light from one cup illuminating one half and light from the second cup illuminating the other half of the

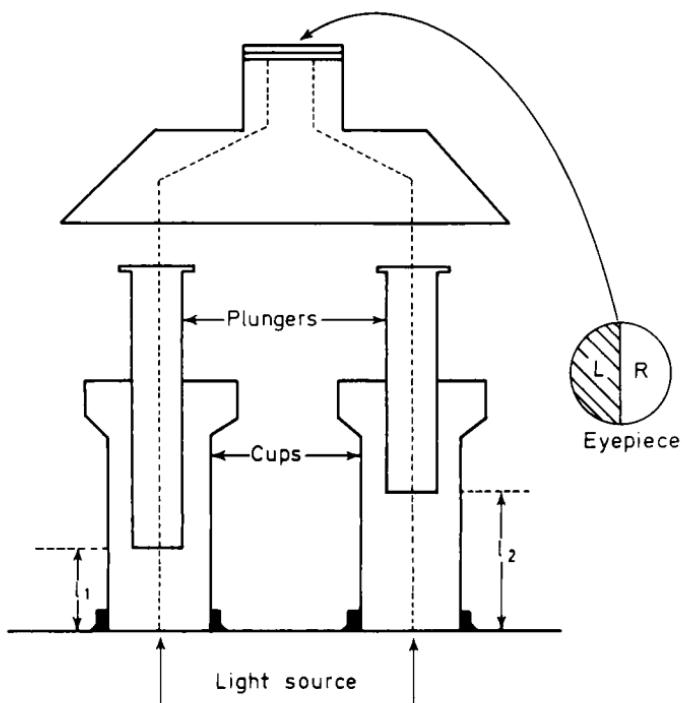


Figure 6.2. Visual colorimeter

field. The depth of solution may be varied by raising or lowering the plungers (or cups) until the two sides of the field are evenly illuminated or matched, i.e. until the dividing line practically disappears. The depth of the solution through which the light passes to the plunger is then read on the vernier scale attached to the plungers or cups. It is usual to set either the test or standard at one particular vernier reading and then raise or lower the other plunger or cup until identical illumination is reached. When this condition is achieved the first law of colorimetry is obeyed.

BEER'S LAW

The intensity of a solution when viewed through monochromatic light is directly proportional to the concentration of the substance (c) and the depth through which the light passes (l).

A 5 per cent solution of copper sulphate when viewed through a depth of 4 cm will have the same amount of colour as a 10 per cent solution viewed through 2 cm.

Then $c_1 l_1 = c_2 l_2$

$$\text{or } c_2 = c_1 \times \frac{l_1}{l_2}$$

i.e. conc. of test = conc. of standard $\times \frac{\text{reading of standard}}{\text{reading of test}}$

3. Photoelectric absorptiometers

These instruments are called absorptiometers since it is the amount of light absorbed which is measured and not colour. In this way many errors are eliminated due to the personal characteristics of each individual. Photoelectric absorptiometers use photoelectric cells either of the barrier layer or emissive type. Light falling on these cells generates an electric current, which can be made to deflect a galvanometer needle, the deflector being proportional to the light density. Light on passing through a coloured solution is absorbed, the amount of absorption depending upon the concentration of the solution. The more light absorbed, the less light is transmitted to the photoelectric cell and the smaller the current generated. From this the second law of colorimetry is derived.

LAMBERT'S LAW

When monochromatic light passes through a transparent medium the rate of decrease in intensity with the thickness of the medium is proportional to the intensity of light.

In combining Lambert's Law with Beer's Law the basic laws of colorimetry and spectrophotometry are obtained.

BEER LAMBERT LAW

When monochromatic light passes through a coloured solution the amount of light transmitted decreases exponentially with the increase in the concentration of the solution and with the increase in the thickness of the layer of solution through which the light passes. (The first statement follows from Beer's Law and the second from Lambert's Law.)

Mathematically, this law can be expressed as:

$$T = e^{-kct} \quad (1)$$

in which T = transmission

k = a constant, the absorption coefficient

c = concentration of solution

t = thickness of the solution or light path

e = base of natural or Napierian logarithms.

Transmission (T) is defined as the ratio of the intensity of the transmitted (emergent) light to that of the incident light, thus equation (1) can be expressed in another way namely:

$$\frac{I_e}{I_i} = e^{-kct} \quad (2)$$

Where I_e is the intensity of the emergent light and I_i is the intensity of the incident light.

Rearrange equation (1)

$$\begin{aligned} T &= e^{-kct} \\ \log_e T &= -kct \\ -\log_e T &= kct \\ -\log_{10} T &= kct \end{aligned} \quad (3)$$

Now $-\log_{10} T$ is the term used for absorbance (A) or the older terms of extinction (E) and optical density (OD). Although transmission is linear (0–100%) and absorbance is logarithmic (0– ∞), there is a relationship between the two, namely:

$$\begin{aligned} A &= \log_{10} \frac{(1)}{(T)} \text{ or } \log_{10} \frac{100}{T} \\ \text{or } A &= 2 - \log_{10} T \end{aligned} \quad (4)$$

Since $A = -\log T$

then by substituting this into equation (3)

$$A = kct \quad (5)$$

In medical laboratory sciences k is usually constant, since the same conditions apply for both the test and standard solutions. As the standard and test solutions are always compared in identical cuvettes or tubes the same light path or thickness of solution is used and therefore t is also a constant value.

Equation (5) can thus be re-written:

$$A = c \quad (6)$$

Now $A_{\text{test}} = c_{\text{test}}$ and $A_{\text{std}} = c_{\text{std}}$

$$\text{Then } \frac{A_{\text{test}} = c_{\text{test}}}{A_{\text{std}} = c_{\text{std}}} = A_{\text{test}} \times c_{\text{std}} = c_{\text{test}} \times A_{\text{std}}$$

$$\text{Therefore } c_{\text{test}} = \frac{A_{\text{test}} \times c_{\text{std}}}{A_{\text{std}}}$$

Expressed another way,

$$\text{Conc. of unknown} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{conc. of standard}$$

This is the standard formula used in photoelectric absorptiometry and spectrophotometry providing Beer's Law is obeyed. Since sets of measurements are made at a constant light path, it is the concentration which varies and therefore it is usual to say that Beer's Law is obeyed or is not obeyed. The absorbance scale is always

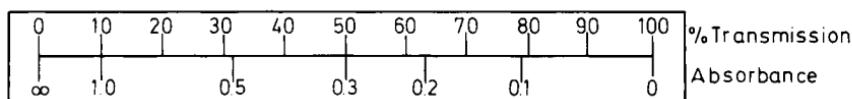


Figure 6.3. Photoelectric absorptiometer scale

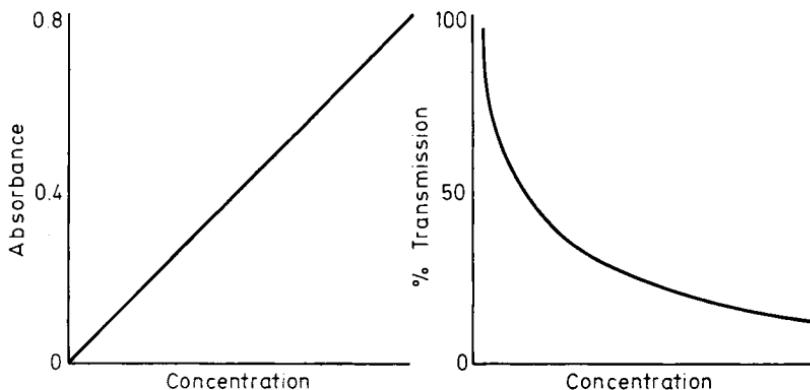


Figure 6.4. Graphs showing the relationships between absorbance and concentration and percentage transmission

used, but some absorptiometers and spectrophotometers are provided with two scales, one from 0 to 100 showing per cent transmission, and the other 0 to ∞ , showing absorption (see Figure 6.3). The 100 per cent transmission always corresponds to zero absorbance, and on the absorbance scale corresponds to zero transmission. When Beer's Law is obeyed and the concentrations are plotted against absorbance, a straight line relationship is obtained. On the other hand if per cent transmission is plotted against concentration a curve is obtained (see Figure 6.4).

Absorptiometers

These have five essential parts.

A. LIGHT SOURCE

This can vary in intensity, depending upon the type of instrument used.

B. WAVELENGTH SELECTION

In most instruments filters are used for this purpose, but in the more expensive type of equipment a diffraction grating or prism is used to obtain approximately monochromatic light.

1. Filters

The filter chosen is usually complementary to the colour of the solution to be measured (*see* Table 6.1).

Table 6.1 COMPLEMENTARY COLOURS

<i>Colour of solution</i>	<i>Usual filter</i>
Blue	Yellow
Bluish-green	Red
Purple	Green
Red	Bluish-green
Yellow	Blue
Yellowish-green	Violet

Filters are made of glass, or dyed gelatin between glass plates, and have a limited transmission band, at which they transmit maximally (*see* Table 6.2). To understand the use

Table 6.2 MAXIMUM TRANSMISSION OF ILFORD SPECTRUM FILTERS

<i>Number</i>	<i>Type</i>	<i>Peak of maximum transmission (nm)</i>
600	Spectrum deep violet	420
601	Spectrum violet	430
602	Spectrum blue	470
603	Spectrum blue-green	490
604	Spectrum green	520
605	Spectrum yellowish-green	550
606	Spectrum yellow	580
607	Spectrum orange	600
608	Spectrum red	680

of light filters, consider a bluish-green solution which absorbs light in the red part of the spectrum. Such a solution when illuminated by white light absorbs red colour wavelengths and emits bluish-green light, together with a small amount of red. The greater the concentration of the solution the smaller the amount of red light transmitted.

The most sensitive readings of the galvanometer will therefore be obtained by allowing only the transmitted red light to activate the photoelectric cell. The red filter achieves this by stopping the transmission of bluish-green light and allowing only the red light to pass through the solution (see Figure 6.5). Before the correct filter is chosen for any investigation, further studies are required with respect to sensitivity and linear relationship (see p. 122).

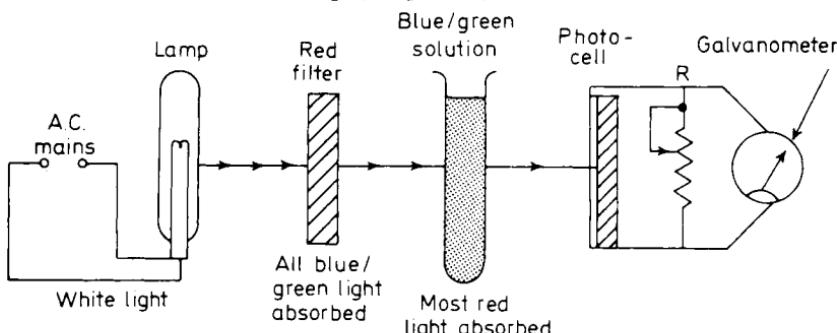


Figure 6.5. Single cell photoelectric absorptiometer: diagram showing light path and the use of a colour filter

2. Diffraction grating or prism

See spectrophotometers.

C. CELLS AND CUVETTES

These are used to hold the coloured solutions and must be scrupulously clean. They have two parallel flat clear sides made of optical glass; the other two parallel sides are opaque and must not be placed in the light path. The set of cells used should be optically matched before placing the cuvettes into the light path; the outside of the cells must be wiped clean with a lens tissue and held up to the light to ensure there are no dirty finger marks or spillage of fluid on the outside of the optical side. Spillage of fluid or dirty finger marks will absorb light and interfere in the measurement of the colour.

The cells must be carefully cleaned and scratches on the optical glass must be avoided. A badly scratched cuvette must be discarded. Never clean cuvettes with chromic acid—always use a good detergent.

Test-tubes are used in some of the simpler instruments but they must be interchangeable. Whilst more convenient to use than cells they are not as satisfactory.

D. PHOTOELECTRIC CELL

There are three types of photoelectric element, the barrier layer cell, the photoemissive tube and the photomultiplier tube. Light falling on these elements generates an electric current which deflects a galvanometer needle, the deflection being proportional to the light intensity.

1. *Barrier layer cell (Selenium cell)*

The commonest form of barrier cell consists essentially of a metal disc upon which is deposited a thin layer of selenium. This is covered by a thin transparent layer of metal, which is lacquered except for a thicker portion near the periphery. At this thick portion of the disc one of the terminals to the galvanometer is connected, the other connection being made to the underside of the disc which is covered by a non-oxidizing metal. When light passes through the thin metal layer to the selenium layer beneath, electrons are liberated which pass across the 'barrier' between the selenium and transparent metal layer to become negatively charged, while the thicker metal disc becomes positively charged. If the cell is connected to a galvanometer, a current will pass, the strength of which is proportional to the intensity of light falling on the selenium. The 'EEL' selenium photocell is an example of the barrier layer cell, sensitive to the visible part of the spectrum.

Absorptiometers using this type of cell may either be direct reading, single photocell instruments or of the double cell type. The double cell or null point instruments have two closely matched selenium cells balanced against each other. Light from a single source reaches both photocells; some light will be absorbed by the coloured solution inserted in front of one of the cells and this will be reflected by the galvanometer needle. By varying a resistance in the circuit, or by closing a diaphragm, the two currents may be balanced, the galvanometer acting as a null point indicator. Unlike the photoemissive tube they do not require the use of a battery.

2. *Photoemissive tubes*

These are similar to radio valves and are composed of an evacuated glass tube or a tube containing an inert gas at low pressure. The tubes are coated internally with a thin sensitive layer of either caesium or potassium oxide and silver oxide to act as the cathode. A metal ring inserted near the centre

of the valve forms the anode and is maintained at a high voltage by a battery. When light penetrates the valve it falls on the sensitive layer, electrons are emitted, thereby causing a current to flow through an outside circuit; this is then amplified by electronic means and is a measure of the amount of light falling on the photosensitive surface.

The current from the phototube is smaller than that from the selenium cell and hence the amplification. They are sensitive to light outside the visible part of the spectrum and are more reliable than the selenium cell.

3. Photomultiplier tube

This is a further development of the photoemissive tube in which the sensitivity is greatly increased by connecting the elements within the tube in series.

E. GALVANOMETERS

The galvanometer measures the output of the photosensitive element, and in most instruments a very sensitive one is used.

SPECTROPHOTOMETERS

These instruments are essentially very similar to photoelectric absorptiometers, the major differences being in the method of producing monochromatic light.

The colour filters are dispensed with and either a diffraction grating or glass prism produces the monochromatic light. A diffraction grating disperses the white light into a continuous spectrum, by turning a wavelength adjustment. The grating is rotated



Figure 6.6. The spectrum of a spectrophotometer

and different parts of the spectrum are allowed to fall onto the photocell.

In the glass prism spectrophotometers, light is focused onto the prism which then passes through and forms an extended spectrum. On adjusting the exit slit (wavelength adjustment) light can pass through the cuvette and illuminate the photocell. This is the cheapest form of selecting monochromatic light and is only usually fitted to a spectrophotometer reading in the visible part of the spectrum (*Figure 6.6*).

Other more expensive spectrophotometers can be used both in the visible and ultraviolet as well as in the infrared range.

FLOW-THROUGH COLORIMETERS

In order to speed up analytical procedures a more recent development in colorimetry is the introduction of flow-through cells. These cells enable colorimetric readings to be speeded up considerably, since the cells or cuvettes can be drained without being removed from the colorimeters. Examples of these types of colorimeters are the EEL Spectra and Pye-Unicam SP1300. The Technicon AutoAnalyser system is another example of a flow-through cuvette.

Maximum absorption

The selection of the correct wavelength or filter is one of the most important steps in colorimetric analysis. This is determined by measuring the absorbance of the coloured reaction throughout the visible spectrum and then deciding the wavelength or filter which gives the highest absorbance; an example of which can be seen in *Figure 6.7*. The absorbances of a solution of methyl red as used as secondary standards in bilirubin estimations have been plotted on a piece of graph paper against the wavelength settings. From the graph it can be seen that maximum absorption is at 525 nm.

Selectivity

Once the correct filter or wavelength has been chosen to give maximum absorption, its selectivity is determined by taking further readings at various wavelengths with two different concentrations of the same solution. These are then plotted on a piece of graph paper and the results can be seen in *Figure 6.8*. Here one can see that the filter or wavelength chosen for maximum absorption also gives maximum selectivity as the 5 mg and 10 mg readings are equidistant from each other, i.e. 5 mg absorbance is 0.160 and the 10 mg absorbance is 0.320.

Linear relationship

With every colorimetric or spectrophotometric analysis carried out in the laboratory it is essential that calibration curves are prepared to confirm that Beer's Law is obeyed.

If a straight line relationship is obtained between absorbances and concentrations Beer's Law is obeyed and therefore a linear relationship has been confirmed.

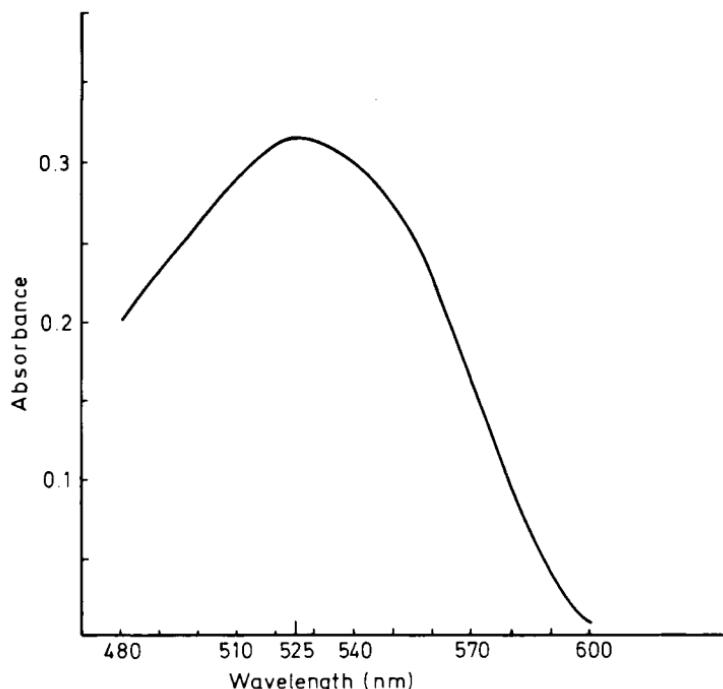


Figure 6.7. Graph showing maximum absorption at 525 nm

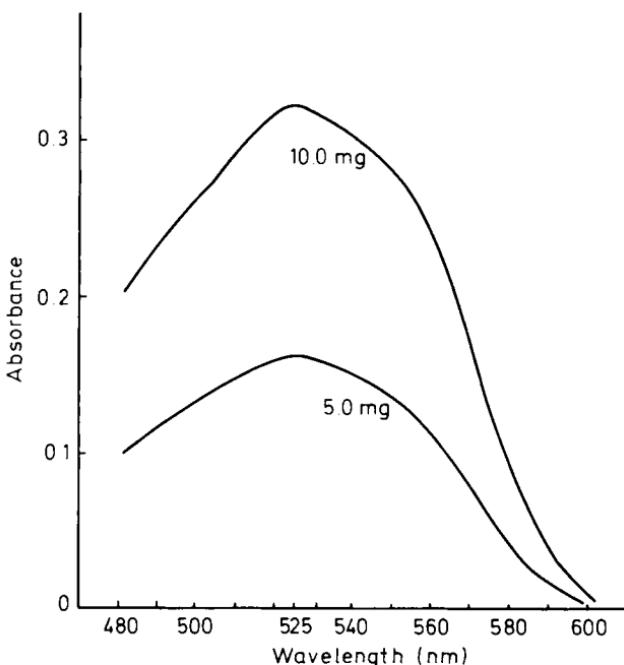


Figure 6.8. Graph showing maximum selectivity

Calibration curves

The greatest care must be taken in the preparation of a calibration curve. Always use freshly prepared reagents and standards and scrupulously clean glassware.

One of the biggest problems in most colorimetric analyses is the presence of protein in biological fluid, since protein will in the majority of cases interfere with the final colour reaction.

Standard preparations are usually prepared in an aqueous medium so there is no problem with protein, but as the standards, tests and blanks must be treated in the same way with the colour reagent, the protein must be removed from either blood, serum or plasma. This step is called protein precipitation and used in the estimation of blood glucose (*see p. 208*).

In this method 2.0 ml of protein-free supernatant are required for colour development. To obtain this protein-free supernatant 0.05 ml blood or plasma is pipetted into 3.9 ml of isotonic sodium sulphate-copper sulphate solution. 0.05 ml of sodium tungstate is then added to the diluted sample and thoroughly mixed. Copper tungstate is formed which acts as a protein precipitant, leaving the glucose and other diffusible components in solution when the protein precipitate has settled out. This is hastened by centrifuging.

In the above-mentioned procedure 0.05 ml of blood or plasma has been diluted to 4.0 ml and therefore 2.0 ml of protein-free supernatant will only contain the equivalent of 0.025 ml of whole blood or plasma.

The unknown solution of glucose is compared against a standard solution of glucose which has been similarly treated (except for protein precipitation). From the absorbances of the standard and unknown, the concentration of glucose can be determined.

Let x = the concentration of glucose in the test sample.

Then 2.0 ml of supernatant (= 0.025 ml of blood or plasma) will contain x mg glucose.

If 2.0 ml of glucose standard solution (containing 0.025 mg glucose per ml) were used for colour comparison, then the standard is equivalent to 0.05 mg of glucose.

Using the standard formula:

$$\frac{\text{Absorbance (A) of unknown}}{\text{Absorbance (A) of standard}} \times \frac{\text{strength of standard used}}{\text{in the colour development}} \times \frac{100}{\text{amount of blood in reaction mixture}}$$

$$\text{or } \frac{\text{A of unknown}}{\text{A of standard}} \times 0.05 \times \frac{100}{0.025} = \frac{\text{mg glucose per 100 ml of blood}}{}$$

$$\text{Then } \frac{\text{A of unknown}}{\text{A of standard}} \times 200 = \text{mg glucose per 100 ml of blood}$$

This then means that when 2 ml of the standard glucose (0.025 mg/ml) solution is treated with the colour reagent in the same way as 2 ml of protein-free supernatant, it will be equivalent to not 0.025 mg/ml but 200 mg/100 ml of blood glucose.

Likewise 0.5 ml, 1.0 ml and 1.5 ml of the same standard glucose solution will be equivalent to 50, 100 and 150 mg of glucose per 100 ml under the conditions of the test.

PREPARATION OF CALIBRATION CURVE

From the above it can be seen that in the estimation of blood glucose (p. 210) a calibration curve can easily be constructed by using the following volumes:

mg glucose per 100 ml ml of glucose standard (0.025 mg/ml)	0	50	100	150	200
ml of distilled water	2.0	1.5	2.0	0.5	0.0

The solutions are thoroughly mixed and the colour development carried out as per test sample.

The absorbances for each standard preparation are recorded and after subtracting the blank (zero value) from each reading, the results are plotted on a piece of graph paper as can be seen in *Figure 6.9*. It is important to label the graph correctly as shown, as this is good clinical chemistry practice.

Figure 6.9 shows a linear relationship between absorbance and concentration and therefore Beer's Law is obeyed, while in *Figure 6.10* there is only a linear relationship up to a concentration of 150 mg per 100 ml and above this concentration Beer's Law is not obeyed. Only when Beer's Law is obeyed must you use the formula shown on p. 210.

When absorbances fall on a non-linear part of a curve, the sample should be analysed again after dilution.

From the calibration curve, the sensitivity of the method can usually be determined. The line through the 0 should be ideally at 45° (see *Figure 6.11a*) and at this angle the method is said to be of ideal chemistry, because for each alteration in absorbance read-

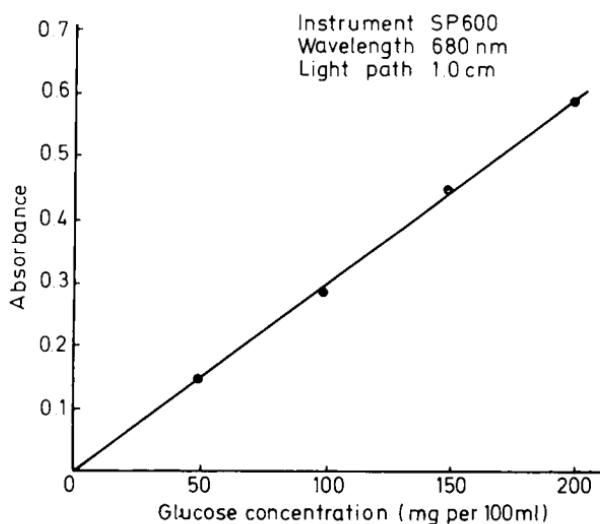


Figure 6.9. A correctly labelled graph showing a linear relationship between absorbance and concentration

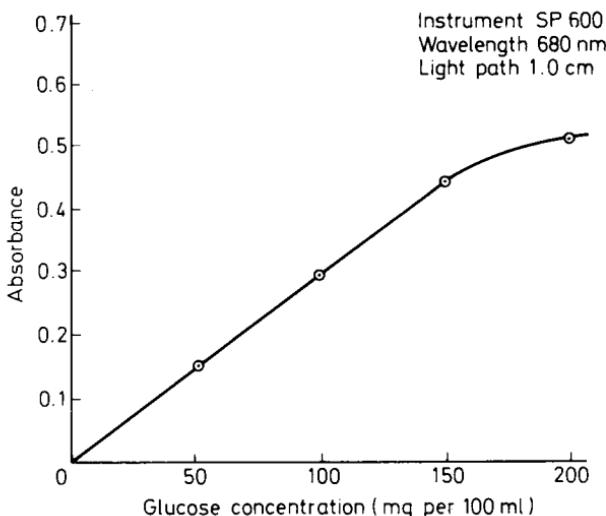


Figure 6.10. A correctly labelled graph showing a linear relationship up to 150 mg per 100 ml

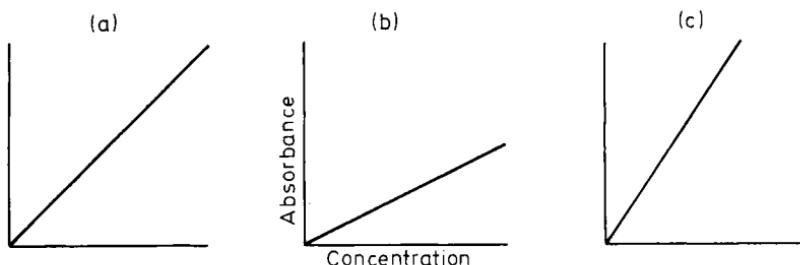


Figure 6.11. Graphs showing the degree of sensitivity of a method

ing, a satisfactory increase in concentration is found. The same cannot be said for *Figure 6.11b* and *Figure 6.11c*. If good linearity is not obtained, the filter or wavelength next to maximum absorption may have to be used.

Excluding personal errors, the accuracy of any result in an analytical procedure using colorimetry depends upon the accurate preparation of a calibration curve. Always use volumetric glassware whenever possible and avoid pipetting small volumes of standard, for example if the following dilutions have to be made:

ml of dilute standard	0.1	0.2	0.4
ml of distilled water	4.9	4.8	4.6

This is bad procedure and is not recommended; it is far more accurate to dilute the standard 1 in 10 so that the following dilutions can be made:

ml of dilute standard	1.0	2.0	4.0
ml of distilled water	4.0	3.0	1.0

Requirements of colorimetric analysis

When colorimetric determinations are made, it is essential to ensure that the colour being measured is only due to the substance under investigation and is not due to any of the reagents used. It is therefore essential to include the following solutions.

1. *Test solution.* This contains the unknown concentration of the substance together with the reagents used in the test.
2. *Standard solution.* This is usually identical to the test solution, except that it contains a known amount of the substance being determined and is approximately equal in concentration to that expected in the test solution.
3. *Blank solution.* This solution is identical to both the test and

standard solution in that it is carried through the complete test procedure and contains all the reagents used, but without any test or standard substance. Any colour given by the reagents used in the analysis can be detected and eliminated.

It is essential to avoid any errors due to dirty glassware, turbidity of solution or air bubbles, as all these factors will seriously interfere with light absorption. It is especially important to remember not to handle the cuvette by its absorptive surfaces, which must be clean and dry.

In order to be sure that the absorbance is due solely to the substance under test, the reading given by the 'blank' solution must be considered with the reading obtained from the 'test' and 'standard' solutions. The photoelectric absorptiometer is set to read zero absorbance with distilled water. The blank, test and standard absorbance readings are recorded, re-checking the zero absorbance between each reading. The blank reading is then subtracted from the test and standard reading as follows:

$$\frac{\text{test} - \text{blank}}{\text{standard} - \text{blank}} \times \text{concentration of standard}$$

This procedure will usually ensure that only the substance under investigation is being measured; however, in certain instances, e.g. in the estimation of protein, the instrument is set at zero absorbance with the blank solution, and not distilled water. Satisfactory results are only obtained with absorbances ranging from 0.2 to 0.8, so that if possible the determination should be modified in order that the lower and upper limits of detection fall within this range. The actual details of using a spectrophotometer or absorptiometer will vary with each instrument, so that the manufacturer's instructions must be followed.

Spectroscopy

When white light is passed through certain coloured solutions, part of the light is absorbed in relatively narrow areas of the spectrum, giving dark regions known as absorption bands. The position of these bands can be used to identify the coloured material in solution.

The absorption bands are conveniently observed through a direct vision spectroscope (*Figure 6.12*) which has some means of determining the position of the bands in terms of the corresponding wavelength of light. Wavelengths are measured in nanometers (nm).

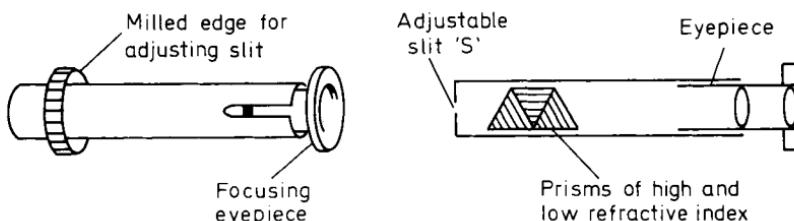


Figure 6.12. The direct vision spectroscope

USE OF THE DIRECT VISION SPECTROSCOPE

1. Place the eye to the eyepiece, and view the sky through the instrument but do not point towards direct sunlight.
2. Close the slit 'S' by turning the milled ring, then re-open the slit slightly until the spectrum is visible.
3. Adjust the eyepiece until the colours are focused and the Fraunhofer lines can be clearly seen.

Note—Fraunhofer lines, which are due to absorption of light by different elements in the sun's atmosphere, are seen as fine vertical black lines across the spectrum. They will be invisible unless a very narrow slit is used.

4. Check that the D line of the sun's spectrum, which occurs at 589 nm in the orange-yellow, corresponds with the position of the 589 reading on the scale.
5. Place the solution in a test-tube or glass cup. (When examining blood, a dilution greater than 1 in 50 is usual.)
6. Position the tube in front of the slit, and observe through the eye piece. Record the position of any absorption bands seen in relation to the spectral colours and Fraunhofer lines. If possible check against a solution of known composition.

Note—A graduated centrifuge tube makes a suitable fluid container as varying depths of solution may be viewed through the spectroscope.

Spectral colour	Wavelength in nm
Red	760–620
Orange	620–595
Yellow	595–560
Yellow-green	560–540
Green	540–500
Blue-green	500–470
Blue	470–430
Violet	430–380

FLAME EMISSION SPECTROSCOPY (flame photometry)

In the clinical chemistry laboratory of today the estimations of the elements sodium and potassium are probably two of the most commonly requested investigations. The determination of these two elements must be simple, accurate and precise, and these criteria are achieved using the technique of flame emission spectroscopy.

Principle

When an element, in its atomic state, is placed in a flame the atoms increase in energy, become 'excited' and emit energy in the form of light in order to attain their original energy state. The light energy emitted is specific in wavelengths for each element, e.g. sodium emits light of wavelength 589 nm and potassium emits light of wavelengths 404 and 767 nm. The light emitted is proportional to the number of excited atoms present.

Basis of the technique

A dilution of the sample, usually in de-ionized water, is converted to an aerosol. This aerosol is mixed with gas used as a fuel, usually propane. The mixture is then ignited in a burner chamber; the heat from the flame releases free atoms from the molecular vapour and increases their energy state. As the atoms return to their ground state they emit energy in the form of light; the emitted light is then quantitated in the same way as a photoelectric absorptiometer.

Instrumentation

Although design varies from manufacturer to manufacturer, all the instruments have a similar layout, which can be seen in *Figure 6.13*.

Nebulizer-Burner System—this consists of a nebulizer (atomizer), cloud chamber with condensation vanes, and a burner suitable for the fuel to be employed.

Detector System—this consists of a monochromator in the form of a filter, orange for sodium and deep red for potassium; a photodetector which can vary from a simple photoelectric cell to photomultiplier tubes depending on design. The output from the photodetectors is fed to either a galvanometer or digital display electronic tubes.

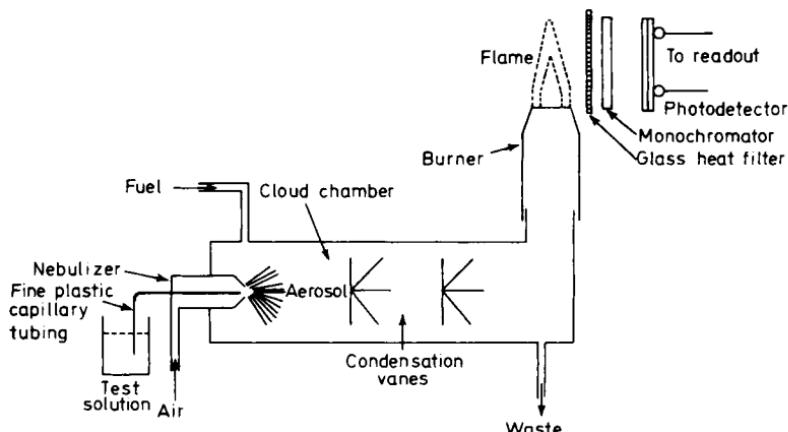


Figure 6.13. Diagram of a flame emission photometer

Method

Again this varies from instrument to instrument; some manufacturers include an automatic diluter in the system, which simply requires the sample to be presented to the instrument for suitable dilution, whereas others require accurate manual dilution, of sample, e.g. sodium 1 in 500 and potassium 1 in 25.

THE MEASUREMENT OF pH

The pH meter is designed to measure the concentration of hydrogen ions in a solution. Basically three parameters are involved in the effective measurement of pH, the actual molar concentration of hydrogen ions, the dissociation constant of the acid (pK_a) and temperature.

pH is defined as the negative value of the logarithm to the base 10 of the hydrogen ion concentration.

$$\text{pH} = -\log_{10} (\text{H}^+)$$

Water at 25 °C has 0.0000001 moles of hydrogen ions per litre (10^{-7}), that is the log of the hydrogen ion concentration is -7 , therefore the pH is 7.0 at 25 °C. A strong alkaline solution such as sodium hydroxide would have a higher concentration of hydroxyl ions and a lower concentration of hydrogen ions. Such

a solution might have 0.00000000000006 moles of hydrogen ions per litre and this would therefore have a pH of 13. The stronger the acid the lower the pH.

The measurement of pH is called potentiometric analysis. Since the early twentieth century various workers have reported that a difference in electrical potential could be measured between two solutions of different pH separated by a thin glass membrane. The potential thus produced varies with the hydrogen ion concentration of the two solutions, i.e. the glass membrane is H⁺ ion-sensitive.

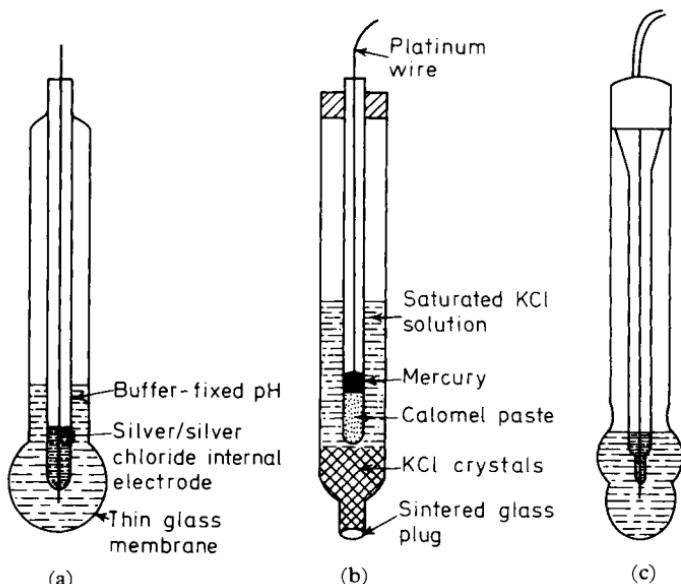


Figure 6.14. (a) Simple glass electrode. (b) Calomel reference electrode. (c) Modern glass electrode

tive. It is on this principle that the glass electrode is constructed. The type of glass is important—it must be a soft, hygroscopic glass, with a relatively low electrical resistance. Modern glass electrodes are constructed from glass containing lithium oxide. The inner surface of the glass membrane is in electrical contact with a buffer solution of fixed pH. Into this buffer dips a silver/silver chloride electrode, the internal reference electrode (*Figure 6.14*). It has been suggested that the glass electrode functions like a semi-permeable membrane, permeable only to hydrogen ions (possibly hydrated) which enter the lattice of the glass (i.e. selective for H⁺ ions only). A calomel (mercurous chloride) reference electrode (*Figure 6.14*) consists of mercury in contact with a solution of

potassium chloride saturated with calomel. It is surrounded by an outer vessel containing saturated potassium chloride which acts as a salt bridge between the reference and test solution (this electrode is not dependent upon pH).

Only when the glass electrode is coupled with a calomel electrode is the potentiometric measurement of pH possible. We then have the arrangement as shown in *Figure 6.15*.

The potential difference (or electrical voltage) between the two electrodes depends upon the hydrogen ion concentration of the test solution or standard. Because of the small differences produced an amplifier is included in the circuit to detect the point of balance between the two electrodes. It is a logarithmic response, measured in millivolts, which on the pH meter is calibrated both in mV and pH.

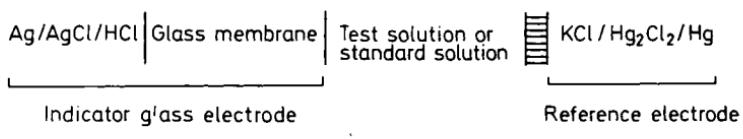
In *Figure 6.14* you can see a simple glass electrode, but the better types of glass electrode are now constructed of two glasses; the working part of the electrode is made of special pH-responsive glass and this is sealed to a stem of a harder high-resistance glass. This will exhibit a much greater resistance to ion transfer and therefore eliminate errors due to various depths of immersion. By this arrangement the working part of the electrode is always immersed in the solution. A good glass electrode will measure up to pH 14.

There is also available for use with most pH meters a combined glass and calomel reference electrode, i.e. two electrodes in one.

pH measurements vary with temperature and all measurements should be made at a temperature of 25 °C. With increasing temperature there will be a fall in pH. Variations of pH with temperature can be seen as follows:

Temperature 0 °C	pH value
0	7.47
10	7.27
25	7.00
30	6.92
50	6.61

It is therefore important to record the temperature of the liquid before measuring the pH, adjusting the dial on the pH meter, and then record the pH. Some pH meters have facilities whereby a temperature thermometer can be incorporated into the circuit and variations above or below 25 °C are automatically corrected by the meter itself.



is the boundary between the two miscible phases (liquid junction)

Figure 6.15. *The arrangement of electrodes in the potentiometric measurement of pH*

Precautions with glass electrodes

The sensitivity of the glass electrode will be affected by:

1. Continuous use, when the electrode may need regenerating.
2. Protein solution: protein solution will poison the glass membrane and therefore must be removed.
3. Dehydrating agents or concentrated acids which dehydrate the membranes. The presence of water in the glass is essential, pH function is impaired when the glass is dehydrated, but can be restored by subsequent immersion in distilled water for several hours or overnight.
4. Temperature—see above.
5. Scratching or fracturing of the glass membrane. Under these circumstances a new glass electrode is required. When not in use the electrode should be left with the membrane immersed in distilled water. New electrodes will need generating by placing them in 0.1M HCl overnight.

Standardization of the pH meter

Standardization should be carried out at least once a day and preferably before a series of measurements are to be made.

Always use two buffer solutions, for example one at pH 4.0 and the other at pH 7.0. If higher or lower pHs are being measured use buffer solutions in the appropriate pH ranges.

RADIOACTIVE ISOTOPES AND THEIR DETECTION

Radioactive isotopes have been used in biochemical analysis for a number of years and are now of increasing importance in most clinical chemistry departments.

Lord Rutherford and his colleagues first discovered radio-

activity in approximately 1920. The Curies in France continued with this work and, following the invention of the cyclotron by Lawrence in 1932, it became possible to apply the use of radioactive isotopes to biochemical analysis.

Isotopes are of two kinds, stable and unstable (radioactive).

Stable isotopes

Isotopes are atoms of the same element, having identical atomic numbers but having different atomic weights. Stable isotopes are permanent substances, which do not disintegrate with time and are not radioactive.

Unstable isotopes

These isotopes can be prepared from stable elements in a cyclotron, when they become unstable because of their radioactivity. Radioactive isotopes disintegrate spontaneously, the rate of decay being proportional to the number of radioactive atoms present.

Radioactive isotopes can occur naturally as, for example, uranium.

The radiation emitted during disintegration can be of three types, alpha (α), beta (β) or gamma (γ).

Alpha particles are helium atoms (He^{2+}) with very low penetrating power. Preparations using these emitting particles are not commonly used. Beta particles are either positive or negative electrons; they have more penetrating power than alpha particles but are stopped by a very light shielding. Gamma particles are electromagnetic radiations similar to X-rays and have a high penetrating power.

The types of radioactive isotopes used in medicine are those which emit β - and γ -type radiations. The naturally occurring isotopes are the source of α -radiation.

Units of activity

The activity of a radioactive isotope preparation is measured by the number of nuclear disintegrations per second. The curie (c) is the unit of activity commonly used.

One curie is 3.7×10^{10} disintegrations per s. This is a great deal of activity and smaller units such as a millicurie (mc) or microcurie (μc) are much more commonly used.

Radioactive half-life

The half-life of a radioactive substance refers to the length of time required for the isotope to decay to half its activity. This can vary

from a few seconds to one of 1000 years. Isotopes used in medicine usually have a half-life of about six weeks, e.g. I^{125} .

<i>Stable isotopes</i>	<i>Radioactive isotopes</i>	<i>Particles</i>
Iodine I^{127}	I^{125}	gamma
Carbon C^{12}	C^{14}	beta
Calcium Ca^{40}	Ca^{45}	gamma
Cobalt Co^{59}	Co^{57}	gamma
Tritium H	H^3	beta

Biological half-life

The biological half-life of a compound is the measure of the disintegration of the compound itself and has no relation to its radioactive properties.

Methods of measurement

The simplest way of detecting radiation is the use of photographic films or plates. The familiar radiation protection badges worn by all staff in isotope departments are the commonest application.

This method of detection, however, cannot be used for quantitative estimation of radioactive disintegrations; in that type of assay a gamma or beta counter is required.

The essential part of a counter is the Geiger-Muller tube, a tube consisting of a fine wire anode surrounded by a coaxial cylindrical metal cathode. These are completely encased into a glass casing filled with gas at low pressure. Radiations entering the counter strike the Geiger-Muller tube, ionizing molecules of gas contained in it, and the negative ions move towards the positively charged anode. This causes a drop in potential which, when connected to a measuring device, is recorded as a pulse. By counting the number of pulses produced in a specific time, the radioactivity of a compound can be calculated. This is usually expressed in terms of counts per min.

I^{125} , for example, emits high-energy radiations and the conventional gamma counter is satisfactory, but for low-energy radiation of, for example, C^{14} and H^3 a scintillation (beta) counter is required. In the method the compound to be estimated is dissolved in a suitable solvent (toluene), along with another compound (organic phosphor) which then undergoes a scintillation reaction. In this reaction the phosphor fluoresces or scintillates, i.e. emits light photons which are collected by the photomultipliers and converted into a pulse and recorded in the usual way.

Isotope laboratory design and safety

This is adequately covered in the DHSS booklet *Safety in Pathology Laboratories*.

COULOMETRIC ANALYSES

This form of analysis carried out in the clinical laboratory is in the estimation of chloride in biological fluid. Potentiometric titrations (coulometric titrations) are used every day in the form of chloride meters, automatic instruments based on the application of Faraday's law of electrolysis. The chloride meter is an easily operated instrument, containing a digital readout, various operating controls and a measuring head. Into the measuring head are fitted four silver electrodes; two silver generating electrodes (one an anode, the other a cathode) and two end-point detection electrodes. A linear current flows between two silver generating electrodes. Silver ions are released from the anode and combine with the chloride ions in the sample during titration. The digital display starts registering as the silver and chloride ions combine ion to ion until all the chloride has been precipitated as silver chloride. The free silver ions in the solution cause a change in conductivity which is sensed by the two end-point detecting electrodes. This abrupt change in conductivity stops the digital readout, displaying the result directly in mEq of chloride per litre.

OSMOMETER

An osmometer is an instrument for measuring the concentration of solutes in a solution. When solutes are dissolved in a solvent the resulting solution changes because the freezing point is lowered and the osmotic pressure is increased. These are called colligative properties and they have a specific value since they are closely related to the osmolality of a body fluid.

Measurement of osmolality

The instruments available measure osmolality of body fluids by indirect means, by measuring the depression of freezing point, since the freezing point of a liquid is dependent upon its solute concentration. Water is first cooled without stirring to below freezing point. At a definite degree of supercooling, a vibration is activated to induce freezing and the temperature of the water then

rises to the freezing point of 0 °C, which is recorded on a galvanometer. In biological fluids the freezing point is below 0 °C; this depression of freezing point is therefore a measure of the osmolality of the solution. The galvanometer is usually calibrated to read directly in milliosmols (mOsm), 1 mOsm being equivalent to a depression of freezing point of 0.00186 °C.

AUTOMATED ANALYSIS

Most medical laboratory scientists are thankful that many of the boring repetitive tests in the clinical laboratory have now been automated in one form or other. If the routine tests are not completely automated some form of semi-automation is used, such as the use of diluters, automatic pipettes and burettes, dispensers, mixers, shakers, etc.

Since the introduction of a complete form of automated analysis after Skeggs (1957), many more tests are now carried out per patient than was even thought of several years ago.

Automation has a two-fold advantage:

1. it increases the speed of the investigation; and
2. increases the accuracy and precision of the estimation when compared with the conventional manual method. However, many laboratories tend to forget that there should always be a similar manual method available in cases of emergency.

Automation can be of two different forms: namely, continuous flow or discrete analysis.

Continuous flow systems

Historically the first real continuous flow system in clinical chemistry was manufactured by Technicon Instruments Corporation of New York and this system was given the trade name AutoAnalyser.

The AutoAnalyser uses a combination of modules where a sequence of standards and test samples are 'picked up' in a stream of fluid moved along a tube by a peristaltic pump, mixed at appropriate stages with a series of reagents, then passed through the various modules and finally analysed colorimetrically. To separate the samples in the moving stream and to assure that cross-contamination does not occur between samples, a non-wettable plastic tube is used into which air bubbles are introduced at regular inter-

vals. The air segments being large enough to completely fill the lumen of the tube.

With manual procedures every reaction must generally be brought to completion, but with the AutoAnalyser system it is never necessary, since it continuously measures and compares on a moving graph the level of concentration of a given component in the test solution against a known concentration of that component in a standard control solution (*see Figure 6.16*). The peak

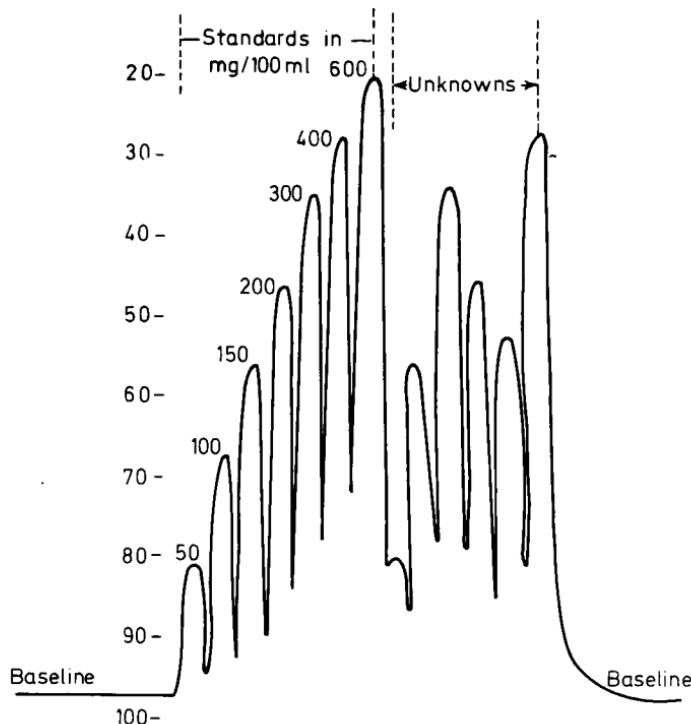


Figure 6.16. The peak heights of standards and unknowns recorded on a moving graph

heights of the standards are plotted against concentration and the peak heights of the unknown compared, from which the unknown concentration is obtained (*see Figure 6.17*). An example of the modules used to determine a single substance in an AutoAnalyser I system (AAI) is as follows: (1) sampler, (2) proportioning (peristaltic) pump, (3) dialyser (to remove protein), (4) constant temperature heating bath, (5) colorimeter and (6) recorder. This arrangement can be shown in *Figure 6.18*, which refers to a flow diagram.

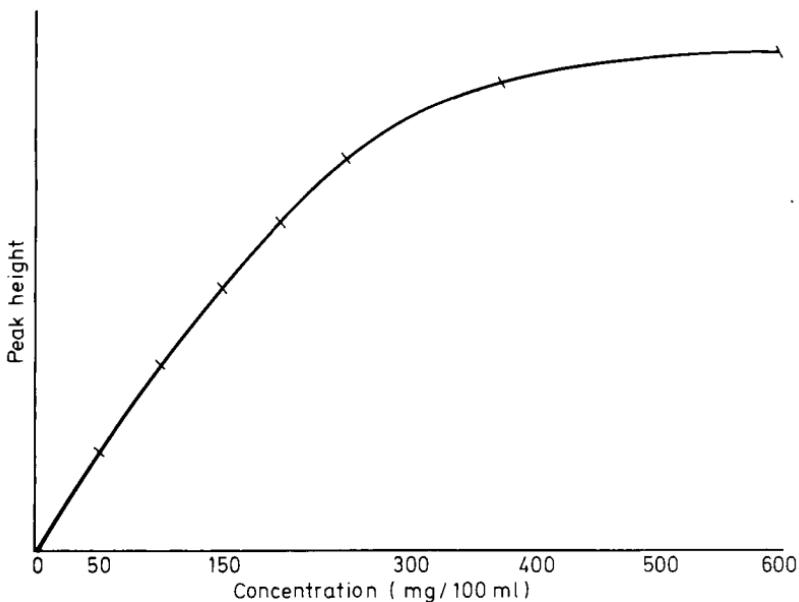


Figure 6.17. Peak heights plotted against concentrations

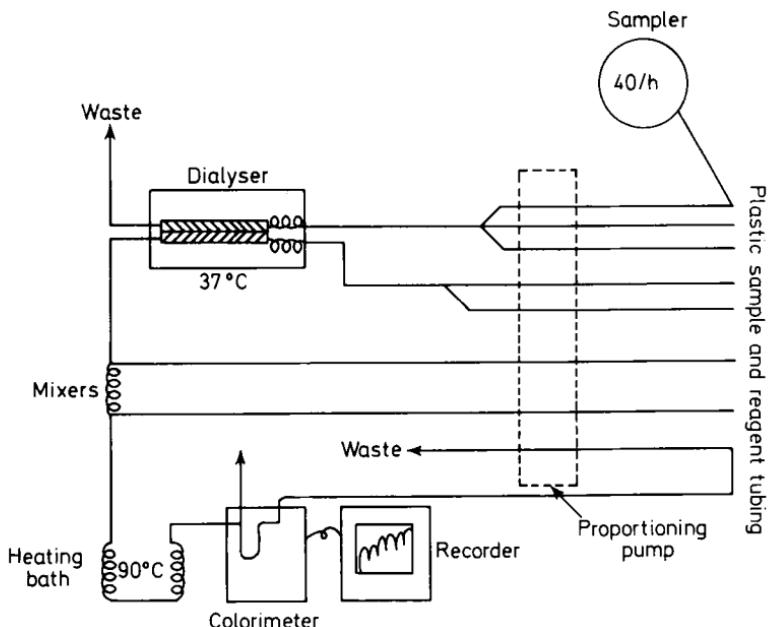


Figure 6.18. Flow diagram for an AAI system

SEQUENTIAL MULTIPLE ANALYSIS SYSTEMS

The increasing demands on the clinical laboratory for faster rates of analysis, flexibility of procedures along with increased accuracy of results led to a newer generation of AutoAnalysers, the Sequential Multiple Analysis system (SMA). This system, the SMA 12/60, is capable of performing 12 simultaneous colorimetric tests on one sample and can introduce samples at the rate of 60 per h. Although the AAI system could be so constructed to estimate 4 different parameters at the same time (e.g. Na, K, CO₂, urea), the SMA systems give steady state conditions in which the concentration of the sample in the colorimeter flow cell remains constant for a period of time, and the result can therefore be reported directly into concentration on the recorder chart paper (*see Figure 6.19*).

Tomorrow's generation of AutoAnalysers will be the SMAC, a computer-controlled SMA system achieving a high speed of analysis of 150 samples per h with a total of 20 test profiles from the same sample.

AUTOANALYSER II SYSTEM (AAII)

After the experience of the SMA system, the second generation of the AAI system was developed. This analyser combines many virtues of the AAI system along with the many features of the SMA.

Discrete (discontinuous) systems

In these systems each sample is processed separately and the reactions take place in individual tubes. The chemistries used either involve protein precipitation or else the amount of protein in the sample reaction mixture is so minute that it does not interfere with the test.

The AutoAnalysers have one significant advantage over other systems in that protein is removed by dialysis, whereas other instrument makers have difficulty in breaking the patent on the Technicon dialyser and therefore have to use other means of deproteinization. Basically the main units in a discrete analyser are (1) the preparation unit, (2) centrifuge, (3) incubator, (4) automatic colorimeter and printer, and (5) automatic pipettes and dispensers. The samples and reagents are dispensed or removed by means of the fifth unit, the manipulations or preparations stages being processed on a continuous tube transport system, very much like a belt on a caterpillar tractor. While they are on top of the belt the tubes are upright and contain the test materials;

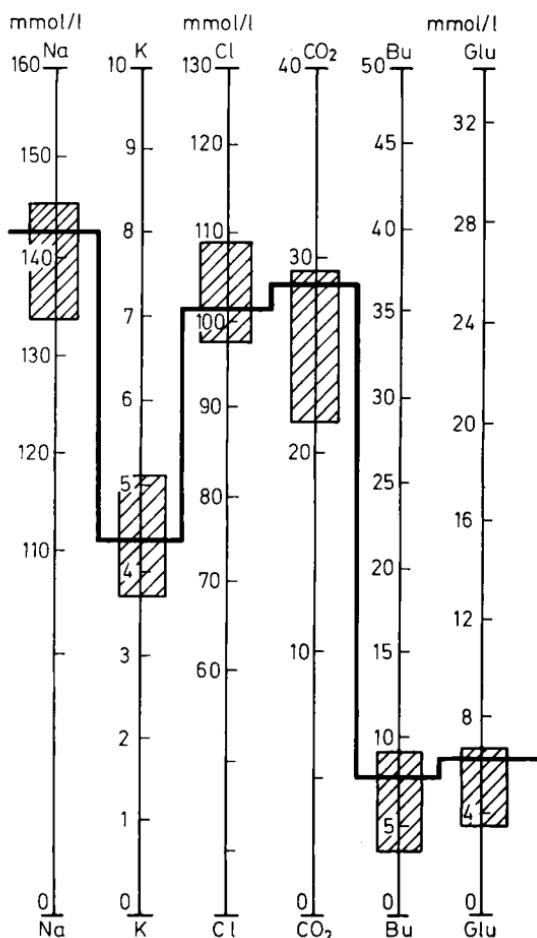


Figure 6.19. Recorder chart paper of the SMA system of analysis. Shaded areas are the 'normal' ranges

as they move to the end they tilt forward, pouring out their contents into the next stage, and then pass along the bottom of the belt in an inverted position during a washing cycle. At timed intervals the tubes advance one position, as clean tubes come to the upright position, sample and reagents are added, incubation, colour development, and automatic readings follow.

Although not all the discrete systems are completely based on this principle, some of the discrete analysers are the Vickers Multi-channel 300, Hycel Corporation's Mark X, and the Analmatic System.

CHROMATOGRAPHY

Early in the twentieth century a Russian botanist, Tswett, was working on plant pigments and devised a system very similar to paper chromatography. In his work he was able to separate the various plant pigments on a column of calcium carbonate. Since he worked primarily with coloured solutions, Tswett called this technique chromatography, a term now used today, even though many of the separations commonly used have nothing to do with colour.

General principle

A mixture of substances in solution can be separated when applied to a support medium. This can either be paper (paper chromatography), a thin layer of silica (thin layer chromatography) or a column packed with an adsorbent or ion exchange resin (column chromatography). Tswett adsorbed a mixture of plant pigments on the calcium carbonate and then separated the components into a series of coloured bands by washing or eluting the pigments with solvents. The pigments separated depending upon their solubility in the solvent; if, however, all the pigments were completely soluble in the solvents, there would be no separation.

Terminology

Elution—The use of a solvent to separate components.

Eluate—the solvent containing the component, sometimes called the fraction.

Origin—the point of application of substance to chromatogram.

Loading—the amount of substance applied to the paper or support.

Solvent front—the level at which the elution fluid has reached.

Stationary phase—usually a solid or liquid adsorbent, e.g. paper or water.

Mobile phase—a solvent or gas used to separate the component.

Column—a cylindrical tube usually made of glass for holding either the adsorbent or ion exchange resin.

Polarity—a polar compound is one that is held by the stationary phase, whereas a non-polar compound tends to move forward in the mobile phase.

Adsorption chromatography is when the stationary phase is a solid, while the mobile phase can either be a gas or liquid.

Paper partition chromatography is when the stationary phase is a liquid, frequently water held onto an inert, porous support

(paper), and the mobile phase can either be a liquid or gas. In this type of chromatography more than one solvent is usually present in a liquid mobile phase, since it is always saturated with the stationary phase, thus making the two phases immiscible. Separation occurs between two components of a mixture when one component is more strongly retained than the other by the stationary phase.

Thin layer chromatography is a variant of chromatography in which the support medium is applied to a glass plate or plastic film.

One-dimensional chromatography is when the solvent runs into one direction only.

Two-dimensional chromatography is after the solvent has run in one direction, the paper is dried and turned through 90°, replaced in the tank and developed in the new direction (see Figure 6.20).

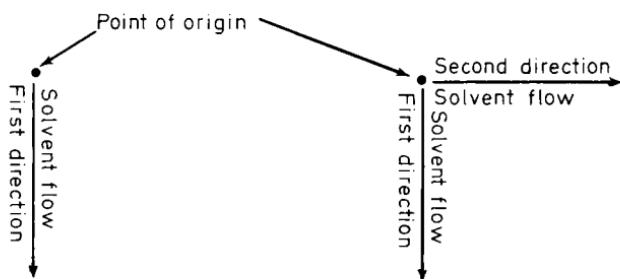


Figure 6.20. Directions of flow in chromatography

Multiple development is when the development is re-run a number of times in a solvent system to improve resolution.

Development is the process of allowing the solvent to move along the column or paper.

Resolution is the degree of separation of the component after development.

Location is the detection of the components after development, either by using a specific or general reagent or ultraviolet light.

Tanks are airtight containers in which development takes place.

Rf value (relative fraction) is defined as a ratio of the distance the solute has travelled from the point of origin to the distance travelled by the solvent front.

Sometimes it may be necessary to allow the solvent to run off the support in order to obtain good separation of the components. In this case it is not possible to measure the solvent front, so a standard substance is used and R_g values are obtained in the same way.

$$R_g = \frac{\text{distance solute has moved from origin}}{\text{distance standard has moved from origin}}$$

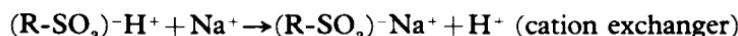
In certain cases the R_f or R_g values are multiplied by 100.

Ion exchange chromatography. An increasing number of laboratories now produce water free from ions, although not all non-electrolyte contaminants may be removed, hence the water is not pyrogen-free. There may also be some extraction of organic impurities from the resins, but under normal circumstances the water obtained by this method is purer than that obtained by distillation. Water purified by ion exchange resins is sometimes called 'conductivity water', as it has such a low electrical conductivity that it is suitable for use in such measurements.

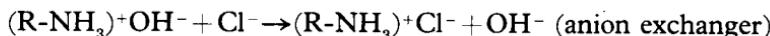
Principles of ion exchange resin. Ion exchange resins are usually cross-linked polymers containing ionic groups as part of their structure. They have negligible solubility but porous enough for ions to diffuse through the resin. During polymerization (manufacture), the polystyrene resins are formed by condensation between the vinyl benzyl (styrene) and small quantities of divinyl benzene to give cross-linked polymer chains. This is important because the amount of cross-linking determines the insolubility of the resin and also the amount of swelling that occurs when the resin is mixed with water and its capacity for exchanging ions.

Ion exchange resins are either (a) anion exchange resins ($R-NH_3^+$) OH^- which are bases or (b) cation exchange resins ($R-SO_3^-$) H^+ which are acids. R represents a polystyrene resin. Anion exchangers are usually supplied in the Cl^- form because they are more stable, then they are converted into the OH^- form which is the exchangeable ion. Cation exchangers are supplied in the H^+ form.

If water containing sodium chloride is passed through a column of cation exchange resin, the Na^+ cations replace the H^+ cations of the resin.



The water now contains H⁺ ions (obtained from the resin) together with the original Cl⁻ anions. If the water is passed through an anion exchange resin, the Cl⁻ replaces the OH⁻ anion of the resin.



The water now contains H⁺ and OH⁻ ions which combine to form H₂O. In this way the water is prepared ion-free.

Conductivity water is usually produced by passing the water through a 'mixed-bed' de-ionizer, i.e. mixing the two resins together in one column.

Ion exchange resins may be regenerated by passing HCl through the cation resin, followed by washing well with water and passing NaOH through the anion resin, and washing with water. If the resins are of the 'mixed-bed' type, it is necessary to separate the two resins first by passing an upward flow of water through the mixture. The two resins, being of unequal density, will separate out.

Paper partition chromatography

The solvent can either run down the length of the chromatography paper (descending) or percolate upwards (ascending chromatography). In the descending form the upper end of the paper dips into a narrow trough containing the solvent; the chromatography paper then passes over a glass rod to hang down in the tank (*Figure 6.21*) in which there is also a container with the stationary solvent to keep the atmosphere saturated.

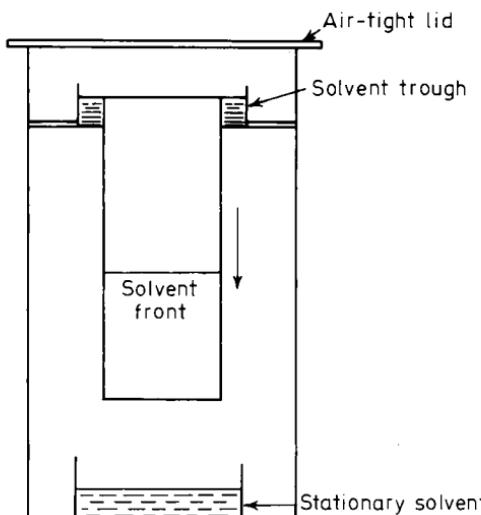


Figure 6.21.
Chromatography tank—
descending technique

In the ascending technique the solvent is in the bottom of the tank and the paper dips into it. By the side of the trough containing the mobile phase is a small reservoir containing the stationary phase. Chromatography papers vary in size and thickness, the most commonly used in this country are Whatman 1, 4 and 3MM. They should be comparatively free from impurities, which can hinder the resolution and cause irregularities in development.

TECHNIQUE FOR ONE-DIMENSIONAL CHROMATOGRAPHY (descending)

1. Take a sheet of Whatman No. 1 chromatography paper of suitable length and width for the tank in use and draw a pencil line parallel to and about 8 cm from one end.
2. 10 µl of test solution is then loaded on the line by allowing the sample to spot the paper several times on the pencil mark (see *Figure 6.22*).

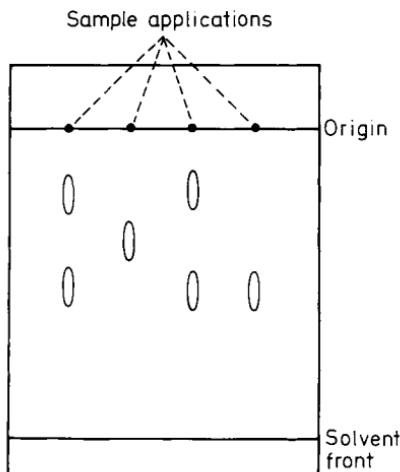


Figure 6.22. One-dimensional chromatography—sample applications

3. Always allow the spot to dry at room temperature, or a little above, before adding the next volume. Do not overheat.
4. The smaller the area of application, the more compact will the component be after development.
5. Depending upon the sample being chromatographed, the volumes loaded onto the chromatogram will vary.
6. The standard samples are now applied in the same way.
7. Place the chromatogram in the tank, and align the sheet so that the solvent will run correctly and not irregularly.

8. Replace the lid, run the solvent into the trough and allow development to proceed.
9. The time of development will depend upon the component being investigated but this can vary from 16 to 36 h.
10. Carefully remove the paper and mark the position of the solvent front if prolonged development has not been used.
11. Hang the paper in a fume-cupboard and blow off the solvent in a stream of cold air, until completely dry.
12. The paper is now ready for locating the position of the separated components.
13. Place the locating reagent in a glass spray or atomizer and spray the area quickly and evenly until the paper is damped.
14. Re-hang the paper in the fume-cupboard and allow most of the reagent to dry.
15. It may now be necessary to re-suspend the chromatogram in a hot-air oven for a few minutes at 95–100 °C.
16. Examine the paper every few minutes until all the individual components can be seen.
17. Overheating can char the paper with particular reagents and therefore the paper must be prevented from ‘breaking up’.
18. Compare the spots of the unknown components with that of the standards.
19. Calculate the *Rf* value if necessary.

Thin layer chromatography

In 1938 two Russian scientists made a great advance in the field of adsorption chromatography by introducing thin adsorption chromatography on carrier plates. Approximately 20 years later Stahl in 1958 introduced thin layer chromatography (TLC) and demonstrated its many applications. TLC gives much more rapid results and is a far more sensitive technique. These are only two of the advantages over paper chromatography; on the other hand some separation techniques still require paper chromatography. The technique is gaining popularity and most laboratories can easily adapt it for routine use by constructing their own apparatus (*see Progress in Medical Laboratory Technique, Vol. 3*), or by obtaining the basic equipment from the usual commercial firms.

PREPARATION OF HOME-MADE THIN LAYER SLATES

Flat plates of ordinary window glass (2.5 mm) can be used, the size depending upon the airtight tank available. The three usual sizes are 10 cm², 20 cm² or 10 × 20 cm.

All plates must be thoroughly cleaned before use, preferably

by washing in a good detergent, followed by hot water and finally running in distilled water before drying.

ABSORBENTS

Materials used are generally silica gel, alumina and cellulose. The required quantity of absorbent is mixed with distilled water to form a slurry; 30 g of silica gel and 60 ml of water, shake thoroughly for 1–2 min in a stoppered flask and then use promptly as described below.

PREPARATION OF CHROMATOPLATES

The following method of preparing plates has the advantage of being simple, versatile, and plates of various sizes can be coated. After thoroughly cleaning the glass plates, a narrow strip of adhesive tape (Elastoplast) is applied to the apparatus edges of the plate. The thickness of the tape determines the thickness of the absorbent coating. To prevent the plates moving during the coating process, the tape is lapped over onto the supporting surface. Sufficient slurry to cover the plate is prepared as described above, then poured along the untaped edge of the plate. Using a thick uniform glass rod, the slurry is drawn along the plate in one continuous glide along the tape. Care should be taken not to roll. If the glass plate is placed on a large piece of filter paper, any excess slurry falling over the edge of the plate is dried immediately and therefore will not creep back onto the plate. After the plate has dried for about 30 min, the tape is carefully removed and the plate activated at 110 °C for 30 min. Very small plates can be taped together and coated at the same time.

APPLICATION OF THE SAMPLE

The sample is applied with a micropipette to the plate under a stream of warm air (hair drier) on a line about 2–5 cm from the lower edge of the plate. A plastic spotting plate can be used for the correct spacing of the various samples along the starting line. The distance between spots is usually 1–3 cm, the diameter of the spots should not exceed 8 mm (*see Figure 6.23*). To ensure standard development it is usual to score-mark the plate about 12–15 cm from the bottom; in this way the absorbent is removed from the plate and no more solvent can move above the score line (*see Figure 6.23*).

After evaporation of the solvent in which the sample was applied, the plate is placed in a chromatographic jar containing

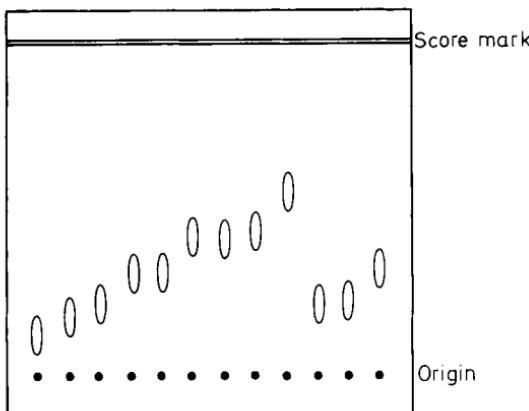


Figure 6.23. Sample applications to a thin layer chromatogram

enough solvent to cover the bottom of the jar, but without reaching the surface of the absorbent.

To obtain rapid equilibrium, one side of the jar should be lined with filter paper, which extends into the solvent at the bottom. When the jar has become saturated with solvent vapours, additional solvent is carefully poured down one side of the jar until about 2 cm of the chromoplate is covered, thus starting the development. When the solvent has completely reached the score line, the plate is removed from the tank and dried.

VISUALIZATION

The locating agent is usually sprayed onto the dried plate after development with one of the spray guns available for this purpose. The Shandon spray gun utilizing the disposable canisters of propellant is very satisfactory.

IDENTIFICATION

After visualization by spraying with locating agent, the individual components can then be identified by their characteristic colour and R_f value.

ELECTROPHORESIS

The technique of electrophoresis is similar in many ways to chromatography in that the moving force is now replaced by an electric current. The technique is basically used for the representation of

protein molecules in biological fluid, but it can be applied to other components providing they have or can carry an electrical charge.

Electrophoresis of serum or urine proteins using cellulose acetate strips

PRINCIPLE

Electrophoresis is the migration of charged particles in an electric field. At a pH above or below their isoelectric points, proteins carry net negative or positive charges and therefore can be made to migrate under these conditions. At a pH alkaline to its isoelectric point, a protein will carry a net negative charge and therefore

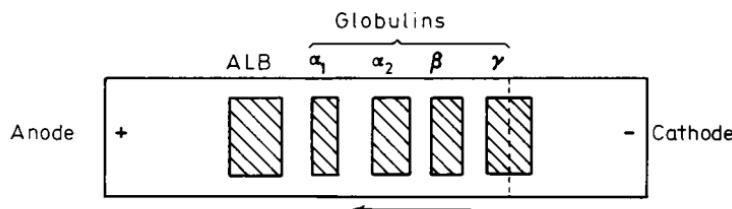


Figure 6.24. Normal electrophoretic pattern in a buffer of pH 8.6

migrates to the anode when a current is passed. Different proteins vary markedly in their isoelectric points and therefore differ in electric mobility at any given pH value.

Serum proteins vary in their isoelectric points from 4.7 (albumin) to 7.3 (γ -globulin) and thus each protein will migrate at a different rate when in a buffer of pH 8.6 (Figure 6.24).

EQUIPMENT

1. Electrophoresis tank, e.g. Shandon Electrophoresis Apparatus after Kohn SAE 3225.
2. Polarity Controller and Safety Switch complete with lead for power supply SAE 2692.
3. Power Unit—Vokam Constant Voltage/Constant Current D.C. Supply SAE 2761.

A layout of an electrophoresis apparatus can be seen in Figure 6.25.

REAGENTS

1. Cellulose Acetate Strips 12×2.5 cm.
2. Barbitone Buffer pH 8.6 ionic strength 0.05, 9.2 g barbitone, 15.5 g sodium barbitone and 23 ml 5 per cent thymol in

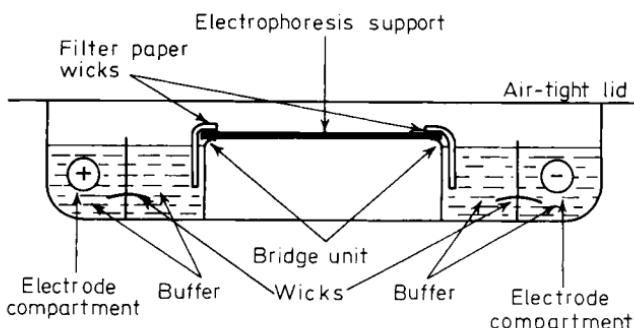


Figure 6.25. Horizontal strip electrophoresis tank

isopropanol is dissolved in warm distilled water, cooled and diluted to 5 litres.

3. Ponceau S, 0.5 per cent *w/v* in 5 per cent trichloracetic acid.
4. Acetic Acid, 5.0 per cent *v/v* in water.

METHOD

1. The cellulose acetate strips are marked in pencil at the point of application of the sample, approximately 4 cm from one end (which will be the cathode end).
2. They are then labelled for later identification and then placed lightly on the surface of the buffer in a shallow plastic tray, allowing the buffer to impregnate from the strip from below by capillarity before being submerged completely.
3. The impregnated strip is removed from the buffer (using forceps) and lightly blotted between two sheets of filter paper, to remove excess moisture, but ensuring that no opaque, white spots are present. If this happens the strips should be returned to the buffer solution for a short time before being blotted again.
4. The strips are then positioned into the tank, with the point of application on the cathode side, and a wick of filter paper is placed over either end of the strips, dipping into the buffer in the tank.
5. Approximately 5 µl of the same sample is applied to each of two of the strips, slowly, leaving a margin down either side and in as fine a streak as possible along the pencil line. A sheet of perspex placed on top of the tank helps by providing support for the hand.
6. The lid is replaced on top of the tank and the power supply connected.

7. Switch on the current and adjust it to 0.4 millamps per cm width of strip; this usually gives a voltage in the region of 185 volts.
8. Leave for 2 h.
9. The strips are removed from the tank and dried.
10. Stain with ponceau S solution for 10 min, again floating the strips on the surface of the dye to ensure complete impregnation before submerging.
11. After staining is complete the strips are placed in a small tank containing 5 per cent acetic acid.
12. Replace the acid several times to ensure the dye has been removed from the background completely.
13. Dry and Sellotape one strip to the laboratory record card for reference, the second one for dispatch to the ward.
14. The fractions can be eluted from the strip by cutting out the individual fractions and placing the cut strip into a test-tube containing 4.0 ml 10 per cent *v/v* teepol in water.
15. The absorbance can then be read at 520 nm.

Notes—(a) Reverse direction of the current after each run. (b) If poor separation of the protein fractions is found, check the pH of buffer. (c) Serum from well-clotted unhaemolysed blood must be used. (d) Fresh serum may show an extra band in the α_2 to β region. (e) Strips are run from the cathode to anode.

Cellulose acetate versus paper electrophoresis

For many years the only support used in electrophoresis was paper, but with the introduction of cellulose acetate as a support a more rapid and far better separation of the protein fractions was achieved. Paper electrophoresis used to take about 16–18 h for complete separation but now with the better types of cellulose acetate supports and improved electrophoresis tanks, a protein separation can now be accomplished in about 20 min.

The information given here is only a guide. Different stains for identifying the protein can be used. If higher currents are used, a cooling system is necessary to prevent the protein becoming denatured.

7

Introduction to Clinical Chemistry

INTRODUCTION

It is important for every medical laboratory scientist to have an intelligent understanding of the laboratory investigations he or she carries out. This can only be accomplished by having a basic knowledge of physiology—the science of bodily functions, and further reading is recommended after consulting the bibliography at the end of the book.

The human body develops from a single initial cell which grows and reproduces, forming millions of cells which in turn arrange themselves into tissues. The tissues in turn arrange themselves into organs, which form a new being. The cell is described on p. 299. Chemical functions within these organs are taking place all the time, and it is the role of the clinical chemistry department to observe any chemical changes.

METABOLISM

This is a general term applied to various changes whatever their nature taking place in living cells or in the body. Ceaseless chemical activity takes place within the protoplasm as materials are built up (anabolism) from simple units to complex ones (e.g. the synthesis of protein from amino acids), while simultaneously others are broken down (catabolism) from more complex substances to simple forms, such as the hydrolysis of glycogen to glucose units.

Since both these processes occur side by side it is convenient to use the term metabolism when referring to both processes. When exogenous catabolism takes place it refers to the breakdown of the diet; on the other hand cellular breakdown is described as endogenous catabolism.

Metabolic processes are accompanied by the production of heat and energy, the necessary energy and heat required for the bodily processes being acquired by the metabolic utilization of foodstuffs. Since these chemical reactions are under the control of enzymes,

the cells can only survive within certain limits of temperature. If the temperature is too high these organic catalysts are destroyed, while at low temperatures reactions are retarded and finally cease.

Metabolic rate

The amount of energy liberated by the catabolism of food in the body is the same as the amount liberated when food is burned outside the body. This finding enabled the physiologists to understand more about the metabolic rate of the body. The output of energy by the body, and the rate of oxygen consumption, depend upon muscular activity, environmental conditions, body temperature and so on.

In order to reduce these factors to a minimum, the metabolism of one individual can be compared with that of another by keeping the subject in bed, warm, completely relaxed both mentally and physically for 12 to 15 h after the last meal. The output of the body under these conditions is then called basal metabolism, which is the amount of energy expenditure of the body at complete rest, assessed from the oxygen consumed in kcal per m^2 of body surface per h. The basal metabolic rate (BMR) was determined by using an indirect form of calorimetry using the Benedict-Roth BMR apparatus. This apparatus is basically an oxygen-filled spirometer and a carbon dioxide absorber. The spirometer bell is connected to a pen which writes on a rotating drum and records the amount of oxygen consumed during the experiment. Nowadays the determination of BMR has been replaced by much more reliable laboratory investigations, such as serum thyroxine, since this hormone from the thyroid gland is concerned with the regulation of the BMR.

Calories

Basal metabolism is expressed in terms of calories. There are large calories used in nutrition and also small calories used in physics. The unit commonly used in nutrition is the *calorie* or *kilocalorie* (kcal), this being the energy required to raise the temperature of 1 kilogram of water by 1 °C. On the other hand, a calorie is the amount of heat required to raise the temperature of 1 g of water 1 °C. With the introduction of SI units the calorie is now replaced by the *joule* (J), when $4.2 \text{ kJ} = 1 \text{ kcal}$.

The energy necessary to carry out metabolic processes is dependent upon the nutritional materials.

The energy-producing ability of three foods in the body are as follows:

1 g of carbohydrate provides 4 kilocalories	(16.8 kJ)
1 g of protein provides 4 kilocalories	(16.8 kJ)
1 g of fat provides 9 kilocalories	(37.8 kJ)

Regulation of body temperature

In the body, heat is produced by muscular exercise, utilization of foodstuffs and all vital processes that contribute to basal metabolism. It is lost from the body through respiration, by the skin and small amounts are lost in the faeces and urine. The skin is by far the most important regulator of the body temperature.

The balance between heat production and heat loss determines the body temperature which is maintained within very narrow limits.

Normal body temperature

The temperature of the body ranges from 35.8 °C (96.4 °F) to 37.3 °C (99.2 °F), the mean being 36.7 °C (98.1 °F) with a standard deviation of 0.22 °C (0.4 °F). Many normal individuals have temperatures differing from 36.7 °C by more than 0.56 °C and therefore to refer to a normal temperature is clearly illogical. In temperate climates the body temperature is nearly always higher than the environmental temperature, when there is a continuous loss of heat through the skin.

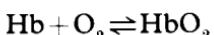
BLOOD GAS ANALYSIS

Respiration exchange

Respiration is the term generally used in the interchange of two gases, oxygen and carbon dioxide, between the body and the environment. Two processes are involved; external respiration when the lungs absorb oxygen and remove carbon dioxide from the body, and internal respiration when the uptake of oxygen and the formation and liberation of carbon dioxide by the cells are involved.

In external respiration, air is drawn into the lungs (inspired) by breathing in through the nostrils. This air is drawn down the windpipe or trachea, which divides into two tubes called bronchi, each of which passes into the substance of the lung and divides into a number of smaller bronchioles. Branching of the bronchioles continues until each one ends in a minute air sac, called an

alveolus. The walls of the alveoli are covered with a film of water, which because of the constant supply of air is saturated with oxygen. Each alveolus is within, and in close contact with, a mesh-work of capillaries. The oxygen diffuses through the alveolar walls into the blood capillaries, first into the plasma and then into the red cells (erythrocytes), where it combines with the haemoglobin to form an unstable compound, oxyhaemoglobin. This is a reversible process because the dissociation of oxyhaemoglobin to release oxygen is dependent upon the tension of the oxygen in the medium surrounding the haemoglobin.



Hb = deoxygenated haemoglobin

HbO_2 = oxyhaemoglobin

Simultaneously, the carbon dioxide in the plasma and red cells diffuses out of the blood capillaries and dissolves in the watery covering of the alveolus. This carbon dioxide now diffuses into the alveolus and it is removed as air is breathed out (expired). In internal respiration the oxygen is transported by the blood from the lungs to the tissues as oxyhaemoglobin, as mentioned above. The red colour of deoxygenated haemoglobin is darker than the bright red colour of oxyhaemoglobin, and this is why arterial blood is always brighter than venous blood. In carbon monoxide poisoning, haemoglobin combines with carbon monoxide in coal gas to form cherry-red carboxyhaemoglobin. This combination is far more readily carried out than with oxygen, being about 210 times as fast.

The dissociation of oxyhaemoglobin is dependent upon temperature, electrolyte concentration and carbon dioxide concentration.

Carbon dioxide is carried by the blood both in the cells and plasma, but as with oxygen it exists in three main forms: (1) a small amount of carbonic acid (2) in combination with proteins, mainly haemoglobin, to form carbamino-bound carbon dioxide; and (3) as bicarbonate combined with either sodium or potassium cations.

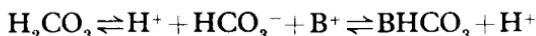
The amount of carbon dioxide dissolved in the blood is not large but it is important because any change in its concentration will cause the following reaction to shift from its equilibrium.



The enzyme carbonic anhydrase specifically catalyses the removal of CO_2 from H_2CO_3 ; however, the reaction is reversible,

as the formation of carbonic acid in the tissues from CO_2 and H_2O is accelerated by the enzyme.

It is estimated that in 24 h the lungs remove the equivalent of 20–40 litres of normal acid in the form of carbonic acid. This acidity is transported by the blood with hardly any variation in blood pH, since most of the carbonic acid formed is rapidly converted to bicarbonate.



(where B^+ represents cations in the blood, principally sodium, Na^+).

Acid-base balance

BUFFERING SYSTEM OF THE BLOOD

The normal venous blood pH is within the range 7.36 to 7.42. The buffer systems of the blood are so efficient that the pH of venous blood is more acid than arterial blood by only 0.01–0.03 pH units.

These blood buffer systems are the plasma proteins, haemoglobins, the carbonic acid–bicarbonate system and inorganic phosphates.

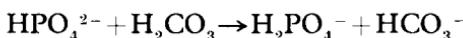
A small decrease in pH which occurs when CO_2 enters the venous blood at the tissues had the effect of altering the ratio of acid to base in all these buffer anion systems.

1. $\text{H}^+ + \text{Hb}^- \rightleftharpoons \text{HHb}$ Hb = haemoglobin
2. $\text{H}^+ + \text{Prot}^- \rightleftharpoons \text{HProt}$ Prot = protein
3. $\text{H}^+ + \text{HCO}_3^- \rightleftharpoons \text{H}_2\text{CO}_3$
4. $\text{H}^+ + \text{HPO}_4^{2-} \rightleftharpoons \text{H}_2\text{PO}_4^-$

Anions such as bicarbonate which can filter through the glomerulus enter the renal tubules accompanied by cations, especially sodium. Renal tubular cells can actively secrete H^+ , the net effect being exchange of one H^+ for one Na^+ from the glomerular filtrate. As the secreted H^+ comes from cellular carbonic acid, HCO_3^- is left behind, thus replenishing buffer anion. Some of the H^+ secreted by the tubular cells combines with filtered bicarbonate to form carbonic acid; this dissociates to water and CO_2 , which diffuses back into the tubular cells. Thus the acid is excreted, cations conserved and buffer anion replenished.

In the plasma the phosphate concentration is too low to be a quantitatively important buffer, its significance being in the urinary control of acidity. The urine normally contains little HCO_3^- since it is almost completely reabsorbed by the tubules.

In the plasma the ratio of $\text{H}_2\text{PO}_4^- : \text{HPO}_4^{2-}$ is about 1.5, but in the urine it is 9 : 1 and may rise as high as 50 : 1 when large amounts of acid have to be eliminated. This is because most of the phosphate in the glomerular filtrate is in the form of monohydrogen phosphate (HPO_4^{2-}). This accepts H^+ to become dihydrogen phosphate (H_2PO_4^-), which is excreted in the urine accompanied by cations and therefore conserves the valuable HCO_3^- .



Blood gas analysis

In the clinical laboratory when blood gases are requested, the estimations usually carried out are pH, pCO_2 and pO_2 . From these parameters we are able to obtain some idea of acid-base balance and respiration exchange.

There are a number of blood gas analysers on the market and some laboratories prefer one particular model to another, but on the whole they are all basically of the same design. When blood gases are requested, an arterial blood specimen collected into a pre-heparinized syringe anaerobically is placed into a thermos flask containing ice and water for transport to the clinical chemistry laboratory. The blood gas analyser contains three specific electrodes all equilibrated to 37°C. The pH electrode is standardized by using two different buffer solutions, e.g. pH 6.84 and pH 7.38. After standardization, the arterial blood is placed into the pH electrode and three measurements are made, all of which should agree to within 0.01 of a pH unit.

While the pH measurements are being made, the pCO_2 and pO_2 selective electrodes are being equilibrated with known gas mixtures for standardization. When adequate equilibration and standardization is obtained, the gas mixtures which have been 'flowing through' the electrodes are stopped and the pCO_2 and pO_2 content of the arterial blood measured. Three readings are again made and providing they agree between certain limits (depending upon the instrument), the mean value is obtained. From the pH and pCO_2 parameters, the standard bicarbonate and base excess can be calculated.

pH is a measure of hydrogen ion concentration, e.g. pH 7.3
 pCO_2 and pO_2 are measured in kilopascals, e.g. 6.0 kPa

Normal arterial pH 7.38–7.45

Normal arterial pCO_2 4.7–6.0 kPa

Normal arterial pO_2 11.3–14.0 kPa

THE ENDOCRINE SYSTEM

The endocrine system adjusts and correlates the various activities of the body, making adjustments to the body systems by the changing demands of the external and internal environment. This integration is brought about by the secretions of chemical substances directly into the blood stream to regulate the metabolic processes of various 'target' tissues. The organs manufacturing the chemical substances are the endocrine or ductless glands; their secretions, the hormones, have diverse metabolic processes, yet have several characteristics in common:

1. They are only required in very small amounts.
2. They are produced in an organ other than that in which they perform their action.
3. They are secreted into the blood stream prior to use.

Structurally, they are not always protein in nature; the known hormones include proteins or polypeptides or single amino acids and steroids.

A steroid is a hormone derived from the tetracyclic hydrocarbon perhydrocyclopentane phenanthrene as shown in *Figure 7.1*, examples of which are cortisol or testosterone. Non-steroid hormones are substances such as adrenaline or thyroxine (*Figure 7.2*).

The most widely recognized hormones are the ones commonly referred to as the 'sex hormones'. However, they represent only a very small fraction of the hormones of interest in clinical chemistry and medicine.

In addition to the ovary and testis, hormones are secreted by the adrenal cortex and medulla, anterior and posterior pituitary, parathyroid, thyroid and the islets of Langerhans in the pancreas. Calcitonin, a calcium-reducing hormone, is generally thought to be produced by cells in the thymus as well as the thyroid and parathyroid, so the thymus should be included in this list. Other tissues such as those of the gastrointestinal tract are known to be concerned with hormone production.

The pituitary gland (often described as the master gland or the leader of the endocrine orchestra) is reddish-grey in colour, roughly oval in shape and is situated in the base of the brain. The gland is attached to the brain by a stalk which is continuous with the part of the brain known as the hypothalamus. The pituitary has three lobes or parts:

1. The anterior lobe or pars anterior
 2. The mid-lobe or pars intermedia
 3. The posterior lobe or neurohypophysis
- } both of these are also called the adenohypophysis

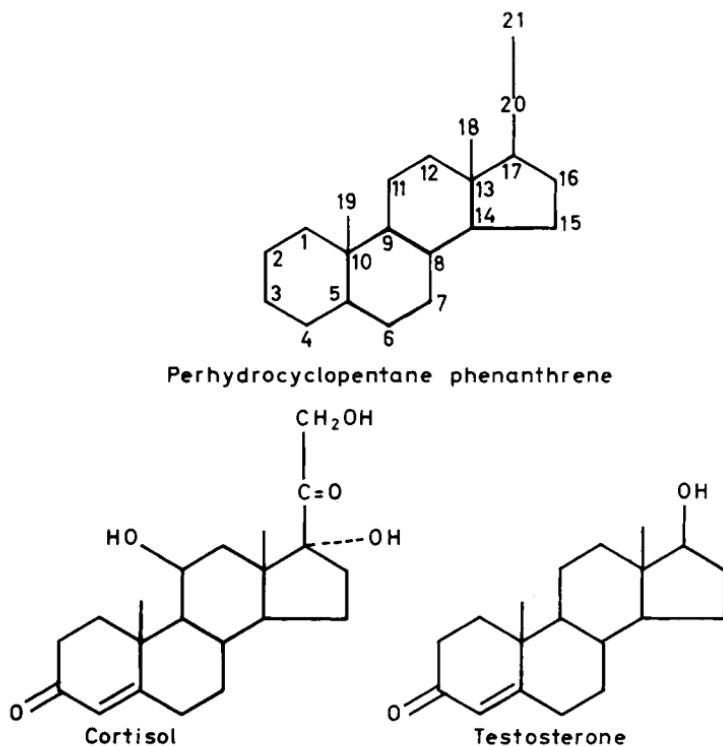


Figure 7.1. Steroid hormones

Anterior pituitary

The anterior pituitary secretes six separate trophic hormones ('trophic' is derived from the Greek word *trophein*, which means to nourish), each of which have a specific action in the endocrine system.

They are growth hormone (somatotrophic hormone), adrenocorticotropic hormone (ACTH), thyrotrophic stimulating hormone (TSH), prolactin (lactogenic hormone) and the two pituitary gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) or interstitial cell stimulating hormone. All these hormones except growth hormone do not generally affect

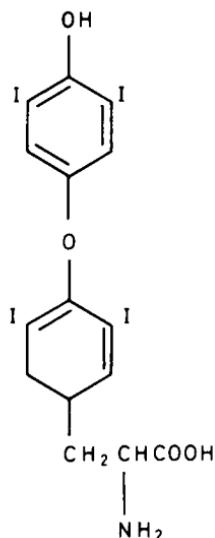
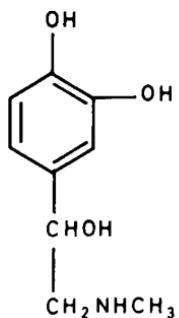


Figure 7.2. Non-steroid hormones

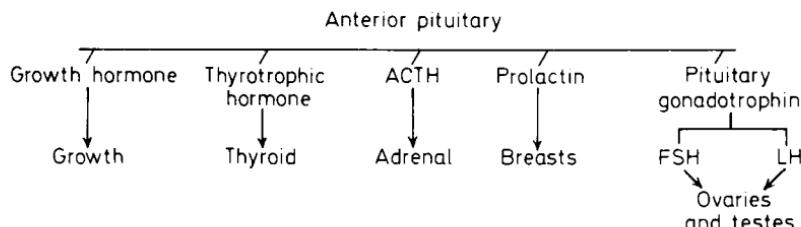


Figure 7.3. Scheme showing the various hormones secreted by the anterior lobe of the pituitary

metabolic processes, but normally act on other endocrine glands to stimulate the production or release of secondary hormones; for example, ACTH stimulates the release of cortisol. An increase in concentration of a secondary hormone, whether secreted by the endocrine glands or administered for medical reasons, will depress the secretion of the appropriate trophic hormone; for example, the administration of excess exogenous thyroxine will decrease the secretion of TSH.

Function of hormones

GROWTH HORMONE

Growth hormone acts directly on tissue to promote nitrogen retention and growth, probably through the control of protein metabolism. It is an antagonist to insulin, causing hypoglycaemia.

ADRENOCORTICOTROPHIN

ACTH acts directly on the adrenal cortex to predominantly stimulate the formation of glucocorticoids (hormones involved in carbohydrate metabolism) such as cortisol and adrenal androgens such as dehydroepiandrosterone (androgens stimulate male secondary sexual characteristics). It does also have some effect on aldosterone, a mineralocorticoid concerned with electrolyte balance.

THYROTROPHIC STIMULATING HORMONE

TSH acts on the thyroid gland to promote the production and release of the thyroid hormones, thyroxine and triiodothyronine. In physiological concentrations thyroxine is a protein anabolizer (build up) but in excess is a protein catabolizer (breakdown). Excess thyroxine lowers the blood cholesterol and stimulates the breakdown of bone.

PROLACTIN

In conjunction with other hormones, the lactogenic hormone is responsible for milk formation and mammary (breast) development. A hormone of the placenta also stimulates growth of the mammary glands but inhibits production of prolactin. After delivery of the baby the placental inhibition is removed, prolactin is secreted and the secretory cells are stimulated to secrete milk.

FOLLICLE STIMULATING HORMONE

FSH induces growth in the ovarian follicle during the first half of the menstrual cycle and together with LH stimulates production of oestrogens. In the male it stimulates the production of spermatozoa (male egg cells).

LUTEINIZING HORMONE

LH induces ovulation and stimulates the development of cells in the ruptured follicle to form structures called the corpora lutea, which in turn secrete a second ovarian hormone progesterone. In the male, LH stimulates the testes to secrete testosterone, the most potent androgen.

THE MID-LOBE OF THE PITUITARY

The intermediate lobe of the pituitary secretes a hormone called melanocyte stimulating hormone (MSH), which physiologically causes dispersion of the melanin pigment in the skin.

POSTERIOR PITUITARY

Two hormones are secreted by the posterior lobe, oxytocin or pitocin and anti-diuretic hormone or vasopressin. Principally, vasopressin increases the blood pressure and exerts its effect on the kidney tubules by controlling the reabsorption of water (anti-diuretic action), while oxytocin stimulates uterine contractions, particularly at the time of labour.

PINEAL GLAND

The pineal gland is a small body situated in the brain below the corpus collosum and posterior to the third ventricle. It is approximately 10 mm in length and its functions are somewhat vague, but it has been reported to be involved in aldosterone secretion.

THE PLACENTA

In addition to permitting the exchange of materials between the embryo and mother, the placenta is an endocrine organ of pregnancy producing steroid hormones such as oestrogens and progesterone and the non-steroid hormones, human chorionic gonadotrophin (HCG) and human placental lactogen (HPL). The presence of HCG in early pregnancy is used as the basis of the immunological pregnancy test, but this hormone can also be produced by certain tumours of the uterus and testes.

The adrenal glands

There are two adrenal glands which are situated on each side of the vertebral column on the posterior abdominal wall behind the peritoneum. They are in close association with the kidneys, each being attached to the upper pole of the kidney.

The glands are divided into two distinct parts which differ both

in anatomy and in function. The outer part is called the cortex and the inner the medulla.

FUNCTION OF THE ADRENAL CORTEX

It secretes a large number of steroids, of which there are three main groups:

1. The mineralocorticoids.
2. The glucocorticoids.
3. The sex hormones.

The sex hormones, androgens and oestrogens, influence both the development and maintenance of the secondary sex characteristics and the utilization and excretion of nitrogen. The glucocorticoids regulate carbohydrate metabolism, in that they are antagonistic to insulin, stimulating gluconeogenesis and decreasing carbohydrate utilization. They are also protein catabolizers. The mineralocorticoids, the most potent of which is aldosterone, have their main effect on the renal tubules by retaining sodium, chloride and water and exchanging potassium, thus maintaining their balance in the body.

The final stage of aldosterone secretion is, however, stimulated by the renin-angiotensin system, renin being produced in the juxtaglomerular cells of the kidney.

FUNCTION OF THE ADRENAL MEDULLA

The hormones of the adrenal medulla are adrenaline and nor-adrenaline. Both hormones prepare the body to withstand rapid physiological responses to emergencies such as cold, fatigue, shock, etc., by mobilizing the 'fight and flight' mechanisms of the sympathetic nervous system. In addition the two hormones induce metabolic effects such as glycogenolysis in the liver and skeletal muscle, mobilization of free fatty acids and stimulation of the metabolic rate.

Endocrine function tests

A variety of hormones (or other parameters) are estimated under this classification, ranging from simple hormones such as the steroid cortisol to more complex hormones like prolactin.

Cortisol, a steroid hormone directly related to the adrenal cortex, is estimated by fluorimeter or the now more recent technique of radioimmunoassay.

In the fluorimetric procedure, the steroid is extracted into a solvent dichloromethane, the solvent is shaken with ethanolic

sulphuric acid reagent and the resulting fluorescence read. A pure cortisol standard is similarly treated. From the two fluorimetric readings the level of cortisol in the unknown is calculated. A level below normal is usually found in Addison's disease (hypoadrenocortical activity) and elevated values can be found in Cushing's syndrome (hyperadrenocortical activity).

These tests usually give us some idea of the activity of the adrenal cortex and similar tests can be done to assess the activity of other hormones or endocrine glands. Although this may not be as straightforward as a cortisol estimation, most of the hormones can be estimated directly or indirectly; for example, vasopressin by osmolality, the placenta by HPL or oestriol determinations and the parathyroid gland by calcium and phosphate levels.

The thyroid gland

The thyroid gland consists of two lobes, one on each side of the trachea with a connecting portion, the isthmus, making the entire gland more or less H-shaped in appearance. In the adult the gland weighs about 25–30 g.

FUNCTION

The principle function of the gland is to secrete two hormones, thyroxine and triiodothyronine, and to store iodine (*see also* Thyrotrophic hormone).

Thyroid function tests

The commonest parameter in the investigation of thyroid dysfunction is the estimation of serum thyroxine. Serum thyroxine can now be measured by radioassay, either by an antigen–antibody reaction (radioimmunoassay) or by using a binding protein, e.g. thyroxine binding globulin (TBG), when it is called competitive protein binding assay. The antigen–antibody reaction is far better and more reliable providing of course you have a specific antibody to thyroxine.

Briefly, in the antigen–antibody reaction, a fixed and known amount of antibody is mixed with an excess of antigen (test thyroxine and radioactive thyroxine). After a fixed equilibration period, the antigen–antibody complex is separated from the excess radioactive thyroxine by using dextran-coated charcoal and the radioactive counts in the supernatant compared against standard thyroxine preparations similarly treated. From the counts obtained, the thyroxine level in the unknown is calculated.

Values below normal are found in hypothyroidism and values above normal are found in hyperthyroidism.

The parathyroid glands

There are four parathyroid glands which usually lie on the posterior aspect of the lobes of the thyroid gland and are arranged in two pairs.

FUNCTION

The principle function of the parathyroids is to secrete parathormone (PTH), a hormone responsible for the metabolism of calcium in the body, and therefore the maintenance of a constant level of plasma calcium. Besides raising the plasma calcium, PTH also lowers the plasma phosphate. Calcitonin, a hormone which lowers the plasma calcium, is also thought to come from the parathyroids but is primarily of thyroid origin.

WATER AND BODY FLUIDS

Water plays an important part in the structure and function of the body. In the healthy adult approximately 60 per cent of the body consists of water; even bone contains approximately one-third and adipose tissue contains a large amount in the connective tissue and in the spaces between the fat cells.

Body fluid is divided into functional compartments, separated from one another by cell membranes. Fluid within these cells is called intracellular fluid (ICF), and fluid outside, extracellular fluid (ECF). Extracellular fluid is further divided into intravascular fluid (plasma water) and interstitial fluid. Interstitial fluid is tissue fluid and includes cerebrospinal fluid (CSF), lymph, amniotic fluid, water in the gastrointestinal tract, aqueous humour, pleural fluid, etc. The ECF is the immediate environment of the organism and the aqueous medium which surrounds the tissue cells is the transport mechanism for nutrient and waste materials. Besides this service it also provides stability of physico-chemical conditions such as temperature, osmotic pressure and pH (*see Figure 7.4*).

MEASUREMENT OF BODY FLUIDS

The direct measurement of body fluids involves complicated procedures which are not practical in man. Indirect methods are therefore used which involve the administration of substances

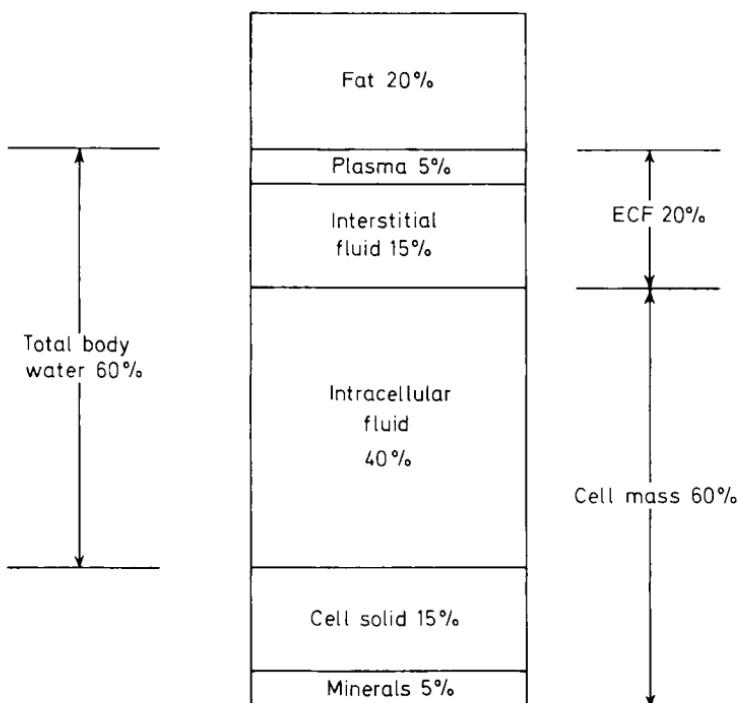


Figure 7.4. Distribution of different phases of body fluids as percentage of body weight

which are believed to diffuse into the various compartments of the body.

Water loss and balance

A normal subject's intake and output of water is usually in equilibrium. Any minor short-term discrepancies are balanced by an exchange of water from the intracellular pool. During one day a normal adult will lose about 2.6 litres of water, made up approximately as follows:

Sweat	100 ml
Lungs	500 ml
Skin	400 ml
Faeces	200 ml
Urine	1400 ml
	<hr/>
	2600 ml

The water intake is therefore approximately 2600 ml, of which about 2000 ml is from moist food and drinks; the remaining 600 ml is manufactured by oxidation during metabolic processes. There are approximately 45 litres of water in the body, 3 litres in the plasma, 12 litres in interstitial fluid and 30 litres in the intracellular fluid, therefore any short-term discrepancies in the water balance will be drawn from this water pool. All body fluids contain electrolytes.

The normal water balance of the body can be upset in various

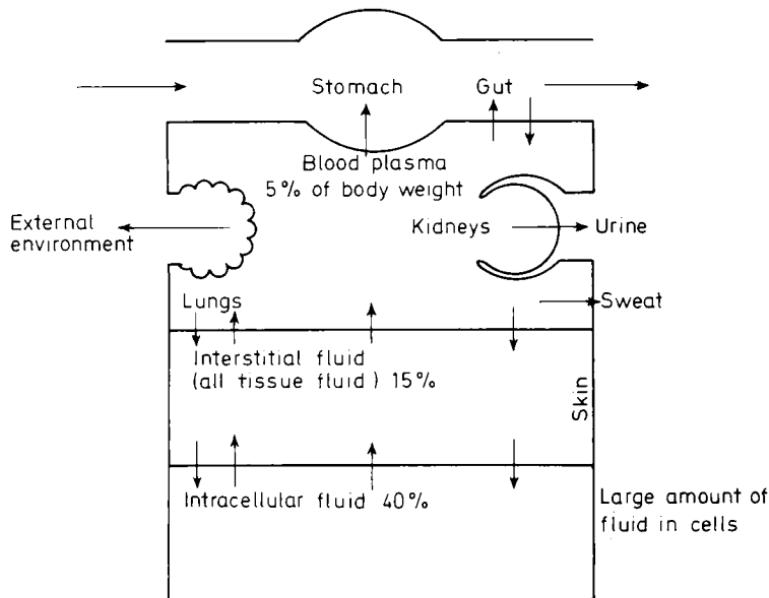


Figure 7.5. Interrelationship of body fluids

ways. Water depletion is the commonest and disturbances in both water and electrolytes will then occur. When fluid intake is restricted, the plasma sodium concentration rises above normal and the ECF becomes hypertonic. Water passes into the ECF (5 litres) from the ICF (30 litres) by osmosis, but the volume of the ICF is relatively little altered. Water intoxication occurs when large volumes of water are drunk, or when too much hypotonic fluid has been given intravenously, the reverse process will then occur (*Figure 7.5*). Salt depletion is the result of Na^+ (and Cl^-) loss from the ECF in excess of water loss, e.g. diarrhoea, vomiting. Potassium depletion also occurs; this may result from disturbed

function of the renal tubule, for example the effect produced by certain diuretic drugs.

Hormonal control of water output

The hormone involved in water regulation is ADH or anti-diuretic hormone released by the posterior pituitary. Its main purpose is to retain water, i.e. reduce the urinary output. This is done in the distal, convoluted and collecting tubules of the kidney. In the presence of ADH water flows back into the interstitial fluid which is hypertonic until equilibrium has been reached (osmosis). The production of the hormone is stimulated by changes in plasma osmolarity or blood volume, and is released by the hypothalamus, the posterior pituitary acting as a storage centre for ADH. When the blood is diluted by, for example, large amounts of water, the ADH secretion is inhibited, more water is excreted in the distal and collecting tubules and a large volume of dilute urine results. In contrast, when blood becomes more hypertonic and the osmolarity increases, ADH is stimulated, retains more water in the tubules, resulting in concentrated urine of small volume being excreted.

A number of drugs, including alcohol, will suppress ADH and thus increase urine flow.

ELECTROLYTE STUDIES

The direct or indirect measurement of body fluids cannot be carried out on a routine basis. The estimation of electrolytes are the usual alternative, since the control of sodium and water balance are so closely related. Sodium (Na^+) is widely distributed in the body; it is predominantly present outside the cell while potassium (K^+) is almost intracellular. Salt depletion is the result of Na^+ (and Cl^-) loss from extracellular fluid as in the case of diarrhoea and vomiting. Potassium depletion also occurs from disturbed function of the renal tubules produced by diuretic therapy. Chronic potassium depletion can be accompanied by a high plasma bicarbonate and disturbed renal tubular function can also be assessed by plasma urea levels.

Electrolyte requests can therefore involve the clinical chemistry department in the estimation of Na^+ , K^+ , HCO_3^- and urea levels. Chlorides are not usually estimated now except in very special circumstances.

A specimen of heparinized blood is collected with as little stasis as possible and quickly sent to the laboratory to separate the cells from the plasma. Red cells contain a lot of potassium and rapid

separation of the cells from the plasma prevents the leakage of potassium into the plasma. Haemolysis must also be avoided.

Sodium and potassium are estimated by flame emission spectroscopy (flame photometry) as described in Chapter 6. Urea levels can be determined by any of the methods described in Chapter 10, while plasma bicarbonate can be estimated on the Auto-Analyser or by the following manual method.

PLASMA BICARBONATE

The Harleco CO₂ apparatus is a convenient way of measuring carbon dioxide content (bicarbonate) in approximately one minute. The sample is placed into the reaction vessel, and after acidifying with lactic acid the CO₂ liberated is measured in the CO₂ syringe. A sodium bicarbonate standard is used to calibrate the apparatus to the varying atmospheric temperature and pressure under which the test is being performed.

The normal

plasma sodium	ranges from	135–146	mmol/l
plasma potassium	ranges from	3.5–5.2	mmol/l
plasma bicarbonate	ranges from	23–30	mmol/l
plasma urea	ranges from	3.3–6.7 (20–40)	mmol/l mg/100 ml)

8

Gastric and Pancreatic Function Tests

It is important before discussing tests concerned with the digestive and pancreatic system to have an understanding of the nutritional needs of the body. The food we eat is composed largely of animal and plant materials or of products derived from them, and includes the essentials of the diet such as carbohydrates, proteins, fats, vitamins, mineral salts, water and roughage. If the cells of the body are to function efficiently these essential nutrients must be available in the correct proportions.

Most foodstuffs are *ingested* in forms which are unavailable to the body, since they cannot be absorbed from the gastrointestinal tract until broken down to basic units. The breakdown of the naturally occurring foodstuffs into absorbable units is the process of *digestion*, helped by the *secretion* of enzymes and juices in the tract. The products of digestion, along with fluids, minerals and vitamins, cross the mucosa of the intestines and enter the lymph or blood, a process known as *absorption*. Certain constituents of the diet which cannot be digested and absorbed are excreted from the bowel in the form of faeces, a process of *elimination*. These five processes—ingestion, secretion, digestion, absorption and elimination—are all activities of the digestive system.

Food and absorption

The diet contains various foods which are classified according to their chemical structure and physical properties.

CARBOHYDRATES

Carbohydrates are found in sugar, jams, cereals, bread, potatoes, fruit, vegetables and milk. They consist of carbon, hydrogen and oxygen, the ratio of hydrogen to oxygen being the same as that in water, the exception being in the deoxy (one less oxygen) sugars.

The three main dietary carbohydrates are monosaccharides, disaccharides and polysaccharides.

Function

In the body the carbohydrates are utilized to provide energy and heat, and help to maintain the normal blood glucose level. They are stored as glycogen in the liver and muscle, and any excess remaining is converted to fat and stored in the fat depots.

There are three main groups:

1. *Monosaccharides*—these are simple sugars which can be directly absorbed from the small intestine.

Hexoses ($C_6H_{12}O_6$)—the main one is glucose which plays an important role in supplying energy to the cells. Fructose and galactose are further examples.

Pentoses ($C_5H_{10}O_5$)—are simple sugars, widely distributed in plant material such as fruits and gums, the chief ones being ribose, xylose and arabinose. All animal cells contain ribose and deoxyribose as constituents of nucleic acids.

2. *Disaccharides* ($C_{12}H_{22}O_{11}$)—these cannot be absorbed directly but must be hydrolysed to monosaccharides by their appropriate enzyme (disaccharidase) before absorption can take place. The enzyme is present on the surface of the intestinal cell, where the hydrolysis takes place. Examples of disaccharides are sucrose, found in cane sugar; lactose, found in milk sugar; and maltose, an intermediate product in the breakdown of starch to glucose. From Table 8.1 it can be seen that the appropriate disaccharide is hydrolysed by its specific enzyme and the resulting monosaccharide is then absorbed in the usual way.

Table 8.1

<i>Disaccharide</i>	<i>Disaccharidase</i>	<i>Monosaccharide</i>
lactose	lactase	galactose + glucose
maltose	maltase	glucose + glucose
sucrose	sucrase	fructose + glucose

3. *Polysaccharides* ($C_6H_{10}O_5)_x$ —their structure is known and a considerable amount of digestion is required before they can be absorbed. The most important polysaccharides are starch, glycogen, inulin and cellulose. Starch and glycogen are

hydrolysed by amylase to mainly maltose and glucose; maltase finally breaks it down to glucose before it can be utilized by the body.

Thus it can be seen that although the diet may contain adequate carbohydrate, absorption will depend upon normal pancreatic function (amylase), the presence of disaccharidases and normal mucosal cells for transport across the intestinal wall.

PROTEINS AND NITROGENOUS FOODS

Proteins are the chief nitrogenous constituents of the tissues of the body and of the food we eat. They are obtained chiefly from meats, eggs, milk, cheese, fish, cereals and certain vegetables such as peas and beans. Proteins are complex compounds containing carbon, hydrogen, oxygen, nitrogen, sulphur and phosphorus. Before they can be absorbed they are broken down by proteolytic enzymes to their simplest constituents, the amino acids, because it is only as such that protein can be absorbed into the venous capillaries of the villi for transportation to the liver. Normal absorption will therefore depend upon normal pancreatic function to provide the proteolytic enzymes and normal mucosal cells for the transport mechanism. Amino acids are divided into two groups, essential and non-essential. Essential amino acids are those which are not synthesized by the body out of the materials ordinarily available at a speed commensurate with normal growth, e.g. valine, methionine, threonine, leucine, isoleucine, phenylalanine, tryptophan and lysine. They are essential for the repair of body tissue, the maintenance of the osmotic equilibrium between blood and tissue fluids and for providing energy and heat, when there is an insufficient supply of carbohydrate. Non-essential amino acids are those readily synthesized from —NH₂ groups and simple carbon compounds, e.g. glycine, tyrosine, alanine, glutamine, serine, etc.

Proteins are usually classified as (a) simple or (b) conjugated. The simple proteins on hydrolysis yield only amino acids and include albumin, globulin, glutelins and gliadins (plant proteins), scleroproteins, protamines and histones, whereas a conjugated protein is a protein to which is attached a non-protein substance known as a prosthetic group.

<i>Conjugated protein</i>	<i>Prosthetic groups</i>	<i>Example</i>
Chromoproteins	Haem	Haemoglobin
Lipoproteins	Lipid	Chylomicrons
Glycoproteins	Carbohydrate	Pituitary gonadotrophins
Nucleoproteins	Nucleic acid	Viruses

This classification is arbitrary, but for descriptive purposes is quite convenient.

FATS (lipids)

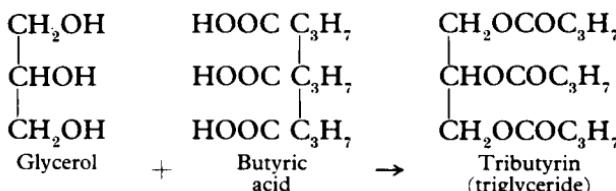
Fat in the diet is important not only for its high energy, but because it contains the fat-soluble vitamins A, D, E and K, and certain essential fatty acids, linoleic and linolenic acid. Fat is also necessary for nerve sheaths, cholesterol in the bile, and to support and cushion certain organs in the body, e.g. the kidneys and eyes.

Dietary fat is digested by the action of pancreatic lipase, partially to glycerol and fatty acids and partially to split products mono-glycerides and diglycerides. With the aid of bile salts, these products of digestion enter the mucosal cells of the small intestine where the fats are completely digested by the action of intestinal lipase. The lipid material is eventually converted to chylomicrons, a material which can easily pass through the intestinal cell wall into the lymphatic system.

It can therefore be seen that normal fat absorption will depend upon the presence of bile salts, pancreatic and intestinal lipase and normal intestinal mucosa for the formation of chylomicrons.

Chylomicrons are triglycerides, cholesterol, cholesterol esters and phospholipids coated with a layer of lipoprotein.

Lipids are divided into two groups, animal and vegetable. Animal fat is obtained chiefly from dairy produce such as milk, butter and cheese, from eggs, meat and bacon and from oily fish such as cod, halibut and herring. Vegetable fat is found in margarine, olive oil, groundnuts and hazel nuts.



Lipids can be divided structurally into (a) simple or (b) compound lipids. The simple lipids are either (1) fats, which are esters of glycerol with fatty acids, e.g. the mono-, di- and triglycerides (the major portion of human depot fat is made up of triglycerides, the minor portion being the mono- and diglycerides); or (2) waxes, which are esters of alcohols other than glycerol, e.g. beeswax, which is an ester of palmitic acid with myricyl alcohol. The compound lipids are (1) the phospholipids (lecithin, cephalin, sphingomyelin) and (2) the glycolipids (cerebrosides, which occur in large amounts in brain tissue).

Cholesterol is a sterol and not a lipid, but it and its esters, being lipid-soluble, are usually considered along with the lipids.

VITAMINS

Vitamins are organic compounds essential for life, health and growth. They are not eaten as such in the diet, but are widely dispersed in the food we eat. Their absence causes the so-called 'deficiency' diseases. They are divided into two groups, fat-soluble and water-soluble vitamins. Vitamins A, D, E and K are fat-soluble, while the water-soluble ones are the B group vitamins and vitamin C.

For the absorption of the fat-soluble vitamins it is essential to have a normal bile secretion while in the absorption of B_{12} the intrinsic factor secreted by the stomach is essential. In the presence of intrinsic factor a complex is formed with B_{12} which can bind to the intestinal wall where it is absorbed. Most of the other water-soluble vitamins are absorbed in the upper small intestine.

Fat-soluble vitamins

VITAMIN A

This vitamin is essential for normal mucopolysaccharide synthesis, and a deficiency causes drying up of mucus-secreting epithelium. Rhodopsin, the retinal pigment, which is a protein (opsin) combined with a derivative of vitamin A, is necessary for vision in dim light. It is found in fish oils and animal fats. It can be formed in the body from β -carotene, of which the main dietary sources are green vegetables and carrots.

VITAMIN D (calciferol)

Vitamin D is necessary for normal calcium absorption. A deficiency causes rickets in children and osteomalacia in adults, hence the name 'antirachitic' vitamin. Like vitamin A, it is found

in fish oils and animal fats. In the skin, the precursor or pro-vitamin, 7-dehydrocholesterol can be converted into active vitamin D₃ (cholecalciferol) by ultraviolet light.

VITAMIN E (tocopherol)

In experimental animals a deficiency of vitamin E causes failure in reproduction, but there is no proof that the same holds good for man. Its function is not clearly understood, but the sources of this vitamin are peanuts, lettuce, wheat germ, oil and dairy produce.

VITAMIN K

Vitamin K is necessary for prothrombin synthesis in the liver, which in turn is essential for blood coagulation. Other coagulation factors also need vitamin K for their synthesis. It is found in fish, green vegetables, liver and spinach. It cannot be synthesized by man, but it can be formed by the bacterial flora of the colon.

Water-soluble vitamins

The B complex—

VITAMIN B₁ (thiamine or aneurine)

Thiamine pyrophosphate is an essential coenzyme in the enzyme system needed for the decarboxylation of α -oxoacids, one of the reactions involved being the conversion of pyruvate to acetyl coenzyme A. Besides being involved in carbohydrate metabolism, it regulates the normal functioning of the nervous system.

Thiamine is present in many plants, and is in particularly high concentration in wheat germ, oatmeal and yeast. Adequate amounts are present in a normal diet, but the deficiency syndrome is still prevalent in rice-eating areas.

VITAMIN B₂ (riboflavine)

This vitamin is concerned with biological oxidation systems, necessary for growth and catabolism in all tissues in man and animals. It is a component of flavin adenine dinucleotide (FAD), a coenzyme involved in oxidation-reduction reactions. It is found in yeast and vegetables, such as beans and peas, and in wheat, milk, cheese, eggs, liver and kidney.

NICOTINAMIDE

Nicotinamide is necessary for the metabolism of carbohydrates, being an important constituent in the coenzyme, nicotinamide

adenine dinucleotide (NAD) and its phosphate (NADP). Besides being involved in carbohydrate metabolism, it is essential for the normal function of the gastrointestinal tract, and for satisfactory function of the nervous system. It can be formed in the body from nicotinic acid. Both substances are plentiful in animal and plant foods. Nicotinic acid is present in high concentrations in yeast, bran, fresh liver and fish.

VITAMIN B₆ (pyridoxine)

In the same way as thiamine and nicotinamide, pyridoxine can also be phosphorylated to yield a coenzyme, pyridoxal phosphate, which is a coenzyme for the amino transferases (transaminases) and for the decarboxylation of amino acids. Pyridoxine is found in egg-yolk, beans, peas, yeast and meat. Dietary deficiency is very rare, but the anti-tuberculous drug isoniazid produces a picture of pyridoxine deficiency, probably by competition with it in metabolic pathways.

PANTOTHENIC ACID

This B group vitamin is a component of coenzyme A which is essential for carbohydrate and fat metabolism, promoting fatty acid oxidation and the oxidation of pyruvate. It is also used in detoxication mechanisms involving acetylation. It is widely distributed in foodstuffs such as liver, kidney, meat, wheat, bran and peas.

BIOTIN

Biotin is essential for the growth of many micro-organisms and is a coenzyme in carboxylation reactions (i.e. the conversion of pyruvate to oxaloacetate). It is another vitamin belonging to the B group which is found in eggs. A high concentration of uncooked egg-white is toxic to rats and man, causing loss of hair and dermatitis. This is because the protein avidin, present in egg-white, combines with biotin and prevents its absorption.

FOLIC ACID (pteroylglutamic acid)

Folic acid plays an important role in cellular metabolism, especially in the transfer of one carbon unit such as the aldehyde group —CHO. It is necessary for the normal maturation of erythrocytes. It is also included in the B group, and is found in green vegetables and some meats. It is easily destroyed in cooking but a dietary deficiency is very rare.

VITAMIN B₁₂

Deficiency of B₁₂, like folic acid, causes megaloblastic anaemia, but unlike that of folate it can result also in sub-acute combined degeneration of the spinal cord. Cyanocobalamin is found in beef, kidney and liver. Fresh milk and dairy produce contain a small amount. A dietary deficiency is very rare, but absorption of the vitamin depends upon its combination with intrinsic factor secreted by the stomach.

VITAMIN C

Ascorbic acid is necessary for erythropoiesis, for healthy bones and teeth, for normal collagen formation and for the maintenance of the strength of the walls of the blood capillaries. It is found in fresh fruit, especially blackcurrants and citrus fruits. Green vegetables also contain vitamin C.

MINERAL SALTS

Mineral salts are necessary in the diet for all body processes, and although required in varying quantities, the amount is usually small.

Calcium and magnesium are absorbed in the small intestine with the help of vitamin D, and normal fat absorption. Sodium and potassium, which occur as chlorides and phosphates in body tissues and fluids, are absorbed in the small intestine by an active process in the same way as they are in the renal tubule. Sodium-potassium exchange is also regulated by the hormone aldosterone.

Iron present in haemoglobin is absorbed in the duodenum and upper jejunum, the absorption being stimulated by the presence of anaemia. All the minerals are present in adequate amounts in a normal diet.

Calcium

Calcium is the chief constituent of teeth and bones and is absorbed in the small intestine with the help of vitamin D. It plays an important part in the coagulation of blood, in the contraction of muscles, and in the permeability of cell membranes. It is found principally in milk, cheese, eggs and green vegetables.

Phosphate

Phosphate combines with calcium in the formation of bone and teeth and helps to maintain the normal composition of body fluid.

Bone salt is mainly hydroxy apatite $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{Ca}(\text{OH})_2$. Phosphate is found in cheese, liver, kidney and oatmeal.

Sodium

Sodium is present mainly in tissue fluids and therefore plays an important part in cell activity and in the fluid balance of the body. The amount of sodium in our normal diet far exceeds the requirements of the body. It is found in fish, meat, eggs, milk and in table salt, and especially in prepared foods such as bacon and sausages.

Potassium

Potassium is an essential constituent of all cells, and is necessary also for the normal activity of cardiac, skeletal and smooth muscle. It is present mainly in cells. It is widely distributed in all foods.

Iron

Iron is necessary for the formation of the cytochromes which are involved in tissue oxidation and is of course essential for the formation of haemoglobin. It is found in liver, kidney, beef and green vegetables. About 12 mg are considered to be the average daily requirement.

Iodine

Iodine is essential for the formation of the thyroid hormones, thyroxine and triiodothyronine. It is found in salt water, fish and in vegetables grown in soil containing iodine.

WATER

Water has many functions, some of which are the formation of urine and faeces, transport of water-soluble substances, e.g. vitamins and the dilution of waste products and poisonous substances in the body. It is absorbed passively as in the renal tubule along an osmotic gradient created by the absorption of sodium and other solutes. Any water remaining for absorption after passing through the osmotic gradient, will be absorbed in the colon. Many foods contain at least 75 per cent by weight of water. Some water is taken also in the diet as liquid and some is derived from the oxidation of foods.

ROUGHAGE

Roughage gives bulk to the diet and stimulates peristalsis and bowel movement and is the undigested part of the diet.

In addition to providing all the above-mentioned components the diet must altogether provide a sufficient number of calories for the energy needs of the body, otherwise the body's protoplasm is utilized to provide energy, the body weight falls and the tissues waste, as may happen during a period of illness.

ALIMENTARY TRACT

The alimentary tract (*Figure 8.1*) is a convoluted tube where the food is ingested, digested, absorbed and eliminated. It extends from the mouth to the anus and consists of the mouth, pharynx, oesophagus, stomach, small intestine and large intestine, including the rectum and anus. Various secretions are poured into the

- R.L. = Right lung
- L.L. = Left lung
- H. = Heart
- L. = Liver
- S. = Stomach
- P. = Pancreas
- D. = Duodenum
- S.I. = Small intestine
- A.C. = Ascending colon
- T.C. = Transverse colon
- D.C. = Descending colon
- B. = Bladder
- R. = Rectum

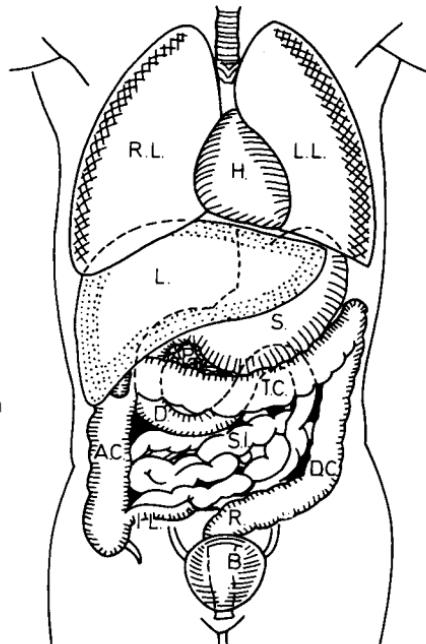


Figure 8.1. Diagram showing the position of the main organs of the body, including the alimentary tract

alimentary tract, some by the lining membranes of the organs mentioned and some by glands situated outside the tract. The accessory organs are the salivary glands, pancreas, liver and biliary tract, their secretions being poured into the tract through various ducts.

Digestion

Digestion starts in the mouth where the lips, cheeks, muscles and teeth are all involved in the ingestion and mastication of food. The food is moistened by saliva, the secretion from the salivary glands, to form a bolus or soft mass of food ready for deglutition or swallowing. The three pairs of salivary glands, two parotid, two submandibular and two sublingual, secrete about 1500 ml of serous fluid rich in enzyme into the oral cavity per day, the only stimulus for its secretion being the sight and smell of food. Saliva consists of water, mucin, mineral salts and the enzyme α -amylase or ptyalin, which starts the digestion of starch to maltose.

This digestive action is of secondary nature since food remains in the mouth for only a short time. Mucin, a glycoprotein, lubricates the food, and the saliva, besides keeping the mouth and teeth clean, may also have some antibacterial action. When mastication is complete and the bolus formed it is pushed backward into the pharynx by the upward movement of the tongue. The pharynx, the cavity between the mouth and tongue, is divided into three parts, the naso-pharynx, the oro-pharynx and the laryngopharynx, which is then connected to the oesophagus, the narrowest part of the tract. The bolus is then carried down the gullet or oesophagus by peristalsis, which propels the food through the cardiac orifice into the stomach. The stomach is a J-shaped dilated portion of the alimentary tract; its size and shape varies with each individual and with its contents. From *Figure 8.2* it can be seen that it is divided into the fundus, body and pyloric antrum. It has three layers of muscle tissue: an outer longitudinal layer, a layer with circular fibres and an inner layer of oblique fibres. This arrangement allows for the churning motion characteristic of gastric activity.

The stomach is lined with a mucosal layer which is fairly flexible, called the gastric mucosa. When the stomach is in a state of contraction, the mucous membrane lining is thrown into longitudinal folds or rugae, and when the stomach is dilated the lining is smooth and velvety. The gastric mucosa contains many deep glands; in the pyloric and cardiac region the glands secrete mucus, while in the fundus and body of the stomach the glands contain

the parietal or oxytic cells, which secrete hydrochloric acid, and chief or peptic cells which secrete pepsinogens. Besides these secretions, gastric juice will also contain the intrinsic factor secreted by the gastric mucosa, gastric lipase and possibly rennin. The hormone *gastrin*, normally produced by the pyloric glands, in response to the presence of food, is carried by the blood stream to the stomach, where it stimulates the secretion of gastric acid and pepsin. The upper two-thirds of the stomach act as a reservoir, in that it holds the food for a sufficient period of time to allow

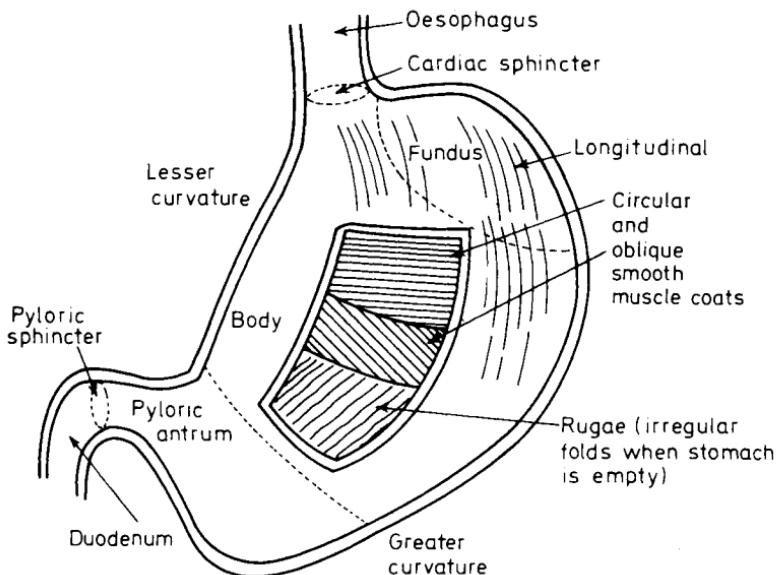


Figure 8.2. The stomach

thorough mixing with the gastric juice, so that the HCl and pepsinogen can act on the constituents of the food. The time the food is held will depend upon its nature and consistency, but it is normally for 2 to $2\frac{1}{2}$ h. The lower one-third of the stomach churns the food to a semi-fluid mass of uniform consistency called chyme, which is slowly passed through into the duodenum in small jets, when the pyloric sphincter relaxes and the muscular walls of the stomach contract. Although the main digestive processes begin in the duodenum, food will be digested by salivary amylase until it is stopped by the concentration of hydrochloric acid, while proteins are broken down to proteases and peptones by the action of pepsin. Hydrochloric acid also limits the growth of micro-organisms entering the stomach.

The duodenum is part of the small intestine, which starts at the pyloric sphincter and leads into the large intestine at the ileo-colic valve, the other two sections being the jejunum and ileum. The duodenum is the C-shaped section of the gut, which goes round the head of the pancreas and contains the ampulla of the bile duct where the pancreatic duct joins the bile duct, the opening of which is controlled by the sphincter of Oddi. It is through the sphincter of Oddi that the pancreatic juice and bile enter the duodenum to continue the digestion of food. The partially digested food is gradually transferred from the duodenum into the second part of the small intestine, the jejunum and then into the ileum, the latter part of the small intestine, by muscular contractions

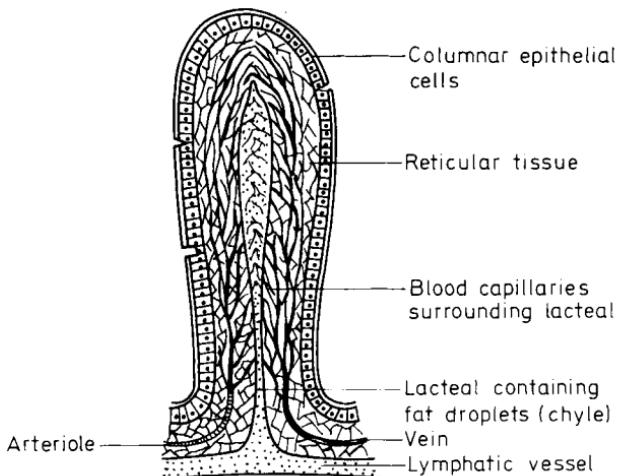


Figure 8.3. A diagrammatic illustration of a villus

known as peristalsis. The ileo-colic valve controls the flow of the contents of the ileum into the large intestine and also prevents the backflow of contents from the large intestine. During its passage through the small intestine the acid chyme comes in contact first with the alkaline pancreatic juice and bile, then with intestinal juice, secreted by glands in the mucosa of small intestine induced by the hormone enterocrinin. It is this juice which completes the digestion of nutritional materials after the pancreatic juice and bile have had their effect. Carbohydrates, proteins and fats in their undigested or partly digested state cannot pass through the mucosa of the gut into the body, but in the form of glucose, amino acids and fatty acids they can permeate through the minute projections in the wall of the small intestine, called villi (see Figure 8.3). The

villi create an enormous surface area for absorption which is said to be about five times that of the skin surface of the body. Glucose and amino acids are absorbed into the blood capillaries, fatty acids and glycerol are absorbed into the lacteals. Other nutritional materials such as vitamins, minerals and water, are absorbed from the small intestine into the blood capillaries. The ileum leads into the large intestine, the terminal part of the alimentary tract which commences at the caecum and terminates at the anal canal. It is divided into the caecum, the ascending, transverse, descending and pelvic colons, rectum and anal canal. When the contents of the ileum pass through the ileocolic valve into the caecum they are fluid, even though some water has been absorbed in the small intestine. Water absorption therefore continues in the colon, along with the absorption of glucose, some minerals and drugs. Mucin and certain minerals such as calcium, copper, iron, in excess of body needs are secreted.

Cellulose is broken down by bacterial action, mainly in the caecum, and micro-organisms present in the large intestine have the ability to synthesize some vitamins, for example vitamins D and K. The elimination of all the waste solidified matter, called faeces, is carried out by the muscular action of the rectum.

INTESTINAL JUICE OR SUCCUS ENTERICUS

This is the digestive juice which completes the digestion of the nutritional materials. It is difficult to obtain uncontaminated juice but it is basically alkaline in pH and consists of water, mineral salts, mucus and the enzymes, enterokinase, peptidases, lipase, sucrase, maltase and lactase.

Accessory organs and juices in digestion

Besides the salivary glands, the other organs are the pancreas, liver and biliary tract.

THE GALL BLADDER AND BILE DUCT

Bile from the liver flows through the right and left hepatic ducts to join up to form the hepatic duct (*see Figure 8.4*). The hepatic duct passes downwards where it is joined at an acute angle by the cystic duct from the gall bladder. The cystic and hepatic ducts join together to form the bile duct, which passes downwards to the head of the pancreas to be joined by the pancreatic duct at the ampulla of the bile duct. The two together open into the duodenum at the sphincter of Oddi.

The gall bladder is a pear-shaped sac, which acts as a reservoir

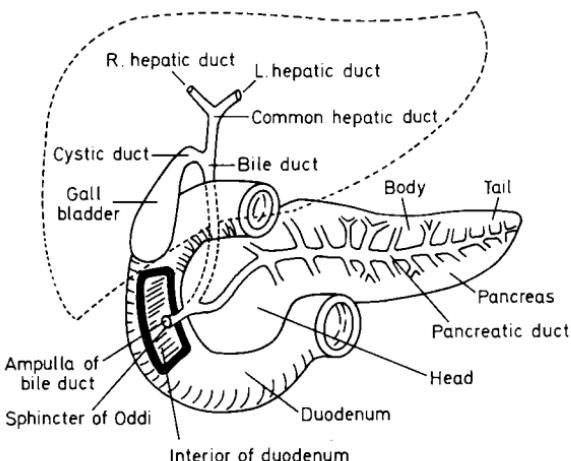


Figure 8.4. The gall bladder and pancreas with outlines of the liver

for the bile before it is discharged into the duodenum. It is divided into a fundus, body and neck.

Functions of the gall bladder

Besides being a reservoir for bile it concentrates the bile and regulates its discharge into the duodenum. During its stay in the gall bladder the bile is concentrated by absorption of water and at the same time its viscosity is increased by the secretion of mucus from the epithelial cells of the wall of the gall bladder. The contraction of the muscular walls of the gall bladder and the relaxation of its sphincter is initiated by the hormone cholecystokin. Cholecystokin is secreted by the mucosa of the upper intestine in response to the presence of food, mainly meats and fats, in the duodenum.

THE PANCREAS

The adult pancreas is a pale yellowish gland about 20–25 cm long and weighs between 60 and 160 g. It is situated in the epigastric and the left hypochondriac region of the abdominal cavity. It is divided into a broad head which lies in the curve of the duodenum, a body which lies behind the body of the stomach, and a narrow tail which lies in front of the left kidney and which just reaches the spleen. It is a dual organ, having both endocrine (internal) and exocrine (external) secretions or functions.

Exocrine functions

The gland consists of a number of lobules which are made up of

small alveoli lined with secretory cells. The secretion from each cell is drained by a tiny duct which unites with other ducts and joins the main pancreatic duct. The main duct passes the whole length of the organ to open into the duodenum about 10 cm from the pyloric sphincter. Just before entering the duodenum the main duct joins with the bile duct at the ampulla of the bile duct and discharges the secretion into the duodenum through the sphincter of Oddi. The secretion is formed by the cells of the pancreatic acini and cells of the intralobular ducts and its composition is divided into organic and inorganic constituents, both of which assist in the digestive processes of the tract.

Pancreatic juice

The inorganic constituent is the alkaline juice of pH 7.5–8.0 or higher, which is mainly bicarbonate used to render the acid chyme entering the duodenum alkaline. The chloride content rises and falls inversely with the bicarbonate level, while the sodium and potassium levels are almost identical with the levels found in plasma. The alkaline reaction is also necessary for the effective function of the pancreatic enzymes which are the organic constituents of the juice. Some of the pancreatic enzymes are trypsin, chymotrypsin, carboxypeptidase, amylase and lipase. Trypsinogen, an inactive enzyme, is activated to trypsin by enterokinase, an enzyme secreted by the duodenal mucosa. Trypsin also converts chymotrypsinogen into chymotrypsin. This powerful protein-splitting enzyme (proteolytic) eventually converts all the proteins and proteoses into peptides. Carboxypeptidase, which is secreted as procarboxypeptidase but activated by trypsin, splits peptide bonds located at the lower end of polypeptide chains into free amino acids when as such they are absorbed by the intestinal mucosa. Pancreatic amylase, an enzyme identical with salivary amylase, converts all starches not affected by the salivary amylase into maltose and maltase completes the breakdown to glucose. Some pancreatic amylase is absorbed into the blood and excreted into the urine, therefore possessing diastatic properties. Lipase is a powerful enzyme which hydrolyses fat into a mixture of lower glycerides and fatty acids. Bile salts emulsify the fats and therefore assist in the breakdown.

Secretion of pancreatic juice

The sight, smell and thought of food stimulates the production of pancreatic juice via the vagus, but this nervous phase is a minor one compared to the humoral control. The presence of acid chyme

from the stomach activates the duodenum to produce secretin, a hormone which stimulates the production by the pancreas of a thin, watery fluid, high in bicarbonate but low in enzymes. The upper jejunum produces a hormone, pancreozymin, which stimulates the production of a viscous fluid low in bicarbonate but high in enzyme content. While the acid chyme stimulates the secretion of secretin, the products of digestion, particular protein, are the stimulus for pancreozymin production.

Endocrine function

The pancreatic islet tissue, first described by Langerhans, consists of a large number of discrete cells widely distributed throughout the pancreas. The islet tissue cells function independently of the acini and are of two types, α -cells and β -cells, each producing a hormone which affects carbohydrate metabolism. The islet of Langerhans makes up 1–2 per cent of the pancreatic tissue. The α -cells (25 per cent of islets) secrete glucagon directly into the circulating blood to promote glycogen breakdown (glycogenolysis), which increases the blood glucose and utilization of glucose by the tissues. Besides the rapid mobilization of hepatic glucose it also utilizes to a lesser extent fatty acids from adipose tissue. The effects of glucagon are overshadowed by the β -cells (50–75 per cent of islets) which also secrete insulin directly into the circulating blood. Insulin increases the permeability of cells to glucose, accelerates carbohydrate oxidation, and at the same time gluconeogenesis (formation of glucose or glycogen from non-carbohydrate sources) is depressed and the conversion of glucose to fat is increased. Glucagon is therefore a hyperglycaemic agent (elevates blood glucose), while insulin is the hypoglycaemic hormone. In spite of the irregular intake and uneven utilization of carbohydrates, the level of blood and tissue glucose is maintained at a fairly constant level by these two hormones, the most important of which is insulin. Other hormones concerned in the regulation of blood glucose but not secreted by the pancreas are: cortisol (adrenal cortex), adrenaline (adrenal medulla), thyroxine (thyroid) and ACTH (anterior pituitary).

Diabetes mellitus in man is due to a deficiency of insulin, which is characterized by hyperglycaemia and glycosuria (glucose in the urine), while insulinoma (hypoglycaemia) is caused by a functioning β -cell tumour of the islets producing too much insulin.

GASTRIC FUNCTION TESTS

The stomach is an organ of digestion and gastric juice is secreted by the cells in the walls of the stomach in response to (1) gastrin, a hormone secreted by the gastric antrum mucosa when food is in the stomach; (2) psychic factors (sight, taste or smell of food); and (3) the presence of some products of digestion in the intestine. One of the commonest investigations, until quite recently, for gastric function, was the collection of gastric juice before and after drinking a pint of oatmeal gruel. This has now been replaced by histamine and pentagastrin stimulation tests. This direct method of gastric analysis is carried out by removing the gastric contents by intubation, but there is an indirect method, which involves the use of diagnex blue and collecting urine samples.

Composition of normal gastric juice

Volume 2–4 litres per day, but there is always a small quantity (about 50 ml) present in the stomach even when it contains no food.

Appearance colourless-grey fluid.

pH 1.0–1.5 due to the HCl produced by the parietal cells.

Water content about 97–99 per cent.

Inorganic constituents sodium, potassium, chloride, calcium, magnesium, phosphate and sulphate.

Organic constituents pepsins, mucin, intrinsic factor, rennin (said to be absent from stomach of the adult), gastric lipase, albumin and globulin.

The composition may vary considerably, depending upon the physiological state of the stomach.

Stimulants of gastric juice secretion

1. Tea and toast.
2. 1 pint of oatmeal gruel.
3. Alcohol—100 ml 7 per cent alcohol plus methylene blue used as an indicator for the emptying time of the stomach.
4. Histamine, given subcutaneously 0.01 mg per kg body weight.
5. Pentagastrin, given intramuscularly 6 µg per kg body weight.

Anything entering the stomach can constitute a humoral agent, but some meals act as a greater stimulus than others. If a meal is given, there are two variables, one being the rate of emptying of the stomach and the other being the rate of secretion of stomach

juices. This is why the older forms of test meals (oatmeal, alcohol) have now been dropped in favour of other stimulants.

Direct stimulus of parietal cells

(a) HISTAMINE

Histamine, produced by the decarboxylation of the amino acid, histidine, acts directly on the parietal cells and stimulates the secretion of gastric juice. It has undesirable side effects and, therefore, should be given with anti-histamine cover, which does not block the gastric stimulating effect of histamine. Anti-histamine does, however, cause drowsiness.

(b) PENTAGASTRIN

Pentagastrin is a synthetically produced pentapeptide consisting of the physiologically active part of the gastrin molecule. It is used in the same way as histamine but in smaller doses. Although it has fewer side effects compared with histamine it is expensive to use.

Augmented histamine test

In the 'augmented' histamine test much larger doses of histamine are given, thereby providing a more reliable proof of an ability to secrete acid. The test has two purposes, firstly to show the inability to secrete acid, and secondly to assess the maximum possible acid secretion as a diagnostic aid to surgical treatment.

1. Prepare the patient by fasting for 12 h, to ensure the stomach is completely emptied.
2. Pass a number 14 Ryle's tube pernasally into the stomach (preferably under X-ray control) until it lies in the pyloric antrum. The tube has markings on it to indicate how far the tube has been swallowed. The position of the tube is maintained by strapping it to the cheek with plaster and insisting that the patient does not alter his or her posture until completion of the test.
3. 8.00 am. All the gastric juice is aspirated from the stomach by means of a syringe attached to the end of the Ryle's tube and placed in a bottle marked 'Resting Juice'.
4. 8.00–9.00 am. Aspirate all the gastric juice at 15-min intervals for the next 60-min period and place in the bottle marked 'Basal Secretion'. (Sometimes the basal secretions are divided into pre- and post-Anthisan collections.)
5. 100 mg mepyramine (Anthisan) is given by *intramuscular* in-

jection half-way through the basal collection period at 8.30 am.

6. 9.00 am. Inject 2 mg histamine acid phosphate (average adult dose) *subcutaneously* 30 min after the Anthisan injection.
7. 9.00–9.15 am }
9.15–9.30 am }
9.30–9.45 am }
9.45–10.00 am } Aspirate gastric juice at 5-min intervals for 60 min and place in bottles marked with the appropriate times. Thereby obtaining four 15-min samples or alternatively obtain three 20-min samples.

(Since the stomach juices are equivalent to the secretion by the glands, the secretory rate can be determined.)

8. All specimens are sent to the laboratory for analysis.

LABORATORY PROCEDURE

1. Measure and record the volume of each collection.
2. Examine visually for blood, mucus and bile.
3. Measure the pH of each specimen using a pH meter.
4. Determine the titratable acidity of each collection by titration with standardized sodium hydroxide to pH 7.4 (blood pH) electrometrically or with phenol red as indicator.
5. Express the results as total acid concentration (titratable acidity) per specimen in mmol/l and also as hydrogen ion concentration in mmol per specimen and total hydrogen ion concentration in mmol per basal hour and post-histamine hour.

ESTIMATION OF TITRATABLE ACIDITY

Reagents

- (a) Phenol red indicator 0.1 g phenol red, 5.7 ml 0.05M NaOH diluted to 250 ml with distilled water.
- (b) 0.02M sodium hydroxide solution—prepare fresh for use by diluting 20.0 ml stock solution of 0.1 mol sodium hydroxide to 100 ml with distilled water in a volumetric flask. Standardize before use on each occasion.

Method

1. Some laboratories will be in the position of determining the titratable acidity by using an automatic burette and pH meter, a method which will not be dealt with in this book.
2. If the pH is greater than 7.4 no further examination is necessary.

3. All specimens with a pH less than 7.0 are treated as follows.
4. Centrifuge or filter the gastric contents.
5. Pipette 1.0 ml or 2.0 ml of clear gastric juice into a 50 ml conical flask, add about 5 ml distilled water and 2-3 drops of phenol red indicator.
6. As a control for end point determination use 1.0 ml or 2.0 ml of pH 7.4 buffer instead of the gastric contents.
7. Titrate each specimen carefully with 0.02 mol standardized NaOH from a 10 ml burette until a faint pink end point is obtained.
8. Note the titre and record the result.

Notes—

- (a) *Titratable acidity* is the sum of the hydrogen ion concentration and un-ionized hydrogen ion concentration. This is now used instead of the obsolete terminology, total acidity, which was the free acidity plus combined acidity (determined by using Topfer's reagent and phenolphthalein which should be abandoned).
- (b) *Hydrogen ion concentration* as measured by pH is not the same as titratable acidity, for example, 0.1 mol HCl has a pH of 1.00 and 0.1 mol CH₃COOH has a pH of 2.6, but these two acid solutions are of identical titratable acidity, 100 mmol/l.
- (c) *Old units* The old units, 'degrees of acidity'; ml 0.1 NaOH per 100 ml, gastric juice and 'mM' are also numerically equal to mEq/l and to the new mmol/l.

Calculation

0.02 mol NaOH contains 20 mmol/l.

Therefore 1.0 ml 0.02 mol NaOH is equivalent to 20 mmol/l.
Let x = titre of gastric juice, y = volume of sample taken and Y = volume of specimen collection.

$$\text{Titratable acidity} = \frac{x \cdot 20}{y} \text{ in mmol/l}$$

$$\text{Hydrogen ion concentration per specimen } \frac{\text{mmol/l} \cdot Y}{1000} \text{ in mmol}$$

For example:

$$x = 4.3 \text{ ml} \quad y = 1.0 \text{ ml} \quad Y = 24 \text{ ml}$$

$$\text{Titratable acidity} = \frac{4.3 \cdot 20}{1.0} = 86.0 \text{ mmol/l}$$

$$\text{Hydrogen ion concentration per specimen} \frac{86.0 \times 24}{1000} = 2.064 \text{ mmol/l}$$

During an augmented histamine test the following results were obtained when 1.0 ml of gastric juice was titrated:

	<i>Time</i>	<i>Volume</i>	<i>pH</i>	<i>Titre</i>	<i>Titrat- able acidity</i>	<i>Comments</i>
1. Basal secretion	8.00–9.00 am	40.0 ml	6.0	0.4	8.0	
2. Post histamine	9.00–9.15 am	47.0 ml	2.5	1.2	24.0	bile present
3.	"	97.0 ml	1.5	4.5	90.0	bile present
4.	"	88.0 ml	1.2	5.8	116.0	
5.	"	82.0 ml	1.3	5.7	114.0	flecks of blood present

Hydrogen ion concentration per specimen

$$\left. \begin{array}{l} 1. \frac{8.0 \times 40}{1000} = 0.32 \text{ mmol} \\ 2. \frac{24.0 \times 47}{1000} = 1.13 \text{ mmol} \\ 3. \frac{90 \times 97}{1000} = 8.73 \text{ mmol} \\ 4. \frac{116 \times 88}{1000} = 10.2 \text{ mmol} \\ 5. \frac{114 \times 82}{1000} = 9.35 \text{ mmol} \end{array} \right\}$$

The sum of the hydrogen ion concentration in samples 2–5 will be total hydrogen ion concentration per post-histamine hour = 29.42 mmol.

Results

Resting juice is of little significance clinically.

Basal secretion is used for the fasting gastric secretion and is helpful in diagnosing duodenal ulcer, since about one-quarter of patients have a significantly high output. The basal secretion has a poor repeatability.

Maximum secretions are of excellent repeatability and this is why the augmented histamine test has completely replaced the fractional test meal.

Bile staining usually invalidates the tests unless the specimen has a pH of less than 3.5. When found in the gastric contents it is usually due to regurgitation from the duodenum into the stomach, or the Ryle's tube was in the duodenum.

Blood should not be present. Small amounts of bright red flecks of blood usually indicate trauma during aspiration. Quantities of altered blood which is usually brown or reddish-brown in colour

(HCl in the gastric contents plus red cells are haemolysed to form acid haematin) are usually found in gastric ulcer or gastric carcinoma.

Mucus is normally only present in small amounts, but can be increased in gastric carcinoma.

Normal values

The normal values for the augmented histamine test depend upon age and sex.

Post-histamine hour values:

Normal males

(All ages)	less than 30 yr	greater than 30 yr
0.1–42.1	14.1–42.1	0.1–33.3

Normal females

(All ages)		
0.3–28.2	12.6–28.2	0.3–10.8

expressed as total hydrogen ion concentration in mmol.

The acidity of the gastric contents yields valuable diagnostic information.

Hypersecretion of gastric juice may be associated with duodenal ulcers. Acid secretion by the stomach is very high.

Hyposecretion of gastric juice occurs in pernicious anaemia and the achlorhydria is usually 'histamine fast'. Gastric carcinoma and chronic gastritis may also fall into this group.

Hyperchlorhydria—excessive secretion of hydrochloric acid.

Achlorhydria—a complete absence of hydrochloric acid.

Pentagastrin test

This is carried out in a similar manner to the augmented histamine test, except that 6 µg/kg body weight is injected intramuscularly.

INDIRECT METHOD (TUBELESS TEST)

This test makes use of a dye, azure A (Diagnex Blue) and, when given by mouth, the HCl in the stomach liberates the dye (blue) which is excreted in the urine. The test is only designed as a screening procedure and cannot replace the standard procedure because a negative result must be confirmed by the augmented histamine test.

Faeces

OCCULT BLOOD

Blood or its breakdown products may be present in the faeces, and its detection is another test used in the investigation of the gastrointestinal tract.

It can be found in the faeces from patients with carcinoma of the stomach, or an ulcer of the alimentary tract. Rectal or menstrual bleeding may contaminate the surface of the specimen with fresh blood; if this is seen, a report is issued stating that blood was detected macroscopically. Minute quantities may not be visible to the naked eye and the blood is said to be 'occult' or hidden. It may be detected by chemical means and microscopical examination may reveal intact red cells.

In 1969 the Department of Health issued two circulars, HM(69)57 and HM(69)74, directing laboratory and clinical hospital staff to the implications of using solutions containing amines controlled or prohibited under the Carcinogenic Substances Regulation 1967: SI No. 879. Owing to the carcinogenic properties of benzidine and orthotolidine, routine laboratory procedures for occult blood testing now use a non-carcinogenic chromogen, such as guaiacum, reduced phenolphthalein and dichlorophenol-indophenol. The Okokit*, a tablet test for occult blood, also contains a non-carcinogenic chromogen, and appears to be generally used throughout the UK.

PREPARATION OF PATIENT

It used to be essential for the patient to be kept on a meat-free diet for three days prior to the examination of the faeces for occult blood, but nowadays the sensitivity of the tests are adjusted so this restriction is unnecessary, although foods such as liver and black puddings should be avoided. The sensitivity of the test should indicate blood loss greater than 2.5–4.5 ml per day and should be strongly positive for the characteristic black and glistening tarry specimen.

Irrespective of whether a meat-free diet is adhered to or the sensitivity of the tests adjusted, it is usual to examine three daily specimens before the presence or absence of occult blood is confirmed.

* Hughes and Hughes Ltd, Romford RM3 0HR, Essex.

(a) THE OKOKIT

Principle

The peroxidase activity of haemoglobin and its iron-containing derivative catalyse the oxidation of the non-carcinogenic chromogen to form a blue colour in the presence of hydrogen peroxide. Other peroxidases in the faeces with a similar action can be destroyed by heat. Boiling and heating may denature some of the peroxidases of the haemoglobin as well as that of the bacterial and plant origin.

Materials supplied in the kit are (1) the diluent, (2) Okokit tablets, and (3) test papers.

Method (modified April 1972)

1. A small amount of the sample to be tested (faeces, boiled faeces, urine, etc.) is placed in the centre of the test paper, as a thin smear.
2. One Okokit tablet is then placed in the centre of the smear.
3. Three drops of diluent is then applied onto the tablet.
4. After 2 min add a further three drops of diluent.
5. Read after $5\frac{1}{2}$ min.

Results

Positive A specimen of faeces containing blood will show a blue reaction around the tablet. The intensity of the reaction will be proportional to the concentration of blood in the specimen.

Trace For trace amounts it is advisable to read the reverse side of the test paper held up to a direct light.

Negative A specimen of faeces containing no blood will show no reaction around the tablet.

Notes

1. The present form of the Okokit is sensitive to a 1 in 40 000 dilution of whole blood (manufacturer's comments), but a subjective element is inherent in any test which depends upon the judgment of the colour developed. It is therefore important that intending users should determine for themselves whether the test fulfils their requirements.
2. Check each time by using a positive control of a 1 in 40 000 dilution of whole blood.
3. When stored at 5–15 °C, the kit is stable for a minimum of two years.

(b) REDUCED PHENOLPHTHALEIN TEST

Principle

As for Okokit except that reduced phenolphthalein is the chromogen.

Reagents

1. 4.8 per cent acetic acid.
2. Reduced phenolphthalein.
Dissolve 2 g phenolphthalein and 20 g potassium hydroxide in 100 ml distilled water. Add approximately 10 g zinc powder and boil until the pink colour has completely disappeared. Decant from the zinc and dilute to 100 ml. Add a speck of zinc dust to keep the solution reduced and filter or centrifuge before use. The reagent becomes less sensitive with keeping.
3. Hydrogen peroxide solution—1 per cent of 20 vols hydrogen peroxide. Prepare freshly.
4. Control solution—0.1 ml whole blood in 500 ml water.

Method

1. A suspension of faeces in dilute acetic acid (5 ml acid and a pea-sized portion of faeces) is homogenized.
2. Place one drop of the suspension on a square piece of No. 3 MM Whatman filter paper.
3. Add one drop of reduced phenolphthalein reagent followed by one drop of hydrogen peroxide solution.
4. Within 90 s note colour reaction.
5. The time for colour development is established by putting 5 ml of the control solution through the method with the tests when a pink colour should appear within 90 s.

Results

Positive A specimen of faeces containing blood will give a pink colour within 90 s.

Negative A specimen of faeces containing no blood should give no pink colorization.

Notes—

1. This method gives results comparable with the Kohn and Kelly orthotolidine technique (*see Introduction to Medical Laboratory Technology*, 4th Ed.).
2. There does not appear to be any need to boil the specimen of faeces, but if boiling of the specimen is essential, this should be carried out for 5 min, cool and then repeat the test as above.

PANCREATIC FUNCTION TESTS

TESTS OF EXOCRINE FUNCTION

Changes in the external secretion of the pancreas can be studied by either direct or indirect procedures. The *direct* studies are usually the quantitative estimation of the various enzymes of pancreatic juice, bilirubin, bicarbonates and fluid volume obtained by duodenal intubation following pancreatic stimulation with pancreozymin and secretin. As this procedure is out of the scope of this book, we will be mainly concerned with some of the *indirect* studies which can be divided into the following groups.

1. Determination of serum enzymes

Unfortunately, tests of pancreatic function are not very satisfactory, as serum amylase levels usually show little change from normal in pancreatic disease except in acute pancreatitis, when they may be raised. Serum lipase estimations are technically not very suitable for routine use and urinary amylases are very rarely carried out nowadays, so only the estimation of serum amylase will be discussed.

SERUM AMYLASE

Principle

A small amount of plasma or serum is incubated at 37°C with a solution containing 0.4 mg of starch. The loss of blue colour which the starch gives with iodine solution is taken as a measure of the extent to which the starch has been digested by the amylase.

Reagents

1. Buffered Starch Substrate (pH 7.0)

Dissolve 13.3 g of dry anhydrous disodium hydrogen phosphate (or 33.5 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) and 4.3 g benzoic acid in 250 ml of water. Bring to boil. Mix 0.2 g of soluble starch in 5–10 ml of cold water in a beaker and add it all to the boiling mixture, rinsing the beaker out with additional cold water. Continue boiling for 1 min, then cool to room temperature and dilute to 500 ml. Keep the solution at 4°C and prepare freshly each month.

2. Stock Iodine Solution

Dissolve 13.5 g of pure sublimed iodine in a solution of 24 g of potassium iodide in about 100 ml of water and make to 1 litre with water.

3. Working Iodine Solution

Dissolve 50 g of potassium iodide in a little water, add 100 ml of stock iodine solution, and dilute to 1 litre with water.

4. 0.9 per cent Sodium Chloride

Method

Test

1. Serum or plasma is diluted 1 in 10 with 0.9 per cent sodium chloride solution.
2. Add 1.0 ml of buffered substrate to a 150 × 15 mm test-tube and place in 37 °C water bath for 2 min.
3. 0.1 ml of diluted serum is added, mixed and kept at 37 °C for 15 min.
4. The tube is removed, cooled and 8.5 ml of water is added.
5. 0.4 ml of dilute iodine is now added and the solution mixed.
6. A blank is prepared similarly to the test, except that the 0.1 ml of diluted serum is added last, i.e. after the addition of the dilute iodine solution.
7. The absorbances of the test and blank are read within 5 min at 660 nm, zeroing the spectrophotometer with distilled water.

Calculation A Somogyi amylase unit is the amount of amylase which will destroy 5 mg starch in 15 min.

Since 1 ml of buffered substrate contains 0.4 mg starch and 0.1 ml of diluted serum is equivalent to 0.01 ml of undiluted serum,

then: Let $A = \text{absorbance}$

$$\frac{A \text{ of blank} - A \text{ of test}}{A \text{ of blank}}$$

is equivalent to the amount of starch digested by 0.01 ml of serum in 15 min, and as 1 amylase unit will destroy 5 mg of starch (but only 0.04 mg of starch was used)

$$\frac{A \text{ of blank} - A \text{ of test}}{A \text{ of blank}} \times \frac{0.4}{5.0} \times \frac{100}{0.01} = \frac{\text{amylase units per } 100 \text{ ml}}{\text{serum}}$$

$$\text{or: } \frac{A \text{ of blank} - A \text{ of test}}{A \text{ of blank}} \times 800 = \frac{\text{amylase units per } 100 \text{ ml}}{\text{serum}}$$

Results

Normals, up to 200 Somogyi units per 100 ml.

In acute pancreatitis values over 1000 Somogyi units can be expected.

Notes—

- (a) As salivary amylase will invalidate the results, contamination from saliva must be avoided.
- (b) When values over 350 units are obtained, the determination should be repeated with a higher dilution of serum.

2. Faecal studies

In severe pancreatic disease, evidence of impaired digestion of foodstuffs can be obtained by a microscopical examination of the faeces, and in cases of pancreatic obstruction and/or in fibrocystic disease of the pancreas in infants, trypsin activity may be of importance. In fibrocystic disease of the pancreas a sweat test is a better laboratory procedure to use; this will be discussed later.

MICROSCOPICAL EXAMINATION

Faeces contain various crystals, cells, bacteria and foreign bodies such as hairs. Also present are the various food residues, both digested and undigested. These include fats, muscle fibres, starch granules and cellulose structures. For a description of the various crystals, cells, ova and parasites, other works should be consulted. Only fats and the various food residues will be considered.

PREPARATION OF SLIDE

1. On one end of a microscope slide place 1 drop of saline solution. At the other end place 1 drop of Lugol's iodine (see p. 455) solution.
2. Using a swab stick, first emulsify a small portion of fresh faeces in the saline solution, and another portion in the iodine.
3. With a little experience it is easy to avoid making a suspension either too thick or too thin.
4. Carefully apply a coverslip to each drop.
5. Using the 16 mm objective examine the saline suspension to obtain a general impression of the specimen.
6. Use the 4 mm objective to obtain a detailed view of the various constituents.
7. Now examine the iodine preparation. Confirm whether any structures thought to be starch granules in saline preparation have stained blue in the presence of the iodine and have stained cells and muscle fibres etc. brown.

MICROSCOPICAL APPEARANCE*Cellulose structures*

These structures form the skeletal wall of plant cells. As cellulose is not usually digested, it tends to be excreted intact, and appears in some bizarre forms. Cellulose appears as a clear structure with a sharply defined wall. Some of the forms seen are illustrated in *Figure 8.5*.

Starch granules

These granules, when found intact and undigested, are usually seen within a cellulose sac (*Figure 8.5*). The intake of raw vegetables results in more starch granules in faeces than when the vegetables are eaten cooked, as this softens the cellulose, releasing the

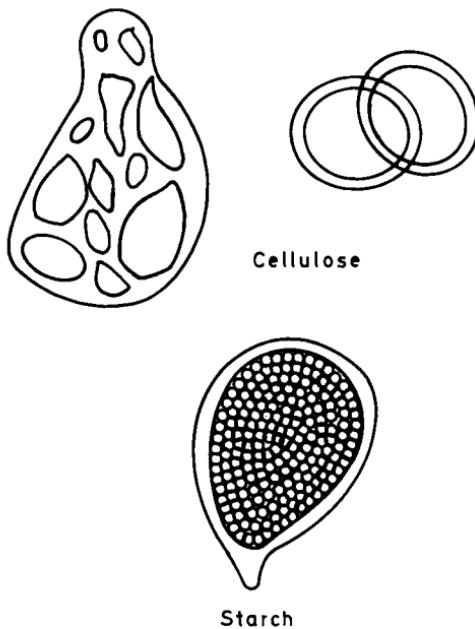


Figure 8.5. Cellulose and starch granules

starch granules which become digested. Under ordinary conditions starch granules are uncommon in the faeces of adults but are more often seen in the faeces of infants. The most important cause of an increased quantity of starch in faeces is because of the increased rate of passage through the intestine.

Muscle fibres

Muscle fibres, which are derived from meat, are stained yellow-brown by faecal stercobilin. They are normally excreted fully or partially digested. The presence of undigested fibres indicates digestive impairment, for example pancreatic disease. The various stages of muscle digestion are as follows, but it must be realized

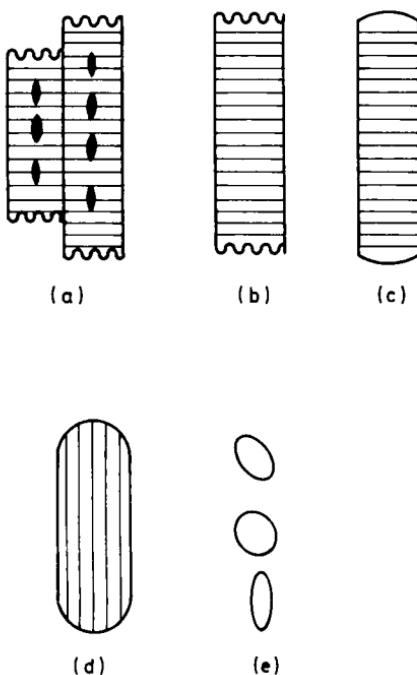


Figure 8.6. Muscle fibres showing various stages of digestion

that there is no sharp demarcation between the types; it is a gradual transition.

Undigested muscle fibres as seen in the faeces are shown in *Figure 8.6a*. They have irregular ends, nuclei and transverse striations; an isolated undigested fibre is seen in *Figure 8.6b*. Note the irregular ends and transverse striations. When acted upon by the digestive juices, the nuclei disappear first and then the ends become rounded. This partially digested fibre can be seen in

Figure 8.6c. In *Figure 8.6d* the muscle fibre has been further digested when it can be seen that the ends are still round, but now it has longitudinal striations. *Figure 8.6e* shows fragments of fibres free from striations with rounded ends, the digestion being more complete than in *Figure 8.6d*.

Soaps appear as plaques with rolled-over edges or as masses of needle-shaped crystals (*Figure 8.7*) and are often seen as a mass

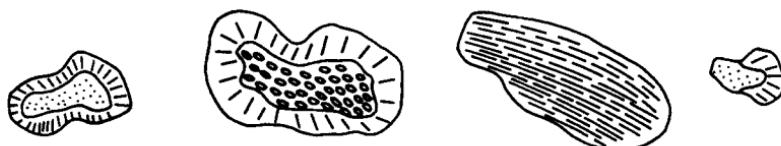


Figure 8.7. Various forms of soap plaques

of soap crystals. They are insoluble in ether and ethanol, whereas fats and fatty acids are soluble. A simple method of identification consists of making a suspension of the faeces in a few drops of saturated copper nitrate on a slide, covering with a coverslip and examining after a few minutes. Soaps are stained green owing to their conversion to copper soaps. Fatty acid crystals are not stained. In a normal specimen of faeces an occasional soap plaque may be seen. Excessive amounts of soaps indicate defective absorption. *Fat globules* tend to rise to the surface of the preparation. They are called neutral fats and vary in size, are highly refractile and look oily. But before deciding that the oil globules are neutral fat (stearin, palmitin and olein) it is important to make sure the patient is not receiving liquid paraffin or other oily drugs (*Figure 8.8*).

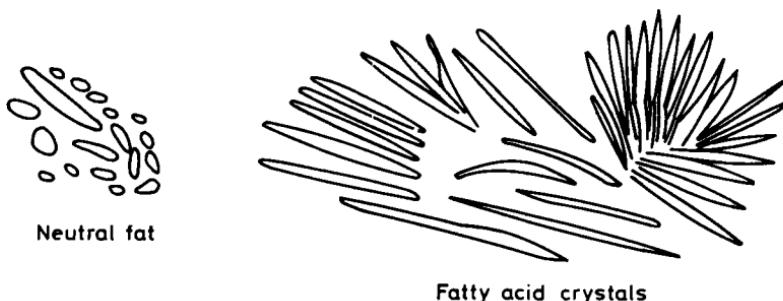


Figure 8.8. Neutral fat and fatty acid crystals

Fatty acid crystals appear as colourless needle-shaped crystals. They are longer than bacilli and are often slightly curved. Usually seen in groups of two, three or more. They are unaffected by aqueous copper nitrate but are soluble in ether and ethanol.

PANCREATIC ENZYMES IN FAECES

Trypsin is normally found in faeces and is the only enzyme which is assayed. It is of little value in the diagnosis of chronic pancreatic disease in adults, as the ranges found in the normal are very wide. In cases of fibrocystic disease of the pancreas in infants it is reduced or completely absent when a fresh specimen is examined. Trypsin will liquefy gelatin; it is active at an alkaline pH and as the test must be carried out on freshly passed faeces, specimens more than 1 h old are unsuitable.

PROTEOLYTIC ACTIVITY

Principle

Gelatin is incubated with several dilutions of the faeces in sodium hydrogen carbonate. If trypsin is present the gelatin is digested to water-soluble products causing liquefaction of the gelatin. The more enzyme there is in the faeces, the higher the dilution at which liquefaction takes place.

Reagents

1. 5 per cent sodium hydrogen carbonate.
2. 7.5 per cent gelatin solution. Prepare by dissolving the gelatin in warm water, then diluting with distilled water to 100 ml. Store in the refrigerator and warm to about 40 °C to liquefy when required for the test.

Method

1. Prepare a 1 in 10 dilution of faeces. Place 9 ml of the sodium hydrogen carbonate solution into a tube marked at 10 ml, then add faeces to the mark and emulsify with a glass rod.
2. To a series of 10 tubes add 5 ml of the sodium hydrogen carbonate solution.
3. Add 5 ml of the 1 in 10 faecal suspension to the first tube, mix and deliver 5 ml of this to the next tube.
4. Repeat the doubling dilutions until the ninth tube, then discard this 5 ml (approximately 1 in 2560), leaving the last tube containing sodium hydrogen carbonate alone to act as a control.

5. Add 2 ml of the warmed gelatin to each tube and mix well.
6. Place in the 37 °C water bath for 1 h.
7. Remove the rack from the water bath and place it in the refrigerator overnight.

Results

The presence of trypsin is indicated by the liquefaction of the gelatin, so record the highest dilution at which the gelatin is liquefied. Check that the control tube is still a complete gel; if not, discard the test.

X-ray film method

It is also possible to use undeveloped, unfixed X-ray film as the substrate for this test. Simply cut a piece of film to fit a petri dish containing damp filter paper, and continue as follows:

1. Beginning with the highest dilution of faeces, place one drop from each tube on the film. Add one drop of sodium hydrogen carbonate to act as control.
2. Close the lid of the dish and incubate at 37 °C for 30 min to allow any trypsin present to digest the gelatin off the film.
3. Place the dish in the refrigerator for about 10 min to harden the gelatin.
4. Carefully wash the film in cold water.

Results Digestion of the film emulsion, shown by a clear area, indicates the presence of trypsin. Where the surface of the film is merely crinkled, as with the control, there has been no proteolytic activity.

Note the highest dilution of faeces which contained trypsin.

After the first three days of life, the faeces of babies and young children have a high proteolytic activity. By the age of 12 years, the activity has decreased markedly. It has been shown that the faeces of most normal babies digest gelatin at a dilution of 1 in 100 or greater, whereas infants with fibrocystic disease of the pancreas digest it only with a dilution of less than 1 in 50.

Fibrocystic disease of the pancreas (cystic fibrosis: mucoviscidous)

This disease of the pancreas is the commonest inborn error of metabolism, usually presenting in earlier childhood and is a general dysfunction of the exocrine glands. The pancreatic and bronchial secretions are viscid, which block the pancreatic ducts and bronchi, causing obstructions of these organs. Sweat glands

are also affected and the diagnostic feature is a high content of sodium and chloride in the sweat, often to about twice the normal level.

Although pancreatic trypsin is deficient it is not always diagnostic, and a far better procedure is to estimate the sodium and chloride content of the sweat. Sweat can be collected in a number of ways, some of which are described below.

COLLECTION OF SWEAT

A. *Local stimulation using methacholine chloride*

This procedure is usually carried out by medically qualified staff.

1. A subcutaneous injection of 2 mg methacholine chloride (mecholyl) is given in the forearm.
2. Wash the injection site with ether and distilled water, taking care not to cause any leakage of mecholyl.
3. The area around the injection is then covered with a pre-washed and dried disc of Whatman No. 40 filter paper.
4. The filter paper must be weighed beforehand in a flask and neither the flasks nor filter paper must be touched by the fingers. Forceps should, therefore, be used to apply the filter paper to the forearm.
5. Cover the filter paper with a square of plastic and completely seal the plastic to the arm with adhesive plaster.
6. The filter paper is removed after 1 h and weighed in the flask.
7. Add 5–10 ml of distilled water to the flask, mix well and determine the sodium content by means of the flame photometer (p. 130) and the chloride content by the chloride meter (p. 137).
8. Between 100 and 300 mg of sweat are obtained by the method, more than 100 mg being sufficient for the test.

B. *Pilocarpine iontophoresis*

A direct current of 1.5 mA is passed for 25–35 min between two electrodes. The positive electrode is filled with 0.5 per cent aqueous pilocarpine nitrate solution and the negative with 1 per cent aqueous sodium nitrate solution. Under the surface of the positive electrode is a circle of ashless filter paper saturated with pilocarpine nitrate, and the negative electrode with a gauze saturated in the sodium nitrate solution. The positive electrode is strapped to the flexor surface and the negative electrode on the extensor surface of the forearm.

After 5 min the area covered by the positive electrode is washed

with distilled water and covered with a Whatman No. 40 filter paper of known weight. The paper is carefully handled with forceps, covered with parafilm and the sweat collected for 25–35 min. After this the paper is removed, weighed, placed in a flask and the electrolytes eluted with 10 ml distilled water. The sodium and chloride concentrations are measured as before.

Results In normal children the upper limit for sweat sodium is 70 mmol/l, while the upper limit for chloride is 65 mmol/l. In adults the sodium concentration may exceed 90 mmol/l.

In fibrocystic disease the values for sweat sodium can vary from 80 to 150 mmol/l and for chloride from 70 to 140 mmol/l. The distinction between normal and fibrocystic subjects is very good in young children.

TESTS OF ENDOCRINE FUNCTION

Changes in the internal secretions of the pancreas are usually assessed by estimating the blood glucose (sugar), or for diagnostic purposes carrying out a glucose tolerance test is of greater value. In the blood, in addition to glucose, there are small amounts of other sugars such as lactose, fructose, pentoses and a negligible amount of galactose. Urine contains very small amounts of glucose, lactose, fructose, galactose and sucrose.

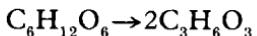
Blood glucose and blood sugar

The estimation of carbohydrates in the blood can be divided into two steps: (1) the estimation of blood glucose and (2) blood sugar. As the main carbohydrate present in the blood is glucose, the term 'blood sugar' is loosely used to include glucose, other sugars, and the other reducing substances which may be present in the blood (glutathione). The vast majority of methods for sugar analysis until quite recently depended upon the reducing power of glucose and involved using reagents such as copper and ferricyanide in an alkaline medium. Methods are now available for the specific estimation of blood glucose using the enzyme glucose oxidase, which gives results up to 30 mg/100 ml lower than techniques which estimate blood sugar. On the other hand there are methods available for estimating 'true' glucose values which eliminate the non-specific reduction and measure only that due to sugars. In the latter methods the 'true' glucose values are approximately 5 mg/100 ml higher than the specific glucose methods.

It is therefore important to report blood levels as 'glucose', 'true glucose' or sugar depending upon the method used.

Glycolysis

Glucose disappears from whole blood on standing as a result of glycolysis due to its conversion into lactic acid.



It is an enzymatic reaction; the rate decreases with respect to temperature. At 37 °C the loss of glucose is about 20 mg/100 ml per h, while at 4 °C the decrease is about 5 mg/100 ml per h. Glycolysis can be prevented by using preservatives, the commonest being sodium fluoride, used in combination with the anti-coagulant potassium oxalate; 2 mg sodium fluoride and 6 mg of potassium oxalate per ml of blood will act as a preservative and anti-coagulant for 2–3 days. Although fluoride is an enzyme poison, it can be used for glucose oxidase methods up to a concentration of 5 mg sodium fluoride per ml of blood. Fluoride is an inhibitor of both erythrocyte metabolism and of bacterial growth. In erythrocyte metabolism it inhibits the enzyme enolase involved in the glycolytic pathway, but has less effect on the bacterial growth. Saturated benzoic acid used in the preparation of glucose solutions is more effective as a bactericide, but is not necessary in preserving blood samples.

Estimation of blood glucose and blood sugar

There are three main methods for estimating the 'sugar' content of body fluids: (a) the reduction of cupric to cuprous salts; (b) reduction of ferricyanide to ferrocyanide; and (c) glucose oxidase methods. Of these three methods only (a) and (b) will be described here. Method (b) is commonly used in automatic procedures. *Dextrostix* is a reagent strip for the semi-quantitative estimation of blood glucose, which should be used in conjunction with the Ames Reflectance Meter. It is far better to use a conventional blood glucose or sugar method as described rather than this reagent strip.

DETERMINATION OF 'TRUE GLUCOSE'

Principle

After protein precipitation with copper tungstate, the supernatant is heated with alkaline tartate reagent, under standard conditions. The cuprous oxide so formed is then estimated by the blue-green compound produced upon the addition of arsenomolybdate solution. This colour is measured in the absorptiometer or spectrophotometer against standard glucose solutions treated in a similar way.

Reagents

1. Isotonic sodium sulphate-copper sulphate solution—Mix 320 ml of 3 per cent sodium sulphate ($\text{Na}_2\text{SO}_4 \cdot 10\text{H}_2\text{O}$) with 30 ml 7 per cent copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$).
2. Sodium tungstate—10 g per 100 ml distilled water.
3. Alkaline tartrate reagent—25 g sodium hydrogen carbonate are dissolved in a minimum amount of distilled water (about 300 ml). When in solution add 20 g anhydrous sodium sulphate with constant stirring. After the carbonate has dissolved, a solution of 18.4 g potassium oxalate in 60 ml warm distilled water is added. Finally add 12 g sodium potassium tartrate dissolved in about 50 ml distilled water. Dilute to 1 litre and mix well.
4. Arsenomolybdate solution—25 g ammonium molybdate are dissolved in 450 ml of distilled water, 21 ml concentrated sulphuric acid are added slowly, mixed and then followed by 3 g sodium arsenite ($\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$) dissolved in 25 ml water. Mix and keep at 37 °C for 2 days. If the reagent is needed quickly the mixture can be heated to 55 °C but should be stirred well to prevent local overheating. It is better to keep at 37 °C for 2 days. Keep in a dark bottle. For use dilute 1 volume of this reagent with 2 volumes of water.
5. Stock standard glucose solution—100 mg glucose dissolved in saturated benzoic acid (0.3 per cent) and made up to 100 ml. Store at 4 °C. The stock solution should be prepared 24 h before use to allow for equilibrium between mixtures of α - and β -glucose to be attained. Solid glucose is usually predominantly the α form in commercial preparations. This is important when using glucose oxidase (see p. 212).
6. Working standard glucose solutions—prepared by diluting 1.0 ml, 2.5 ml and 5.0 ml of stock standard to 100 ml with isotonic sodium sulphate-copper sulphate solution (equivalent to 0.01, 0.025, and 0.05 mg glucose per ml). Store at 4 °C.

Method

1. Pipette 0.05 ml blood or plasma into a centrifuge tube containing 3.9 ml isotonic sodium sulphate-copper sulphate solution.
2. To ensure accurate measurement of blood, wipe the outside of the blood pipette carefully. Allow the blood to run out of the pipette at the bottom of the tube. Raise the pipette

to the top of the diluent and rinse out the pipette well with the clean diluent.

3. Add 0.05 ml sodium tungstate solution.
4. Mix well and centrifuge at 2500 rpm for 5 min.
5. Into suitably labelled boiling tubes (6" x 1") pipette the following:

	<i>Test</i>	<i>Blank</i>	<i>Standard</i>
Supernatant	2.0 ml	—	—
Isotonic $\text{Na}_2\text{SO}_4\text{-CuSO}_4$ soln	—	2.0 ml	—
Standards	—	—	2.0 ml
Alkaline tartrate	2.0 ml	2.0 ml	2.0 ml

6. Mix well, stopper with cotton wool and place in the boiling water bath for 10 min.
7. Remove from bath and cool immediately.
8. Add 6.0 ml arsenomolybdate to each tube, mix well and then
9. Add 5.0 ml of distilled water to all tubes.
10. Mix well and read absorbance at 680 nm (red filter) in a spectrophotometer or absorptiometer.

Calculation

The calculation can be worked out in two ways.

(a) Draw a calibration curve (*see p. 126*) using the three standards, which will also confirm that Beer's Law is obeyed and from this the concentration of glucose in the test sample is found. This is the correct approach and should be done every time, and more especially when a new reagent has been prepared.

Let x = concentration of glucose in test.

Then 2.0 ml of supernatant (=0.025 ml of whole blood) will contain x mg of glucose.

$$\text{Therefore } x \times \frac{100}{0.025} = \text{mg glucose per 100 ml}$$

$$\text{or } x \times 4000 = \text{mg glucose per 100 ml}$$

(b) If the first method is not used then it is most important that two standards are carried through the test procedure. Then the standard which gives an absorbance nearest to the test sample is used in the calculation. Subtract the blank reading from both the test and standard absorbances.

Let Y = the concentration of standard in mg per ml

Then as 2 ml of supernatant will contain the equivalent of 0.025 ml of whole blood or plasma.

Let A = absorbance

$$\text{then } \frac{A \text{ of unknown}}{A \text{ of standard}} \times \frac{100}{0.025} \times Y = \text{mg glucose per 100 ml}$$

$$\begin{aligned} \text{e.g. let } A \text{ of test (T)} &= 0.32 & A \text{ of standard (S)} &= 0.42 \\ A \text{ of blank (B)} &= 0.02 & \text{Standard concentration} &= \\ && 0.025 \text{ mg per ml} & \end{aligned}$$

$$\text{Then } \frac{T - B}{S - B} \times \frac{100}{0.025} \times 0.025 = \text{mg glucose per 100 ml}$$

$$\text{or } \frac{0.32 - 0.02}{0.42 - 0.02} \times \frac{100}{0.025} \times 0.025 = \text{mg glucose per 100 ml}$$

$$\frac{0.32}{0.40} \times 100 = 75 \text{ mg glucose per 100 ml}$$

Notes—

1. If the results are higher than 350 mg per 100 ml repeat the test using less supernatant, i.e. 1.0 ml supernatant and 1.0 ml isotonic sodium sulphate–copper sulphate. Not forgetting to include a factor of 2 in the calculation.
2. The use of an isotonic sodium sulphate–copper sulphate solution for protein precipitation has enabled a ‘true glucose’ to be estimated.
3. Copper in the supernatant and standard acts as a preservative and anti-glycolytic agent.
4. S.I. units are progressively being introduced into clinical chemistry and therefore to convert mg glucose per 100 ml into mmol per l use the following calculation.

$$\text{mg per 100 ml} \times \frac{1}{18} = \text{mmol per l}$$

DETERMINATION OF GLUCOSE

The method mentioned above is not specific for glucose, but greater specificity can be obtained by using an enzyme, glucose oxidase. This enzyme, which is specific, oxidizes glucose to gluconic acid. It acts on β -D-glucose, but has a negligible effect on α -D-glucose. The two isomers exist in solution in equilibrium with the chair form (*Figure 8.9*) and the shift from one to the other in solution is called mutarotation. Mutarotation is important, as it must be complete during the oxidation of glucose in the sample. This step can be speeded up in the presence of the enzyme gluco-

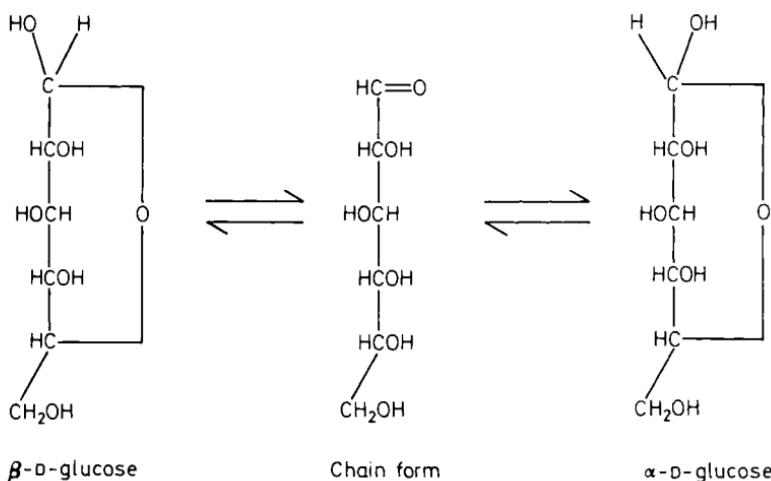
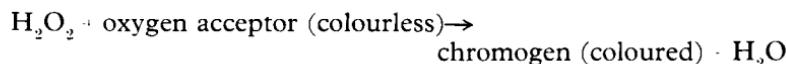
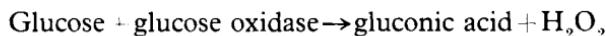


Figure 8.9. Mutarotation of glucose

mutarotase, which is present in most commercial glucose oxidase preparations.

In the estimation of glucose using the enzyme oxidase it is important that the stock standard glucose solution is prepared at least 24 h before use (to allow for equilibration between mixtures of α - and β -glucose to be attained) as solid glucose is usually predominantly the α -form in commercial preparations. Since glucose oxidase reacts only with β -glucose, a freshly prepared solution will not produce maximum colour if there should be insufficient glucomutarotase in the sample of glucose oxidase used.



The chromogen is measured photometrically. Some of the oxygen acceptors now used are reduced 2:6-dichlorophenolindophenol, guaiacum, 4-aminophenazone and perid (2'2-diazo-di(3-ethyl benzthiazoline-6-sulphonic acid)). Most of the methods use either guaiacum or 4-aminophenazone; the latter procedure will be described.

BLOOD GLUCOSE METHOD

Principle

Phenol in the presence of an oxidizing agent, in this case hydrogen

peroxide, gives a pink colour with aminophenazone. Two solutions are used: a protein precipitant (phosphotungstic acid containing phenol) and the enzyme solution (glucose oxidase, peroxidase) containing the 4-aminophenazone.

Reagents

Use AR reagents wherever possible.

1. Protein precipitant—10 g sodium tungstate, 10 g disodium hydrogen phosphate (Na_2HPO_4) and 9 g sodium chloride are dissolved in 800 ml distilled water. Add 125 ml N HCl to bring the pH to 3.0 (check with narrow range indicator paper). Add 1 g phenol and dilute to 1 litre with distilled water. Keeps indefinitely at 4 °C.
2. Colour reagent—to 75 ml of 4 per cent disodium hydrogen phosphate (Na_2HPO_4) add 215 ml distilled water, 5 ml Fermcozyme 653AM glucose oxidase*, 5 ml 0.1 per cent peroxidase RZ*. Mix well then add 300 mg sodium azide and 100 mg 4-aminophenazone. Mix again and store at 4 °C. Keeps for at least 2 months.
3. Stock standard glucose solution—1000 mg glucose per 100 ml in saturated (0.3 per cent) benzoic acid. Prepare at least 24 h before use.
4. Working standard glucose solution—prepare by diluting 5.0 ml, 10.0 ml and 20.0 ml of stock standard to 100 ml with saturated benzoic acid. These standards are then equivalent to 50, 100 and 200 mg glucose per 100 ml.

Method

1. Into suitably labelled centrifuge tubes add the following:

	<i>Test</i>	<i>Standards</i>
Protein precipitant	2.9 ml	2.9 ml
Working standard preparations	—	0.1 ml
Blood or plasma	0.1 ml	—

2. Mix well and centrifuge at 2500 rpm for 5 min.
3. Transfer 1.0 ml supernatant to another test-tube and add 3.0 ml colour reagent.
4. Use 1.0 ml protein precipitant and 3.0 ml colour reagent for the blank.

* Hughes and Hughes.

5. Mix well and incubate all tubes at 37 °C for 10 min, shaking occasionally to ensure adequate aeration.
6. Remove from water bath, cool and measure the absorbances against the blank at 515 nm (green filter) in 1 cm cells.

Calculation

The calculation can be worked out in the same way as for 'true glucose', i.e. by using (1) a calibration curve or (2) a straightforward calculation, using the standard which gives an absorbance nearest to the test reading. As the standards are taken through the whole procedure, there are no dilution factors to be taken into consideration.

Hence

$$\frac{\text{A of unknown}}{\text{A of standard}} \times \text{strength of standard} = \frac{\text{mg glucose per}}{100 \text{ ml}}$$

e.g. if the 200 mg per 100 ml standard is used and the absorbance of the test and standard are 0.210 and 0.420 respectively,

then $\frac{0.210}{0.420} \times 200 = 100 \text{ mg glucose per 100 ml}$

Notes—

1. Beer's Law is obeyed up to about 400 mg per 100 ml.
2. The colour is stable for at least 30 min.
3. 5 mg sodium fluoride per ml of blood has no effect on glucose values.
4. For CSF glucose use 0.2 ml CSF and 1.8 ml protein precipitant; this will give a three-fold increase in sensitivity.

Results

The normal range of fasting venous blood glucose taken not less than 3.4 h after the last meal usually lies between 50–90 mg per 100 ml, but values outside this range are occasionally found. In capillary blood the value is usually about 5 mg per 100 ml higher. The range for children and infants is the same as for adults. In methods which estimate blood sugar the normal range can vary between 70 and 120 mg per 100 ml (*see p. 207*).

Fasting hyperglycaemia is defined as a blood glucose value above 100 mg per 100 ml or more than 120 mg for other methods.

The diagnosis of diabetes mellitus cannot be confirmed on a single estimation, but further investigations such as a glucose tolerance curve have to be carried out.

Fasting hypoglycaemia is defined as a blood glucose value below 50 mg per 100 ml. A value 10–30 mg higher would be obtained with blood sugar techniques. As in the case of hyperglycaemia,

a single value does not diagnose hypoglycaemia; further tests are required.

BLOOD GLUCOSE VALUES AFTER MEALS

A rise and fall in blood glucose values occurs after having a meal containing carbohydrates in any form. The increase in blood glucose will depend upon the amount of glucose produced as a result of carbohydrate digestion, the rate of digestion, the rate of absorption and with the rate of removal of glucose from the circulating blood.

It is therefore important to state the time the blood was taken for glucose estimation on the report form, along with the time the last meal was taken.

ORAL GLUCOSE TOLERANCE TEST

As mentioned above there is a temporary rise in blood glucose when a subject ingests glucose, or a meal containing carbohydrate; the extent and duration of rise will depend upon the type of food taken. This effect of ingested carbohydrate, when studied under standard conditions, is the basis of the glucose tolerance test. It is used for investigating abnormalities of carbohydrate metabolism and in cases where glycosuria has been found.

The response to a carbohydrate load depends upon the previous carbohydrate diet and on the amount of glucose ingested. It is important therefore that the subject is placed on a normal carbohydrate diet for about 3 days before the test is carried out. When the subject is placed on a low carbohydrate diet for some time before the test an impaired glucose tolerance test can be obtained (the blood glucose level rises higher than normal and the rise is more prolonged). Conversely, if placed on a very high carbohydrate diet (or non-fasting before the test) there can be an enhanced glucose tolerance (diminished rise in blood glucose level).

The changes in glucose tolerance with alteration of the diet are probably caused by alteration in tissue oxidation of glucose, changes in insulin and growth hormone secretions and in liver glycogen metabolism.

Method

1. The subject must be on a normal hospital diet for about 3 days prior to the test.
2. The patient has nothing to eat after supper and no breakfast is given.

3. In the morning, urine is collected before commencing the test.
4. Take a fasting sample of blood for glucose estimation and then give 50 g of glucose dissolved in 150–200 ml water by mouth. (For children the dose is usually 1 g of glucose per kg body weight up to a maximum of 50 g.)
5. Take further samples of blood at 30-min intervals for 2½ h.
6. Urine samples are taken at intervals during the test. Some workers collect half-hourly, but this is not always possible.
7. Two specimens 1 h and 2 h after taking the glucose can usually be obtained.
8. Estimate the blood glucose levels of each specimen using the methods previously described, and test the urines for the presence of glucose by the method described on p. 253.

Results

Normal response A typical normal response is shown in *Figure 8.10*, when the fasting level is within the normal limits, and at no stage of the test should the level exceed 160 mg per 100 ml, and it usually returns to the fasting level within 2 h of drinking the glucose solution. Glucose is not found in the urine.

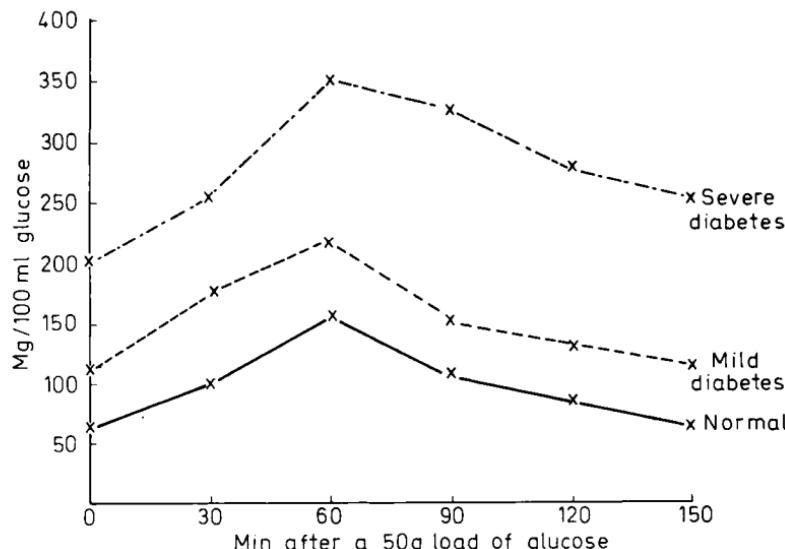


Figure 8.10. Glucose tolerance curves

Diabetic response In diabetes mellitus, the fasting level and the peak of the curve may be well above normal limits and considerably longer may elapse before the blood sugar returns to the fasting level (*Figure 8.10*). Glucose may be found in one or more of the urine specimens (glucosuria).

RENAL THRESHOLD FOR GLUCOSE

Renal threshold for glucose is the highest level the blood glucose reaches before glucose appears in the urine and is detectable by routine laboratory tests.

In normal persons, providing there is normal renal function, the renal threshold for blood glucose is about 180 mg per 100 ml. This is because the renal tubules are able to absorb all glucose passed through the glomeruli. At higher blood levels tubular re-absorption may not be sufficiently rapid, and glucose may appear in the urine. The renal threshold may be somewhat lower than 180 mg per 100 ml in some subjects. In such cases glucose may be found in the urine whenever the blood glucose levels exceed the low threshold value, that is glycosuria may occur without the blood glucose level rising to abnormal heights. This condition is known as 'renal glycosuria', and it can be distinguished from diabetes mellitus by examination of a glucose tolerance curve.

During a glucose tolerance test, because of the delay in urine reaching the bladder, maximum glycosuria is found in the next specimen after the highest blood glucose concentration is reached.

9

Liver Function Tests

The liver is the largest organ in the body and from a metabolic standpoint is the most complex. Tests of its many functions have been devised in the hope that they will serve as diagnostic aids when a metabolic process has been disturbed. Unfortunately, these liver function tests differ in sensitivity and many give normal results, even when only about 15 per cent of the liver parenchyma are functioning. The only liver function tests described here are those for the detection of urinary bilirubin and its derivatives.

Gross structure

The adult liver weighs about 1500 g and is situated underneath the diaphragm (*Figure 9.1*). It has four lobes. The right lobe is the largest, the left lobe is smaller and wedge-shaped, the quadrate lobe is nearly square in outline and the caudate lobe has a tail-like appearance. The latter two lobes are very small and can only be distinguished on the undersurface of the liver. Attached to the undersurface of the liver by connective tissue is a pear-shaped sac, the gall bladder, which acts as a reservoir for the bile before it is discharged into the duodenum.

The portal fissure is the name given to a cleft on the under-surface of the liver where various structures enter and leave the organ. Entering the liver is the portal vein carrying blood from the stomach, spleen, pancreas and intestines, and the hepatic artery carrying arterial blood. Leaving the liver are the hepatic veins carrying blood to the inferior vena cava, and the right and left hepatic ducts carrying bile to the gall bladder (*Figure 8.4*).

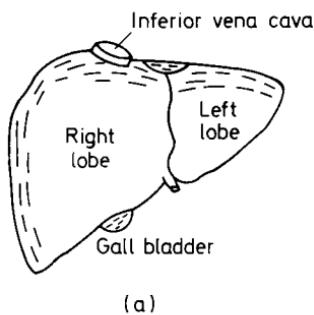
Anatomic structure

The basic structure is the hepatic lobule, which is hexagonal in outline and formed by cords of cells arranged in columns which radiate from a central vein. Between the column of cells run the sinusoids (a system of capillaries and open spaces) containing blood derived from the portal vein and hepatic artery. In this way each hepatic cell is provided with an adequate amount of blood containing essential nutrients. After the blood has been in contact

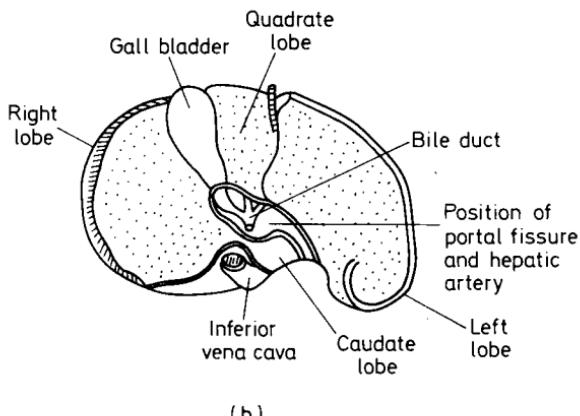
with these cells it drains into the central vein and eventually into the hepatic vein.

Bile secretion

Bile is secreted by the cells of the lobules into the biliary canaliculi which in turn drain eventually into the right and left hepatic ducts.



(a)



(b)

Figure 9.1. (a) Liver showing the right and left lobes. (b) The undersurface of the liver

Between meals the sphincter of Oddi (Figure 8.4) is closed and bile is therefore stored and concentrated in the gall bladder. The sphincter, at the sight, taste or smell of food, relaxes and when the gastric contents enter the duodenum, cholecystokinin, a hormone secreted by the intestinal mucosa, causes the gall bladder to contract and bile enters the duodenum.

Composition and functions of bile

There is a slight difference between the composition of bile produced by the liver and that entering the duodenum. In the gall bladder the liquid is slightly alkaline (pH 7.0–7.6), viscous and golden yellow or greenish in colour. As it acts as a transport medium for the biliary constituents during excretion from the body, its composition varies from time to time. In the main, bile contains mucus, bile salts, bile pigments, cholesterol, fatty acids, fats and inorganic salts. It can also contain drugs, toxins, dyes and heavy metals removed from the blood stream by the liver cells. The bile salts are the sodium and potassium salts of bile acids conjugated by the liver with glycine or taurine. In this form the bile acids are water-soluble and the four found in human bile are cholic, deoxycholic, chenodeoxycholic and lithocholic acids. Deoxycholic acid is the main bile acid found in the faeces of a normal adult. Conjugation of cholic acid with glycine or taurine will form glycholic and taurocholic acids respectively. Bile salts react with water-insoluble substances such as stearic acid (fatty acid), cholesterol and fat-soluble vitamins (A, D, E and K) to form water-soluble complexes, sometimes called micelles. This hydroscopic power of bile salts is important in promoting absorption of water-insoluble substances, and because they reduce surface tension their emulsifying action within the intestines helps in the digestion of fats. Lipases are also activated by bile salts and when bile is excluded from the intestines as much as 25 per cent of the ingested fat appears in the faeces.

Bile, because of its alkalinity, helps also to neutralize the acid chyme from the stomach.

Functions of the liver

These are numerous but for convenience may be classified into seven main groups.

1. EXCRETION

This is basically the production and excretion of bile into the intestine.

2. CARBOHYDRATE METABOLISM

In periods of availability, the principal monosaccharides resulting from the digestive processes (p. 182) are synthesized to glycogen and stored in the liver. During a fast, blood glucose levels are maintained within normal limits by breakdown of stored glycogen

(glycogenolysis), while non-carbohydrates (amino acids and fats) are converted into glucose (gluconeogenesis).

3. LIPID METABOLISM

Besides the esterification of cholesterol, the synthesis of cholesterol, phospholipids, endogenous triglycerides and lipoproteins occurs mainly, but not exclusively, in the liver.

4. PROTEIN SYNTHESIS

Many of the plasma proteins, but not the γ -globulins, are synthesized in the liver. Many of the coagulation factors, fibrinogen, prothrombin, factors V, VII, IX, X, XI and XII, are also manufactured there. Vitamin K is required for the production of prothrombin, factors VII, IX and X. The liver contains a little heparin, most of the heparin being found in the granules of circulatory basophils and in the granules of most cells.

5. STORAGE

In addition to glycogen and many vitamins, the liver is the main storage site for iron.

6. DETOXICATION AND PROTECTIVE FUNCTION

Drugs and toxic substances are detoxicated by conjugation, methylation, oxidation or reduction. Ammonia derived from amino acids and protein metabolism, or produced in the gut by bacteria, is converted to urea and rendered non-toxic.

7. HAEMATOPOIESIS

The Kupffer cells lining the sinusoids form part of the reticuloendothelial system and are involved in the normal destruction of erythrocytes.

BILE PIGMENT METABOLISM

Erythrocytes at the end of their life span, which is usually about 120 days, are removed from the circulation by the reticuloendothelial system (RES). The protoporphyrin ring of the haem group of haemoglobin is opened up and the resulting bilirubin-iron complex (choleglobin) is formed. The protein and iron are then removed; the former is catabolized to amino acids which enter into new protein synthesis, while the latter is used for the synthesis of new haemoglobin and is retained in the RES in the

form of haemosiderin and ferritin. Ferritin is a soluble iron-protein complex, while haemosiderin is an insoluble iron storage complex which gives an intense blue colour with potassium ferri-cyanide (Prussian blue reaction). After the removal of the protein and iron, the remaining green pigment, biliverdin, is reduced by the tissue enzymes to a yellow pigment, bilirubin. Bilirubin, a water-soluble substance, enters the plasma and is bound to plasma albumin, which on passing into the parenchymal cells of the liver is conjugated principally with glucuronic acid to form the water-soluble mono- and diglucuronides of bilirubin. This conjugation takes place under the influence of uridyl diphosphate glucuronyl transferase. Conjugated bilirubin may enter the circulation and is then also bound to albumin, but principally is transported out of the liver cell into the bile canaliculi and excreted in the bile. The two conjugated forms of bilirubin are present in normal plasma and are responsible for a normal bilirubin level of up to 1 mg per 100 ml. The unconjugated bilirubin (water-insoluble) is also present in plasma during jaundice.

After passing into the gall bladder, the bilirubin glucuronides may be re-oxidized to biliverdin, thus giving rise to the green colour of gall bladder bile. From the gall bladder the bilirubin passes via the bile duct into the small intestine where it is ultimately reduced by bacterial enzymes to a colourless compound, stercobilinogen (faecal urobilinogen). This colourless compound is then oxidized to a brown pigment, stercobilin, the chief pigment of faeces. A small amount of stercobilinogen is reabsorbed and passes via the portal vein to the liver, where it is re-excreted in the bile. A small amount escapes removal from the blood by the liver and is excreted by the kidney. The urinary excretory product, although identical to stercobilinogen, is called urobilinogen and when exposed to air is oxidized to urobilin, which is identical to the stercobilin of faeces. *Figure 9.2* shows the schematic formation of bilirubin and its derivatives. A normal adult may excrete up to 4 mg of urobilinogen per day in the urine, while the faecal excretion of the pigment may be up to 250 mg per day.

Bile salts

Bile salts enter the duodenum with the bile and are normally found in the faeces in small amounts, but a normal urine should contain very little. During the process of absorption from the intestine, the bile salts are almost completely reabsorbed via the portal vein, removed by the liver and re-excreted in the bile. Bile salts are usually present along with bile pigments in the urine in obstructive

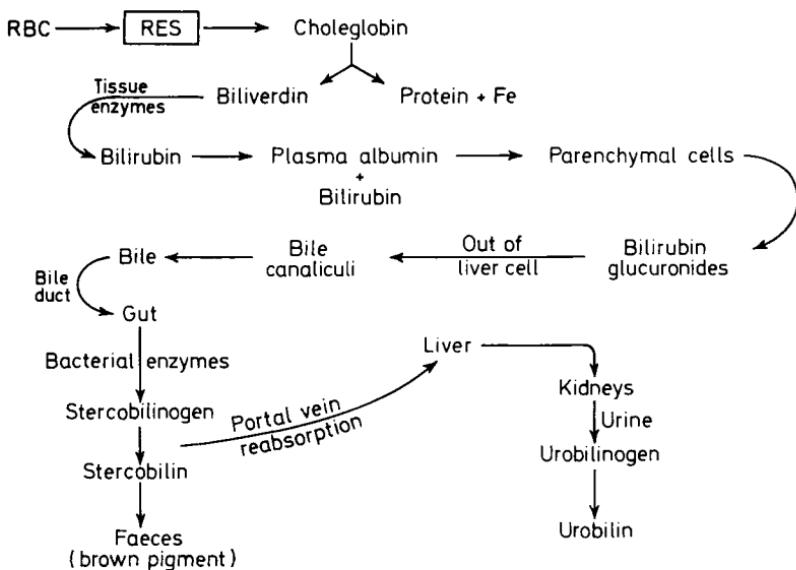


Figure 9.2. Schematic formation of bilirubin and its derivatives

jaundice, but they can be absent when pigments are still present, during recovery.

Jaundice

Bilirubin is normally present in the plasma and is partly responsible for the yellow colour. In certain conditions, the concentration of pigment increases and causes jaundice (icterus) when the skin, sclera of the eye and the body fluids become pigmented (yellow). Some of the causes of jaundice are:

1. An obstruction to the bile duct by either a gall stone within the lumen or by a tumour (usually pancreatic) exerting pressure from outside. In these disorders there is an increase usually of the conjugated bilirubin.
2. An excessive breakdown of erythrocytes as in haemolytic anaemia, giving an increase mainly in unconjugated bilirubin.
3. Diminished function of the liver cells, as in hepatocellular or toxic jaundice, giving an increase of both types of bilirubin.

Jaundice, when present, results not only in a raised plasma or serum bilirubin level, but in the excretion of bilirubin and/or its

derivatives in the urine in a greater concentration than that normally present. Table 9.1 indicates the type of pigment found in the urine in the three causes of jaundice listed above. In haemolytic jaundice the unconjugated bilirubin is absent from the urine because the bilirubin-albumin complex does not pass through the glomeruli.

Table 9.1 INCREASED AMOUNTS OF PIGMENTS FOUND IN URINE DURING JAUNDICE

Cause	Bile salts	Pigments	Urobilin	Urobilinogen
Haemolytic jaundice	—	—	+	+
Obstructive jaundice	+	+	—	—
Hepatic or toxic jaundice	±	+	+	+

DETECTION OF BILIRUBIN AND ITS DERIVATIVES

1. Bile pigments (bilirubin)

PLASMA OR SERUM

Bilirubin in the plasma has been determined for many years by using the Van den Bergh diazo reaction. This involves treating the plasma with diazotized sulphanilic acid, and the red azobilirubin complex so formed at an acid pH is estimated quantitatively in the spectrophotometer at 540 nm against a suitable standard.

URINE

Unconjugated bilirubin is unable to pass through the glomerulus and therefore does not appear in the urine. This is generally true with normal and elevated amounts of plasma unconjugated bilirubin, whereas the conjugated bilirubin is filtered at the glomerulus and is not, when the plasma levels are raised, reabsorbed fully by the tubules. The detection of bilirubin in the urine indicates that excess conjugated bilirubin is present in the plasma.

It is advisable to see the notes on urine testing on p. 246, before carrying out these tests.

FOUCHET'S TEST

Principle

Barium chloride reacts with the sulphate radicals in the urine to form a precipitate of barium sulphate. Any bile pigment present adheres to the precipitate and is detected by the oxidation of bilirubin (yellow) to biliverdin (green) on treatment with ferric chloride

in the presence of trichloracetic acid. A blue colour is given by bilicyanin.

Reagents

1. 10 per cent barium chloride.
2. Fouchet's reagent.

Trichloracetic acid	25 g
Distilled water	50 ml
10 per cent ferric chloride	10 ml
Dilute to 100 ml with distilled water.	

Method

1. Test the reaction of the urine, and if alkaline, acidify with 33 per cent acetic acid.
2. Add 5 ml of 10 per cent barium chloride to 10 ml of urine and mix well. If the precipitate formed is insufficient, add a drop of dilute sulphuric acid or ammonium sulphate solution.
3. Filter through Whatman No. 1 filter paper.
4. Carefully unfold the filter paper and place on top of another dry filter paper, and add 1 drop of Fouchet's reagent onto the precipitate in the centre of the paper. If bile is present, a green or blue colour develops, the colour intensity being proportional to the amount of bile pigment present.

ICTOTEST (REAGENT TABLETS*)

These reagent tablets are quick, simple, standardized colour tests for bilirubin in urine, based on a diazo reaction (see p. 268 on the procedures to be adopted using impregnated strips or tablets).

Composition

The tablet contains *p*-nitrobenzene diazonium *p*-toluene sulphonate, salicylsulphonic acid and sodium hydrogen carbonate.

Principle

When urine is placed on the special mat, bilirubin is adsorbed on its surface. The slight effervescent properties of the tablet partially disintegrate it and cause the reagent to wash onto the surface of the mat, where the bilirubin couples with the diazo compound in the presence of salicylsulphonic acid, forming a bluish-purple compound.

* Ames Company, Stoke Court, Stoke Poges, Slough, Berks.

Directions

1. Place five drops of urine on square of special test mat provided.
2. Place a tablet in middle of moist area.
3. Flow two drops of water over tablet.
4. Observe colour of mat around tablet exactly 30 s later.

Results

Negative The mat around the tablet remains unchanged at 30 s or turns slightly red or pink.

Positive The mat around the tablet turns a bluish-purple within 30 s. The concentration of bilirubin is roughly proportional to the intensity of the bluish-purple colour and to the speed with which it develops.

Precautions

1. Make sure that the container for the urine is absolutely clean and free from contaminants, for example disinfectants, detergents.
2. Recap the Ictotest bottle tightly as soon as the tablet has been removed, to avoid uptake of moisture.

Sensitivity

These reagent tablets are very sensitive and can detect 0.05 mg to 0.1 mg bilirubin per 100 ml of urine, which coincides with the lower limit of accepted pathological significance.

Specificity

Bilirubin is the only substance known to give the characteristic bluish-purple colour with the reagent tablets (*see Handbook of Routine Diagnostic Tests*, published by Ames Company).

FAECAL BILIRUBIN

Meconium, the material excreted during the first few days of life, contains biliverdin. The faeces of very young infants generally contain unaltered bilirubin, but with the development of the bacterial flora bilirubin is gradually reduced to stercobilin. In adults all bilirubin reaching the intestine is reduced to stercobilin unless there is a rapid intestinal movement, when bilirubin may be excreted. Bilirubin can also be found in the faeces of patients receiving antibiotics such as neomycin, which sterilizes the gut. Biliverdin is oxidized to bilirubin on exposure to air. Bilirubin can

be detected by emulsifying a portion of the faeces in distilled water, about 1 in 20 suspension. Treat this with an increasing amount of Fouchet's reagent, but not more than an equal volume is required and usually much less. Bilirubin is oxidized quite rapidly to green biliverdin or blue bilicyanin.

2. Urine urobilinogen

As can be seen from Table 9.1, alterations in urobilinogen excretion can be of some value in assessing jaundice or monitoring its progress.

Freshly passed urine contains a trace of urobilinogen which is colourless, but on standing this is oxidized to urobilin, an orange-yellow pigment. This pigment, along with urochrome, contributes to the normal colour of urine, but when there is an excess of the urobilin pigment, the urine is orange-yellow in appearance.

QUALITATIVE TEST FOR UROBILINOGEN (Wallace and Diamond reaction)

Principle

Urobilinogen is detected by the red colour it gives with Ehrlich's reagent; porphobilinogen also gives the same colour reaction. Urobilin will not give a colour with this reagent.

Reagents

1. Ehrlich's reagent:

4-dimethylaminobenzaldehyde	2.0 g
Concentrated hydrochloric acid	20 ml
Distilled water	80 ml

2. 20 per cent *v/v* hydrochloric acid.

Method

Urine samples must be freshly voided.

1. To 10 ml of urine add 1 ml Ehrlich's reagent.
2. To 10 ml of urine add 20 per cent hydrochloric acid (control).
3. Mix by inversion and stand for 3–5 min at room temperature.
4. Note the colour produced and warm to 50 °C if no colour developed at room temperature.

Results

Normal urines should give a faint red colour with Ehrlich's reagent but not with 20 per cent HCl.

A similar colour is also given by porphobilinogen (see p. 231), and other substances may produce colours ranging from yellow to orange-pink (e.g. *p*-aminosalicylic acid). A red colour with 20 per cent HCl is probably due to a dye such as methyl red.

	<i>Room temperature</i>	50°C
Urobilinogen absent	no red colour	no red colour
Urobilinogen in normal amounts	faint red colour	
Urobilinogen in excess	distinct red colour	

When the urobilinogen is present in excess, dilute the urine from 1 in 10, in steps of 10 up to and beyond 1 in 100 if necessary. Repeat the test on 10 ml aliquots of these dilutions until the last dilution showing the faintest red colour is found.

Normal urine will give no colour at room temperature at a dilution of 1 in 20. Abnormal concentrations may give positive results up to and beyond 1 in 100 dilution.

Bilirubin, if present, must be removed before carrying out Ehrlich's test. Use either the filtrate from Fouchet's test or add 1 volume of 10 per cent calcium chloride to 5 volumes of urine. Mix well and filter through Whatman No. 1 filter paper. Use the clear filtrate for the test. The bilirubin is adsorbed onto calcium phosphate in the same way as it adheres to barium sulphate in Fouchet's test.

UROBILISTIX REAGENT STRIPS FOR UROBILINOGEN*

These reagent strips are firm plastic strips with the reagent impregnated into an absorbent area at the tip of the strip to provide a rapid, convenient test for urinary urobilinogen.

Composition and principle

The reagent, 4-dimethylaminobenzaldehyde, is stabilized in an acid buffer resulting in the formation of a brown colour with urobilinogen.

Directions

1. Dip reagent area of strip in fresh, well-mixed freshly voided uncentrifuged urine.
2. Remove strip from urine. Tap edge of strip against urine container to remove excess urine.

* Ames Company, Stoke Court, Stoke Poges, Slough, Berks.

3. Allow reaction to continue for exactly 60 s from dipping.
4. Immediately compare colour of reagent area with colour chart, holding reagent area close to the chart. Interpolate if colour produced falls between two colour blocks. Avoid glare.

Results

The test is read in Ehrlich units per 100 ml, the colour varying from yellow to more intense shades of brown with increasing concentrations of urobilinogen. Colour blocks representing 0.1, 1, 4, 8 and 12 Ehrlich units are provided. 1 Ehrlich unit is equivalent to 1 mg urobilinogen. Values up to 1 Ehrlich unit should be considered marginal and must be left to the clinician to interpret in the light of clinical evidence.

Sensitivity

Urobilistix reagent strips will detect urobilinogen in concentrations of approximately 0.1 Ehrlich unit per 100 ml urine. The absence of urobilinogen cannot be determined with the product. No substances are known to inhibit the reaction of the reagent strips.

Specificity

The reagent strips are not specific for urobilinogen. They will react with some of the substances known to react with Ehrlich's reagent, e.g. *p*-aminosalicylic acid, but not with porphobilinogen or haemoglobin (*see Handbook of Routine Diagnostic Tests*, published by Ames Company).

3. Urine urobilin (Schlesinger's test)

Principle

After first oxidizing any urobilinogen present to urobilin, a greenish-yellow fluorescent compound of zinc urobilin is formed, the fluorescence of which is more definite in ultraviolet light than daylight.

Reagents

1. Absolute ethanol.
2. Zinc acetate (powdered).
3. Tincture of iodine.

Method

1. To 10 ml of urine in a test-tube add a few drops of tincture of iodine to oxidize the urobilinogen to urobilin. Bilirubin must be removed beforehand, as for urobilinogen.
2. Into another test-tube place about 10 ml of ethanol and add approximately 1 g of powdered zinc acetate.
3. Pass the solutions backwards and forwards from one tube to the other, until nearly all the powder is dissolved.
4. Filter the mixture through Whatman No. 1 filter paper into a clean test-tube.
5. View the filtrate from above. It is better to examine by using reflected light with the tube held against a black background. A greenish-yellow fluorescence is present if the urobilin is present. Examine under an ultraviolet light when the fluorescence is more noticeable. Next examine with a direct-vision spectroscope. Zinc urobilin shows an absorption band in the green portion of the spectrum centred at 506.5 nm. Urobilin itself, in acid urine, also exhibits an absorption band, centred at 490 nm (green blue), but this band is more diffuse and less easy to identify than the zinc urobilin absorption band.

Results

Normal urine should give no more than a barely detectable amount of fluorescence.

URINE

Detection of bile salts

Bile salts lower the surface tension and can be responsible for increased foaming when urine is shaken. The reduction of surface tension is used in their detection.

HAY'S TEST

Reagents

Flowers of sulphur.

Method

Place some fresh clear urine in a small beaker at room temperature. Sprinkle a little finely powdered flowers of sulphur onto the surface. Sulphur particles sink in the presence of bile salts, but remain on the surface of the urine if absent. Urines preserved with thymol may give a false positive.

Modification

Into a clean beaker place some distilled water and carefully sprinkle some flowers of sulphur on top of the surface in the centre of the beaker. With a clean pasteur pipette add a drop of urine to the centre of the sulphur. If bile salts are present, the sulphur will be dispersed towards the side of the beaker. In the absence of bile salts, the sulphur will remain in the centre of the beaker.

Treat a normal urine in a similar manner for both methods.

It has been suggested that this test is of little diagnostic value.

Detection of porphobilinogen

In acute intermittent porphyria, porphobilinogen, a colourless compound and an intermediate product in the biosynthesis of haem, is found in the urine. Porphobilinogen, like urobilinogen, gives a red colour with Ehrlich's reagent and the two compounds must be differentiated from each other.

TEST FOR PORPHOBILINOGEN*Principle*

Urine is treated with Ehrlich's reagent, when a red colour is given by porphobilinogen. To distinguish this colour from that given by urobilinogen, the addition of saturated sodium acetate solution, which alters the pH, intensifies the colour given by urobilinogen but not that by porphobilinogen and makes the urobilinogen more soluble in the extracting solvent.

Reagents

1. Ehrlich's reagent.

4-dimethylaminobenzaldehyde	0.7 g
Concentrated hydrochloric acid	150 ml
Distilled water	100 ml

2. Saturated sodium acetate.

Hydrated sodium acetate about	100 g
Distilled water	100 ml

3. Chloroform or amyl alcohol : benzyl alcohol mixture (3 : 1 v/v).

Method

Urine samples must be freshly voided.

- To 5 ml of urine add 5 ml of Ehrlich's reagent, mix and allow to stand for 3–5 min.

2. Add 10 ml saturated sodium acetate solution, mix and leave for a few minutes.
3. Add 2 ml of chloroform or amyl alcohol : benzyl alcohol mixture and shake thoroughly. Allow the two layers to separate.

Results

Urobilinogen is soluble in the organic layer, so any red colour remaining in the aqueous phase after extraction constitutes a positive test for porphobilinogen.

Rimington found that the amyl alcohol : benzyl alcohol mixture gave a more complete removal of the coloured complex formed by urobilinogen from the aqueous phase than did chloroform.

It may therefore be necessary to repeat the extraction with chloroform to confirm a positive test for porphobilinogen.

10

Renal Function Tests

The kidneys are the primary organs involved in the excretion of waste products, the other organs involved being the lungs, skin and intestines. In the urinary system there are two kidneys which form and secrete urine by peristalsis; it is conveyed from each kidney through a ureter to the urinary bladder. The bladder provides temporary storage for about 300ml urine, which is

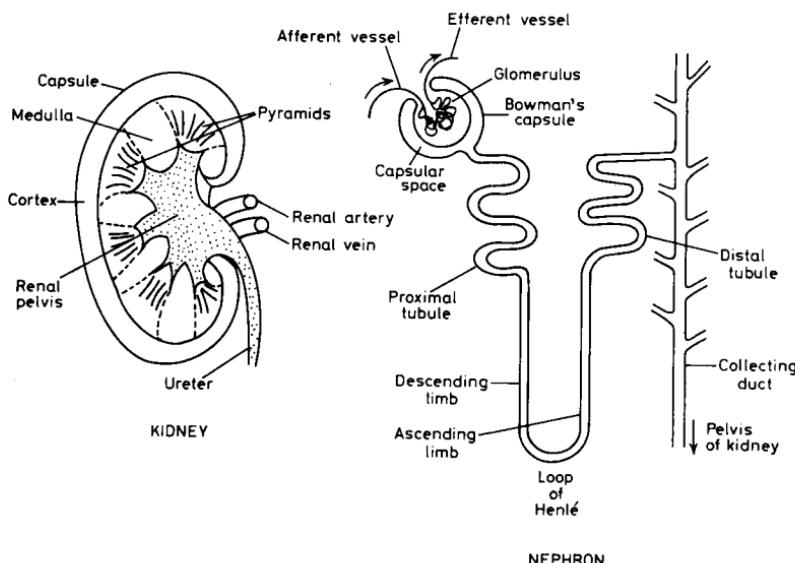


Figure 10.1. The kidney and nephron

eventually voided through the urethra to the exterior. The kidneys are situated at the posterior of the abdomen, one on either side of the vertebral column, the right kidney a little lower than the left, due to the space occupied by the liver.

Each kidney, which is bean-shaped, is enclosed in a capsule of fibrous tissue, which is easily stripped off. Underneath the capsule lies the cortex, followed by the medulla, which is made

up of renal pyramids, then the hilum, where the renal arteries and nerves enter, and the renal vein and ureter leave the kidney.

The microscopical structure of the kidney is composed of nephrons and collecting tubules. There are approximately one million nephrons to each kidney, this being the functional unit. The nephron consists of the glomerular or Bowman's capsule, proximal convoluted tubule, loop of Henle and the distal convoluted tubule (*Figure 10.1*).

FUNCTIONS OF THE KIDNEY

1. MAINTENANCE OF WATER AND ELECTROLYTE BALANCE OF THE BODY

When the amounts of water and/or electrolytes in the body fluctuate, their excretion is regulated by the kidney with the help of anti-diuretic hormone and aldosterone, so that the body fluids are restored to their normal composition and volume. This is best explained by referring to the osmotic pressure of plasma. Under normal conditions, the osmolarity of the plasma varies only slightly, despite wide variations of the fluid and electrolyte intake of the body. When an excess of water is taken, it will tend to dilute the plasma and reduce its osmotic pressure, producing a renal response which results in the excretion of an increased volume of urine with an osmolarity less than that of the plasma. By excreting water in excess of the solutes, the kidney maintains the water balance of the body. On the other hand on restricted fluids, an increase in plasma osmolarity will be corrected by the excretion of a 'concentrated' urine with an osmolarity higher than that of plasma, showing that the solutes are being excreted in excess of water (*see selective reabsorption*).

2. MAINTENANCE OF pH OF THE BLOOD

The normal pH of blood in health is slightly on the alkaline side, pH 7.36–7.42, and the kidneys are responsible for removing substances which cause it to become acid or more alkaline. Substances of acid reaction are the waste products of protein metabolism—urea, uric acid and creatinine—which are constantly being formed and therefore must be excreted. Salts of sodium, potassium, calcium, magnesium and phosphorus are alkaline in reaction, and therefore must be excreted if their concentration reaches too high a level in the blood.

3. EXCRETION OF DRUGS AND TOXINS

Drugs after completing their action in the body leave waste products which are excreted by the kidneys. If the drugs are not metabolized they themselves are excreted. Toxins in the body are rendered harmless by detoxication in the liver, and the conjugated compounds so formed excreted in the urine.

Formation of urine

Urine is formed in the nephrons by a combination of two processes, simple filtration and selective reabsorption.

SIMPLE FILTRATION

This process takes place between the glomerulus and glomerular capsule. The blood supply to the kidneys averages 1200 ml per min (25 per cent of the heart's output per min). A higher pressure in the glomerulus meets negligible pressure in the Bowman's

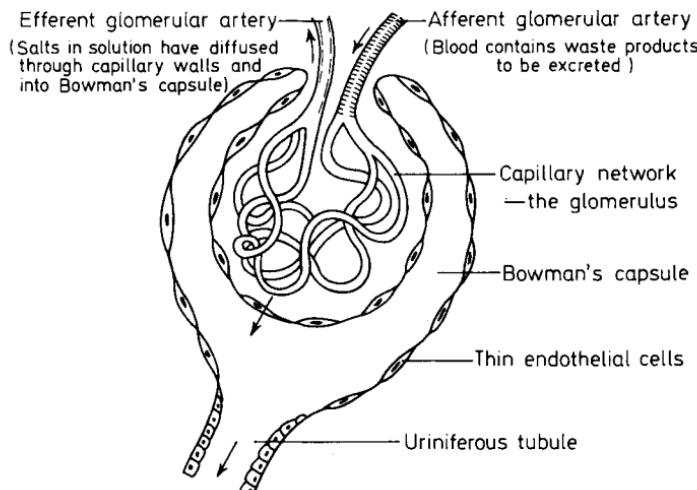


Figure 10.2. Diagram of a glomerulus

capsule, resulting in the passage of substances in solution through the semi-permeable membrane at the rate of about 120 ml per min (glomerular filtrate rate). The filtrate contains salts, glucose, urea, uric acid and other substances of small molecular size. Cells and plasma proteins which have a large molecular size do not pass through the semi-permeable membrane (Figure 10.2).

SELECTIVE REABSORPTION

When the filtrate enters the tubule, the tubular epithelium reabsorbs water and selected essential substances into the peritubular capillaries. By passive and active reabsorption the cells adjust the composition of urine to meet the body requirements. Approximately 80 per cent of the water and sodium chloride content, together with glucose, phosphate and amino acids, are absorbed in the proximal tubule. About 20 per cent of the tubular fluid enters the loop of Henle where water is passively absorbed, 6 ml per min of concentrated tubular fluid now enters the distal tubule, where there is an active reabsorption of sodium. The fluid leaves the distal tubule at the rate of approximately 1 ml per min, passing into the collecting ducts in the form of urine. Over a period of 24 h this will give a urine volume of 1-1½ litres.

Renal threshold

The constituents of the glomerular filtrate have either a high, medium, low or no-threshold value. This means that the threshold of a given substance in the plasma is the highest level at which the constituent is present in the blood before it appears in the urine. Glucose, a high threshold substance, is completely reabsorbed from the filtrate and only appears in the urine when the blood level is about 150 mg per 100 ml, this being the normal threshold value. Urea has a threshold value of zero, because only small amounts of it are reabsorbed in the tubules, and it is always present in the urine no matter what the blood level happens to be. Finally, creatinine is a no-threshold substance, as it is not reabsorbed and is always present in the urine. (Tubular cells can also secrete some creatinine into the filtrate.) The threshold of any substance can be altered by impaired renal function.

Urine analysis

COMPOSITION

Urine is water containing the water-soluble waste products removed from the blood stream via the kidneys. Normal urine consists of approximately 95 per cent water, the remainder being made up of urea, uric acid, creatinine, sodium, potassium, chloride, calcium, phosphate, etc. The composition varies widely from day to day, depending upon the food and fluid intake.

Microscopical and chemical examination of urine may yield useful information in many abnormal conditions. Infections of the kidneys, ureters, bladder and urethra may result in the presence of

pus, red blood cells and organisms in the urine. When the kidneys become inflamed various casts may appear in the urine (*see p. 243*). Reducing substances, ketones, bile and protein can be found in a variety of pathological conditions.

VOLUME

Of the 75–150 litres of glomerular filtrate produced by the normal adult kidney, tubular reabsorption reduces this volume to between 1 and 2 litres per day. This varies with fluid intake and diet, as well as other physiological factors. As already mentioned copious drinking will increase the volume of urine passed, whilst excessive perspiration will decrease the volume. In certain pathological conditions, the output will differ considerably from the normal volume. An increased output (polyuria) can be found in diabetes mellitus, and also in diabetes insipidus, when 10–20 litres may be excreted. A reduced output (oliguria) may be found in acute nephritis or fevers, while a complete suppression (anuria) can be occasionally encountered in blood transfusion reactions.

APPEARANCE

Urine is normally clear and pale yellow in colour, due to a pigment, urochrome, said to be a compound of urobilin, urobilinogen and a peptide substance. Urochrome is a product of endogenous metabolism, and is fairly constant in amount from day to day. In concentrated urines there is the same amount of urochrome, thereby giving a much darker appearance, ranging from dark yellow to brown red in colour.

<i>Colour of urine</i>	<i>Cause</i>	<i>Conditions</i>
Milky	Fat globules Pus	Chyluria Infection of the urinary tract
Greenish-yellow greenish-brown dark brown	Bile pigments	Jaundice
Orange yellow	Excess urobilin	Jaundice
Red or reddish	Haemoglobin myoglobin porphyrins beetroot	Haemoglobinuria Trauma Porphyria
Brown to brown-black	Haematin methaemoglobin melanin	Haemorrhages Methaemoglobinuria Melanotic tumours

Urines may be abnormally pigmented by dyes, for example the administration of methylene blue by mouth results in a greenish-blue urine, phenols cause a dark, almost black urine, and certain foods and drugs can result in various colours and odours in the urine.

ODOUR

Freshly passed urine has a characteristic aromatic odour, said to be due to volatile organic acids. When the urine is allowed to stand, decomposition of urea by bacteria occurs, and ammonia is evolved.

REACTION

Freshly passed normal urine is usually slightly acid with a pH about 6.0 and a range of 4.8–6.8. Due to alkaline fermentation cloudiness occurs and phosphates are precipitated; amorphous urates may also be deposited on standing. The urates are insoluble in dilute hydrochloric acid, while the phosphates are soluble.

It is usually sufficient to indicate whether the urine is acid or alkaline by using indicator papers. British Drug Houses supply indicator papers in reel dispensers suitable for this purpose. These cover the range for urine pH being from pH 4–6, 6–8 and 8–10. Other pH ranges covered are from pH 1–4, 10–12 and 12–14. A full range indicator from pH 1–14 is also available. The pH is then determined by simply dipping the indicator strips into the urine and then comparing the change of colour of the strip against the colour guide supplied with the indicator papers.

For a more accurate determination of pH, a pH meter should be used. Alternatively a colorimetric method can be used, similar to the determination of pH when preparing media.

SPECIFIC GRAVITY (Sp. gr.)

The specific gravity of a normal urine is within the range of 1.016 to 1.025. It is subject to wide fluctuations. For example, after drinking a large quantity of water, the sp. gr. may be as low as 1.001, while it can reach 1.040 after excessive perspiration. In diabetes mellitus due to high concentrations of glucose, the sp. gr. may be as high as 1.040. The presence of protein in large amounts will also increase the sp. gr. A high sp. gr. is found in acute nephritis due to the concentrated urine, whereas in chronic nephritis the reverse is obtained.

Measurement of specific gravity

Specific gravity is the relative proportion of the weight of a volume

of urine to that of an equal volume of distilled water, water being a standard unit.

$$\text{i.e. sp. gr.} = \frac{\text{weight of urine}}{\text{weight of same volume of distilled water}}$$

The standard method for determining sp. gr. is by using the specific gravity bottle, or by weighing. The urinometer technique is not as accurate.

1. *Specific gravity bottles* Specific gravity bottles (density bottles) can be supplied in sizes varying between 10 and 100 ml. They are designed to allow identical volumes to be weighed.

Technique for using the specific gravity bottle

1. Weigh the clean, dry, stoppered bottle accurately.
2. Fill the bottle with the test liquid.
3. Carefully insert the ground-glass stopper. The excess liquid is ejected from the central hole in the stopper.
4. With a clean tissue remove all traces of liquid from the outside of the bottle, and wipe carefully across the top of the stopper. Make sure that there is no air underneath the stopper, or in its capillary.
5. Weigh the bottle of test liquid.
6. Empty the bottle, and rinse it out several times with distilled water.
7. Fill the bottle with distilled water and replace the stopper as before, then wipe the outside and weigh.
8. Subtract the weight of the empty stoppered bottle from both total weights, and divide the weight of the test liquid by that of the distilled water.

Both liquids should be at the same temperature, as volume is altered by temperature variations.

2. *Weighing* If there is only a small volume of urine weigh a known quantity of urine; by using a volumetric pipette and using the same pipette, weigh the same volume of distilled water.

$$\text{sp. gr.} = \frac{\text{weight of urine}}{\text{weight of same volume of distilled water}}$$

3. *Urinometers* Urinometers are designed for estimations of specific gravity. Their use in medical laboratories is mainly confined to measurement of the specific gravity of urine. The urinometer is so calibrated that it sinks in distilled water, until the mark '0' on the stem is level with the surface of the water. This denotes

a specific gravity of 1.000. In urine, which is more dense than water, the instrument is more buoyant, and the specific gravity is read off the scale at the level of the surface of the urine.

Precautions when using the urinometer

1. Make absolutely certain that the instrument floats centrally in the liquid by rotating it, and that it is not in contact with the bottom or sides of the container.
 2. Take readings at the lowest point of the meniscus, which should be viewed at eye-level. Errors will result if this is not done.
 3. Always check the accuracy of new urinometers in distilled water. Sometimes a correction factor is necessary; for example, if the reading in distilled water is 1.004, then 0.004 must be subtracted from test readings.
 4. As these instruments are usually calibrated at 15°C, one must add 0.001 for every 3°C above 15°C, and subtract 0.001 for every 3°C below 15°C. For every 1 g per 100 ml albumin present, subtract 0.003.
4. *Osmometry* A newer technique introduced in the laboratory is that of osmometry, which tends to replace the specific gravity procedures. It is a technique in which the total solute content of body fluids is measured by using instruments called osmometers (*see p. 137*). In body fluids the osmotic pressure is of significance and this depends upon the total concentration of molecules and ions in solution (solutes). This concentration cannot be expressed either in mEq/l or mmol/l, and the unit in which this concentration is expressed is the osmol. An osmol is the amount of substance in one litre of solution which under ideal conditions exerts an osmotic pressure of 22.4 atmospheres and will therefore depress the freezing point by 1.86°C.

The measurement of the depression of freezing point below that of pure water by the total solutes is therefore a measure of the total osmotic pressure of the body fluids.

The osmol is a very large number and to avoid the use of fractions, a milliosmol is used (mosmol), which is one-thousandth of an osmol and is equivalent to a freezing point depression of 0.00186°C.

Osmolarity is the osmotic pressure of 22.4 atmospheres exerted by one mole per litre of un-ionized substance, while osmolality is the osmotic pressure of 22.4 atmospheres exerted by one gram mol per kg of un-ionized substance—the latter is preferred.

Normally the urine osmolality varies between 700 and 1500 mosmol per kg, but this depends upon the diet taken.

Osmolality is usually affected in disease, as is specific gravity, but because of the variation in the nature of the solutes, one cannot be calculated from the other. For example a urine with a specific gravity of 1.016 can give values of between 550 and 910 mosmol per kg.

Collection of urine

Sterile specimens of urine are not necessary for biochemical analysis, but the urine should be collected in a clean dry bottle. If a timed specimen is required, the patient should empty the bladder at, for example, 8.0 am, this urine being discarded. All the urine passed up to and at 8.0 am the following morning is saved and placed in a labelled bottle. This collection will then be a 24-h sample. The same procedure applies for a 2-, 4- or 6-h specimen of urine. If the patient wishes to defaecate during the collection period, the bladder should be emptied beforehand, to avoid losses of urine. The collection of timed urine specimens is very important and it is surprising how badly this is often done.

PRESERVATIVES

Sometimes it may be necessary to keep the urine for 24–48 h before the analysis can be carried out, or bacterial action during the collection period may affect the constituent to be analysed. However, if the urine is collected into a clean, dry container, little change will take place in a 24-h specimen by the time it is received in the laboratory. Keeping the urine container cool and stoppered during collection will also help to preserve the urine. Urea is the most labile constituent in urine, as bacteria will convert it to ammonium carbonate; the ammoniacal odour of grossly bacterially contaminated urine is well known. Because of bacterial action, the urine would be unsuitable for the determination of pH, urea and ammonia.

The choice of preservative is often influenced by the estimation required on the urine. As a rule acid is quite satisfactory, when 20 ml 2N HCl can be added to the container prior to the collection. Urines preserved in this manner are suitable for the estimation of urea, ammonia and calcium.

Chloroform, toluene and thymol have also been used. A few crystals of thymol will preserve the urine quite satisfactorily for the estimation of sodium, potassium, chloride, urea, protein, reducing substances and amylase.

MICROSCOPIC EXAMINATION

Urine should be examined as soon as possible after it has been voided. Delay may result in disintegration of cells, and the deposition of amorphous urates or phosphates.

Method

1. Centrifuge approximately 10 ml of urine, and decant the supernatant fluid.
2. Tap the bottom of the centrifuge tube to loosen the deposit, and place one small drop on a clean slide.
3. Apply a coverslip, avoiding the formation of air bubbles.
4. Examine the field with the $\frac{2}{3}$ in objective to obtain a general impression of the deposit, then use the $\frac{1}{6}$ in objective to identify all the constituents. (Illumination of the field should not be too bright, as casts and other structures may not be seen.)

METHOD OF REPORTING DEPOSITS

Note—The best way of learning to identify urinary deposits is to seek constant guidance from an experienced technician. Many extraneous inclusions may be mistaken for casts by the beginner, for example cotton wool, hair and scratches on the slide or coverslip. Oil or air bubbles may be mistaken for red blood cells (*Figure 10.3*). Sometimes the entire deposit may be missed through incorrect focusing of the microscope.

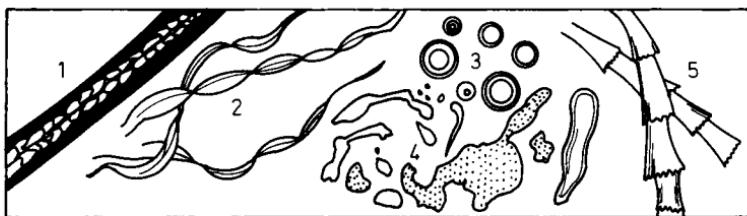


Figure 10.3. Some miscellaneous inclusions. (1) Hair, (2) cotton wool, (3) oil droplets, (4) air bubbles, (5) feather barbs

Deposits may be divided into two main groups as follows:

ORGANIZED DEPOSIT

Cells

1. Epithelial cells of the squamous type are present in many normal urine specimens, especially in non-catheter samples from female patients (*Figure 10.4*).
2. Red blood cells are not present in the urine of normal males.

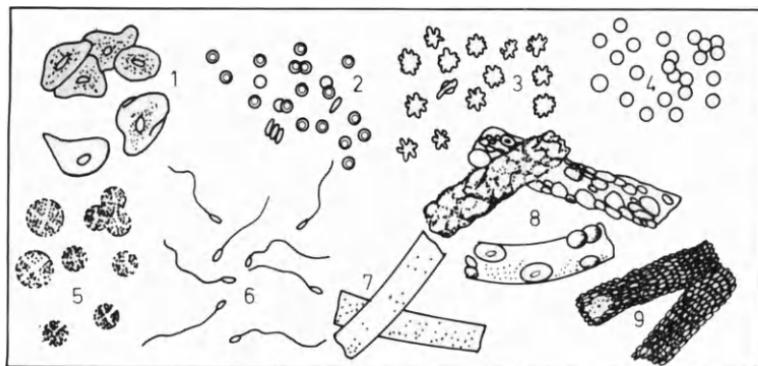


Figure 10.4. (1) Epithelial cells. (2) Normal red blood cells. (3) Crenated red blood cells. (4) Swollen red blood cells. (5) Leucocytes. (6) Spermatozoa. (7) Hyaline casts. (8) Cellular casts. (9) Granular casts

In samples from female patients they may be of menstrual origin. Red cells may be normal, crenated or swollen, depending on whether the urine is isotonic, hypertonic or hypotonic (*Figure 10.4*).

3. Leucocytes in normal urine are found only occasionally, generally not more than 2 per field using the 4 mm objective. Depending on the tonicity of the urine, they may be normal, swollen, or shrunken in size. When degenerate, they are sometimes called 'pus cells' (*Figure 10.4*).

Casts

Casts of the renal tubules are not present in normal urine. They indicate renal dysfunction, and are usually associated with albuminuria. The sides of a cast are parallel, and the end may be either rounded or broken off (*Figure 10.4*). They are of three main types, but these are not necessarily clearly defined. For example, a hyaline cast may have cellular inclusions.

1. Hyaline casts are transparent and homogeneous.
2. Cellular casts are partially or wholly composed of pus, epithelial or red blood cells.
3. Granular casts are degenerated cellular casts, and are granular in appearance.

Organisms

Organisms are of no significance when they are found in urine samples that have been standing overnight. The presence of

bacteria and pus cells in freshly voided urine is indicative of infection and the findings should be reported.

Spermatozoa

Spermatozoa (*Figure 10.4*) should be reported when present in large numbers, which may suggest a lesion in the genito-urinary tract.

Mucus

Mucus is derived from the mucous glands of the urinary tract, and appears as long translucent shreds. The presence of small amounts of mucus is considered normal.

UNORGANIZED DEPOSIT

A knowledge of the reaction of urine samples is of great assistance in identification of deposits. In samples with an acid reaction, the commonest crystalline deposits likely to occur may be calcium oxalate, sodium urate or uric acid. In alkaline specimens, deposits are more likely to be phosphates, calcium carbonate or ammonium urate (*Figure 10.5*).

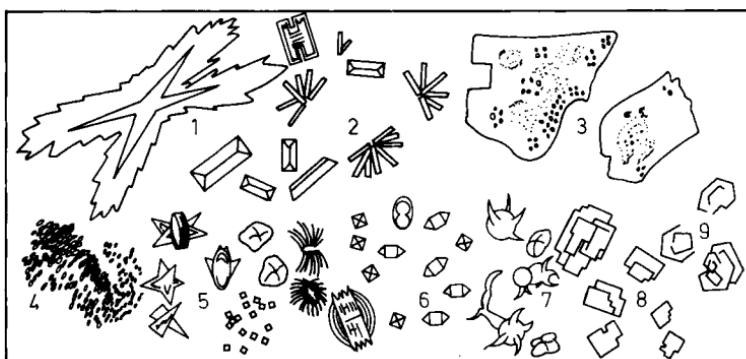


Figure 10.5. Various crystals. (1) Triple phosphate (ammonium magnesium phosphate). (2) Stellar phosphate (calcium phosphate). (3) Calcium phosphate (plate form). (4) Amorphous phosphates. (5) Uric acid (various forms). (6) Calcium oxalate. (7) Ammonium and sodium urate. (8) Cholesterol. (9) Cystine

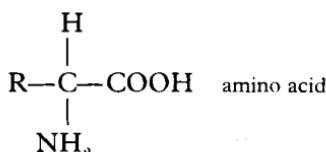
BLOOD

In some kidney diseases, or if the urinary tract is damaged, blood can be passed into the urine (haematuria). This can often be detected macroscopically, or, if the condition is only slight, microscopical examination will reveal intact blood cells. In intravascular

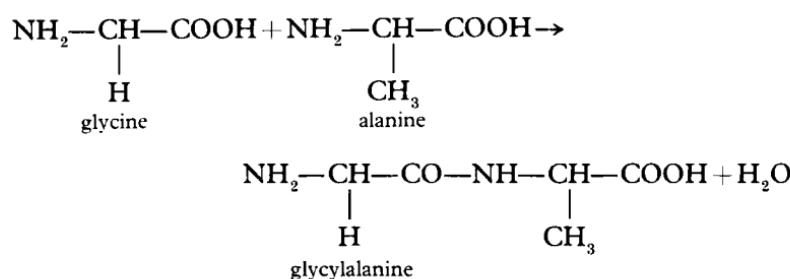
haemolysis, which can occur after an incompatible blood transfusion or in haemolytic anaemia, there are no intact red cells, but free haemoglobin is present (haemoglobinuria). Microscopic detection of red blood corpuscles is not as sensitive as the chemical method. The techniques given on p. 198 can be used.

PROTEIN

Proteins are one of the most important, as well as the most complicated groups of biological substances. They are built up of amino acid units which can be liberated during acid and basic hydrolysis. Twenty-two different amino acids can be identified in such a way.



When the carboxyl group of one amino acid reacts with the amino group of another amino acid, a peptide is formed. The linkage joining the amino acids together —CO—NH— is known as a peptide bond, and this bond is important when proteins are estimated quantitatively by the biuret reaction.



Normal urine contains traces of protein material, but the amount is so slight that it escapes detection by any of the simple laboratory tests. In various pathological conditions when protein is found in the urine (proteinuria) it may be derived from plasma albumin, plasma globulin, haemoglobin, and related products from red cells. Bence-Jones protein and protein from pus and mucus (from urinary tract lesions) may also be present. Semen and vaginal secretions can also give rise to proteinuria. Contamination of urine samples, for example, by vaginal discharge or faeces must be avoided. To prevent this, catheterization may be essential in

the female patient; a mid-stream specimen from the male is satisfactory.

Qualitative tests

1. BOILING TEST

Principle

Proteins are coagulated and denatured by heat.

Reagent

33 per cent acetic acid.

Method

1. If the urine is alkaline, make slightly acid with 33 per cent acetic acid.
2. Filter or centrifuge if not clear, and fill a test-tube three-quarters full with the urine.
3. Hold the tube at an angle and heat the upper layer of urine.
4. Protein, if present, is coagulated and turbidity occurs.
5. Add several drops of 33 per cent acetic acid and boil again.
6. When protein is present, the turbidity remains; if it disappears on adding acetic acid, it is due to the precipitation of phosphates.
7. During heating of the urine, carbon dioxide is driven off, so that the degree of alkalinity increases with resultant precipitation of phosphate. Acidification reverses this reaction.
8. It is a good policy to add acetic acid after boiling, even when there is no turbidity, because sometimes an alkaline urine will give a turbidity on the addition of acid. This is due to metaprotein, which in alkaline solution is uncoagulable, but when the pH is altered to neutral or slightly acid, the metaprotein is precipitated and immediately coagulated by heat. Excess acid must be avoided, as this keeps the protein in solution, preventing its coagulation.

Notes—

1. The boiling test is not quantitative, but the amount of protein may be roughly assessed. Report protein as trace, +, ++ and +++, depending on the amount of precipitate produced.
2. This test is sensitive to about 5 mg protein per 100 ml and is a more reliable method than the sulphosalicyclic acid test. However, most laboratories appear to use the Albstix method, which will give both positive and false negative results.

2. SULPHOSALICYLIC ACID TEST*Principle*

Sulphosalicylic acid is an anionic precipitant, and therefore the neutralization of the protein cation results in the precipitation of the protein.

Reagents

25 per cent sulphosalicylic acid in distilled water.

Method

To 5 ml of clear urine add 0.5 ml sulphosalicylic acid. In the presence of protein a white precipitate appears, the turbidity being proportional to the amount of protein present. Compare against untreated urine.

This test is sensitive to as little as 10 mg per 100 ml of protein; although uric acid may give a positive reaction, the turbidity will disappear on warming the tube. Radio-opaque substances also give false positive reactions with the reagent (see p. 248).

3. ALBUSTIX*

Albustix reagent strips are firm, plastic strips with the reagent system at one end. The reagent is an indicator system and a citrate buffer.

Principle

Tetrabromophenol blue, at pH 3, is yellow, but in the presence of protein and at the same pH, the indicator changes to a shade of green.

Method

Dip the test end into freshly passed uncentrifuged urine, remove immediately and compare at once with the colour chart. If the test end remains yellow, the urine contains no protein.

Precautions

1. Make sure that the container for urine is absolutely clean and free from contaminants, particularly disinfectants and detergents, including quaternary ammonium compounds. Acid used as a preservative reduces the sensitivity.
2. Do not touch test end of strip.
3. Recap bottle tightly as soon as strip has been removed.

* Ames Company, Stoke Court, Stoke Poges, Slough, Berks.

4. Do not leave strip in urine, or hold in or pass through urine stream, to avoid risk of dissolving out reagents.
 5. Read strip in a bright white light; coloured fluorescent lighting may interfere with readings.
- Hold strip very near to colour chart when making readings.

Sensitivity

Positive results are obtained with albumin, globulin, haemoglobin, Bence-Jones protein and mucoproteins. The + colour block represents approximately 30 mg albumin per 100 ml urine. Smaller concentrations of protein than this are detectable, as indicated by the presence of the trace colour block. Markedly alkaline urines can give false positive reactions.

Specificity

The strips are unaffected by urine turbidity, X-ray contrast media, most drugs and their metabolites, and urinary preservatives which may affect other tests.

Quaternary ammonium compounds (e.g. cetavalon) will give false positive results.

Differentiation between protein and a radio-opaque substance

In X-rays of the urinary tract, a radio-opaque substance such as uroselectan is used, and if urine is collected following this test a false positive reaction (pseudo-albuminuria) will be given by the sulphosalicylic acid. The following table will show how the two substances can be distinguished from each other.

	<i>Boiling test</i>	<i>Sulphosalicylic acid</i>	<i>Albstix</i>
Protein	+	+	-
Radio-opaque substance		+	-

Bence-Jones protein

In multiple myeloma abnormal proteins are formed in the bone-marrow; these proteins may be found in the plasma and can be excreted in the urine. The most important member of this group of proteins is Bence-Jones protein, and the recognition of its presence is an important aid to the diagnosis of this disease. Bence-Jones protein can be distinguished from the proteins generally

found in the urine by its behaviour on heating. It will precipitate at temperatures between 40 and 60 °C, whereas the other proteins precipitate between 60 and 70 °C. On raising the temperature to boiling, Bence-Jones protein will re-dissolve, but the other proteins will not. On cooling to 60 °C from boiling, Bence-Jones protein re-precipitates. Bence-Jones protein gives a positive reaction with the sulphosalicylic acid reaction, but can be missed with the boiling test.

Tests for Bence-Jones protein

1. BRADSHAW'S TEST

1. Into a test-tube place a few ml of concentrated hydrochloric acid.
2. Carefully add down the side of the tube a few ml of urine, so as to preserve a sharp junction between the two liquids.
3. If Bence-Jones protein is present a white 'curdy' precipitate occurs at the interface.
4. The test may be positive if other proteins are present in considerable amounts, but if the urine is diluted with distilled water, a true Bence-Jones protein often remains positive.
5. For confirmation the heat test should be performed.

2. HARRISON'S THREE-TUBE TEST METHOD

1. Filter urine, and adjust reaction with 33 per cent acetic acid until just acid.
2. Place 5 ml of this urine into each of three tubes.
3. To the three tubes add 0, 1 and 2 drops of 33 per cent acetic acid respectively, and mix. This will provide variations in the pH of the specimen.
4. Place a thermometer inside the test-tube and immerse the tubes in a beaker of cold water and heat slowly.
5. Carefully observe the temperature and any signs of precipitation. It is advisable to stir the water to maintain a uniform temperature.
6. When Bence-Jones protein is present, at least one tube will go turbid at a temperature between 40 and 60 °C, which disappears on raising the temperature to boiling.

Notes—

1. If protein other than Bence-Jones is present, allow the urine to boil, filter off the precipitate while still hot, and test the clear filtrate as above.
2. In theory this procedure should detect the Bence-Jones protein, but

it is not very reliable as a diagnostic aid. It has been said that up to 1 g per 100 ml of Bence-Jones protein could not be detected by its behaviour on heating. The only satisfactory method for detecting Bence-Jones protein is by electrophoresis (see p. 150), the urine being concentrated about 5 times beforehand.

3. ELECTROPHORESIS METHOD

As there is usually insufficient protein in the urine for detection by this technique, the urine must be concentrated beforehand. One of the methods used for concentration is by using a polyacrylamide hydrogel, Lyphogel*, which will concentrate biological fluids in 5 h. Each Lyphogel pellet expands in aqueous solution to absorb 5 times its own weight of water and low molecular weight substances such as salt, while excluding protein and other substances of molecular weight of 20 000 or more.

Concentration of urine

1. To 10 ml urine in a test-tube, add 1.48 g Lyphogel.
2. Cover the test-tube with parafilm and leave for 5 h or more.
3. Remove the gel pellets from the solution with forceps.
4. Any fluid still adhering to the swollen cylinders should be drained back into the concentrate by touching them to the side of the tube.
5. As 1 g of Lyphogel absorbs 5 ml water, this technique will remove 8 ml water.

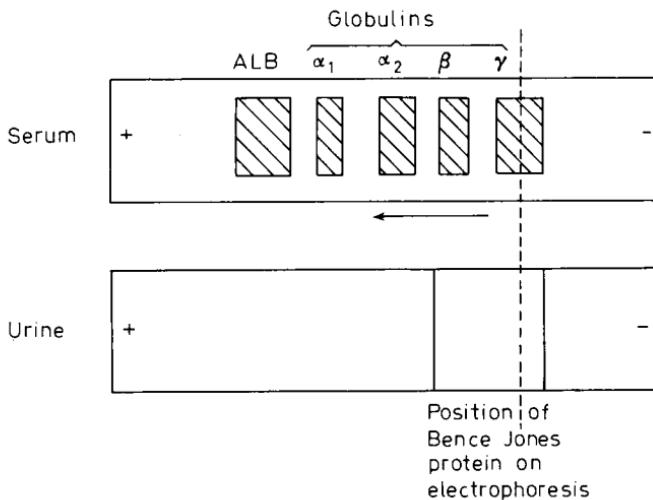


Figure 10.6. Position of proteins on electrophoresis

* Gelman Instrument Co., UK Agents Hawksley & Sons, Lancing, Sussex.

6. Apply the concentrate to an equilibrated electrophoresis strip as described in Chapter 6, p. 151, along with a fresh specimen of patient's serum if possible.
7. If Bence-Jones protein is present, the abnormal sharp protein band will, on electrophoretic analysis, migrate between the β and γ globulin positions.

Proteinuria

It is generally thought that the glomerular filtrate contains a small amount of protein and the tubular cells reabsorb most of this protein, so that in the normal individual the kidneys may excrete up to 50 mg of protein per day. Pathological proteinuria (a common finding in renal disease) usually implies glomerular damage causing increased filtration of protein, but it may be due to defective tubular reabsorption. However, proteinuria is sometimes found when there is no renal disease. Increased pressure on the renal veins accounts for proteinuria in 5 per cent of young healthy adults and also in pregnancy.

REDUCING SUBSTANCES

A reducing substance is one which will reduce blue alkaline cupric sulphate to red cuprous oxide. The most important substances being the carbohydrates, glucose, lactose, fructose, galactose and pentoses (e.g. ribose, xylose and arabinose).

Alkaline cupric sulphate can also be reduced by substances which are not carbohydrates, such as glucuronic acid, salicyluric acid, uric acid, creatinine and homogentisic acid, if present in the urine in sufficiently large concentration. Sucrose will not reduce the alkaline copper reagent. Normal urine contains small amounts of reducing substances, but the concentration is too small to be detected by Benedict's qualitative reagent.

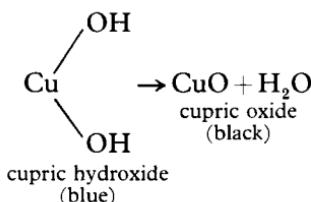
Detection of reducing substances

1. BENEDICT'S QUALITATIVE TEST

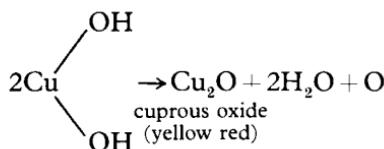
Principle

The aldehyde or ketone group of the carbohydrates reduces blue cupric hydroxide to an insoluble yellow or red cuprous oxide. If no carbohydrate is present in the urine, the cupric hydroxide when heated is converted to an insoluble black cupric oxide, but the

presence of sodium citrate in the reagent prevents this spontaneous reduction.



Reaction in absence of reducing agent



Reaction in presence of reducing agent.

Reagent

(A) Dissolve by heat 173 g sodium citrate and 100 g anhydrous sodium carbonate in about 600 ml distilled water. Also 17.3 g copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) are dissolved in about 100 ml of distilled water and then added slowly, with stirring, to solution (A). When cool transfer to a 1 litre volumetric flask and dilute to the mark with distilled water.

Method

Into a test-tube place 0.5 ml urine, followed by 5 ml of Benedict's reagent. Mix well and place in boiling water bath for 5 min. Allow to cool, then observe any colour change due to precipitation of cuprous oxide. Phosphate precipitation may produce a white, turbid appearance, which may be ignored.

If the solution appears green, due to the suspension of a yellow precipitate in a blue solution, report reducing substances present as 'a trace'. If the solution shows a yellow tinge, report result as +, an orange precipitate as ++, and a brick-red precipitate as +++.

Notes—

1. Benedict's quantitative reagent must not be used in place of the qualitative reagent above.
2. Other reducible reagents have been devised, for example those containing bismuth, but these are not widely used.
3. Benedict's copper reduction method has been adapted for use by the diabetic patient in his own home. Using a standard dropper, 5 drops

of urine, 10 drops of water, and a tablet containing copper sulphate and NaOH are added to a test-tube. The heat evolved by the solution of the caustic soda boils the mixture and any glucose present reduces the copper sulphate to cuprous oxide, the colour of which is compared with a standard colour chart. This is known as the Clinitest.*

Identification of reducing substances

Benedict's qualitative reagent will reveal the presence of a number of reducing substances, and it is important to identify the reducing substance.

1. CLINISTIX REAGENT STRIPS*

These reagent strips are a quick, simple, qualitative colour test to detect the presence of glucose only in urine.

Composition

A strip of firm plastic, one end of which is the reagent system, a buffered enzyme preparation and a chromogen system.

Principle

1. Glucose is oxidized by atmospheric oxygen in the presence of glucose oxidase to gluconic acid and hydrogen peroxide.
2. Hydrogen peroxide in the presence of peroxidase oxidizes the chromogen to shades of purple.

Directions

1. Dip test end of the reagent strip in fresh urine and remove immediately or pass briefly through urine stream.
2. 10 s after wetting, compare colour of test area with colour chart. Read the test carefully, in good light and with strip near to colour chart. *Ignore any colour developing after 10 s.*

Interpretation of colour reaction

1. Test end turns purple within 10 s—glucose present.
2. Test end remains cream after 10 s—glucose absent.

Precautions

1. Make sure the container for urine is absolutely clean and free from contaminants, particularly disinfectants and detergents containing oxidizing substances such as hypochlorites and peroxides.
2. Do not touch test end of strip.

* Ames & Co. Ltd.

3. Recap bottle tightly as soon as strip has been removed, to avoid uptake of moisture.

Sensitivity

The smallest concentration of glucose in urine which can be detected with the reagent strips ranges from 10 to 100 mg per 100 ml (0.01 to 0.10 per cent) owing to variations in urinary constituents and pH in different specimens. Ascorbic acid (vitamin C) may decrease the sensitivity of the test. This should be borne in mind when testing the urine of patients receiving therapeutic doses of this vitamin, or parenteral preparations in which ascorbic acid is incorporated as antioxidant, for example, tetracyclines.

Specificity

Glucose oxidase is a specific enzyme for glucose. Thus no substance excreted in urine other than glucose gives a positive result with these reagent strips. In particular they do not react with other reducing sugars, for example lactose, galactose and fructose, or reducing metabolites of some drugs (e.g. salicylates), as copper reduction methods do. Oxidizing agents such as hydrogen peroxide will give false positive results.

Alternative procedure for use with napkins

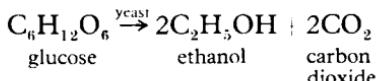
The technique is to press the test end of the strip against a freshly wet napkin, remove it when thoroughly wetted, and observe it exactly 1 min later. The result is interpreted as described above.

Test only a really wet napkin, and avoid using one which is contaminated with faeces. Do not leave the strip in contact with the napkin, because of risk of the reagents being dissolved out and because oxygen from the air is necessary to the reaction.

2. YEAST FERMENTATION TEST

Principle

Glucose can be converted into ethanol and carbon dioxide by the action of living yeast cells.



If yeast is allowed to ferment in urine containing glucose the CO₂ evolved indicates the presence of glucose (or fructose).

Method

This technique is very rarely used nowadays. A few grams of washed yeast are mixed with 50 ml boiled urine and then placed in a small test-tube. The tube is inverted into a beaker of the same urine mixture, without allowing any air to enter the tube. Set up positive and negative controls at the same time. Incubate at 37 °C.

Results

If glucose (or fructose) is present, gas is generally evolved within 3 h.

THE OSAZONE TEST

Some sugars react with phenylhydrazine to give crystalline compounds known as osazones. Phenylglucosazone, which is formed from glucose and also fructose, is in the form of sheaves of needle-like crystals. Phenyllactosazone crystals, on the other

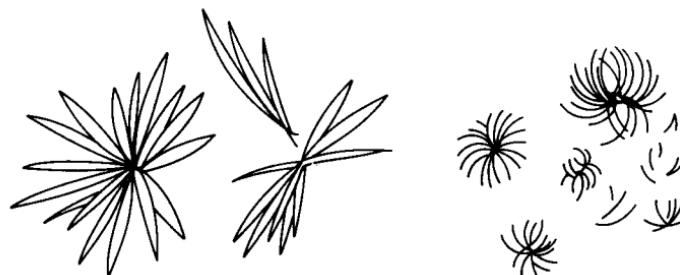


Figure 10.7
Phenylglucosazone crystals Phenyllactosazone crystals

hand, are smaller and more feathery than those obtained from glucose (*Figure 10.7*). The osazone test will give some indication whether a sugar is either glucose or lactose, but the solubility of the crystals formed should be determined to verify the microscopical appearance, as so many factors influence the crystalline structure.

Reagent

Crystalline sodium acetate—2 parts.

Phenylhydrazine hydrochloride—1 part.

Method

1. Place 5–10 ml of urine in a test-tube (the urine should be slightly acid in reaction. Add acetic acid if necessary).

2. Add 0.5–1.0 g of the reagent to the urine
3. Place the tube into boiling water, and allow to boil for 45 min.
4. Turn off the gas and leave the tube in the water to cool. Do not remove the tube or cool rapidly, as the osazones will not be formed.
5. Examine a few crystals microscopically. Compare their appearance with the crystals obtained with known samples of glucose and lactose, prepared in parallel with the test.
6. Verify results by testing the solubility of the crystals in water. Phenylglucosazone crystals are insoluble in water, while phenyllactosazone crystals are soluble and may be recrystallized.

Notes—

1. Lactosazone crystals are difficult to prepare from the small quantities of lactose encountered in urine.
2. Glucosazone crystals form readily and tend to be greenish in colour. Pentosazone crystals are like glucosazone crystals, but smaller.
3. This procedure is very rarely used nowadays. Chromatography is far better.

SELIWANOFF'S TEST FOR FRUCTOSE

Principle

Fructose, when boiled in the presence of hydrochloric acid, yields a derivative of furfuraldehyde which condenses with resorcinol to form a red coloured compound.

Reagent

Resorcinol	0.05 g
Concentrated hydrochloric acid	33 ml
Distilled water	to 100 ml
Stable for about 6 weeks	

Method

Add 0.5 ml urine to 5 ml of the reagent in a test-tube. Mix well and bring to the boil. Treat a normal and positive control urine in the same way.

Results

Fructose gives a red colour in about 30 s. The test is sensitive to 0.1 per cent fructose, but if more than 2 per cent glucose is present, this will also give a red colour on further boiling. Interpretation should therefore be based on colour development time.

BIAL'S TEST FOR PENTOSE*Principle*

Pentoses when boiled in the presence of hydrochloric acid yield aldehydes of furfural type, which in the presence of orcinol condense to form green-coloured compounds.

Reagent

Dissolve 300 mg of orcinol (*m*-dihydroxytoluene) in 100 ml concentrated HCl to which is added 5 drops of 10 per cent ferric chloride. Stable for up to 1 week.

Method

Add 0.5 ml of urine to 5 ml of the reagent in a test-tube. Mix well and place in boiling water bath until liquid begins to boil.

Results

Pentoses give a green colour, with a sensitivity of 0.1 per cent. Considerable quantities of pentoses will give a blue/green precipitate. Fructose gives a red colour. Glucuronates give a similar greenish colour if the boiling is prolonged.

FEARON'S METHYLAMINE TEST FOR LACTOSE*Principle*

Alkaline hydrolysis opens the carbohydrate ring to form an enediol, which in the presence of methylamine hydrochloride forms a red product. Alkaline hydrolysis opens the carbohydrate ring, exposes either the ketone or aldehyde group which is rearranged to form an enediol. Enediol then reacts with the methylamine hydrochloride to form a red-coloured product.

Reagents

0.2 per cent methylamine hydrochloride.

10 per cent sodium hydroxide.

Method

1. To 5 ml urine add 1 ml methylamine solution and 0.2 ml sodium hydroxide solution.
2. Mix by inversion and place tube in a 56°C water bath for 30 min (boiling for 5 min can also be used).
3. Remove tube from bath and allow to cool to room temperature.
4. Compare colour with a 'blank' consisting of the reagents and unheated urine, and a control tube containing water instead of methylamine hydrochloride.

Results

0.5 per cent lactose will give an intense red colour in less than 30 min at 56 °C, 0.05 per cent lactose a slight but definite colour after standing at room temperature for 20 min. Glucose, fructose, galactose, xylose and sucrose in large amounts give a yellow colour. The only sugars which give a red colour are the reducing disaccharides, lactose and maltose—maltose is not usually found in urine.

Chromatographic identification of urinary sugars

Galactose is found in the urine only very rarely, and the only satisfactory means of confirming its presence is by chromatographic analysis. Paper chromatography has been used for many years and gives very satisfactory results (see p. 146), but thin layer chromatography is quicker and more sensitive. The application of thin layer chromatography has already been discussed in Chapter 6, so the technique of sugar chromatography will only be described here.

THIN LAYER CHROMATOGRAPHY OF SUGARS

Reagents

1. Solvent mixture *n*-butanol–acetic acid–water 75/25/6, *v/v*.
2. Aniline–diphenylamine locating agent.
Solution 1. 1 per cent aniline *v/v* and 1 per cent diphenylamine *w/v* in acetone.
Solution 2. 85 per cent phosphoric acid.
Before use mix 10 volumes of solution 1 with 1 volume of solution 2.
3. Standards. Prepare standard solutions in 10 per cent aqueous isopropanol so that 5 µl contains 5 µg of sugar. Solutions of glucose, lactose, fructose, galactose and xylose are suggested. After several runs it will be possible for a mixture of the glucose, galactose and lactose to be used as a routine.
4. Silica gel G.
Prepare a 20 × 10 cm thin layer plate as on p. 148 or purchase pre-coated 250 µm 20 × 10 cm plates from Merck or Camlab.

Technique

Determine the amount of reducing substance present (p. 251) and calculate the volume required to be added so that the sample will contain 5–10 µg sugar (usually 2–5 µl).

Apply the required volumes along the point of application at

1 cm intervals, with standards either side of the urine spots. It is not necessary to desalt the urine.

Place the plate in an airtight tank and allow the solvent front to rise 12–15 cm; this usually requires 2–3 h. Remove the plate

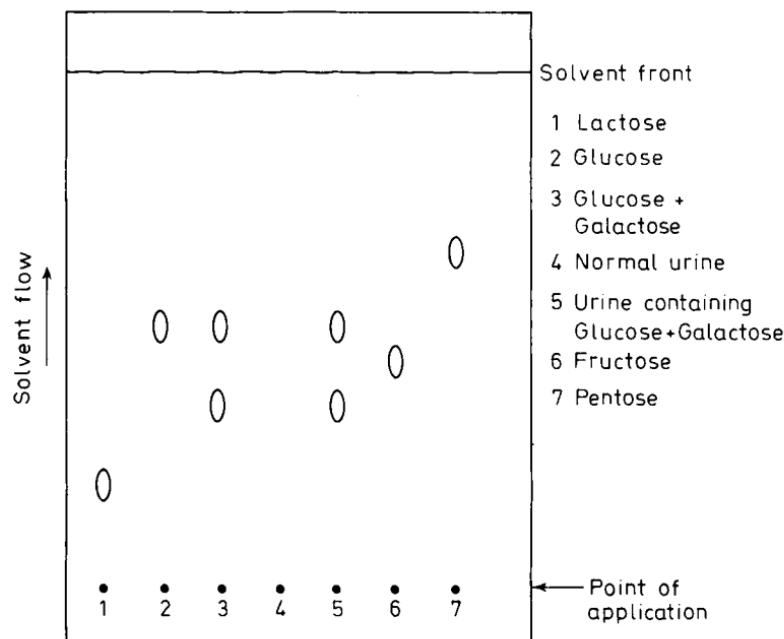


Figure 10.8. TLC of various sugars

from the tank and allow the chromatogram to dry in a fume-cupboard under hot air. Spray with the locating agent and heat for 5 min at 120 °C in hot-air oven.

Figure 10.8 shows the position of various sugars and Table 10.1 shows the approximate *Rf* values (see p. 144) and characteristic colours obtained with the locating agent. The sensitivity of the

Table 10.1

<i>Sugar</i>	<i>Characteristic colour</i>	<i>Approx. <i>Rf</i></i>
Lactose	grey	0.14
Galactose	grey	0.34
Fructose	pink	0.39
Glucose	grey	0.42
Pentoses	grey brown	>0.5

method is in the order of 0.1 µg of glucose and it will occasionally detect glucose in normal urine.

The above tests can now be tabulated and the results obtained will be as follows:

Table 10.2

Sugar	Benedict's qual. reagent	Clinistix	Seliwanoff	Methylamine	Bials'	Yeast fermenta- tion
Glucose	-					
Lactose	-					
Fructose	-					
Pentose	-					
Galactose	-			Confirm by chromatography		

Glycosuria

Although glucose (and other carbohydrates) is freely filtered through the glomeruli in the kidney, it is almost completely reabsorbed by the renal tubules. The capacity of the tubules to reabsorb glucose is, however, limited, and when the blood glucose concentration rises above about 150 mg per 100 ml (renal threshold) the tubules cannot reabsorb all the glucose and therefore glycosuria occurs. The reabsorptive capacity of the tubules varies from person to person and with age.

Glucose (glycosuria)

With rare exceptions, glucose is the only carbohydrate found in the urine in pathological conditions. The most important of these is diabetes mellitus, when as much as 5 g per 100 ml of glucose can be found in the urine. Glycosuria can also occur in endocrine hyperactivity and severe liver disease.

Lactose (lactosuria)

Sometimes found in the urine towards the end of pregnancy and during lactation. This condition is harmless and it is important that a positive test for reducing substances during pregnancy is further investigated to ensure it is lactose and not glucose.

Fructose (fructosuria)

Found in the urine after eating fruits, honey, jams. If glucose is found as well it is indicative of diabetes. Fructose can also be

found in the urine in a rare metabolic condition, due to a congenital defect—'essential fructosuria'. Fructosuria is a harmless condition, but it must be identified and not wrongly classified as diabetes.

Galactose (galactosuria)

Very rare condition occurring in infants, due to a deficiency of enzyme, galactose-1-phosphate uridyl transferase, which catalyses the conversion of galactose-1-phosphate to glucose-1-phosphate. It is important that galactose is identified as quickly as possible after birth, as the child must be placed on a galactose-free diet. The accumulation of galactose in brain tissue will mentally retard the infant.

Galactose can also be found in the urine after taking large amounts of galactose or lactose; in this case galactosuria is harmless.

Pentose (pentosuria)

Occurs under similar conditions to fructose.

KETONES

In diabetes mellitus, if there is a depression of carbohydrate metabolism, or in starvation, there is an increased oxidation of fat to provide energy. Under these conditions the liver yields acetoacetic acid, some of which is decarboxylated to give acetone and some reduced to β -hydroxybutyric acid. This results in the accumulation of ketone bodies in the blood (ketonaemia), some of which are excreted in the urine (ketonuria).

Ketone bodies

1. $\text{CH}_3\text{CO} \cdot \text{CH}_2\text{COOH}$ acetoacetic acid
2. $\text{CH}_3\text{CHOH} \cdot \text{CH}_2\text{COOH}$ β -hydroxybutyric acid formed from the reduction of acetoacetic acid
3. $\text{CH}_3\text{CO} \cdot \text{CH}_3$ acetone formed in the body from acetoacetic acid by the loss of carbon dioxide

It is important to test for these substances as part of the routine urine examination of diabetic patients.

Acetoacetic acid**GERHARDT'S TEST***Principle*

Ferric chloride reacts with many substances to give characteristic colours, one of which is acetoacetic acid.

Reagents

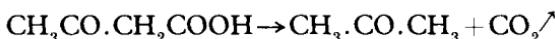
10 per cent ferric chloride.

Method

1. Add the ferric chloride solution drop by drop to 5 ml of urine in a test-tube.
2. A red or purplish colour is given by acetoacetic acid.
3. When the urine contains a large amount of phosphate, a precipitate of ferric phosphate is produced.
4. Filter or centrifuge the urine and add a few more drops of ferric chloride to the clear sample.

Salicylates

A similar colour is given by salicylates and they can be differentiated from acetoacetic acid by their behaviour on heating. If the urine is boiled, acetoacetic acid loses carbon dioxide and is converted into acetone. Acetone will not give a positive reaction with ferric chloride. Salicylates, on the other hand, are unaffected by boiling.



If the heating is carried out after adding the ferric chloride, the acetoacetic acid colour will disappear, while the salicylate colour persists.

Phenylpyruvic acid (phenylketonuria)

On the addition of ferric chloride solution to the urine in phenylketonuria, a green or blue colour is obtained, which fades in a few minutes to a yellowish colour. This is a rare condition and must be reported immediately. Phenylketonuria is an inborn error of metabolism, whereby the body is unable to convert phenylalanine to tyrosine by its usual enzymatic pathways.

Phenylalanine accumulates in the blood, urine and CSF, transamination converts the phenylalanine into phenylpyruvic acid, which is excreted in the urine as early as two to three weeks after birth. Early recognition of phenylpyruvic acid is important,

as dietary control is necessary to prevent the infant from becoming mentally retarded.

Phenistix are used for detecting the presence of phenylpyruvic acid and *p*-aminosalicylic acid (PAS).

Composition

The test area of the strip is impregnated with ferric ammonium sulphate, magnesium sulphate, and cyclohexylsulphamic acid.

Principle

Ferric ions at a suitable pH react with phenylketones (notably phenylpyruvic acid) to give a greyish-green colour, and with PAS and its metabolites to give a brownish-red colour. The desired acidity (pH 2.3) is provided by cyclohexylsulphamic acid, and the magnesium salt minimizes interference by phosphates.

Directions

1. Press test end of strip against freshly wet napkin (not merely damp), or dip in urine and remove immediately, so as to avoid dissolving out test reagents.
2. Compare colour of test end with colour chart exactly 30 s later, read in good light and ignore any colour developing after 30 s.

Interpretation of colour reactions

1. Test end turns greyish-green within 30 s—phenylpyruvic acid positive.
2. Test end turns off-white or cream within 30 s—phenylpyruvic acid negative.
3. When the test end turns brownish-red at once, this indicates the presence of ingested PAS.

Precautions

1. Do not leave strip in contact with wet napkin or urine to avoid risk of dissolving out reagents. For same reason also do not place strip in dry napkin and read it when this has been used.
2. Phenylpyruvic acid decomposes on standing, especially in a warm atmosphere, therefore:
 - (a) Always test *fresh* urine.
 - (b) Use only a really wet napkin; a partially dried one or one that has been re-wetted with water gives unreliable results.

- (c) Do not use a napkin contaminated with faeces; certain faecal bacteria specifically and rapidly destroy phenylpyruvic acid.
- (d) Do not leave the strip to be read some time later; a positive result will fade.
- 3. Do not touch test end of strip. Recap bottle tightly as soon as strip has been removed.

Sensitivity

8–10 mg per 100 ml phenylpyruvic acid.

Specificity

The test is not specific for phenylpyruvic acid as the following substances also give a positive reaction: PAS, *p*-hydroxyphenylpyruvic acid, *o*-hydroxyphenylpyruvic acid, imidazolepyruvic acid, tetracycline and a few phenothiazine tranquillizers or their metabolites.

Note—

Until quite recently the Phenistix test was used as a method of 'screening' infants for phenylketonuria. The MRC Working Party on Phenylketonuria have recommended that the ferric chloride phenistix test should no longer be used and the Guthrie test (Bacterial Inhibition Assay) be substituted in its place.

ROTHERA'S NITROPRUSSIDE TEST

Principle

Nitroprusside in alkaline solution reacts with a ketone group to form a purple colour.

Reagents

Ammonium sulphate, sodium nitroprusside and concentrated ammonia.

Method

1. Saturate about 5 ml of urine with ammonium sulphate and add a small crystal of sodium nitroprusside.
2. Mix well and then add 0.5 ml of concentrated ammonia.
3. A purple colour indicates the presence of acetoacetic acid, acetone, or both, and is maximal in 15 min.
4. The rate of colour development is a better quantitative guide than the intensity of colour. The reaction is more sensitive than Gerhardt's test for acetoacetic acid and is less sensitive with acetone than it is with acetoacetic acid.

Modifications

Several workers have modified the test by using a powdered reagent.

1. Mix 100 parts of ammonium sulphate and 1 part of sodium nitroprusside and use this for saturating the urine. Mix well and then add the concentrated ammonia.
2. Prepare a powdered mixture of 1 g fine sodium nitroprusside, 20 g ammonium sulphate and 20 g anhydrous sodium carbonate. The powder is mixed completely and if kept dry will keep for at least 1 year. A small pinch of powder is placed on a white tile; add one drop of urine and note colour development. Acetone and acetoacetic acid will give a violet colour.

ACETEST

This is Rothera's test in a tablet form.

Composition

The tablet contains sodium nitroprusside, aminoacetic acid (glycine), lactose and disodium phosphate.

Principle

The ketone group of acetone and acetoacetic acid react with sodium nitroprusside at the optimum alkaline pH provided by the buffer system to give a lavender or purple colour. Lactose enhances the colour.

Directions

1. Place the reagent tablet on a clean white surface, preferably a piece of filter paper.
2. Put one drop of urine on tablet.
3. Compare colour of tablet with colour chart exactly 30 s later.

Interpretation of colour reaction

1. Tablet turns lavender or purple within 30 s—ketones present.
2. Tablet remains white at 30 s (or turns cream)—ketones absent.

Precautions

1. Make sure that container for urine is absolutely clean and free from contaminants, for example acids, disinfectants and detergents.

2. Test only fresh specimens if possible; refrigeration is required if the specimen has to be kept.
3. Recap the bottle tightly as soon as a tablet has been removed, to avoid uptake of moisture.

Sensitivity

In ketosis, the urine contains a considerable preponderance of the acetoacetic acid over acetone, and as the tablets have a greater sensitivity for the former, the colour produced is almost entirely due to acetoacetic acid.

Specificity

The only substance besides acetone and acetoacetic acid likely to be present in the urine which is known to give colours with these tablets is phenolsulphonphthalein (used occasionally in renal function tests). The colour is not the same as those shown on the colour chart, but it can be mistaken for it.

KETOSTIX

Composition

A strip of firm plastic, one end of which is the reagent system. This is a buffered mixture of sodium nitroprusside and glycine, for testing for ketones in urine, plasma, serum and milk (not whole blood).

Principle

As for the Acetest, except there is no lactose in this strip.

Directions

1. Dip test end of the reagent strip in fresh specimen and remove immediately, or pass briefly through urine stream.
2. Briefly touch tip of strip on container to remove excess liquid.
3. Compare colour of test end with colour chart exactly 15 s later.

Interpretation of colour reaction

1. Test end turns lavender or purple within 15 s—ketones present.
2. Test end remains off-white at 15 s—ketones absent.

Precautions

1. Make sure that container for specimen is absolutely clean

and free from contaminants, for example acids, disinfectants and detergents.

2. Test only fresh specimens if possible; refrigeration is required if specimen has to be kept.
3. Do not touch test end of strip.
4. Recap the bottle tightly immediately after removing a strip to avoid uptake of moisture. Do not remove desiccant.
5. Do not leave strip in specimen to avoid risk of dissolving out reagents.

Sensitivity

The sensitivity is similar to the Acetest. In milk, however, the ratio of acetoacetates to acetone is rather small, though the former is still the major component.

Specificity

Besides acetoacetic acid and acetone, phenylketones, bromosulphthalein, phenolsulphonphthalein and phenolphthalein will also turn the strip shades of red or purple which, while not matching the chart, could cause confusion.

β -Hydroxybutyric acid

There is no test in everyday use for this ketone. Although it occurs together with the other ketones, it can be tested for by boiling the urine at an acid pH to remove the acetone and acetoacetic acid. The β -hydroxybutyric acid is oxidized to acetoacetic acid with hydrogen peroxide and then tested with Rothera's reagent. A positive reaction indicates the presence of β -hydroxybutyric acid in the urine.

LABSTIX

These reagent strips combine five rapid convenient tests for pH, protein glucose, ketones and blood in urine.

Composition

A strip of firm plastic with the various reagent systems attached. The pH reagent area is impregnated with methyl red and bromthymol blue. This permits the differentiation of pH values to half a unit within the range 5–9. For the detection of blood, the reagent strip is impregnated with a buffered mixture of organic peroxide and a chromogen. The remaining reagent systems have already been discussed.

Directions

1. Dip test areas of strip in fresh, well-mixed, uncentrifuged urine. Remove immediately so as to avoid dissolving out test reagents.
2. Tap edge of strip against container to remove excess urine.
3. Compare test areas closely with corresponding colour charts at times specified. Read in good light and ignore colours developing on a test area after the specified reading time of the test.

Precautions to be adopted when using Ames reagent strips or tablets

1. Accurate and reliable results are dependent upon strict observance of the manufacturer's directions and careful following of recommended procedures for handling and testing.
2. The specimen container must be absolutely clean and free from contaminants, e.g. antiseptics or detergents.
3. Do not acidify the specimen prior to using Ames reagents.
4. Fresh specimens of urine should be tested whenever possible because chemical changes may take place on storage. Please note the remarks on preservatives given for individual tests.
5. Remember that when using Ames reagent strips, they should be dipped into the urine cleanly and quickly and where applicable they must be compared with the colour blocks provided at the times stated.
6. Accurate and reliable results depend upon strict observance of manufacturers' directions and the careful following of the recommended procedures for handling and testing.
7. Ames tests all involve colour reactions and it is not necessary to filter the urine before testing. In order to maintain Ames tests in perfect condition it is important always to replace the cap tightly immediately after use and to store away from excessive heat and moisture. Do not store in a refrigerator. Do not store above 30 C (86 F).
8. Deterioration of reagent strips result in a brownish discolouration of the test area. When this occurs the strips should not be used.

Before commencing examination of specimen the following points should be noted:

Appearance

Note colour of specimen

e.g. straw, amber, red, black, etc.

Note nature of deposit e.g. pus, blood, etc.

Note any odour that may be present e.g. acetone, ammonia

Specific gravity Measure specific gravity. If there is insufficient urine to float hydrometer, either

1. use a narrower container, or
 2. after chemical testing dilute the urine with an equal quantity of water and double the last two figures of the hydrometer reading.

(For further information regarding the colour reactions and precautions see *Handbook of Chemical and Biological Information Systems*, published by Ames.)

There are other additional reagent strips available such as Bili-Labstix for pH, protein, glucose, ketones, bilirubin and blood in urine, and Uristix for protein and glucose in urine.

Quantitative blood and urine analysis

In some cases it may be necessary to estimate quantitatively glucose, protein and urea.

URINE GLUCOSE

Principle

Glucose or any other reducing carbohydrate reduces Benedict's quantitative alkaline copper solution to a white precipitate of cuprous thiocyanate instead of the usual reddish-brown cuprous oxide.

Besides the usual ingredients of the qualitative reagent, Benedict included potassium thiocyanate and potassium ferrocyanide in the quantitative reagent. The ferrocyanide helps to keep the cuprous oxide in solution. The disappearance of the blue colour and the formation of a white precipitate indicate complete reduction of the copper, which is much easier to gauge. Sodium carbonate is used as the alkali, as it is less likely to cause destruction of small amounts of sugar during the titration.

Reagents

- #### 1. Benedict's quantitative reagent.

(B) Copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) 18 g Dissolve in about 100 ml of distilled water

When cooled, add solution (B) to solution (A), stirring constantly. Add 5 ml of 5 per cent potassium ferrocyanide, and dilute to 1000 ml with distilled water in a volumetric flask. Filter if necessary. Keeps indefinitely.

2. Anhydrous sodium carbonate.

Standardization of reagent

See below.

Method

1. Measure 25 ml of Benedict's quantitative solution into a conical flask containing several 'anti-bumping' granules.
2. Add 3–4 g of sodium carbonate.
3. Bring the solution to the boil over a bunsen burner, using a gentle flame, and keep it boiling.
4. Using a burette clamped to a stand, allow the urine to run slowly into the boiling solution. A white precipitate is formed, and the blue colour gradually disappears.
5. Continue adding the urine until the blue colour has completely disappeared.
6. Repeat the test in duplicate, but this time add the bulk of the urine rapidly, and when the end-point is near, add further urine drop by drop, boiling for 30 s between addition. Record titre.
7. The ideal titre is one requiring between 8 and 12 ml of urine. It may be necessary to dilute the urine after the preliminary titration (or after the Benedict's qualitative test).

Note—A greenish-blue colour will reappear if the flask is allowed to stand. This is a re-oxidation process of the air and must be ignored.

Calculation

0.05 g of glucose will reduce 25 ml Benedict's quantitative reagent. If the titre of the urine was 8.0 ml then 8.0 ml of urine must contain 0.05 g of glucose.

Therefore

$$\text{g of glucose per 100 ml urine} = \frac{0.05 \times 100}{8.0}$$

or 0.625 g per 100 ml

The correct calculation will therefore be as follows:

$$\text{g of glucose per 100 ml urine} = \frac{0.05 \times 100}{\text{titre of urine}} \times \text{dilution of urine (if any)}$$

Standardization of Benedict's quantitative reagent

Prepare a standard glucose solution containing 0.5 g per 100 ml. Titrate the reagent with this solution as above. Note titre.

Example:

25 ml of Benedict's reagent are reduced by 10 ml of glucose solution and 0.5 g per 100 ml glucose is equivalent to 0.005 g per ml.

Therefore 10 ml of glucose solution will be equivalent to 0.05 g glucose.

Thus 25 ml of Benedict's reagent will reduce 0.05 g of glucose.

Estimation of other reducing sugars

This is carried out in the same way except that a different factor is substituted in the calculation, e.g. 25 ml Benedict's quantitative reagent reduces 0.067 g of lactose. The factor can be obtained by standardizing the reagent against the appropriate reducing sugar.

Urine glucose estimations are sometimes of use in the control of diabetic patients, but nowadays blood glucose estimations have replaced this type of analysis. If, however, urine glucose estimations are required it is far more convenient to use the following procedure.

GLUCOSE OXIDASE METHOD

The urine is first of all shaken with a few grams of activated charcoal to remove interfering substances. Filter through a fine filter paper and dilute 5 ml of the clean filtrate to 50 ml with distilled water in a volumetric flask. Take 0.1 ml of the diluted urine and estimate the glucose concentration in the same way as for the blood glucose on p. 208. As the urine has already been diluted 1 in 10, this factor must be incorporated into the calculation.

Urine proteins

TURBIDIMETRIC METHOD

This technique is similar to that given for cerebrospinal fluid protein (Chapter 11). It may be necessary to filter the urine before analysis. If after filtration the urine is still cloudy, put up a blank consisting of urine plus water.

COLORIMETRIC METHOD (Biuret reaction)*Principle*

Alkaline copper solution reacts with the peptide bonds in the protein molecule, producing a violet colour which is directly proportional to the amount of protein present.

Reagents

1. 20 per cent trichloracetic acid.
2. 1N (M) sodium hydroxide, 40 g per litre of water.
3. Standard protein solution. 500 mg per 100 ml in 0.90 per cent NaCl. Store distributed into small volumes below - 18 °C. The standard can be prepared from diluted pooled serum, or by making suitable dilutions of commercial control serum. The protein content of the standard should be checked beforehand against a standard solution of accurately known protein nitrogen content (Armour's standard protein solution).
4. Stock Biuret reagent. Dissolve 45 g of sodium potassium tartrate in approximately 400 ml of 0.2N (M) NaOH. Add with constant stirring 15 g copper sulphate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), when in solution add 5 g of KI and dilute to 1 litre with 0.2N (M) NaOH.
5. Working Biuret solution. Dilute 200 ml of stock solution to 1 litre with 0.2N (M) NaOH containing 5 g KI per litre.

*Method**Test*

1. To 1 or 2 ml of urine add an equal volume of trichloracetic acid.
2. Mix well and allow to stand for a few minutes.
3. Centrifuge.
4. Decant the supernatant fluid without disturbing the deposit.
5. Dissolve the precipitated protein in 1 ml of 1N (M) NaOH.
6. Add 2 ml of distilled water.

Blank

3 ml of distilled water.

Standard

3.0 ml of standard protein solution.

To all three tubes add 5 ml of working Biuret reagent, mix thoroughly and place them in the 37 °C water bath for 10 min. After colour development allow the tubes to cool and compare the absorbances in the photoelectric absorptiometer, using a green

filter or absorbance at 540–560 nm. Use the blank to zero the instrument.

Calculation

Mg protein per 100 ml urine =

$$\frac{\text{Absorbance of test}}{\text{Absorbance of standard}} \times \text{strength of standard} \times \frac{100}{\text{amount of urine taken}}$$

Let absorbance of test = 0.25 and absorbance of standard = 0.50. The standard protein solution contained 500 mg per 100 ml (5 mg per ml), then 3 ml of protein solution will contain 15 mg protein. If 2.0 ml of urine was used in the precipitation stage, then:

$$\text{Mg of protein per 100 ml urine} = \frac{0.25}{0.50} \times \frac{15 \times 100}{2} = 375$$

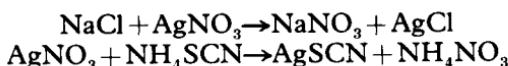
Divide the result obtained by 1000 to convert into g protein per 100 ml. 24 h excretion of protein may be required when following the treatment of nephrotic patients.

Urine chlorides

VOLHARD'S METHOD

Principle

Chlorides in the urine are precipitated with an excess of silver nitrate in the presence of nitric acid. The excess silver nitrate remaining after the complete precipitation of chlorides is measured by back titration with ammonium thiocyanate, using ferric alum as the indicator. When the reaction is complete, the slightest excess of thiocyanate produces a reddish-brown colour due to the formation of the complex ferri-thiocyanate ion (*see also* Chapter 5, p. 105).



Reagents

Standard 0.1 N (M) silver nitrate.

16.988 g of AR silver nitrate are dissolved in about 400 ml of distilled water in a litre volumetric flask. 85 ml concentrated nitric acid are added, mixed and the contents are diluted to 1 litre.

1 ml of 0.1 N (M) $\text{AgNO}_3 \equiv 0.1 \text{ mEq (mmol) NaCl}$.

Standard ammonium thiocyanate solution

Dissolve 9.0 g of ammonium thiocyanate AR in 1000 ml of distilled water. Titrate the silver nitrate solution against the ammonium thiocyanate, and dilute the latter accordingly, so that 25 ml of thiocyanate is exactly equivalent to 25 ml of silver nitrate solution. This is then 0.1N (M) solution of ammonium thiocyanate; alternatively a factor can be incorporated into the calculation (see Chapter 5, p. 106).

$$\begin{array}{l} 1 \text{ ml } \text{AgNO}_3 = 1 \text{ ml } \text{NH}_4\text{SCN} \\ \text{or} \quad \quad \quad 1 \text{ ml } \text{AgNO}_3 = 1 \text{ ml } \text{NH}_4\text{SCN} (xF) \end{array}$$

Ferric alum indicator

4 g ferric ammonium sulphate (ferric alum) are dissolved in 100 ml distilled water to which a few drops of 6N (mol) HNO_3 have been added.

Method

1. Measure 10 ml of urine into a 100 ml volumetric flask.
2. Add approximately 25 ml of distilled water and exactly 20 ml of the standard silver nitrate solution.
3. Make up to the mark with distilled water.
4. Filter the mixture and transfer 50 ml of the solution to a 250 ml conical flask. Add 5 ml ferric alum solution and 3 ml nitrobenzene AR.
5. Titrate against the ammonium thiocyanate solution until the permanent faint reddish-brown colour is produced (ferric thiocyanate). Note titre.

Calculation

$$1 \text{ ml } 0.1\text{N } \text{AgNO}_3 \equiv 0.1 \text{ mEq (mmol) NaCl.}$$

Let x = ml of thiocyanate used.

20 ml of silver nitrate was used to precipitate the chlorides, this was then diluted to 100 ml and 50 ml of filtrate was used in the titration ($\equiv 10 \text{ ml } \text{AgNO}_3$ and 5 ml urine).

Therefore 50 ml filtrate $\equiv 10 \text{ ml } \text{AgNO}_3$

$$(10 - x) \times 0.1 = \text{mEq (mmol) NaCl in 5 ml urine}$$

$$(10 - x) \times 0.1 \times \frac{1000}{5} = \text{mEq (mmol) NaCl/litre of urine.}$$

Notes—

1. To convert mEq/l (mmol) of NaCl into mg per 100 ml multiply by 5.85.

2. Plasma chloride determinations are more reliable than urinary chloride estimations.
3. Some workers prefer to use the qualitative test to control salt therapy. This can be done by simply placing 3–4 ml of urine into a test-tube, add an equal volume of silver nitrate reagent (AgNO_3 , 3 g, conc. HNO_3 , 17 ml, distilled water 10 ml) and compare the precipitate obtained against a normal urine sample. Report the chloride content as normal, diminished or absent.

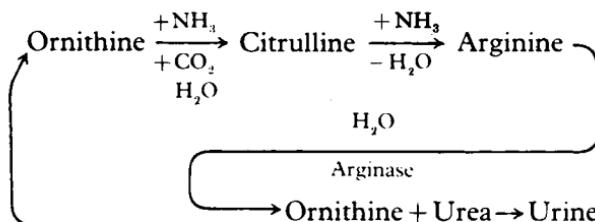
CHLORIDE METER METHOD

Urine chlorides can also be estimated by using the chloride meter as described in Chapter 6, p. 137. As the chloride excretion can vary depending upon dietary conditions, the volume of urine taken for the estimation may have to be adjusted accordingly. When there is a variation from the standard procedure as described on p. 106, the appropriate factor must be incorporated into the calculation.

The average adult excretes about 200 mmol of sodium chloride per day, but this value is greatly influenced by the salt content of the diet. Urinary chloride determinations do not always reflect the true chloride balance of the body. After surgical operations a reduced chloride excretion may be found in cases of hyperchloraemia, while patients with hypochloraemia may excrete a lot of chloride (see also Sweat Tests in Chapter 8).

Urea

Urea is one of the end products of protein metabolism. It is formed in the liver from deaminated amino acids, most probably by way of the ornithine–arginine cycle. The enzyme arginase is found in large quantities in the liver.



Any excess urea in the circulation is eliminated from the blood stream by the kidneys and passes out into the urine.

In health, blood always contains some urea; the level varies but ranges from 15 to 40 mg per 100 ml. In the elderly values slightly higher than these are found, even without significant renal

dysfunction. In general, a blood urea of over 50 mg per 100 ml is suggestive of impaired renal function.

As urea is one of the principal end products of protein metabolism, it follows that the urea content of the blood and urea is influenced over a period of time by the amount of protein in the diet. People on low-protein diets tend to have lower blood ureas.

Urea diffuses very readily through body fluids. For this reason, similar results are obtained if the estimation is carried out on whatever samples are most readily available; for example, cerebro-spinal fluid, oedema fluid, plasma, serum or whole blood.

The estimation of blood urea is valuable not only in cases of renal failure but in a wide variety of conditions which are not primarily renal. Less frequent causes of raised blood urea are diarrhoea and vomiting, and circulatory failure. In childhood and pregnancy low values are often found.

BLOOD UREA ESTIMATION

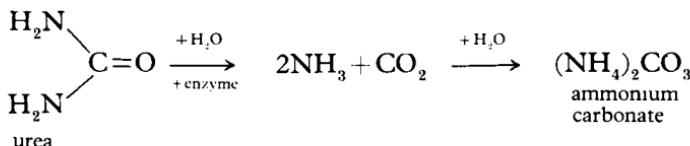
Collection of samples

The estimation can be carried out on whole blood, plasma or serum. Any of the routine anticoagulants may be used, except the following:

1. Sodium fluoride, generally not used as an anticoagulant, but as an enzyme inhibitor, and therefore unsuitable for urease methods.
2. Ammonium oxalate, which must never be used, as most of the routine methods depend upon the measurement of ammonia.

Principles of blood urea determinations

1. One of the methods of estimating urea is based on the action of the enzyme urease, which decomposes urea to form ammonium carbonate.



The ammonia produced may be measured either by using Nessler's reagent or the phenol-hypochlorite reaction.

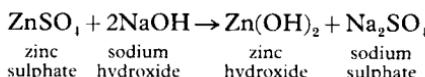
2. When urea is heated with diacetyl monoxime a coloured compound is formed which is used as the basis of its estimation. This is a direct method which does not depend upon the enzyme urease, and there is no interference from ammonia or acetone.

ESTIMATION OF BLOOD UREA USING NESSLER'S REAGENT

Reagents

1. Isotonic sodium sulphate solution (3 per cent crystalline).
2. Powdered urease (soya bean meal, Jack bean meal).
3. Zinc sulphate: 10 g ($ZnSO_4 \cdot 7H_2O$) per 100 ml of distilled water.
4. Sodium hydroxide 0.5 mol solution.

Note—Proteins are removed with zinc hydroxide, which is formed by the interaction of zinc sulphate and sodium hydroxide as follows.



The proteins adhere to the gelatinous precipitate of zinc hydroxide, and the product is separated in the centrifuge.

The two compounds must be accurately balanced for this purpose. If the proportions are incorrect, clouding may occur on subsequent Nesslerization. When the two solutions are prepared, the zinc sulphate should be titrated against the sodium hydroxide. A few drops of phenolphthalein indicator is added to 10 ml of zinc sulphate, and the sodium hydroxide is added from a burette, until a permanent pink colour appears. 10 ml of zinc sulphate solution should react with 10.8 to 11.2 ml of the sodium hydroxide solution.

5. Standard ammonium chloride. Dissolve 0.153 g of ammonium chloride (AR) in distilled water and make up the volume to 100 ml. Dilute 25 ml of this solution to 1000 ml with ammonia-free distilled water.
1 ml of standard ammonium chloride 0.01 mg of nitrogen
6. Standard urea solution can, with advantage, be used instead of the ammonium chloride standard above. It is carried through the entire test in the same way as the blood sample and is a good check that the urease is active; 50, 100 or 150 mg per 100 ml urea solution is suitable. Prepare monthly and store at 4 °C.
7. Nessler's reagent. The addition of Nessler's reagent to a

solution containing ammonia results in the formation of a yellow colour. The intensity of this colour is directly proportional to the amount of ammonia present. The reagent being very sensitive, all distilled water used must be ammonia-free, and ammoniacal solutions in the laboratory should not be open while Nessler's reagent is in use.

For different purposes, Nessler's solution may be prepared in various ways. The best formula for use in routine clinical chemistry is that described below (Koch and McMeekin). Commercially prepared solutions are often unsatisfactory.

- (a) Dissolve 30 g of potassium iodide in 20 ml of distilled water, and add 22.5 g of iodine (*see Notes 1 and 2 below*).
- (b) Shake until completely dissolved and add 30 g of pure metallic mercury.
- (c) Continue shaking, keeping it cool by frequent immersions in cold water, until the supernatant fluid loses its brown colour and becomes greenish-yellow.
- (d) Decant and keep the supernatant fluid. Discard the sediment (*see Note 3 below*).
- (e) Add one drop of the supernatant fluid to a small tube containing 0.5 ml of 1 per cent starch solution.
- (f) If a blue colour is formed, the supernatant is ready for the addition of distilled water, to bring the total volume up to 200 ml. This is then mixed thoroughly with 975 ml of accurately prepared 10 per cent sodium hydroxide solution.
- (g) If no blue colour is formed, add one drop of potassium iodide/iodine mixture (*see (a) above*), to the supernatant fluid. Mix well and test again with the starch solution. This procedure is repeated, one drop at a time, until just enough iodine has been added to give a positive test with starch. When positive, continue as in (f) above.

Notes—

1. Dissolve the potassium iodide first, to facilitate the subsequent solution of the iodine. To prevent vaporization of the iodine, heat must be avoided.
2. As it may be necessary to use more iodine/potassium iodide mixture, it is advisable to prepare two quantities of this solution. The second batch is kept for possible addition to the supernatant fluid as in step (g) above.
3. The mercury and iodine have combined in the supernatant fluid; any excess of mercury, however, must be eliminated by the addition of further iodine. The iodine additions must be minimal and frequent

tests must be made with starch solution, which produces a blue colour in the presence of free iodine (iodine test for starch).

Method

The estimation, like all clinical chemistry analyses, should be carried out in duplicate. It is essential to carry through a 'blank', in order to exclude any false increase in absorbances due to the presence of ammonia in the distilled water or the reagents.

1. Pipette the following into centrifuge tubes and mix the contents of the tubes thoroughly.

	<i>Test</i>	<i>Blank</i>
Isotonic sodium sulphate	3.2 ml	3.4 ml
Whole blood, serum or plasma	0.2 ml	—
Urease	approx. 20 mg	20 mg

2. Incubate in a water bath at 40–50 °C for about 15 min.

Note—Avoid prolonged incubation, which may produce clouding of the Nessler's reagent, due to the liberation of interfering substances from the red blood cells.

3. To the tubes add 0.3 ml zinc sulphate solution and 0.3 ml sodium hydroxide solution. After each addition mix well by inversion. Allow to stand for 30 s to ensure that protein precipitation is complete, and centrifuge the tubes.
4. Set up three tubes, and add the following solutions to them, using volumetric pipettes.

	<i>Test</i>	<i>Standard</i>	<i>Blank</i>
Distilled water	5 ml	5 ml	5 ml
Supernatant fluid	2 ml	—	2 ml
Standard solution (equivalent to 0.02 mg of nitrogen)	—	2 ml	—

5. Add a pinch of sodium citrate to each tube. This helps prevent cloudiness on addition of the Nessler's solution.
6. Immediately before reading the colour, add 1 ml of Nessler's reagent to each tube, with a safety pipette, and mix. View the tubes from above in order to detect any cloudiness following Nesslerization. Should this occur, results are valueless, and the test must be discarded. If the solution is clear, read

immediately in an absorptiometer using a blue filter or at 480 nm zeroing the instrument with distilled water.

Notes—

1. Use the protein precipitant in the above order to avoid lysis of the red cells.
2. Do not filter off the protein-free filtrate, for many filter papers contain ammonium residues.
3. When using Nessler's solution, do not disturb the precipitate which settles in the stock bottle, and never use a pipette containing traces of acetone, or cloudiness will result.

Calculation

Urea $\text{CO}(\text{NH}_2)_2$ has a molecular weight of 60. 1 mol contains 2 nitrogens, i.e. $2 \times 14 = 28$ parts by weight of nitrogen.

Therefore 1 mg nitrogen is contained in $60/28$ or 2.14 mg urea

Now 2 ml standard $\therefore 0.02$ mg nitrogen

2.14 mg urea $\therefore 1$ mg nitrogen

0.2 ml blood is diluted to 4.0 ml which is a 1 in 20 dilution
2.0 ml of the supernatant will therefore contain 0.1 ml undiluted blood.

If T = test reading

S = standard reading

B = blank reading

Then $\frac{T-B}{S-B} \times$ strength of standard

$$\times \frac{100}{\text{amt. of blood used}} \cdot 2.14 \text{ mg urea/100 ml}$$

$$\text{or } \frac{T-B}{S-B} \times 0.02 \times \frac{100}{0.1} \times 2.14 \therefore \frac{T-B}{S-B} \cdot 42.8 \text{ mg urea/100 ml}$$

ESTIMATION OF BLOOD UREA USING DIACETYL MONOXIME

The relationship between the colour reaction and urea concentration is not linear for low values. To overcome this, a small amount of urea is added to the diacetyl reagent to raise all the colours onto a linear part of the graph.

Reagents

1. Diluent. 43 ml of 10 per cent zinc sulphate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) diluted to 1 litre with either a solution containing 13.2 g

- anhydrous sodium sulphate or 30 g hydrated sodium sulphate.
2. Sodium hydroxide. 0.05 M, see p. 101.
 3. Stock urea solution. 200 mg per 100 ml. Store in the refrigerator.
 4. Working urea solution containing 0.08 mg per ml. Dilute 4.0 ml of stock solution to 100 ml with a solution containing 40 mg phenylmercuric acetate per litre of 0.02 mol (0.01N) sulphuric acid.
 5. Stock diacetyl monoxime solution. 2.5 g diacetyl monoxime dissolved in and made up to 100 ml with 5 per cent (*v/v*) acetic acid.
 6. Working diacetyl reagent. Mix 100 ml of diacetyl monoxime stock solution with 25 ml of urea solution (5 mg per 100 ml) and 75 ml distilled water.
 7. Phosphoric-nitric acid reagent. Mix 600 ml of syrupy phosphoric acid with 400 ml distilled water and 10 ml concentrated nitric acid.

Method

1. Into a centrifuge tube pipette the following and mix the contents thoroughly after each addition.

Diluent	4.6 ml
Blood (or serum or plasma)	0.2 ml
0.05 ml sodium hydroxide	0.2 ml

2. Centrifuge at 3000 rpm for 10 min.
3. Label four tubes and add the following solutions. Mix the contents after each addition.

	<i>Test</i>	<i>Blank</i>	<i>Standard (low)</i>	<i>Standard (high)</i>
Supernatant	2.0 ml			
Distilled water		2.0 ml	1.0 ml	
Standard urea solution (0.08 mg/ml)			1.0 ml	2.0 ml
Working diacetyl reagent	2.0 ml	2.0 ml	2.0 ml	2.0 ml
Phosphoric-nitric acid	1.0 ml	1.0 ml	1.0 ml	1.0 ml

5. Place in a boiling water bath for 30 min and cool.
6. Protect from bright light during this stage.
7. Compare the colours at 480 nm or against a blue filter, zeroing the instrument against distilled water.

8. Subtract the reagent blank reading away from the test and standard reading.

Calculation

In the calculation use the standard which gives an absorbance nearest to the test reading.

If the high standard is used, then 2 ml of working standard solution will contain 0.16 mg urea.

The sample of blood, serum or plasma used is 0.2 ml. 2.0 ml of supernatant is used in the colour development from a total volume of 5.0 ml

$$\text{Then } \frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times 0.16 \times \frac{100}{0.2} \times \frac{5.0}{2.0} =$$

$$\frac{\text{absorbance of unknown}}{\text{absorbance of standard}} \times 200 = \text{mg urea per 100 ml}$$

Notes—

1. If the test solution is higher than the highest standard, the colour development is repeated using 1.0 ml of supernatant and 1.0 ml of distilled water. The result is then multiplied by 2.
2. S.I. units are progressively being introduced into clinical chemistry and therefore to convert mg urea per 100 ml into mmol per l use the following calculation.

$$\text{mg per 100 ml} \times \frac{1}{6} = \text{mmol per l}$$

Urine urea

It is often necessary to know the urea concentration in urine, either on a single specimen or as part of a timed collection. This can be an important estimation in cases of renal failure. As urine urea is greatly in excess of the blood level, the estimation can be performed by a similar method as those for blood analysis after first diluting the urine about 1 in 20.

Urea excretion in a normal adult is about 30 g per day or approximately 2 g per 100 ml. The excretion, however, depends upon the protein content of the diet. A low-protein diet results in low urea excretion while a high-protein diet is accompanied by a high urea excretion. Urine urea estimations were most commonly carried out as part of a urea clearance and concentration test, but these tests have now been replaced by a far better parameter, the creatinine clearance test. This estimation measures very closely the glomerular filtration rate of the kidney.

11

Chemical Analysis of Cerebrospinal Fluid

Examination of the cerebrospinal fluid (CSF) is used in the clinical investigation of the central nervous system, which consists of the brain, spinal cord and peripheral nerves.

The brain is about one-fiftieth of the body weight and lies

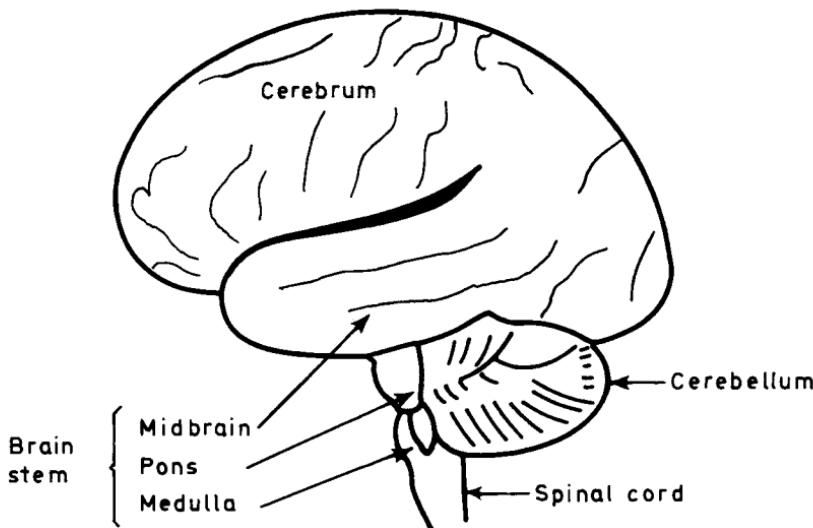


Figure 11.1 The major divisions of the brain

within the cranial cavity. It is divided structurally into the cerebrum (greater brain), the brain stem consisting of the midbrain, pons varolii and medulla oblongata, and lastly the cerebellum or lesser brain (Figure 11.1). The four irregularly shaped ventricles, namely the right and left lateral, and third and fourth ventricle play an important part in the formation of CSF (Figure 11.2a). Completely surrounding the brain and spinal cord, are three membranes known as dura mater (outer membrane), the arachnoid

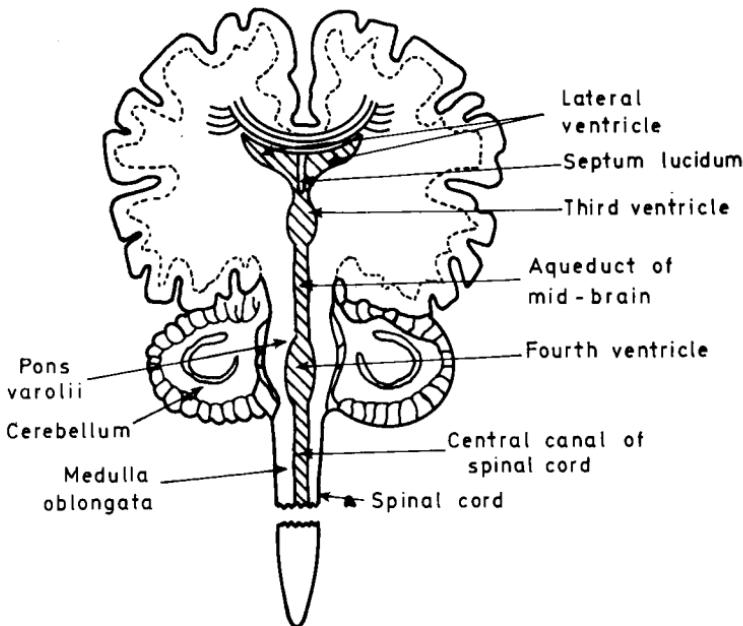


Figure 11.2a Ventricles of the brain, anterior view

mater (middle membrane) and pia mater (the inner membrane). The pia mater and arachnoid mater are separated from each other by the subarachnoid space. Between the tough outer coat (dura mater) and arachnoid mater is the subdural space containing a small amount of tissue fluid (Figure 11.2b).

Formation of CSF

Within the lateral ventricles are the choroid plexuses, where the CSF is formed. They are a network of complex capillaries projecting into the ventricular cavities, covered only by the pia mater and a single layer of cells lining the ventricular system of the brain. The CSF formed by the choroid plexuses passes into the third ventricle via the interventricular foramina (foramen of Munro), then by the aqueduct of the midbrain into the fourth ventricle. From the roof of the fourth ventricle the CSF flows through the foramina into the subarachnoid space to completely surround the brain and spinal cord. At the same time, CSF also flows from the floor of the fourth ventricle downwards through the central canal of the spinal cord. The production of CSF is balanced by an equal absorption of fluid, the reabsorption probably taking place in the

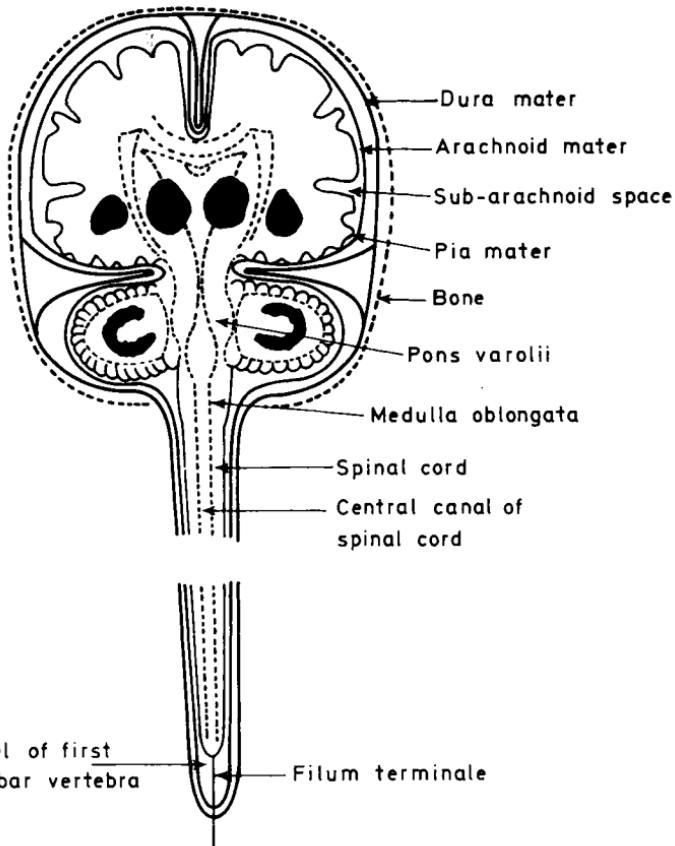


Figure 11.2b The meninges of the brain and spinal cord

blood capillaries of the arachnoid mater. By this process the total volume of CSF will be completely returned to the circulating blood every 6–8 h.

Function of CSF

1. Supports and protects the delicate structures of the brain and spinal cord.
2. Acts as a cushion and shock absorber.
3. Used as a reservoir to regulate the contents of the cranium, i.e. if the volume of the brain or blood increases, CSF drains away; if the brain shrinks, more fluid is retained.
4. Keeps the brain and spinal cord moist.

5. It may act as a medium for the interchange of metabolic substances between nerve cells and CSF.

Obtaining CSF

Specimens of CSF are obtained by introducing a long needle between the third and fourth lumbar vertebrae into the spinal subarachnoid space, with the patient's back flexed to separate the vertebrae. The cord comes to an end at the level of the first lumbar vertebra and cannot be damaged by the needle entering the subarachnoid space an inch or so lower.

A lumbar puncture is far safer than a cisternal puncture, which involves passing the needle between the occipital bone and the atlas into the cisterna magna at the base of the brain.

Composition of CSF

The volume of CSF averages between 120 and 150 ml, and is produced at the rate of about 0.3 ml per min (430 ml/day). It consists of water, dissolved oxygen, carbon dioxide and a number of solids. The sp. gr. is about 1.005, pH 7.4–7.6 and it contains up to 5 lymphocytes per mm³. It is a clear colourless fluid and should show no coagulum or sediment on standing. The composition is very similar to that of plasma, except in protein concentration.

	<i>Plasma</i> <i>mg/100 ml</i>	<i>CSF</i> <i>mg/100 ml</i>
Total protein	6000–8000	15– 45
Glucose	50– 90	50– 80
Chlorides (as NaCl)	560– 620 (100–107 mmol/l)	700–760 (120–126 mmol/l)

CSF can therefore be considered an ultra-filtrate of blood.

A sample of CSF sent to the laboratory for routine examination requires the following investigations: appearance, cell count, total protein, globulin, chloride and sugar. Lange colloidal gold curve is an additional investigation required in certain hospitals, but electrophoresis seems to have taken its place.

The diagnostic importance of CSF examination lies in the cytological and chemical changes produced by certain diseases. Normal ranges and values obtained in various conditions can be seen in Table 11.1.

Table 11.1 CHANGES IN CSF IN VARIOUS CONDITIONS

Conditions	Appearance	No. of leucocytes per mm ³ and type	Protein mg/100 ml	Globulin	Chlorides mg/100 ml	Glucose mg/100 ml
Normal	Clear and colourless	Up to 5 lymphocytes	15–45	No increase	700–760	50–80
Tubercular meningitis	Clear or slightly turbid	Increased lymphocytes and polymorphs	Markedly increased	Increased	500–700	Less than 50
Subarachnoid haemorrhage	May be blood-stained and/or yellow	Normal or increased lymphocytes	Increased	Increased	700–760	50–80
Xanthochromia	Yellow	Normal or increased lymphocytes	Normal or increased	No increase or increased	700–760	50–80

All examinations should be carried out as soon as possible after the specimen is taken. If delay is unavoidable the specimen should be placed in a refrigerator at between 2 and 10 °C and dealt with at the earliest opportunity. Place a small amount of the CSF in a sodium fluoride tube to prevent glycolysis.

Pathological variations and methods of estimation

APPEARANCE

The presence of blood is the main cause of an abnormal colour.

1. Blood

- (a) trauma—while collecting the CSF, some blood may be introduced as a result of trauma; in this case the first few ml should be collected separately, the subsequent fluid should be almost, if not completely, clear. Centrifuging will reveal the presence of a small number of red cells.
- (b) subarachnoid haemorrhage—the CSF will be heavily blood-stained. Furthermore, haemolysis of red cells occurs, liberating haemoglobin, which will eventually be converted into bilirubin. If the CSF is taken a few days after the haemorrhage, the supernatant fluid will be coloured yellow and xanthochromia is present.

2. Turbidity

Turbidity is usually due to an increase in leucocytes or leucocytosis plus organisms. Leucocytes may reach about 400 per mm³ before the fluid appears turbid. In TB meningitis, the fluid, as a rule, is not turbid. For the types of organisms present in CSF see Chapter 27, p. 524.

3. Coagulum

A fibrin clot may form on standing in pathological specimens containing enough fibrinogen. This usually indicates that the protein concentration is greater than 100 mg per 100 ml, but a coagulum can form with a lower protein concentration. Sometimes in TB meningitis a fine web-like clot will form, this usually contains the tubercle bacilli, revealed on microscopic examination.

4. Cell count

The cells in CSF are counted by using a Fuchs–Rosenthal counting chamber (see Chapter 30, 568).

Protein

Turbidimetric methods are the most commonly used techniques for the estimation of total protein, although the colorimetric method is available. In the colorimetric technique CSF is treated with alkaline copper tartrate to form cupric-amino acid complexes. On the addition of phosphomolybdotungstic acid (Folin and Ciocalteau's phenol reagent), the complexes form an intense blue colour due to the reduction of molybdate to molybdenum oxides. The coloured complex is then compared with standard protein solutions similarly treated.

TURBIDIMETRIC PROCEDURES

Principle

Proteins in CSF are precipitated by either dilute trichloracetic acid or dilute sulphosalicylic acid in sodium sulphate solution, and the turbidity of the resultant uniform suspension is measured spectrophotometrically against a standard solution similarly treated.

A. Trichloracetic acid method

Reagents

1. 3 per cent aqueous (*w/v*) trichloracetic acid solution.
2. Stock standard protein solution. 500 mg per 100 ml prepared as on p. 272.
3. Working standard protein solution. 50 mg per 100 ml. Dilute stock standard 1 in 10 with 0.9 per cent NaCl (saline). Prepare fresh each week and store in about 1.5 ml quantities at about -18°C. Thaw a sample for use each day.

Technique

1. *Test.* Add 1.0 ml of CSF dropwise with constant mixing to exactly 4.0 ml trichloracetic acid.
2. *Standard.* Mix 1.0 ml of standard protein solution in the same manner with exactly 4.0 ml trichloracetic acid.
3. *Blank.* Mix 1.0 ml distilled water with 4.0 ml trichloracetic acid.
4. After standing at room temperature for 10 min, remix the turbid solutions.
5. Read the absorbance of the standard and test against the blank at 450 nm or by using a blue filter.

Calculation

T = test reading, S = standing reading, and since the test and standard are treated in the same manner—

$$\frac{T}{S} \times 50 = \text{mg CSF protein per 100 ml}$$

e.g. T = 0.20 S = 0.25

$$\frac{0.20}{0.25} \times 50 = 40 \text{ mg protein per 100 ml}$$

Notes—

1. If the value of the unknown exceeds the upper limit of the method as established by a calibration curve, repeat the determination by using an appropriate saline dilution of the CSF.
2. A calibration curve will show whether a linear relationship holds for increasing protein concentration and should be constructed for each photometric instrument.

Standard calibration curve

Dilute the stock standard protein (500 mg per 100 ml) 1 in 5 with saline and prepare a series of tubes as follows:

mg protein per 100 ml CSF	0	10	20	30	40	50	60	70	80	90	100
ml of standard protein solution											
(100 mg per 100 ml)	0	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0
ml of saline	10	9.0	8.0	7.0	6.0	5.0	4.0	3.0	2.0	1.0	0

Mix well, and treat 1.0 ml of each standard protein solution with 4.0 ml trichloracetic acid as before. If the absorbances are linear, in other words a straight line relationship is obtained, further standards can be prepared up to 250 mg per 100 ml by diluting the stock standard 1 in 2 and setting up the following series of tubes.

mg protein per 100 ml CSF	150	175	200	225	250
ml of standard protein solution (250 mg per 100 ml)	3.0	3.5	4.0	4.5	5.0
ml of saline	2.0	1.5	1.0	0.5	0

Mix well and treat as above.

B. Sulphosalicylic acid-sodium sulphate method**Reagents**

1. 3 per cent sulphosalicylic acid in 7 per cent sodium sulphate.
 - (a) 6 g sulphosalicylic acid are dissolved in 100 ml distilled water.
 - (b) 14 g anhydrous sodium sulphate are dissolved in 100 ml distilled water. Mix equal volumes of (a) and (b).
2. Standard protein solution. 50 mg per 100 ml.

Technique and calculation

As in method A, but substituting sulphosalicylic acid for trichloracetic acid.

C. Method using permanent standards

This method is less accurate than the methods given above, but since it is a rapid technique, many laboratories find it of acceptable accuracy.

Reagents

1. 3 per cent sulphosalicylic acid.
2. Permanent protein standards supplied by Gallenkamp Ltd, London and Widnes.

Technique

1. Add 1.0 ml CSF to a standard tube containing 3.0 ml of sulphosalicylic acid.
2. Mix contents and allow to stand for 5 min.
3. Compare the tube with the turbidity standards, which are marked in mg protein per 100 ml of fluid.

Results

The protein content of normal CSF lies between 15 and 45 mg per 100 ml and is almost entirely albumin in nature. An increase in protein content is the commonest abnormality found, when the protein is a mixture of albumin and globulin, with albumin predominating.

Globulin

Tests for showing an increase in globulin content. Pandy's method is the more sensitive, but is said to be unreliable.

1. PANDY'S METHOD

Principle

If globulin is added to a saturated aqueous solution of phenol, water is absorbed onto the globulin molecules and the phenol is displaced from the solution, causing a fine and persistent turbidity.

Reagents—Pandy's reagent

Saturated aqueous solution of phenol (8–10 g per 100 ml). This solution should be clear and colourless.

Technique

1. Using a pasteur pipette, carefully add 1 drop of CSF to 0.5 ml Pandy's reagent in a small test-tube.
2. The tube is held against the light to detect any turbidity.
3. Normal CSF remains quite clear.
4. A turbidity or precipitate indicates an increase in globulin content.

2. NONNE-APELT'S METHOD

Principle

Globulin is precipitated out of solution by half-saturation with ammonium sulphate.

Reagent

Saturated ammonium sulphate. Dissolve 85 g of ammonium sulphate in 100 ml hot distilled water. Allow to stand overnight, filter and store in well-stoppered bottle.

Technique

1. Pipette 1 ml of saturated ammonium sulphate into a small test-tube.
2. Add 1 ml of CSF and mix.
3. Smaller volumes of sample and reagent can be used providing equal volumes are adhered to.
4. Stand for 3 min and note whether there is any opalescence or turbidity.
5. Normal CSF will remain clear or only show the faintest degree of opalescence.
6. It has been suggested that 1 ml of CSF should be layered on top of 1 ml of ammonium sulphate solution, when a white ring at the junction of the two liquids will be obtained, in increased globulin concentration.

7. This is not recommended as the junction of the two liquids may be greater than 50 per cent saturation.

Results

A small amount of globulin is always present in normal CSF, but this cannot be detected by the above techniques. For a given rise in total protein there is a corresponding rise in globulin content.

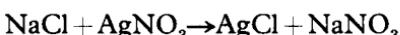
In tabes and disseminated sclerosis, an increased globulin can be obtained with CSF showing a normal total protein content.

Chlorides

1. MOHR'S METHOD

Principle

Chlorides are estimated as sodium chloride by titration against silver nitrate using potassium chromate as indicator. Silver nitrate is added until all the chloride ions present in the CSF have combined with the silver ions. Any further silver nitrate added is now free to combine with the potassium chromate indicator to yield a red precipitate of silver chromate. The solution at this point suddenly changes from pale yellow to faint brick-red colour.



Reagents

1. Standard silver nitrate solution (2.906 g of silver nitrate is dissolved in and made up to 1 litre with distilled water). Keep in an amber bottle and standardize against an accurately prepared sodium chloride solution containing 500 mg NaCl per 100 ml using the method described below. Check at frequent intervals, and determine the factor (*see Chapter 5*).



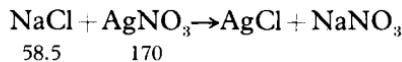
2. 5 per cent potassium chromate solution.

Technique

1. Pipette 1.0 ml CSF into a conical flask containing about 10 ml of distilled water and 2-3 drops of potassium chromate.
2. Slowly add the silver nitrate from a 10 ml burette and continuously rotate the flask, to ensure thorough mixing.
3. As the end-point nears, the silver nitrate solution should be added slowly and carefully to avoid adding excess of the reagent.

4. Note the titre and repeat the titration again if sufficient CSF is available.
5. Normal fluid usually requires between 7.0 and 7.6 ml of silver nitrate.

Calculation



From the above equation it can be seen that 58.5 g of sodium chloride are equivalent to 170 g of silver nitrate.

Therefore $58.5 \text{ mg NaCl} \equiv 170 \text{ mg AgNO}_3$

Now the standard solution of AgNO_3 contains 2.906 g per litre

$$\equiv 2.906 \text{ mg per ml}$$

Hence 2.906 mg or 1 ml of standard AgNO_3 corresponds to

$$2.906 \times \frac{58.5}{170} \text{ mg of NaCl}$$

$$1 \text{ ml AgNO}_3 \equiv 1 \text{ mg NaCl}$$

If the titre was 7.3 ml of AgNO_3 (or $7.3 \text{ ml} \times \text{factor}$), then mg NaCl per 100 ml CSF =

$$\text{titre} \times 1 \times \frac{100}{\text{amt. taken}}$$

$$7.3 \times 1 \times 100 = 730 \text{ mg per 100 ml}$$

Notes—

- (a) It is now desirable to express the NaCl value in terms of mmol per litre, in which case the following formula is used.

$$\begin{aligned} \text{mmol per litre} &= \frac{\text{mg per 100 ml} \times 10}{\text{molecular weight}} \\ &= \frac{730 \times 10}{58.5} = 124.8 \end{aligned}$$

- (b) A solution of AgNO_3 containing 5.812 g/litre can be used in which case 1 ml of $\text{AgNO}_3 = 2 \text{ mg NaCl}$.

2. EEL CHLORIDE METER METHOD

Principle

See p. 137.

Reagents

1. Acid buffer. To approximately 250 ml distilled water add 23 ml glacial acetic acid AR, 1.5 ml concentrated nitric acid AR and dilute to 500 ml.
2. Gelatin solution. 2.5 g of gelatin are dissolved in 250 ml of warm distilled water. When in solution add 0.5 g thymol blue in 100 ml methanol. Stir well until the thymol blue is in solution. Dilute to 500 ml with distilled water and add 0.5 g thymol as a preservative.*
3. Standard chloride solution (100 mEq per litre).

Method

1. *Blank* 13 ml buffer, 5 drops of gelatin solution, 1 magnetic stirrer.
2. *Test* 13 ml buffer, 5 drops of gelatin solution, 0.2 ml CSF and 1 magnetic stirrer.
3. *Standard* As for test, except that 0.2 ml standard chloride solution is used instead of CSF.
4. After adding the required volumes to each beaker, the beaker is placed on the counting platform and the 'titration' button pressed, leaving the beaker in place until the meter has stopped recording.
5. Note the reading in mEq per litre and repeat the process until all samples have been read.
6. The blank should not give a reading, but a reading up to 2 mEq is allowable which must be subtracted from the standard and test values. The standard should read 100 mEq per litre.
7. If not the instrument needs attention (cf. manufacturer's instruction manual) (mEq per litre = mmol per litre).

Results

Normal CSF contains 700–760 mg NaCl per 100 ml (120–130 mmol per litre) and is higher than the plasma level 560–620 mg per 100 ml (96–106 mmol per litre). In meningitis there is usually a fall in chloride content, while an increase can sometimes be found in hypertension.

Glucose

The method for glucose estimation is the same as for blood glucose (p. 208). As the glucose content may be low, it is advisable to carry

* Available from Corning-Eel ready for use.

out the test with twice the volume of CSF, i.e. if blood sample requires 0.1 ml use 0.2 ml of CSF, making sure the diluent is reduced correspondingly, and the calculation is amended to correct for this change.

Note—It is imperative to carry out the assay as soon as possible after withdrawal of CSF. Glycolytic enzymes present in the CSF will cause a reduction in the glucose content, and therefore the estimation becomes valueless after a few hours.

Results

The normal glucose content is between 50 and 80 mg per 100 ml, though 45–100 mg per 100 ml is often allowed. In meningitis the most important pathological change is a decrease in glucose content and in some cases glucose may be absent. Small increases are found in poliomyelitis and raised values may occur in diabetes mellitus.

12

Introduction to Histology

Histology is the microscopic study of the normal tissues of the body whilst histopathology is the microscopic study of tissues affected by disease. The procedures adopted for the preparation of material for such studies are known as histological or histopathological techniques and it is with these techniques that the medical laboratory technician in the pathology department is primarily concerned. The various ways of preparing and examining smears, preserving and processing tissues, cutting and staining sections and the ability to recognize whether or not the procedures have been performed correctly constitute the skills of the medical laboratory scientist in this subject. For the work to be executed competently a knowledge of the structure of cells and the organs and tissues formed by them is essential.

The basic substance of all living things is *protoplasm*, which is contained within small units, called *cells*, many millions of which go to make up the human body.

Protoplasm is the general name given to the main constituents of a cell (of a colloidal nature) together with water, protein, carbohydrates, lipids and inorganic salts. If the cell is studied by histological methods and light microscopy it is seen to contain structures, as shown in *Figure 12.1*. Electron microscopy, however, shows complete tubular structures in the cytoplasm, and detail in the nucleus not seen by ordinary microscopy (*Figure 12.2*).

THE CELL

A cell may be conveniently described as a mass of protoplasm enclosed within a membrane (cell or plasma membrane) containing a subdivision, the nucleus, which is bounded by the nuclear membrane. The portion of cell lying between the plasma and nuclear membranes is known as the cytoplasm. Within the cytoplasm a variety of fine structures called organelles may be identified. These are specialized structures with individual functions and consist of the living material of the cell.

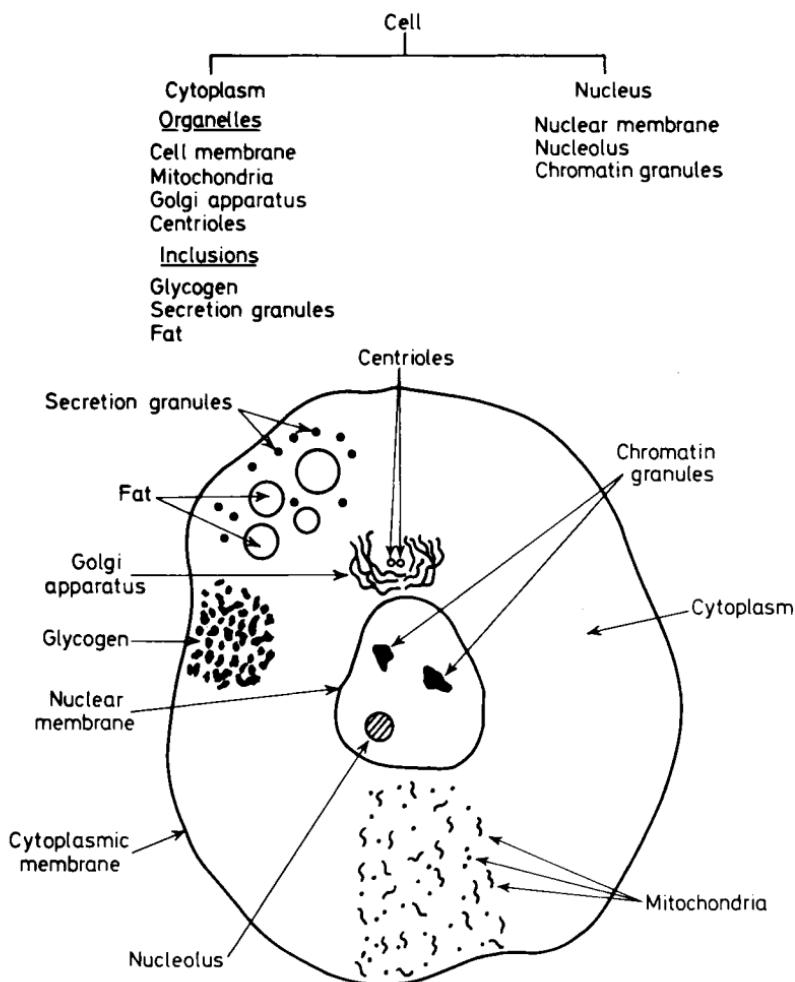


Figure 12.1. Diagram of living cell with its components and inclusions which can be demonstrated by methods for light microscopy. It is unlikely that more than one type would be present but fat, glycogen, pigments and secretory granules are all included

Cell membrane

This is a semi-permeable membrane which permits the selective passage of substances to and from the cell. The exchange of materials through the cell membrane is due to osmotic pressure exerted by the intercellular fluid and cytoplasmic ground substance or, by an active transport mechanism. Electron microscopical

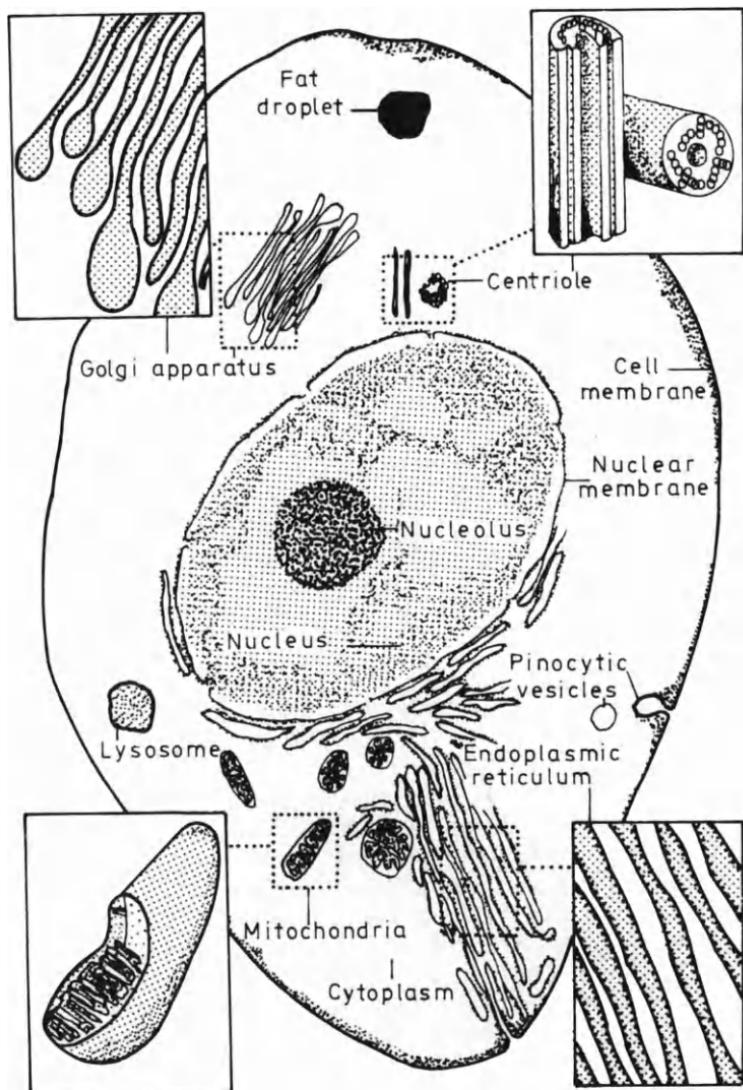


Figure 12.2. Diagram of a normal cell as shown by electron microscopy

studies have shown that the membrane contains three layers which are thought to be composed of protein and lipid molecules.

CYTOPLASMIC ORGANELLES

Endoplasmic reticulum

Cytoplasm is organized into a network of fine branching tubules known as the endoplasmic reticulum (ER). These tubules are lined by a membrane which in places is coated with granules of ribonucleoprotein (ribosomes), and is known as 'rough' or granular ER. It is associated with protein synthesis. Parts of the membrane of the reticulum which are not coated with ribosomes are called 'smooth' or agranular ER and are thought to be associated in some cells with synthesis of fats and similar substances. Although the endoplasmic reticulum cannot be resolved by light microscopy, the amount present in a cell appears to have some effect on the staining reaction of the cytoplasm.

Golgi apparatus

This is a specialized area of smooth endoplasmic reticulum comprising membranous canals and vacuoles. It may be distinguished by its selective reaction with silver salts and osmium tetroxide. Secretory products are concentrated around this area where they may possibly combine with a synthesized carbohydrate component.

Mitochondria

These small filamentous or granular bodies may be distributed evenly throughout the cytoplasm or accumulated in selected sites according to cell type. The number of mitochondria may be very large; as many as 2500 have been found in liver cells. Mitochondria vary in length up to 7 μm and are between 0.5–1.0 μm in diameter. They can be demonstrated in fresh unfixed tissue by special microscopic techniques, or in fixed and stained preparations. Electron microscopy shows these organelles are bound by double membranes. The innermost membrane is reflected to run across the inside of the mitochondria at several points to form shelf-like cristae, and the aggregation and shape of the cristae varies in cells of different functions. Mitochondria have been described as the power houses of the cell and appear to be concerned with cell respiration and enzymatic activity. They are rapidly affected by autolysis being some of the first structures to disappear after the

death of the cell. Acetic acid causes destruction and distortion of mitochondria and should be avoided in fixing solutions.

Lysosome

A minute spherical organelle with a diameter of about $0.25\text{ }\mu\text{m}$. Lysosomes are bounded by a single membrane and contain hydrolytic enzymes, i.e. enzymes which break down large complex molecules into smaller molecules. Rupture of the lysosome membrane releases the enzymes and causes eventual destruction of the cell. This process is known as autolysis. Lysosomes are abundant in leucocytes and macrophages in which they are thought to play an important part in phagocytosis (intracellular digestion) of bacteria and nutrient particles.

Centrosome

The cell centre or centrosome is present in all cells although not readily visible except during cell division. It is seen in sections as a clear area of cytoplasm less than $1.0\text{ }\mu\text{m}$ in diameter, often lying in a concavity of the nucleus, and containing two dark dots, the centrioles. Electron microscopy reveals the centrioles to be short cylindrical bodies whose walls are composed of fine fibres arranged longitudinally like a bundle of twigs. The centrioles are thought to be associated with the formation of fibrillary material, e.g. cilia, the hair-like processes which extend from certain cells and with the spindle of fibrils which extend from the parted centrioles upon which the chromosomes arrange themselves during cell division.

CYTOPLASMIC INCLUSIONS

Non-living substances that may be seen in the cytoplasm of cells are referred to as inclusions. They usually consist of stored nutrients, materials produced by the cell, or ingested particles. The following are those most commonly seen.

Glycogen

Accumulations of glycogen are stored in the cytoplasm of liver cells and skeletal muscle. In stained sections it is seen either as fine granules or as larger amorphous masses.

Fat

Fat is generally stored in fat cells but it may also occur normally or pathologically in other cells. It accumulates in the form of

minute globules which tend to fuse together to form larger globules, often distending the cytoplasm and displacing the nucleus to the periphery of the cell. It is dissolved out when tissues are prepared by the paraffin wax or celloidin techniques unless special fixatives are used, but it is easily demonstrated in frozen sections.

Secretion granules

These are products of cell synthesis and are found in the cytoplasm of specialized cells which have a secretory function. They are dispersed throughout the cytoplasm as small globules which on fixation usually become coagulated to form granules.

Pigments

These are frequently present in the cytoplasm of cells and may be either *endogenous* or *exogenous* in nature. Endogenous pigments such as melanin and haemosiderin are produced within the body; exogenous pigments are particles of foreign matter such as coal dust, which are injected by phagocytosis and absorbed.

Artificial pigments produced as a result of fixation or precipitation of the staining solutions may be present. Such pigments can be easily identified and removed.

THE NUCLEUS

Nuclear membrane

The nucleus contains most of the genetic material of the cell. It is bounded by two membranes each rather similar to the cytoplasmic membrane.

Chromatin

Aggregations of material with an affinity for basic dyes are scattered throughout the nucleus; these are known as chromatin granules. The intense staining reaction of these granules and of the chromosomes which appear during cell division is due to their nucleoprotein content. Nucleoprotein is composed of basic proteins and nucleic acid and the chief nucleic acid present in chromatin is desoxyribonucleic acid (DNA).

Nucleolus

This is a small spheroidal body present within the nucleus of most cells. It contains a high proportion of ribonucleic acid (RNA) and is thought to be concerned with the synthesis of proteins.

Chromosomes

These are small thread-like bodies which are seen within nuclei during cell division. Each chromosome has a bifid structure formed by two *chromatids* lying side by side and linked at one point, the *centromere*.

Normal somatic (body) cells in man contain 46 chromosomes arranged in pairs, one of each pair derived from the father and the other from the mother. Because of this pairing they are known as the *diploid* set and consist of 1 pair of sex chromosomes and 22 pairs of somatic chromosomes (autosomes). The sex chromosomes in the female are similar to each other and are designated by the symbol 'XX', whilst in the male they are dissimilar and are designated 'XY'.

The mature female and male germ cells, namely the *ovum* and *spermatozoon*, contain only a single set of chromosomes, i.e. 23, and these are referred to as the *haploid* set. The chromosomes of the ovum consist of 22 autosomes and 1 'X' chromosome; the chromosomes of the spermatozoon consist of 22 autosomes and 1 sex chromosome which may be 'X' or 'Y', as half the spermatozoon contain an 'X' chromosome and the other half a 'Y' chromosome. On fusion of the ovum and spermatozoon, the diploid set of chromosomes is formed and the sex of the resulting embryo is determined according to the sex chromosome carried by the spermatozoon.

CELL DIVISION

The process by which most human cells divide is called *mitosis* (*Figure 12.3*). Before cell division occurs the amount of deoxyribonucleic acid in the nucleus is doubled so that one half is passed to each new cell. Four stages of mitotic division are recognized, although it should be borne in mind that the whole process is continuous with no intervals between stages.

Prophase

The chromatin of the nucleus becomes concentrated into a tangled mass of filaments which resolve themselves into pairs of chromosomes. The centrioles meanwhile have separated and move towards opposite poles of the cell, drawing with them a number of delicate fibres known as the *achromatic spindle* along which the paired chromosomes become orientated. The nucleolus and

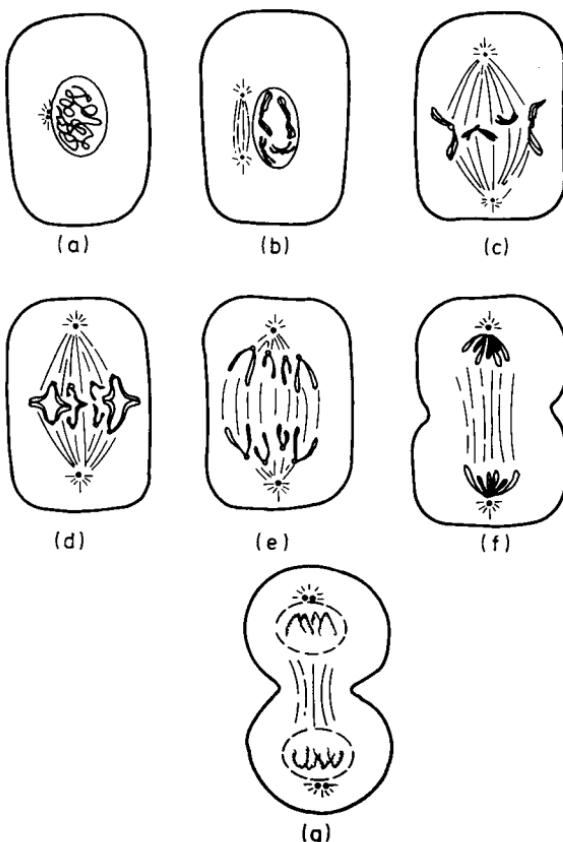


Figure 12.3. The four phases of cell division. (a) Early prophase: Concentration of chromosomes into a tangled mass but constituent chromatids are not apparent. (b) Later prophase: The centrioles have separated and the shortened chromosomes are each seen to be composed of two chromatids. (c) Metaphase: Disappearance of the nuclear membrane and the chromosomes are arranged in the equatorial region of the spindle. (d, e, f) Anaphase: Longitudinal division of the chromosomes, the two halves move apart towards the poles of the spindle. Constriction of the cytoplasmic membrane begins. (g) Telophase: The chromosomes become thread-like and condensed, nuclear membranes form around both groups, and the cell membrane constricts until cleavage into two daughter cells is complete

nuclear membrane disappear and are not seen again until division is complete.

Metaphase

The chromosomes arrange themselves in the equatorial region of the achromatic spindle and each divides longitudinally into two chromatids.

Anaphase

The centrioles move further apart and the two chromatids of each chromosome move away from one another along the spindle towards opposite ends of the cell. The cell now contains two sets of identical chromosomes. The cytoplasmic membrane begins to constrict.

Telophase

In this terminal stage of mitosis each of the two groups of chromosomes are invested with a nuclear membrane. The chromosomes become thread-like again and appear to coalesce to form chromatin granules. The cytoplasmic membrane continues to constrict and finally divides; the two daughter cells are separated from each other to form exact replicas of the parent cell.

INTERCELLULAR SUBSTANCES

Living cells are bound together with other non-living materials, the *intercellular substances*, to form tissues. Some tissues are composed mainly of one type of cell which carries out the particular function of that tissue but most tissues contain in addition other, less specialized cells whose function is to support the main cell type.

The intercellular substances include tissue fluid and various fibres, notably collagen, elastic and reticular fibres. The functions of these substances are to support and strengthen the tissue or to maintain and nourish the cells whose environment they constitute.

Collagen

This is a tough, fibrous protein comprised of fine fibrils of 0.3 to 0.5 μm diameter aggregated to form microscopically visible fibres and ribbons ranging up to 100 μm thick. The fibres are sometimes referred to as *white fibres* and they are the characteristic element of all types of connective tissue.

Elastic fibres

These consist of the fibrous protein elastin. They are long, branching homogeneous threads or ribbons, much thinner than collagen fibres. Known also as *yellow fibres* because of the colour they impart to tissues when present in large numbers, their function is to give such tissues the power of elastic recoil. Elastic fibres are

found abundantly in the walls of blood vessels, trachea, lungs and dermis.

Reticular fibres

Structurally similar to collagen fibres to which they are often connected, these fibres differ from collagen in certain methods of demonstration. The delicate networks formed by reticulum fibres offer support for cells, capillaries, and nerve fibres, and are also found at the junctions between connective and other types of tissue.

EXAMINATION OF TISSUES

Numerous techniques can be used to prepare tissue for microscopical examination, the method selected being governed by a number of factors. These include the structures or inclusions to be studied, the amount and nature of the tissue to be examined, whether the specimen is fresh or preserved and the urgency of the investigation.

Fresh specimens

Fresh specimens may be examined as teased and squash preparations, smears, touch preparations or frozen sections.

TEASED PREPARATIONS

These are prepared by carefully dissecting, with mounted needles, the tissue to be examined. The dissection is carried out while the specimen is immersed in an isotonic solution, such as normal saline or Ringer's solution in a petri dish or watch glass. Selected pieces of the tissue are transferred carefully to a microscope slide and mounted as a wet preparation beneath a coverglass, care being taken to avoid the formation of air bubbles. The preparation is then examined by bright field microscopy, the illumination being reduced either by closing the iris diaphragm or lowering the sub-stage condenser. Many details can be studied in slides prepared by this method, which has the advantage of permitting the cells to be examined in the living state; the preparations, however, are not permanent. The use of the phase contrast microscope greatly increases the structural detail of the cells examined, allowing movement and mitotic division to be observed. The application of certain stains such as methylene blue can be also of great value.

SQUASH PREPARATIONS

The cellular contents of small pieces of tissue not exceeding 1 mm in diameter can be examined by placing the tissue in the centre of a microscopic slide and forcibly applying a coverglass. Staining can be carried out if necessary by making use of capillary attraction; a drop of a vital stain placed at the junction of the coverglass and slide is drawn into contact with the tissue which absorbs it.

SMEARS

The microscopic examination of cellular material spread lightly over a slide in the form of a smear is a technique which has wide application in histopathology. The method of preparing the smear differs according to the nature of the material to be examined, but as a general rule smears are made either by spreading the selected portion of the specimen over the surface of the slide with a platinum loop or, alternatively, by making an apposition smear with the aid of a second slide. Smears may be examined either as fresh preparations in a similar manner to that described for teased preparations, or by using a supravital staining technique in conjunction with a warm stage. Both of these techniques suffer from the same disadvantage, namely, that the preparations are not permanent. Permanent stained preparations can be made from fresh smears by fixing them while still wet, staining to demonstrate specific structures and inclusions and mounting the cleared specimen beneath a coverglass with a suitable mounting medium. Details of these methods are given in Chapter 18.

IMPRESSION SMEARS

These are prepared by bringing into contact the surface of a clean glass slide with that of a freshly cut piece of tissue. Cells transferred to the surface of the slide are examined microscopically by phase contrast or after applying vital stains. Alternatively, the impression smear, which is also known as a touch preparation, can be fixed and stained according to the methods described in Chapter 18.

FROZEN SECTIONS

Sections of 10–15 µm in thickness can be cut from fresh tissue frozen on a microtome with the aid of carbon dioxide or electro-thermal coupling units. The sections are transferred from the microtome knife to a dish containing an isotonic solution, from which they may be either attached to slides prior to staining or carried through the staining solutions by means of a glass rod.

The impetus given to histochemistry in recent years has led to the commercial development of the cryostat, a microtome housed in a form of deep freeze cabinet which permits thin sections to be cut at an atmospheric temperature of -10 to -20°C from previously frozen fresh tissue. The sections are cut by using controls positioned outside the cabinet at room temperature. This instrument, which has become standard equipment in a large number of histological laboratories, definitely facilitates the preparation of sections from unfixed tissues (see p. 364).

Many cell products are soluble in aqueous solutions and for this reason cryostat sections which may be transferred directly from the microtome knife to the microscope slide, are the method of choice for many histochemical investigations. Details of the techniques used for preparing frozen sections are given in Chapter 16.

Fixed tissues

The most effective means of studying normal and diseased tissues of the body microscopically is by the examination of thin sections, previously stained to demonstrate certain structures or inclusions, and mounted on glass slides beneath a thinner glass coverslip. The sections are normally prepared from fixed tissue. Fixation is necessary to prevent the post-mortem changes which occur shortly after death, or on removal from the body.

A number of sectional methods may be used, all of which necessitate that the tissue be supported during the process of cutting the sections. The following factors help to determine the fixative and sectional method to be used.

1. The urgency of the examination.
2. The structures or inclusions to be demonstrated.
3. The material to be sectioned.
4. The staining procedure to be employed.
5. Whether or not serial sections are required.

FROZEN SECTIONS

The use of frozen sections of fresh tissue has already been mentioned. With tissue fixed in formal-saline this method is invaluable as a rapid diagnostic technique and also for demonstrating lipids and the supporting elements of the central nervous system.

PARAFFIN SECTIONS

Paraffin wax is the most widely used embedding medium for preparing histological slides. The fixed tissue not being miscible with

the wax, selected pieces are passed through baths of alcohol of ever-increasing concentration in order to remove all water. The alcohol-saturated tissue is then transferred to an ante-medium which is miscible with both the alcohol and the paraffin wax. Many ante-media raise the refractive index of the tissue, imparting to it a transparent appearance. For this reason, they are commonly referred to as 'clearing agents', but as this is a property not possessed by all of them, the term is incorrect. The ante-medium is eventually replaced by molten paraffin wax and when sufficiently impregnated, the tissue is embedded in fresh wax which solidifies on cooling.

The paraffin wax technique permits thin individual and serial sections to be cut with ease from the majority of tissues. It also allows a multitude of staining techniques to be employed and facilitates storage of the blocks and unstained mounted sections.

CELOOIDIN SECTIONS

Celloidin is a purified form of nitrocellulose and is soluble in a number of solvents. In histology, the solvent generally used consists of equal parts of ethyl alcohol and ether. As an embedding medium, celloidin has certain properties which make it a valuable auxiliary technique.

The use of celloidin permits thicker sections to be cut than is possible with the paraffin wax technique, and for this reason, the method is used for studying the central nervous system. Its rubbery consistency makes it of great value as an embedding medium when sections are required from blocks of tissue that are either very hard or are composed of a number of tissues of varying consistency. As heat is not required during the process of impregnating the tissue, less shrinkage occurs in celloidin sections than in those prepared by the paraffin wax technique.

The disadvantages of the method are that it is slow (impregnating and embedding taking several weeks), the blocks and sections must be stored in 70 per cent ethyl alcohol, sections of less than 10 µm cannot easily be cut and serial sections are difficult to prepare owing to each one having to be handled individually during cutting and staining.

13

Fixation

Shortly after death or removal from the body, cells and tissue begin to undergo changes, which result in their breakdown and ultimate destruction. These are referred to as post-mortem changes, which may be either putrefactive or autolytic in nature.

Putrefaction is due to the invasion of the tissue by bacteria, which generally disseminate from the alimentary tract and spread quickly into surrounding organs. Autolysis is due to the action of enzymes from the dead cell. This phenomenon occurs chiefly in the central nervous system and the endocrine system.

These changes may be retarded by low temperatures or prevented by the use of chemical fixatives. Fixation is the basis of histological technique, and the results of all subsequent procedures depend on the correct selection and use of the fixative employed. It is therefore essential to understand the action which different fixatives have upon the cell and tissue constituents.

A fixative may be described as a substance which will preserve after death the shape, structure, relationship and chemical constituents of tissues and cells. It is mainly due to the action of fixatives on the protein elements of cells and tissues that the structural stabilization is achieved. The preservation should be such that the fixed tissue resembles as closely as possible the form which it had during life. In addition to preserving the tissue and cells the fixing fluid or vapour must also render them insensitive to such subsequent treatment which may be necessary for the production of the final slide or specimen.

A good fixative should be capable of fulfilling the following requirements:

1. It must kill the cell quickly and in so doing, should produce the minimum of distortion.
2. It must penetrate the tissue and cells rapidly and evenly.
3. It must render insoluble the substance of the cell and give good optical differentiation.
4. It must inhibit bacterial decay and autolysis.
5. It must harden the tissue and render it insensitive to subsequent treatment.

6. It must permit at a later date the application of numerous staining procedures in order to render the constituents of the tissue and cells more readily visible.

No single fixing solution has yet been evolved which will comply with each of the conditions outlined above. As a result, it is necessary for the histologist to have at his command a wide range of fixatives in order that he may draw upon the one most suited to his needs as occasion demands.

Temperature has an important effect upon the action of fixatives. A low temperature will retard fixation but will also reduce the autolytic action of the enzymes released after death; a high temperature will decrease the time required in the fixative but will also increase autolysis. Where time is of no object, fixation at a low temperature for a prolonged period is advocated. In cases where fixation is not possible until some time after death, storage at a low temperature (e.g. 2–5 °C) is essential.

SIMPLE AND COMPOUND FIXATIVES

In order to obtain a fixative which will comply as nearly as possible with the conditions previously outlined, it is necessary to mix together several substances, each of which has its own particular effect upon the cell and tissue constituents, in order to obtain the combined effect of their individual actions. These individual substances are known as *simple fixatives*, and the solutions resulting from the mixing of two or more of them are referred to as *compound fixatives*.

Compound fixatives may thus be described as the product of two or more simple fixatives mixed together in order to obtain the combined effect of their individual actions upon the cell and tissue constituents. The formula of some compound fixatives is completely irrational, strong oxidizers being combined with equally strong reducers. Provided that the tissue is only immersed in these irrational solutions for the specified period however, and provided also that it receives the correct treatment subsequent to fixation, excellent results can nevertheless be obtained.

Fixatives are usually grouped under headings according to their action upon the cell and tissue constituents. Those which preserve the tissue in a manner which permits the general microscopical study of the tissue structures and allows the various layers of tissues and cells to retain their former relationship with each other

are termed *micro-anatomical fixatives*; those which are employed for their specific action upon a specific part of the cell structures are termed *cytological fixatives*. This last group may be further subdivided into *nuclear* and *cytoplasmic fixatives* depending upon which of the cell inclusions they act. Generally speaking those cytological fixatives which contain glacial acetic acid or have a reaction of pH 4.6 or less are nuclear fixatives, while those which do not include glacial acetic acid as a constituent and have a reaction above the critical level of pH 4.6 are cytoplasmic fixatives.

Some simple fixatives are used to preserve certain cell products for histochemical demonstration. Chief among these are cold acetone (0–5 °C) and formol-saline buffered to a reaction of pH 7. The cold acetone is used when it is required to demonstrate phosphatases; buffered formol-saline permits the majority of histochemical procedures to be performed. Absolute ethyl alcohol may also be used as a histochemical fixative, usually on sections cut from freeze-dried material or which were prepared by the use of a cryostat.

To preserve an accurate picture of the cell, it is necessary to 'fix' the tissue as soon as possible after it is removed from the body. The specimen is immersed in a large volume of fixative. With routine fixatives, the volume of fluid should be about 50–100 times that of the tissue. In the case of the chrome-osmium fixatives, for example Flemming's fluid, the volume need only be ten times that of the tissue.

SIMPLE FIXATIVES

Actions and properties

Many fixatives have been devised, but only about ten formulae are used in routine work. The action of the simple fixatives upon the cell and tissue constituents will now be described.

FORMALDEHYDE

Formaldehyde (HCHO) is a gas produced by the oxidation of methyl alcohol, and is soluble in water to the extent of 40 per cent by weight. Fats and mucin are preserved, but not precipitated, by formaldehyde. It is a powerful reducing agent, but is often used irrationally in conjunction with certain oxidizing agents (for example, Zenker-formol).

After prolonged storage, formaldehyde often develops a white deposit of para-formaldehyde. The formation of this precipitate is said to be avoided by storage at low temperatures, but its presence does not impair the fixing qualities of the formaldehyde. The

solution is usually acid in reaction, due to the presence of formic acid. Though not harmful, this acid can be neutralized by the addition of a small quantity of magnesium carbonate, or a few drops of sodium hydroxide. Care should be exercised when neutralizing formaldehyde with magnesium carbonate, as carbon dioxide may be released suddenly. Insufficient gas space can result in a violent explosion and it is therefore recommended that neutralization be performed in a wide-mouth vessel. When the immediate reaction between the formic acid and the magnesium carbonate has ceased, the solution may be stored in a Winchester quart bottle.

MERCURIC CHLORIDE

Mercuric chloride ($HgCl_2$) is included in many fixatives and is frequently used in saturated aqueous solution. (At room temperature its solubility in water is approximately 7 per cent.) It precipitates all proteins, but does not combine well with them, and penetrates and hardens tissues rapidly. Fixatives containing mercuric chloride leave a black precipitate in the tissue, and this must be removed by one of the methods described in the section on 'pigments' (p. 395).

OSMIUM TETROXIDE

Osmium tetroxide (OsO_4), commonly known as osmic acid, is a pale yellow powder which dissolves in water (up to about 6 per cent at 20°C), forming a solution which is a strong oxidizing reagent. Osmium tetroxide is extremely volatile and is easily reduced by contact with the smallest particle of organic matter, or by exposure to daylight. It should therefore be kept in a dark, chemically clean bottle. Prolonged exposure to the acid vapour must be avoided since the black oxide OsO_2 , can become deposited in the cornea, resulting in blindness.

Although an expensive reagent, osmium tetroxide is widely used in cytological fixatives. It is the only substance that permanently fixes fat, rendering it insoluble during subsequent treatment with alcohol and xylene. (The Golgi element and mitochondria are also preserved.) Osmium tetroxide is seldom used alone as a fixative, but is usually combined with a chromium salt. After fixation in these solutions, tissues should be washed in running water. Osmium tetroxide is a poor penetrating agent, suitable only for small pieces of tissue. The vapour of osmium tetroxide may be used to fix some tissues, such as the adrenal. The vapour penetrates better than the solution, 'washing out' is unnecessary and the production of artefacts is minimized.

PICRIC ACID

Picric acid ($C_6H_2(NO_2)_3OH$) is normally used in saturated aqueous solution, that is approximately 1 per cent solution. It precipitates all proteins and combines with them to form picrates. These picrates are soluble in water, and the tissue must not come in contact with water until the picrates have been rendered insoluble by treatment with alcohol.

ACETIC ACID

Acetic acid (CH_3COOH) is a colourless solution with a pungent smell. At approximately 17 °C it solidifies, which accounts for its name 'glacial acetic acid'.

Acetic acid is included in a number of histological fixatives. It is not a general protein precipitant but a powerful precipitant of nucleoprotein. When used alone it causes considerable swelling of the tissue, and this property is used in certain compound fixatives to counteract the shrinkage produced by other components. In Heidenhain's 'Susa', for example, the shrinkage produced by mercuric chloride is reduced by the addition of acetic acid. It is often used by cytologists in studying chromosomes, and its chromatin-precipitating properties make it useful in nuclear studies. It destroys mitochondria and the Golgi element, and when used in conjunction with potassium dichromate destroys the lipid-fixing properties of that reagent.

ETHYL ALCOHOL

Ethyl alcohol (C_2H_5OH) is a colourless liquid that is readily miscible with water. It was used extensively by early histologists, but today its use as a simple fixative is confined to histochemical methods. It is frequently incorporated into compound fixatives. Ethyl alcohol is a reducing agent, and should not be mixed with chromic acid, potassium dichromate or osmium tetroxide. As a simple fixative, it is used at concentrations of 70–100 per cent which preserves glycogen, but does not fix it. Ethyl alcohol produces considerable hardening and shrinkage of tissue.

CHROMIC ACID

Chromic acid is prepared by dissolving crystals of the anhydride CrO_3 in distilled water, and is conveniently stored as a 2 per cent stock solution. Chromic acid is a strong oxidizing agent, and should not be combined with reducing agents, such as alcohol and formalin. It is a strong protein precipitant, and preserves carbo-

hydrates. Tissue fixed in chromic acid should be thoroughly washed in running water before dehydration, to avoid the formation of a precipitate of the insoluble sub-oxide.

POTASSIUM DICHROMATE

Potassium dichromate ($K_2Cr_2O_7$) is one of the oldest and most widely used of the simple fixatives. Two entirely different forms of fixation can be produced, depending upon the pH of the solution. At a more acid reaction than pH 4.6 the results are similar to those produced by chromic acid. At a more alkaline reaction than pH 4.6, the cytoplasm is homogeneously preserved and the mitochondria fixed.

One of the most important properties of potassium dichromate is its strong fixative action on certain lipids. This attribute is used particularly in the study of myelinated nerve fibres. If a fixative contains potassium dichromate, tissues preserved in it should be well washed in running water, prior to dehydration.

TRICHLORACETIC ACID

Trichloracetic acid (CCl_3COOH) is sometimes incorporated into compound fixatives. It is a general protein precipitant but has a marked swelling effect on many tissues, a property made use of to counter the shrinkage produced by other simple fixatives. It can be used also as a slow decalcifying agent and the softening effect which it has on dense fibrous tissue is found to facilitate the preparation of sections from blocks of this nature.

COMPOUND FIXATIVES

Compound fixatives may conveniently be considered under two headings: (1) micro-anatomical, and (2) cytological. Micro-anatomical fixatives are used for preserving the various layers of tissue and cells in relation to one another, so that general structure may be studied. Cytological fixatives are usually subdivided into two groups: (a) nuclear, and (b) cytoplasmic. They are used for preservation of the constituent elements of the cells, though this often entails loss of the properties of the micro-anatomical fixatives.

Micro-anatomical fixatives

10 PER CENT FORMOL-SALINE

Formol-saline is a micro-anatomical fixative, but not a compound one. It is described here merely for convenience.

This is recommended for the fixation of material from the central nervous system and general post-mortem tissue. The period of fixation required is 24 h or longer.

Formula

Formaldehyde, 40 per cent	100 ml
Sodium chloride	9 g
Distilled water	900 ml

Advantages

This fixative is excellent for post-mortem material and is consequently very widely used. It causes even fixation and produces very little shrinkage. Large specimens may safely be fixed for an indefinite period provided that the solution is changed every three months. Fixation with formol-saline can be followed by most staining techniques, and it is particularly valuable for work on the central nervous system. Though fat is not fixed, it is preserved and may be demonstrated by suitable staining procedures. This is the only routine fixative which conveniently facilitates the dissection of specimens; 10 per cent formalin is the basis of all museum fixatives, for it is the only fixative that allows the natural colour to be restored to the specimen.

Disadvantages

It is a slow fixative and tissue which has been fixed in formol-saline is liable to shrink during dehydration in alcohol. This shrinkage may be reduced by secondary fixation in formol-saline-sublimate (*see p. 320*). The metachromatic reaction of amyloid is reduced, and acid dyes stain less brightly than they do after mercuric chloride fixation. Formalin has an irritant vapour which may injure the nasal mucosa and cause sinusitis. Rubber gloves must be worn when handling specimens fixed in formol-saline, for dermatitis may be produced by prolonged contact of formalin with the skin. A pigment is often formed in tissue containing a great deal of blood.

10 PER CENT NEUTRAL BUFFERED FORMALIN

This is recommended for the preservation and storage of surgical, post-mortem and research specimens. The period of fixation is 24 h or longer.

Formula

Sodium dihydrogen phosphate (anhydrous)	3.5 g
Disodium hydrogen phosphate (anhydrous)	6.5 g
Formaldehyde, 40 per cent	100.0 ml
Distilled water	900.0 ml

Advantages

This fixative has the same advantages as formol-saline, but in addition it prevents the formation of the troublesome post-mortem precipitate (acid formalin pigment).

Disadvantages

The disadvantages of this fixative are similar to those listed for formol-saline. It does also, however, have the disadvantage of taking longer to prepare and in a busy routine laboratory where large volumes of the fluid are used daily this is an important factor to consider.

HEIDENHAIN'S 'SUSA'

This is recommended mainly for biopsies. The period of fixation required is from 3 to 12 h.

Formula

Mercuric chloride	45 g
Sodium chloride	5 g
Trichloracetic acid	20 g
Glacial acetic acid	40 ml
Formaldehyde, 40 per cent	200 ml
Distilled water	800 ml

Advantages

This fixative penetrates rapidly, producing good and even fixation, with the minimum of shrinkage and hardening. It allows brilliant subsequent staining results with sharp nuclear detail, and may be followed by most staining procedures, including silver impregnations. Large blocks of fibrous tissue may be sectioned more easily after this fixative than after any other. The tissue is transferred directly from the fixative to 95 per cent or absolute alcohol.

Disadvantages

Slices of tissue should not exceed 1 cm in thickness, as prolonged fixation necessary for thicker material produces shrinkage and hardening. Red blood corpuscles are poorly preserved. Some cytoplasmic granules are dissolved.

FORMOL-SUBLIMATE

This is recommended for routine post-mortem material. The period of fixation required is from 3 to 24 h, depending on the thickness of the tissue.

Formula

Saturated aqueous mercuric chloride	90 ml
Formaldehyde, 40 per cent	10 ml

Advantages

This is an excellent routine fixative, and it produces little or no shrinkage or hardening of the tissue. It can be followed by most staining procedures, including the silver reticulum methods, with excellent results. Cytological details and red blood cells are well preserved. The tissue is transferred directly from the fixative to 70 per cent alcohol.

Disadvantages

Slices of tissue should not exceed 1 cm in thickness.

FORMOL-SALINE-SUBLIMATE

Good results are obtained if the formol-sublimate solution (*see above*) is diluted with an equal volume of 10 per cent formol-saline. The results obtained are similar to those following formol-sublimate. The solution is recommended for secondary fixation.

ZENKER'S SOLUTION

This is recommended for the fixation of small pieces of liver and spleen. The period of fixation required is from 12 to 24 h.

Formula

Mercuric chloride	5.0 g
Potassium dichromate	2.5 g
Sodium sulphate (optional)	1.0 g
Distilled water	100.0 ml
Add 5 ml of glacial acetic acid just before use.	

Advantages

Tissue fixed in Zenker's solution permits excellent staining of nuclei and of connective tissue fibres. It is recommended particularly for tissues which are to be stained by one of the trichrome techniques.

Disadvantages

Penetration is poor, and pieces of tissue should not exceed 0.5 cm in thickness. Tissue immersed in the fluid for more than 24 h tends to become brittle. After fixation, the tissue must be washed in running water for several hours. Zenker's solution is not recommended for frozen sections. The solution does not keep well after addition of the acetic acid.

ZENKER-FORMOL (HELLY'S)

This is recommended for the fixation of pituitary tissue and bone-marrow. The period of fixation required is from 12 to 24 h.

Formula

Mercuric chloride	5.0 g
Potassium dichromate	2.5 g
Sodium sulphate (optional)	1.0 g
Distilled water	100.0 ml

Add 5 ml of 40 per cent formaldehyde just before use.

Advantages

Although this fixative contains both oxidizing and reducing agents, it produces excellent nuclear fixation. Staining of nuclei is even more intense than after fixation with Zenker's solution. Cytoplasmic granules are well preserved.

Disadvantages

The disadvantages are comparable with those of Zenker's solution. If material is allowed to remain in the fixative for longer than 24 h, a brown scum is produced on the tissue.

BOUIN'S SOLUTION

This is recommended for the fixation of embryos. The period of fixation required is from 6 to 24 h.

Formula

Saturated aqueous picric acid	75 ml
Formaldehyde, 40 per cent	25 ml
Glacial acetic acid	5 ml

Advantages

This fixative produces very little micro-anatomical distortion and permits brilliant staining results. The tissue should *not* be washed in running water, but transferred directly from fixative to 70 per cent alcohol. Bouin's solution preserves glycogen and may be used for fixing tissue in which this carbohydrate is to be demonstrated. The yellow colour which Bouin's fluid imparts to tissue is useful when handling fragmentary biopsies.

Disadvantages

This fixative penetrates poorly, restricting its usefulness to small pieces of tissue.

GENDRE'S FLUID

A general micro-anatomical fixative which is also widely used for the preservation of glycogen.

Formula

Acetic acid, glacial	5 ml
Picric acid, saturated solution in 95 per cent alcohol	80 ml
Concentrated formaldehyde solution (40 per cent)	15 ml

Advantages

The results produced are very similar to Bouin's solution (*see above*), but the combined action of both the alcohol and high picric acid content make it an excellent fixative for glycogen. When fixation is complete the tissue is washed in several changes of 80 per cent alcohol.

Disadvantages

These are similar to those listed under Bouin's fluid.

Cytological fixatives

As has been previously mentioned, cytological fixatives are usually divided into two groups: (*a*) nuclear, and (*b*) cytoplasmic.

Nuclear fixatives

1. Flemming's fluid
2. Carnoy's fluid

Cytoplasmic fixatives

1. Flemming's fluid without acetic acid
2. Helly's fluid
3. Formalin with 'post-chroming'

Nuclear fixatives**FLEMMING'S FLUID**

This fixative is recommended for the preservation of nuclear structures. The period of fixation is from 24 to 48 h.

Formula

Chromic acid, 1 per cent	15 ml
Aqueous osmium tetroxide, 2 per cent	4 ml
Glacial acetic acid	1 ml

Advantages

This fixative is the most commonly used of the chrome–osmium–acetic fixatives. Excellent fixation of nuclear elements, especially chromosomes, is produced. It is the only fixative which permanently preserves fat. The reagent is costly, but relatively small volumes are required, that is the tissue may be fixed in ten times its own volume of Flemming's fluid.

Disadvantages

Owing to the poor penetrative powers of this fixative it should only be used for small pieces of tissue. The solution deteriorates rapidly, and must be prepared immediately before use. Tissue fixed in Flemming's fluid should be washed for 24 h in running tap water prior to dehydration.

CARNOY'S FLUID

This is recommended for fixing chromosomes, lymph glands, and urgent biopsies. The period of fixation required is from $\frac{1}{2}$ to 3 h.

Formula

Absolute alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

Advantages

This fixative permits good nuclear staining, but is not recommended for detailed nuclear studies. It fixes rapidly and also dehydrates, and is therefore useful for biopsy material. Glycogen is preserved. Following fixation, the tissue is transferred directly to absolute alcohol.

Disadvantages

Excessive shrinkage is caused by this solution and it is only suitable for small pieces of tissue. Red blood corpuscles are haemolysed.

Cytoplasmic fixatives**FLEMMING'S FLUID WITHOUT ACETIC ACID**

This is recommended for mitochondria and the period of fixation required is from 24 to 48 h.

Formula

Flemming's fluid but omitting the acetic acid (*see p. 323*).

The advantages and disadvantages of this solution are similar to those listed for Flemming's fluid. The omission of acetic acid improves the cytoplasmic detail.

HELLY'S FLUID

This is synonymous with Zenker-formol (*see p. 321*).

FORMOL-SALINE 10 PER CENT

Fixation in 10 per cent formol-saline, followed by the post-chroming of the tissue, in 3 per cent potassium dichromate for 3-7 days, permits good cytoplasmic staining, particularly of mitochondria.

THE FIXATION OF SMEARS

Smears which are to be examined for the presence of malignant cells may be fixed in the following solutions. (*See also Chapter 19*).

ALCOHOL-ETHER

This is a widely used cytological fixative, especially recommended for use with the Papanicolaou staining methods. It is highly flammable.

Formula

Absolute ethyl alcohol	1 volume
Ether	1 volume

1. Fix the smears for 15 min or longer.

2. Rinse in alcohol followed by distilled water and continue to stain by the selected procedure.

SCHAUDINN'S FLUID

This is a rapidly penetrating fixative used in diagnostic exfoliative cytology for preserving smears which are to be stained with haematoxylin and eosin.

Formula

Mercuric chloride, saturated aqueous solution	66 ml
Absolute ethyl alcohol	33 ml
Glacial acetic acid	1 ml

1. Fix the smears for upwards of 2 min.
2. Wash in distilled water.
3. Remove the mercuric chloride pigment according to the method given on p. 395.
4. Continue to stain by the selected procedure.

THE FIXATION OF GROSS SPECIMENS

It is often necessary to fix specimens of entire organs. This may be done with 10 per cent formol-saline, or with one of the museum fixatives consisting of formaldehyde in conjunction with various acetates, such as Wentworth's solution:

Formula

Sodium acetate	40 g
Formaldehyde	100 ml
Distilled water	900 ml

The technique of fixation varies with the organ to be preserved. A detailed description of the technique is outside the scope of this book, but the following brief notes will act as a guide.

Central nervous system

Tissue from the central nervous system should be fixed as soon after death as possible, to prevent the autolytic changes which rapidly take place. If the whole brain is to be preserved, it should be suspended in 10 per cent neutral formol-saline by means of a cord passed under the basilar artery. If the spinal cord is required

whole, it should be laid flat on a narrow strip of wood or cork and the dura mater incised along its entire length. The dura mater should now be reflected and pinned onto the board with hedgehog quills or plastic pins (metal pins are not recommended for this purpose, as they rust and leave unsightly holes in the tissue). Fixation is accomplished by floating the pinned specimen, board uppermost, in 10 per cent neutral formol-saline.

Lungs

Formol-saline, 10 per cent, is run into each of the major bronchi from an aspirator placed four feet higher than the specimen. The fluid is run in until the contours of the lung appear sharply outlined. The bronchi should be plugged and the specimen immersed in a large volume of the fixative.

Heart

The heart should be packed with small balls of absorbent cotton wool saturated with 10 per cent formol-saline. The specimen should then be immersed in a large volume of 10 per cent formol-saline.

Liver, kidney and spleen

Such specimens are best fixed by injection. Formol-saline, 10 per cent, is injected into the blood vessel of the organ by means of a Robert's bronchogram syringe, and the specimen then immersed in a large volume of fixative.

Intestine

The method of fixation depends on the pathology to be demonstrated. If the natural shape is to be preserved, as in Crohn's disease, the specimen should be packed with absorbent cotton wool and soaked in 10 per cent formol-saline. If it is desired to demonstrate such parasites as *Trichuris trichiura*, the gut is opened and pinned out, in a similar manner to that described for spinal cord.

SECONDARY FIXATION

Following fixation with formol-saline it is sometimes advantageous to refix the tissue for a further 4 h in a second fixative. The fixatives usually selected for this purpose are formol-sublimate, Zenker-formol and Heidenhain's 'Susa'. This procedure, which

is known as secondary fixation, has the advantage of imparting a firmer texture to the tissue and in many instances improves the subsequent staining results.

POST-CHROMATIZATION

In order to facilitate certain staining procedures fixed tissues or sections can be immersed in 3 per cent potassium dichromate for several hours prior to staining. This procedure is known as post-chromatization or post-chroming, and is used mainly with tissue fixed in formol-saline.

The purpose is to mordant the tissue. Post-chroming should not be confused, however, with post-mordanting (*see p. 390*). This latter procedure is carried out after staining, a classic example being the application of the iodine in Gram's stain.

'WASHING OUT'

Reference has been made to the washing of tissue in running water, after certain fixatives have been used. This may be done in several ways, but whatever the method, it is important to ensure that the specimen is bathed in a constant stream of fresh water. It is important that neither the tissue nor the accompanying label are washed out of the container. It is also important to make sure that the water surrounding the tissue is constantly being changed, preferably by means of a syphon system. Failure to observe this point may result in the fixative being insufficiently removed from the tissue.

The purpose of washing the tissue in running water is to remove oxidizing agents such as potassium dichromate and osmium tetroxide to prevent reduction when coming into contact with the alcohol. It is also important to remove all traces of formaldehyde from tissue to be embedded in gelatin.

14

Decalcification

When heavy deposits of calcium salts are present in tissue the cutting of sections is facilitated by decalcification. Inadequate decalcification results in poor section-cutting and severe damage to the knife-edge. Calcium is normally present in large amounts in bone and teeth but pathologically deposits may be found in varying amounts in other tissues, notably those involved in tuberculous or cancerous changes. Calcified deposits are often present also in the heart valves and walls of large blood vessels, particularly the aorta, of elderly people.

An acid is the essential constituent in most decalcifying solutions, and a second substance is often incorporated to prevent distortion of the tissue, although this should be minimal if adequate fixation has been given. Buffer solutions of pH 4.4 to 4.5 and organic chelating agents, e.g. ethylenediamine tetra-acetic acid (EDTA), can also be used.

A good decalcifying agent should remove all calcium without damage to cells or tissue fibres and with no impairment of subsequent staining or impregnation.

The four acids most commonly used for removing calcium salts from tissues are formic, nitric, hydrochloric and trichloracetic acid.

The speed at which the calcium salts are dissolved out of the tissue is dependent upon the strength, temperature and volume of the decalcifying solution in relation to the size and consistency of the tissue undergoing decalcification. An increase in either the concentration of the acid acting as the decalcifying agent or the temperature at which decalcification takes place, can markedly decrease the time required, but this is usually attended by partial digestion of the tissue and inferior staining results. These adverse effects do not apply to EDTA which may be used successfully at 40 to 60 °C.

SELECTION OF TISSUE

Bone

Blocks of tissue suitable for sectioning are selected from the gross

specimen by means of a sharp, fine-toothed hacksaw after preliminary fixation in neutral 10 per cent formalin. To facilitate fixation and decalcification, the selected block of tissue should not exceed 5 mm in thickness. Damage to the surface of the tissue and impacted bone-dust produced by sawing can be removed by trimming the decalcified tissue with a sharp knife. It is always advisable, however, to discard the first sections cut in order to avoid possible artefacts in the final preparation.

Teeth

Blocks of teeth for sectioning are usually best taken when the specimen is either completely or partially decalcified. They may then be selected with a sharp knife, thereby causing the minimum of damage and distortion to the tissue.

Calcified tissue

Blocks of tissue suitable for processing and sectioning can usually be selected from fixed soft tissues containing calcified areas by means of a sharp knife. If large calcified areas are encountered a hacksaw is gently applied until the deposits are cut through and the surrounding soft tissues are again dealt with by knife. Considerable damage to knife-edge and tissues will occur if the cutting of such areas is attempted by knife alone. The selected tissue blocks should preferably not exceed 5 mm thickness as immersion in the decalcifying solution for too long a period is to be avoided.

Tissues should be completely fixed before commencing decalcification and neutral 10 per cent formalin is the recommended fixative for this purpose. At least 48 h fixation is required for tissue blocks of 5 mm thickness.

Technique of decalcification

1. The selected tissue slice is suspended in the decalcifying solution by means of a waxed thread. This allows the solution free access to all surfaces of the tissue, whilst the wax protects the thread from the action of the acid. With few exceptions, the volume of decalcifying fluid should be approximately 50–100 times the volume of the tissue.
2. The progress of decalcification should be tested at regular intervals, usually daily, but in the final stages more frequent tests are made. The fluid is renewed following each positive test.
3. When decalcification is complete the tissue is transferred directly to 70 per cent alcohol and given several changes over

8 to 12 h. This not only effectively washes out the acid but also establishes the first stage of dehydration for either the paraffin wax or celloidin infiltration techniques (*see* Chapter 15).

4. The tissue is then completely dehydrated and processed according to the required embedding technique. If the paraffin wax method is used it is recommended that at least part of the wax impregnation be carried out in the vacuum oven (q.v.).

Assessment of decalcification

Tissues should be immersed in the acid decalcifying solutions only for as long as is necessary for complete calcium removal. Prolonged immersion beyond this stage will result in deterioration of cell and tissue morphology and the quality of subsequent staining reactions. The stage to which decalcification has progressed and its eventual end-point can be assessed by (1) X-ray examination, (2) a chemical test. The simplicity of the chemical test has fortunately led to the abandonment of several crude methods for decalcification assessment. These included probing of the tissue block by needle, knife or finger nail in an effort to detect residual gritty fragments of calcium. Such malpractices were the direct cause of tissue damage, and small spicules of bone often remained undetected.

1. X-ray examination is the most satisfactory method depending on the availability of facilities and a good relationship between laboratory and radiography department. X-ray is the only means by which tissues treated with EDTA can be adequately controlled, but it cannot be used on material fixed in mercuric chloride because this fixative renders such material radio-opaque.
2. A chemical test (Clayden, 1952) is a simple and reliable expedient when radiography is unavailable. It is a two-stage test which depends on the detection of dissolved calcium in the decalcifying fluid. A positive result at either stage indicates that further decalcification of the tissue in fresh fluid is required and the test should be repeated after a suitable interval.

METHOD

1. Decant 5 ml of the used decalcifying fluid into a clean test-tube and add a small piece of litmus paper.
2. Add strong ammonia (sp. gr. 0.88) drop by drop whilst agi-

tating the tube until the litmus paper just turns blue, indicating alkalinity.

3. If the solution becomes turbid at this stage calcium is present in considerable amounts and the tissue should be transferred to fresh decalcifying fluid.
4. If the solution remains clear proceed with the second stage of the test. Add 0.5 ml saturated aqueous ammonium oxalate, mix and allow to stand for 30 min. Any turbidity developing during this period indicates the presence of calcium and reimmersion of the tissue in fresh decalcifying fluid is necessary.

If the solution remains clear it may be assumed that decalcification is complete.

It is important that sufficient time is allowed between tests to ensure dissolution of calcium by the fresh decalcifying fluid. Intervals of 3–4 h are considered adequate.

When using the chemical test to control the degree of decalcification it is essential that the decalcifying fluid is prepared with distilled water. Failure to observe this precaution may result in false positive readings being produced by the presence of calcium ions in tap water.

Decalcifying solutions

FORMIC ACID (HCOOH)

This is recommended for post-mortem and research tissue. The time necessary for decalcification is from 2 to 7 days.

Formula

Formic acid (sp. gr. 1.20)	5 ml
Distilled water	90 ml
Formaldehyde (40 per cent)	5 ml

Advantages

This solution permits excellent staining results and it is regarded by many workers as being the best decalcifying solution for routine purposes.

Disadvantages

At the above strength decalcification is slow, and the solution is therefore unsuitable for urgent work. Decalcification may be speeded up by increasing the formic acid content up to 25 ml

(Gooding and Stewart's fluid). A disadvantage of using concentrations of formic acid in excess of 8 per cent, however, is that the opacity of the solution interferes with the chemical test used in controlling the degree of decalcification. While the used fluid can be diluted in order to apply this test, the final result is not always as accurate as when used with a sample of the undiluted decalcifying solution.

NITRIC ACID—FORMALDEHYDE

This is recommended for urgent biopsies. The time required for decalcification is from 1 to 3 days.

Formula

Nitric acid (sp. gr. 1.41)	10 ml
Formaldehyde (40 per cent)	5–10 ml
Distilled water	to 100 ml

Advantages

This is a rapidly acting decalcifying solution which permits good nuclear staining.

Disadvantages

Nuclear staining is not as good as that obtained after more slowly-acting solutions. Nitric acid frequently develops a yellow colour when used as a decalcifying agent owing to the formation of nitrous acid. This increases the speed of decalcification but also impairs the subsequent staining reactions. The addition of 0.1 per cent urea to the pure concentrated nitric acid temporarily arrests the discolouration and does not appear to affect the efficiency of the acid.

AQUEOUS NITRIC ACID

A rapidly acting decalcifying solution which is recommended for routine use.

Formula

Nitric acid (sp. gr. 1.41)	5–10 ml
Distilled water	to 100 ml

Advantages

This is a rapid decalcifying solution which causes very little hydrolysis provided that the tissue is not allowed to remain immersed

beyond the stage when decalcification is completed. The subsequent staining results are good.

Disadvantages

The disadvantages of the solution are similar to those given above under nitric acid-formaldehyde. The remarks relating to the use of urea to stabilize the nitric acid apply with this solution also.

PERENYI'S FLUID

This solution was introduced originally as a fixative for ova, but it has gained popularity in recent years as a good routine decalcifying fluid. The time required for decalcification is from 2 to 10 days.

Nitric acid, 10 per cent aqueous solution	40 ml
Absolute ethyl alcohol	30 ml
Chromic acid, 0.5 per cent aqueous solution	30 ml

When freshly mixed the solution is yellow but it rapidly assumes a clear violet colour.

Advantages

No hardening occurs in tissues treated with Perenyi's fluid, indeed it is often used as a softening agent, prior to dehydration, for dense fibrous tissues. Cellular detail is well preserved and subsequent staining is good. When decalcification is complete, tissues do not require washing in water and may be transferred directly to several changes of 70 per cent alcohol.

Disadvantages

It is rather slow for decalcifying dense bone. The chemical test given on p. 330 cannot be used to determine decalcification endpoint because a precipitate is formed when ammonia is added to Perenyi's fluid even in the absence of calcium ions. This difficulty may be overcome, however, by a simple modification:

1. Transfer 5 ml of used decalcifying fluid to a chemically clean test-tube and add a small square of litmus paper.
2. Add ammonium hydroxide solution (sp. gr. 0.88) drop by drop, mixing between drops, until the reaction is alkaline.
3. Add glacial acetic acid drop by drop until the precipitate is dissolved.

4. Add 0.5 ml saturated aqueous solution of ammonium oxalate.

The appearance of a white precipitate within 30 min indicates the presence of calcium, and that the tissue requires further treatment with fresh fluid.

VON-EBNER'S FLUID

The use of this fluid is recommended for teeth and the time necessary for decalcification is from 3 to 5 days. Various formulas have been given for this method, but the following gives good results.

Formula

Saturated aqueous sodium chloride

(36 per cent approx.) 50 ml

Distilled water 50 ml

Hydrochloric acid 8 ml

Advantages

This is a fairly rapid decalcifying solution and subsequent staining results are usually good. It is particularly useful for decalcifying teeth. The excess acid is removed by several changes of 90 per cent alcohol for 24 h. Dehydration is thereby hastened.

Disadvantages

Nuclear staining is not as good as that obtained after formic acid.

TRICHLORACETIC ACID

This is recommended for small pieces of delicate tissue which require decalcification. The time necessary for decalcification is from 4 to 5 days.

Formula

10 per cent formol-saline 95 ml

Trichloracetic acid 5 g

Advantages

It permits good nuclear staining. The excess acid is removed by washing in several changes of 90 per cent alcohol.

Disadvantages

It is a slow decalcifying solution, and is not recommended for use with dense bone.

CITRATE-CITRIC ACID BUFFER (pH 4.5)

This is recommended when speed is not an important factor. The period required for decalcification is approximately 6 days, during which time the solution should be changed daily.

Formula

Citric acid (monohydrate), 7 per cent aqueous solution	5.0 ml
Ammonium citrate (anhydrous), 7.4 per cent aqueous solution	95.0 ml
Zinc sulphate, 1 per cent aqueous solution	0.2 ml
Chloroform, as preservative	a few drops

Advantages

This solution produces no damage to the cells or tissue constituents and permits excellent staining results.

Disadvantages

The method is too slow for routine work.

ION EXCHANGE RESINS

The incorporation of an ion exchange resin (an ammonium form of polystyrene resin) into the decalcifying solution has been claimed to speed up the process of decalcification and to improve staining. The principle of the method is that the calcium ions are removed from the solution by the resin, thereby increasing the rate of solubility of the calcium from the tissue. However, subsequent workers have shown that no obvious improvement in decalcification speed, preservation or staining is achieved by the use of these resins. A layer of the resin, approximately half an inch thick, is spread over the bottom of the vessel being used and the specimen is allowed to rest on it. The decalcifying solution is added, the volume of the solution being approximately 20–30 times that of the tissue. The end-point is determined by radiological examination, the chemical test not being applicable.

The use of ion exchange resins is limited to decalcifying solutions which have a non-mineral acid as their active constituent, formic acid being the usual choice. Two baths of N/10 hydrochloric acid followed by three washes of distilled water will regenerate the used resin for further use.

CHELATING AGENT

This is a very slow decalcifying solution recommended only for detailed microscopical studies. The time required for decalcification is approximately 3 weeks, during which time the solution must be changed at intervals of 3 days, reducing to one day in the final stages.

Formula

Ethylene diamine tetra-acetic acid (EDTA) disodium salt	5.5 g
10 per cent neutral formalin	100 ml

Advantages

Histological artefacts are minimized by the use of this solution, there being no carbon dioxide bubbles produced to destroy the pattern of the remaining organic material. The subsequent staining results are also excellent.

Disadvantages

It is slow and unsuitable for urgent work. The chelating agent also tends to harden the tissue slightly.

SOFTENING OF DENSE FIBROUS TISSUE

Some specimens are composed of dense fibrous tissue which, while not containing calcium salts, is nevertheless too tough for sectioning. Blocks of tissue taken from such specimens may be softened as described by Lendrum by the addition of 4-6 per cent phenol to the dehydrating alcohols.

15

Dehydration, Impregnation and Embedding Techniques

SELECTION OF TISSUE

Following fixation, blocks of tissue for histological examination are selected from the gross specimen. A brief description of the nature of the tissue and site of origin should be recorded either on a working card, or in a book reserved for the purpose. Small cardboard tickets bearing the general laboratory number and pathology number (this information varies according to the system employed) should be written out in waterproof ink or pencil and placed in the compartment of the processing basket or specimen bottle, together with the tissue.

Blocks requiring special attention should have an asterisk marked on the ticket, and details of the special attention to be given to the specimen should be recorded on the working card. Frequently, blocks are to be sectioned from a particular surface. This may be identified by passing a thread through one corner of the opposite surface of the block to that which is to be sectioned. Alternative methods include the insertion of a fine stainless steel pin, a blob of Indian ink, or a V-shaped groove cut into the obverse surface of the tissue block.

At no time after the blocks of tissue have been selected should their identifying label be removed. Failure to observe this rule could lead to a positive malignancy report being issued for the wrong patient.

PARAFFIN WAX TECHNIQUE

DEHYDRATION

The original fixative solutions used are not miscible with paraffin wax, therefore preliminary dehydration is necessary. The three solutions commonly used for this purpose are alcohol, acetone and dioxane.

The alcohol method

This consists in passing the tissue through a series of progressively more concentrated alcohol* baths. Tissues together with their identifying labels are carefully transferred by forceps from one container to another at the appropriate times, allowing them to drain for a few seconds on blotting paper between each change. The containers should be fitted with ground-glass stoppers under which the accompanying labels should be clipped. The more delicate the tissue, the lower is the grade of alcohol suitable for commencing dehydration, and the smaller the intervals there should be between the strengths of the ascending alcohols.

The strength of the initial alcohol and the time required in each grade depend on the size and type of tissue and on the fixative which was used. Table 15.1 may be followed as an approximate guide.

To ensure that the final bath of alcohol is pure, and free from water, it is advisable to keep a layer of anhydrous copper sulphate $\frac{1}{4}$ in in depth and covered with filter paper, on the bottom of the vessel used. This salt *also* acts as an indicator, turning blue when water is present. The alcohol should be discarded if a blue tinge becomes apparent. Iso-propyl alcohol may be used for dehydration purposes.

The period necessary for dehydration may be reduced by processing at 37°C instead of room temperature. This procedure is sometimes of value when sections are required urgently from small fragmentary biopsies. These specimens should be wrapped carefully in filter paper prior to processing.

* The purchase and use of absolute ethyl alcohol is subject to many restrictions for customs and excise purposes. 74° OP spirit (Absolute Industrial Methylated Spirit), which is not subject to these restrictions, is normally used in laboratories.

Proof spirit is legally defined as 'That which, at the temperature of 51°F weighs exactly twelve-thirteenth parts of an equal volume of distilled water.' At 60°F it has a sp. gr. of 0.9198 and contains 57.1 per cent *v/v*, or 49.2 per cent *w/w*, of ethyl alcohol. Spirits are described as so many degrees over-proof (OP) or under-proof (UP). Proof spirit is the standard and is referred to as 100°. A spirit stated as 70 would therefore be 30° UP ($100 - 70$). A spirit stated simply as 160° would be 60° OP ($100 + 60$).

Ninety-five per cent alcohol is equivalent to 66° OP, which means that 100 volumes of this would contain as much ethyl alcohol as 166 volumes of proof spirit.

As proof spirit (100°) contains approximately 57 per cent of ethyl alcohol, 74° OP (174°) would contain

$$\frac{57 \times 174}{100} \text{ per cent ethyl alcohol} \approx \text{approx. 99 per cent}$$

Table 15.1 ALCOHOL METHOD OF DEHYDRATION (TIME IN HOURS)

	<i>10 per cent formol- saline</i>	<i>Zenker or Helly</i>	<i>Bouin's fluid</i>	<i>Susa, Carnoy or formol- sublimate</i>	<i>Flemming's fluid</i>
Running water	—	1-12	—	—	1-12
Alcohol, 30 per cent	—	1-6	—	—	½-3
Alcohol, 50 per cent	—	1-6	—	—	½-3
Alcohol, 70 per cent	3-12	1-6	3-12	—	½-3
Alcohol, 90 per cent	3-12	1-6	3-12	1-6	1-3
Absolute alcohol 1	3-12	1-6	3-12	1-6	1-3
Absolute alcohol 2	3-12	1-6	3-12	1-6	1-3
Absolute alcohol 3	3-12	1-6	3-12	1-6	1-3

The acetone method

This is used for the most urgent biopsies. Only small pieces of tissue should be treated, and dehydration takes from $\frac{1}{2}$ to 2 h. Considerable shrinkage is produced during the process, rendering it unsuitable for routine work.

The dioxane method

Dioxane (diethylene dioxide) is a unique reagent which has the unusual property of being miscible with both water and molten paraffin wax. It produces very little shrinkage and is simple to use. These advantages are offset by the highly toxic vapour, the high cost of the reagent, and the fact that sections are prone to fall out of the surrounding wax. Dioxane should be used only in a well-ventilated laboratory, and any residues should be washed down the sink.

PROCEDURE

The following is Graupner's and Weissberger's dioxane method.

1. Treat the tissue with pure dioxane solution for 1 h; change to fresh dioxane for a further hour, and to yet another bath of dioxane for a final 2 h.
2. Treat in paraffin bath for 15 min, in a second paraffin bath for 45 min, and in the final one for 2 h.
3. Embed in a mould, and cool in water (see p. 352).

As an alternative method, the tissue may be wrapped in a gauze bag and suspended in a jar of dioxane, containing a little anhydrous calcium oxide. Dioxane displaces water from the tissue, which is

absorbed by the calcium oxide. Dehydration takes from 3 to 24 h, depending on the size of the block of tissue.

CLEARING

'Clearing' or 'de-alcoholization' is the term applied to the removal of alcohol from blocks or sections of tissue by immersing them in an ante-medium. Most of the original ante-media raised the refractive index of de-alcoholized tissues, thereby imparting to them a degree of transparency which resulted in this stage of processing being designated the 'clearing stage' and the media used as 'clearing agents'. Not all of the present-day ante-media (e.g. chloroform) cause this transparent effect and the term 'clearing' is therefore strictly incorrect.

Clearing agents must be miscible with both alcohol and paraffin wax. The most common clearing agents are xylene, toluene, chloroform, and cedar wood oil.

Xylene

A rapid clearing agent suitable for urgent biopsies. It is cheap and highly flammable. Tissues are rendered transparent by xylene and it volatilizes readily in the paraffin oven. Biopsies, and tissue blocks not exceeding 3 mm in thickness, are cleared in 15–30 min but some material, notably brain and blood-containing tissues, tends to become brittle if immersion is prolonged.

Benzene

A popular routine clearing agent until recently when its highly carcinogenic properties were recognized. Its use for clearing purposes is therefore strongly discouraged.

Toluene

This appears to have superseded benzene as an ante-medium for routine work because of its lower toxicity. Like xylene it is highly flammable, has similar 'clearing' properties, but without the same brittle effect on tissues. It is somewhat more expensive than xylene. Clearing time is from 15 to 180 min, depending on tissue type and thickness.

Chloroform

An expensive but popular 'clearing agent', being non-flammable and causing minimal shrinkage or hardening of tissues even when the optimum clearing time is exceeded. It is relatively slow in its displacement of alcohol and tissue-blocks are not rendered trans-

parent so that the end-point is difficult to assess. Most tissues of 3 to 5 mm thickness are de-alcoholized in 6 to 24 h. It should be pointed out that chloroform vapour is both anaesthetic and toxic and in addition it may have a deleterious effect on the rubber sealing ring of the vacuum impregnating bath.

Cedar wood oil

Rarely used for routine clearing purposes because of its cost and slow action. This reagent causes little or no damage to even the most delicate tissues. It is therefore of particular value in research laboratories and in embryological procedures. Certain tissues, notably skin and dense fibrous material, benefit from treatment with cedar wood oil in that it imparts to such tissues a consistency which facilitates subsequent section cutting. Tissue-blocks become transparent after alcohol displacement but the oil is difficult to eliminate in the wax oven, several changes of wax being necessary. Alternatively, the cleared tissues may be treated with toluene for 30 min before being transferred to molten paraffin wax. Cedar wood oil for histological purposes is a thin, colourless, slightly yellow fluid distinct from the more viscous type used for oil immersion objectives and which is unsuitable for de-alcoholization.

Other agents

Carbon disulphide, carbon tetrachloride, paraffin oil, cellosolve (2-ethoxyethanol) and methyl benzoate are less commonly used as ante-media. Methyl benzoate, however, dissolves celloidin, and is used in conjunction with it for the double impregnation of tough or fragile objects (p. 356).

IMPREGNATION WITH PARAFFIN WAX

Tissues are transferred from the clearing agent to a bath of molten paraffin wax in the embedding oven. During this stage, the clearing agent is eliminated from the tissues by diffusion into the surrounding melted wax and the wax in turn diffuses into the tissues to replace it. At least one change of wax should be given in order to remove the clearing agent that has been displaced from the tissue and to ensure its replacement with pure wax. The exact number of changes of wax and the time which the tissue requires in each is dependent upon the density and size of the block of tissue and the clearing agent used. A guide to impregnation times suitable for most tissues is given in Table 15.2. The wax used should be of suitable melting point. This varies with the nature of the tissue; hard tissue requires a higher melting point wax than

soft tissue to give the necessary consistency and support during section-cutting. The waxes commonly used have melting points in the range between 50 and 60 °C, the most popular, suitable for both the English climate and most surgical and autopsy material, being m.p. 56 °C..

Table 15.2 IMPREGNATION TIMES IN PARAFFIN WAX TECHNIQUE

<i>Thickness of tissue</i>	<i>Clearing agent employed</i>	<i>Molten paraffin wax</i>	<i>No of wax changes</i>
Under 3 mm	Xylene Toluene	1½ h	One
Under 3 mm	Chloroform Cedar wood oil	2–3 h	Two
3–5 mm	Xylene Toluene	2–3 h	Two
3–5 mm	Chloroform Cedar wood oil	3–5 h	Three
5–8 mm	Xylene Toluene	3–5 h	Two
5–8 mm	Chloroform Cedar wood oil	5–8 h	Three

Complete wax impregnation is necessary for the production of good sections but if tissues are subjected to the high temperatures of the wax oven beyond this point, over-hardening may result, which is thought by some to be detrimental to sectioning. On the other hand, inadequate impregnation leads to ultimate drying and shrinking of the embedded tissue block which, being inadequately supported by wax, cracks or crumbles when section-cutting is attempted.

The wax infiltration oven is an electrically heated cabinet which may be water-jacketed or anhydric, with or without a circulating fan. A reliable thermostat should maintain the internal temperature at 2 to 3 °C above the melting point of the wax. The interior of the oven should be large enough to accommodate an enamel jug and funnel, fitted with Whatman No. 1 filter paper for the filtration of new or reclaimed wax, and a number of glass containers of suitable size for the wax infiltration of tissues. Some purpose-built ovens are fitted with a separate upper compartment enclosed with a hinged lid. This houses several plated metal containers for wax infiltration purposes, thus allowing the entire oven space to be utilized for wax filtration and storage.

The storage and dispensing of molten paraffin wax has been facilitated by the recent introduction of the wax dispenser. This is essentially an electrically heated, temperature-controlled, insulated tank of one imperial gallon (4.5 l) capacity, with an integral outlet filter, heated tap, and loose-fitting lid. Temperature is adjustable up to 70 °C and a safety cut-out device operating at 90 °C prevents accidental overheating of the wax with its attendant fire risk. Only new wax should be stored in the dispenser unless an additional filter, suitable for the reclamation of used wax, has been installed.

Tissue density

Dense tissues require longer immersion in molten paraffin wax to ensure complete impregnation, and therefore structures such as bone, fibromas and brain require approximately twice as long as soft tissues such as kidney or liver. The excessive hardness of dense tissues caused by this increased exposure to hot wax is (with the exception of brain and other CNS material) undesirable because of possible difficulties during section-cutting. Complete wax infiltration of such tissues can be obtained without undue hardening by means of the vacuum impregnation techniques (*see p. 347*).

Size of the block of tissue

The amount of clearing agent carried over into the wax depends on the surface area of the tissue-block. When treating large pieces, the effects of this contamination may be minimized by frequent changes of wax. The time required for thorough impregnation depends on the thickness of the tissue; a piece 5 mm thick, for example, takes an average time of 3 h, whereas a piece 10 mm thick may take up to 10 h.

AUTOMATIC TISSUE PROCESSORS

Automatic tissue processors are an excellent example of the practical application of automation in the medical laboratory. These machines decrease both the time and labour necessary for processing tissue, thereby allowing a more rapid diagnosis to be made while freeing the laboratory staff for work of a more technical nature. The decrease in the processing time is due to the constant agitation which the tissue undergoes, a procedure which also improves the penetration and produces more consistent results.

A variety of these machines is manufactured for use on the laboratory bench. The model described here is the Shandon-Elliott bench-type processor.

GENERAL CONSTRUCTION

The body housing is constructed of resin-bonded fibre glass; the beaker platform and cover plate are made from a laminated plastic material which is impervious to all reagents used for processing. The body is mounted on rollers which permit the platform to be turned, allowing easy access to the beakers and wax baths.

BEAKERS AND WAX BATHS

The machine is designed for twelve individual processing steps and is fitted with ten glass beakers and two thermostatically controlled wax baths. The beakers are of 1 litre capacity, have no lip and fit snugly beneath the plastic cover plate. Each of the wax baths is connected to the processor by means of a three-pin 13 amp plug, thereby allowing them to be removed to the laboratory bench for embedding the tissue. The baths are provided with sensitive thermostats of varying temperature range and incorporate a safety device cut-out switch as protection against overheating. The position of the beakers on the platform is determined by the spring clips which keep them located securely in position.

TRANSFER ARM

The transfer arm, which moves the tissue through the processing reagents, works in conjunction with a number of individual mechanical units, each designed to produce a coordinated movement by means of electrical circuits. The transfer arm can be disengaged from the turning mechanism by raising a spring-loaded plunger in the centre of the cover plate. This allows the tissue to be positioned manually at any point of the processing cycle.

AGITATION

This is provided by means of a continuous vertical movement. The motion of the agitation is transmitted by means of a mechanism which runs through the transfer arm and to which the tissue containers are connected.

TIMING UNIT

This consists essentially of an electric clock to which is connected a metal disc marked to cover a period of 24 h in divisions of 15 min. At the periphery of each graduation is a sliding tab which can be

raised or lowered by a special key. The position of each raised tab is chosen to coincide with an appropriate step in the desired processing cycle. As the disc rotates and each raised tab passes the start point, the transfer arm is lifted and the turning mechanism is set in motion, moving the tissue to the next position in the cycle (*Figure 15.1*).



Figure 15.1. An automatic tissue processor. (Reproduced by courtesy of Shandon-Elliott Ltd)

DELAY MECHANISM

This is a simple trip mechanism by means of which the processing cycle may be delayed for any period up to 24 h thus enabling the processor to be used at weekend periods, the tissue being ready for embedding on Monday morning. A delay lever is positioned on the timing disc in such a manner that, although the disc rotates, no transfer of tissue takes place until the end of the selected delay period. When the predetermined starting time is reached the delay lever engages with a striker pin switch which activates the mechanism for the normal 24 h processing cycle.

SAFETY DEVICES

When the processing cycle is completed and the tissue has reached the second wax bath, a cut-out device operates. The tissue then remains in the wax until removed by hand. A second safety device comes into operation should the first wax bath have solidified. Failure of the transfer arm to return to its normal position results in the tissue containers being carried over to the second wax bath, thereby avoiding damage to the tissues. In addition to the above automatic safety devices, an alarm bell powered by two dry cell batteries can be installed in a convenient position in the laboratory. This is intended to provide warning in the event of a power failure or a fuse being blown.

TISSUE CONTAINERS

Special containers made of either stainless steel or plastic are provided. The stainless steel containers are fitted into a cradle made from the same material. This has a detachable handle and one side hinged, allowing the baskets to be fitted easily. The baskets are designed with one, two, four or six divisions and are supplied with close-fitting lids and with a choice of mesh sizes. Special baskets for curettings and fragmentary tissue are available.

Plastic containers with up to six compartments are also available. These are of value when processing tissues fixed in corrosive sublimate. The plastic containers are loaded onto a central spindle and are designed to interlock. This dispenses with lids with the exception of the top basket.

Procedure for using the automatic tissue processor

1. Select blocks of tissue and place them in the tissue containers, together with their identifying labels.
2. Assemble the individual containers into one unit, making quite sure that all of the lids are fitting correctly.
3. Inspect the processor to ensure that the vessels are full, the correct programme is indicated on the timing disc and the cover plate is free from wax.
4. Raise the transfer arm, lift the release plunger and move the arm until it is in the correct starting position.
5. Attach the tissue containers to the agitating mechanism in the transfer arm.
6. Lower the tissue containers into the first processing reagent.
7. Rotate the timing disc until the selected starting time is opposite the start point.

8. Turn on the switch which controls the automatic mechanism.

Processing schedule for automatic tissue processor

The processing schedule used with the automatic tissue processor will vary according to the type of tissue, the nature of the work, the clearing reagent used and personal preference. Two examples are given in Table 15.3 below, both of which provide good results.

Table 15.3

<i>Schedule I</i>		<i>Schedule II</i>	
<i>Reagent</i>	<i>Processing time</i>	<i>Reagent</i>	<i>Processing time</i>
70 per cent alcohol	2 h	70 per cent alcohol	2 h
90 per cent alcohol	3 h	90 per cent alcohol	2 h
Abs. alcohol 1	3 h	96 per cent alcohol	2 h
Abs. alcohol 2	3 h	Abs. alcohol 1	2 h
Abs. alcohol 3	3 h	Abs. alcohol 2	2 h
Benzene 1	30 min	Abs. alcohol 3	2 h
Benzene 2	1 h	Chloroform 1	2 h
Wax 1	3 h	Chloroform 2	2 h
Wax 2	3 h	Chloroform 3	2 h
Wax 3 (vacuum bath)	1 h	Wax 1	2 h
		Wax 2	2 h
		Wax 3 (vacuum bath)	2 h

Duplex processing

Some automatic tissue processors are obtainable with a twin head at the end of the transfer arm. This enables two tissue carriers to be fitted so that a double load of tissues can be processed on equal immersion time schedules. Provision is also made for a third wax bath which may be substituted for the tenth beaker.

VACUUM-IMPREGNATION TECHNIQUE

The vacuum-impregnation technique depends on the production of negative pressure inside the embedding oven. This pressure reduction hastens the extrusion of air-bubbles and of the clearing agent from the tissue-block, facilitating rapid penetration by the wax.

It is useful for the following tissues: (1) urgent biopsies; (2) dense tissue; (3) lung tissue; and (4) tissue which contains a large amount of fat.

Vacuum-impregnation oven

There are several types of vacuum-impregnation bath (or vacuum-impregnation oven). A type in common use is shown in *Figure 15.2*. The vacuum compartment is a flat-bottomed brass chamber, with a heavy glass lid resting on a thick rubber washer, which creates an airtight junction. The vacuum chamber is immersed in



Figure 15.2. A type of vacuum-impregnation oven. (Reproduced by courtesy of Hearson Ltd)

a thermostatically controlled water-jacket. A valve is fitted on one side of the chamber by means of which air may be admitted when the bath is under negative pressure. On the opposite side of the chamber is a small tube by which the interior is connected to the vacuum pump.

ASSEMBLY OF APPARATUS

1. Fit a Venturi water pump to a cold water tap on the mains supply.
2. Connect the pump to a trap bottle with pressure tubing.
3. Connect the bottle to a glass 'Y' piece, with pressure tubing.
4. Attach one end of the 'Y' piece to a manometer and the other end to a vacuum bath. A glass stop-cock should be inserted in the 'pressure' tubing between the 'Y' piece and the trap bottle.

METHOD OF USING THE VACUUM-IMPREGNATION OVEN

1. Transfer the cleared tissue to a container of molten paraffin wax, and place it in the vacuum bath.
2. Place the heavy glass lid in position and press it firmly down.
3. Close the valve and exhaust the chamber with the Venturi pump, until a negative pressure of 400–500 mm is shown on the manometer.
4. Close the stop-cock between the 'Y' piece and the trap bottle, and turn off the pump.
5. When the tissue has been immersed in the wax for the requisite period, unscrew the valve gradually, allowing the pressure inside the bath to rise to that of the atmosphere.
6. Remove and change the wax, or embed the tissue, as necessary.

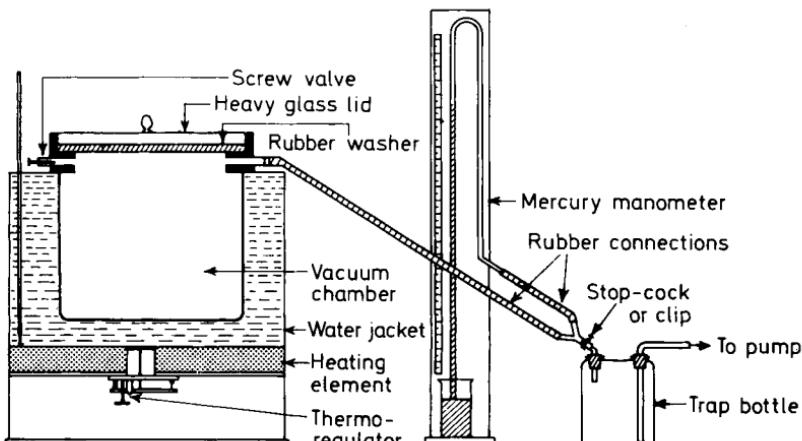


Figure 15.3. Diagram illustrating the assembly of the vacuum embedding apparatus

Note—Never turn off the water pump while the stop-cock between the 'Y' piece and the trap bottle is still open. If this precaution is not observed, water may be sucked back into the trap bottle and the vacuum chamber. An electric pump may be used in place of the water pump.

When the oven is not in use, the rubber washer should be removed. Prolonged exposure to high temperatures causes the rubber to perish.

Moulds for embedding

A variety of moulds is available for 'blocking out' or embedding the tissue in paraffin wax.

Leuckhard embedding boxes

These are convenient moulds for routine work and are widely used. They consist of two L-shaped pieces of metal, usually brass, and may be purchased in a variety of sizes. They are arranged on a glass or metal plate to form a mould of the desired size. When the embedding wax has solidified the moulds and the encased blocks are removed from the base plate and tapped on the bench. The two L pieces immediately come away from the wax block and are ready to be re-used.

Plastic ice-trays

These form convenient moulds for the busy routine laboratory, one block being embedded in each compartment. When set, the wax blocks are easily removed by flexing the plastic tray. This may be facilitated by smearing the inside of the mould with a little glycerine or liquid paraffin.

Watch glasses

These are ideal for embedding fragmentary biopsies. While it is not essential to smear them with glycerine before use, it is a sensible precaution as the blocks are sometimes difficult to remove.

Paper boats

These may be made as shown in *Figure 15.4*. They have the advantages of being cheap to make and allowing blocks to be stored without being removed, but they are not suitable for use in the routine laboratory. A useful method of marking the position of minute pieces of tissue in the paraffin block is to draw a cross with a soft lead pencil on the inner surface of the bottom of the boat. The mark is clearly visible on the wax block when it is removed from the paper boat and the position of the tissue placed within the cross is known.

Plastic embedding rings

This is a new disposable product (Tissue Tek*) available in a number of sizes and intended to speed up the paraffin wax embedding technique. The plastic rings are used in conjunction with special stainless steel base moulds. The tissue to be embedded is positioned in a base mould which has been previously sprayed with an aerosol provided for the purpose. The plastic ring is placed in position and the paraffin wax poured in until it reaches the top.

* Available in Britain from Ames Co.

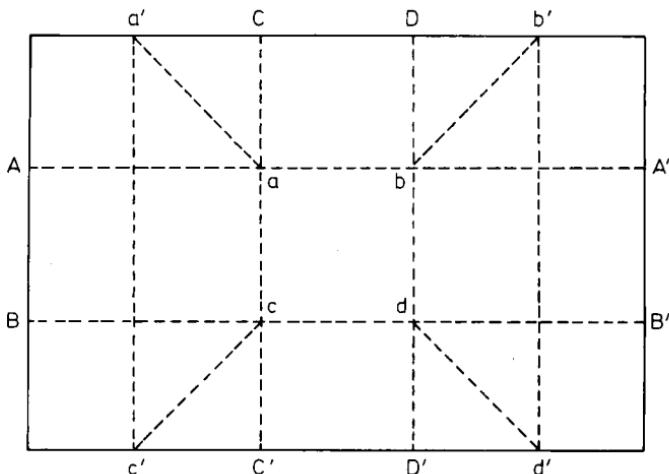


Figure 15.4. Using a piece of thick paper or thin card of suitable dimensions, measure it out in the manner shown. Make sharp folds between AA', BB', CC' and DD', turning all of the edges inwards. Fold along aa', bb', cc' and dd' and turn the resulting projections back against the narrow ends of the box formed. Turn down the two remaining flaps and pinch them securely to ensure that the box maintains its shape

After cooling, the base mould is easily detached, leaving the embedded tissue ready for cutting. No trimming is necessary and the wax-filled plastic ring serves as a block holder. Following sectioning, the blocks are stored in the plastic rings. This method undoubtedly saves a great deal of the technician's time and is of value when blocks have to be re-cut, but it has the disadvantage of requiring a much greater storage space, and the plastic rings are relatively expensive.

Glass tubing

Glass tubing which has one end sealed with a cork is useful for embedding cytological specimens which are to be sectioned. The processing and embedding can be carried out in the tubing, the specimen being centrifuged between each stage. The small particle of tissue will collect at the bottom of the tubing and make the sectioning easier. After the paraffin wax has solidified, the cork may be removed and the block pushed out of the tubing.

Technique for embedding

1. Fill the mould with molten paraffin wax.
2. Warm a pair of blunt-nosed forceps, and use them to transfer the tissue from the paraffin bath to the mould.

3. Warm the forceps again, and orientate the tissue until it is lying in the desired plane. Run the warm forceps round the tissue to ensure that any wax which may have solidified during the transferring from the paraffin bath to the mould, is melted.
4. Remove the corresponding label from the paraffin bath, and place it against the side of the mould adjacent to the tissue.
5. Blow on the surface until a thin film of wax has solidified.
6. Transfer the mould to a container of cold water, and immerse it gently. The mould should remain submerged until the wax hardens. This may take 10 to 30 min, but solidification may be hastened by transferring the mould to running water.

GELATIN EMBEDDING

As a general rule tissue from which frozen sections are to be prepared is not embedded, the freezing of the tissue providing sufficient support for sectioning. When frozen sections are required from tough or friable tissue, however, it is advantageous to embed the tissue in a supporting medium, in order to prevent the tissue from fragmenting.

The usual embedding medium for this purpose is gelatin, and when embedded the blocks of tissue are transferred to formalin in order to harden them. The formalin changes the structure of the gelatin from the hydrosol to the hydrogel condition.

Aschoff's gelatin embedding method

<i>Solution 1.</i>	Gelatin	12.5 g
	Distilled water	87.5 ml
	Phenol crystals, as preservative	1 g
<i>Solution 3.</i>	Gelatin	25 g
	Distilled water	75 ml
	Phenol crystals, as preservative	1 g
<i>Solution 3.</i>	Concentrated formaldehyde solution (40 ^o)	5 ml
	Distilled water	95 ml

MODE OF PREPARATION

Solutions 1 and 2. Heat the distilled water to 37 °C and dissolve the phenol. Add the gelatin and incubate at 37 °C until solution is effected. Filter through surgical gauze, bottle and label.

Solution 3. Add the concentrated formaldehyde solution to the distilled water. Mix well and label.

PREPARATION FOR USE

Melt the gelatin by heating in a water bath or by incubation.

PROCEDURE

1. Place thoroughly washed formalin fixed tissue not exceeding 3 mm in thickness in solution 1 and incubate at 37 °C for 12 to 24 h.
2. Transfer to solution 2 for 12 to 24 h at 37 °C.
3. Embed in solution 2 using a Leuckhart embedding box, cool and trim. Excess gelatin inhibits the freezing.
4. Place the trimmed block in solution 3 for 24 h and then cut frozen sections, according to the technique described on p. 377.

Notes—Following embedding the gelatin block may be cooled in a refrigerator but must not be allowed to freeze.

Excess gelatin may be removed by floating the sections onto paper and trimming with scissors.

Tissues permeated with gelatin take far longer to freeze than unimpregnated tissues of an equivalent size.

By using the above method sections of 5 µm upwards may be obtained.

CELOOIDIN

Celloidin is the trade name given to a purified form of nitrocellulose. It is of particular value as a histological embedding medium for sectioning hard tissues of a mixed consistency, for cutting very thick sections or when the minimum of shrinkage is required and the frozen section technique is not practicable.

Celloidin is usually supplied in the form of wool dampened with alcohol. The working strengths are 2, 4 and 8 per cent, the solvent being equal parts of ether and alcohol.

Necol (Necoloidin), a similar compound available from British Drug Houses, is used in many laboratories in place of celloidin. It is supplied as a solution of about 8 per cent, but for use should be thickened to a 16 per cent solution. Thickening is a simple matter, the solvent being allowed to evaporate in a fume-cupboard until the volume has become reduced by approximately half.

Evaporation is a constant problem when using celloidin and the working solutions should always be stored in bottles fitted with ground-glass stoppers. An ideal bottle for this purpose is a wide-mouthed oil bottle, fitted with a ground-glass stopper and a ground-glass covering cap. It must be remembered that ether

vapour is highly dangerous and celloidin should never be used in the vicinity of an open flame.

Celloidin impregnation and embedding technique

IMPREGNATION

1. Dehydrate the tissue through ascending grades of alcohol, completing the dehydration by using a bath of absolute alcohol containing copper sulphate.
2. Transfer the tissue to a mixture of equal parts of alcohol and ether for 24 h. The purpose of this step is to speed up the subsequent impregnation.
3. Transfer the tissue to a thin (2 per cent) solution of celloidin for 5 to 7 days.
4. Transfer the tissue to a medium (4 per cent) solution of celloidin for 5 to 7 days.
5. Transfer the tissue to a thick (8 per cent) solution of celloidin for 2 to 3 days.

EMBEDDING

1. Half fill a suitable embedding mould with thick (8 per cent) celloidin and place the tissue in position, with the surface to be cut uppermost. Top up the mould with more of the embedding solution. The mould should be considerably deeper than the thickness of the tissue, in order to prevent the tissue from becoming exposed, as the celloidin shrinks on hardening. Paper boats (p. 350) are ideal for this purpose.
2. Place the mould in a desiccator containing ether vapour, in order to remove all air-bubbles. Immediately that all air-bubbles are removed from the embedding medium, invert the tissue so that the surface to be cut is face downwards in the mould. This prevents any air-bubbles from being trapped beneath the tissue.
3. Transfer the mould to a second desiccator containing chloroform vapour, until the celloidin is hardened to the required consistency. This can be tested by pressing the ball of the thumb (not the nail) against the surface of the block, the celloidin being hard enough when no impression is left on the surface.
4. Remove the block from the mould and place it in pure chloroform. The block floats at first but eventually sinks to the bottom of the solution. When the block has sunk, transfer it to a solution of 70 per cent alcohol until required for cut-

ting. The block may now be trimmed with the exception of the cutting surface.

ATTACHING THE BLOCK TO THE HOLDER

Celloidin blocks are attached to wooden or vulcanite holders which have deep serrations cut into them. The block holder is coated with medium (4 per cent) celloidin and the trimmed block pressed firmly into position. Pressure is maintained by means of a lead weight or by winding a piece of thread around the holder and the block. After about 1 h, during which time the block and holder can be returned to the chloroform desiccator, the celloidin is set firm and the block and holder should be re-immersed in 70 per cent alcohol for 30 min. The cutting surface of the block may now be trimmed with a sharp hand razor.

It is a common practice to store both the blocks and holders in 70 per cent alcohol until all work on the sections is finished. Wooden blocks should therefore be made from a hard wood and should be soaked before use in order to ensure that discolouration of the alcohol and block does not occur.

In many laboratories, chloroform is not used to harden the block. Hardening is then done very slowly by placing the mould beneath a bell jar and raising one side slightly, allowing the vapour to escape and the solution to thicken. When using this method the edge of the bell jar that is raised must be changed periodically to ensure that even evaporation takes place and should be lowered overnight and at weekends.

Necol is used in a similar manner to celloidin, but the impregnating solutions are twice as thick, being 4 per cent, 8 per cent and 16 per cent. The tissue is embedded in the stock solution, thickened as described earlier.

LOW VISCOSITY NITROCELLULOSE (LVN)

Low viscosity nitrocellulose is used by some workers as an embedding medium in preference to celloidin. This preference is based upon a harder block being formed with LVN than with celloidin, thinner sections thus being made possible. The sections have a tendency to crack but plasticizers can be incorporated into the medium to overcome this problem. The addition of 0.5 per cent oleum ricini (castor oil) is recommended for embedding chrome mordanted tissues, and this method is described here.

<i>Solution 1.</i>	Low viscosity nitrocellulose	7 g
	Ethyl alcohol, absolute	42 ml
	Ether	50 ml
	Oleum ricini	0.5 ml
<i>Solution 2.</i>	Low viscosity nitrocellulose	14 g
	Ethyl alcohol, absolute	42 ml
	Ether	50 ml
	Oleum ricini	0.5 ml
<i>Solution 3.</i>	Low viscosity nitrocellulose	28 g
	Ethyl alcohol, absolute	42 ml
	Ether	50 ml
	Oleum ricini	0.5 ml

MODE OF PREPARATION

Solutions 1, 2 and 3 Dissolve the LVN in the alcohol and ether. Add the oleum ricini, mix well and label.

PROCEDURE

1. Dehydrate tissue according to the celloidin technique.
2. Place in solution 1 for 4 to 7 days.
3. Place in solution 2 for 4 to 7 days.
4. Embed in solution 3 and continue according to the celloidin technique.

Notes—(a) Sections should be cut dry and collected into 70 per cent alcohol.
 (b) LVN is highly explosive and should be handled with respect. Exposure to direct sunlight should be avoided.

Peterfi's double-impregnation method

This method is a valuable aid for preparing sections from blocks of tissue of varying consistency (e.g. eyes).

Celloidin, dry	1 g
Methyl benzoate	100 ml

Weigh out the dry celloidin and transfer it to a 250 ml flask. Add the methyl benzoate and stopper the flask firmly. Shake several times each day, occasionally inverting the flask, until solution of the celloidin is effected.

PROCEDURE

1. Dehydrate according to the normal schedule.
2. Transfer to the methyl benzoate–celloidin from absolute alcohol and impregnate for 24 to 72 h.

3. Pass through three changes of toluene over a period of 24 h.
4. Impregnate and embed in paraffin wax according to the normal schedule.

Note—Many modifications of the above method have been suggested. Some workers prefer to clear in pure methyl benzoate before impregnating with the celloidin solution; others impregnate for a further 24 h in a second solution containing 2 per cent celloidin in methyl benzoate; the period in toluene is also reduced by many workers.

16

Section Cutting

THE MICROTOME

The microscope is designed to facilitate the study of animal tissue by transmitted light and for this purpose the tissue must be sliced into thin lamellae or 'sections'. These are cut at a predetermined thickness which depends on the character of the tissue. Uniform thickness can only be assured by using a machine known as a 'microtome'.

Microtomes of various designs are made for use with different tissue-supporting media. For preparing paraffin sections, the rocker, rotary, and sliding patterns are normally used.

The Cambridge rocking microtome

This relatively cheap instrument (*Figure 16.1*), although introduced almost a century ago, remains a firm favourite with many

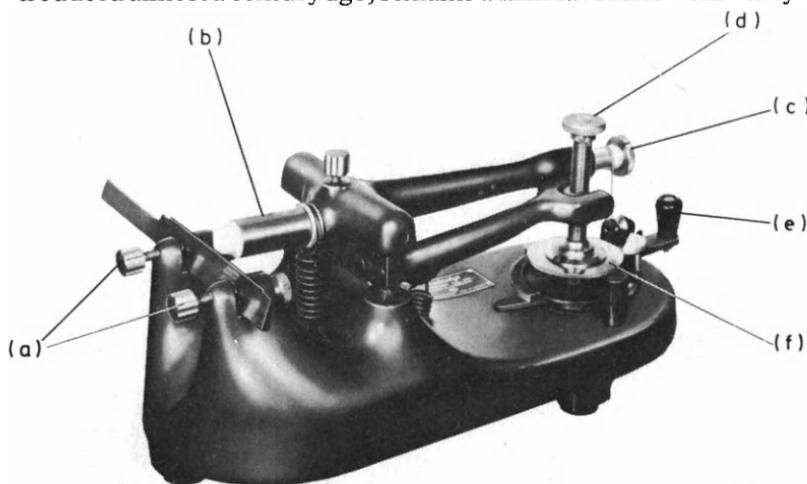


Figure 16.1. The Cambridge rocking microtome: (a) knife clamps; (b) block-holder; (c) tension adjustment; (d) micrometer screw; (e) operating handle; (f) feed mechanism.
(Reproduced by courtesy of Kent Cambridge Medical Ltd)

microtomists because of its simplicity of operation and maintenance and its ability to produce sections of high quality. It consists of a heavy base and two arms; the lower arm rests on a column and supports the upper, both being pivoted on knife edges which act as a fulcrum. The lower arm is located by a spring behind the supporting column and is also attached by means of a trapped nut directly to the micrometer screw, at the base of which is mounted the ratchet wheel of the feed mechanism. The upper arm, which carries the block-holder, is located in front of the supporting column by a second spring and is retained at the opposite end by means of an adjustable cord. The cord passes via a pulley to the operating handle. Preliminary movement of the handle lowers the rear of the upper arm and raises the block to clear the knife prior to producing movement of the feed mechanism. Continued movement of the operating handle causes a pawl to engage in the ratchet wheel of the feed mechanism, turning it according to the predetermined thickness. The turning of the ratchet wheel rotates the micrometer screw and elevates the lower arm to a more obtuse angle, producing a forward movement at the fulcrum. This movement is relayed to the upper arm, thereby moving the block-holder forward. By slowly releasing the pressure on the operating handle, the tension on the locating spring causes the upper arm to return through an arc to its former position, a section being cut as the tissue passes the knife edge. Sections prepared on the Cambridge rocking microtome are thus cut in a slightly curved plane; its feed mechanism is graduated in units of 1 or 2 μm .

The Cambridge rocking microtome was one of the first instruments to be incorporated into a cryostat (q.v.) by British manufacturers for the preparation of sections from unfixed tissue at a temperature of approximately -20°C . The simplicity of its mechanism and the small number of moving parts make it an ideal microtome for low-temperature work.

The rotary microtome

The rotary microtome (*Figure 16.2*) is an excellent machine for research work and is particularly valuable for the preparation of serial sections.

Section cutting is effected by the vertical rise and fall of the object against a fixed knife edge, together with the coordinated advancement of the object controlled by a micrometer screw and set of slides. Both the vertical and advance movements are actuated by rotation of the operating handle. The block-holder is equipped with adjusting screws to ensure that the block is parallel to the

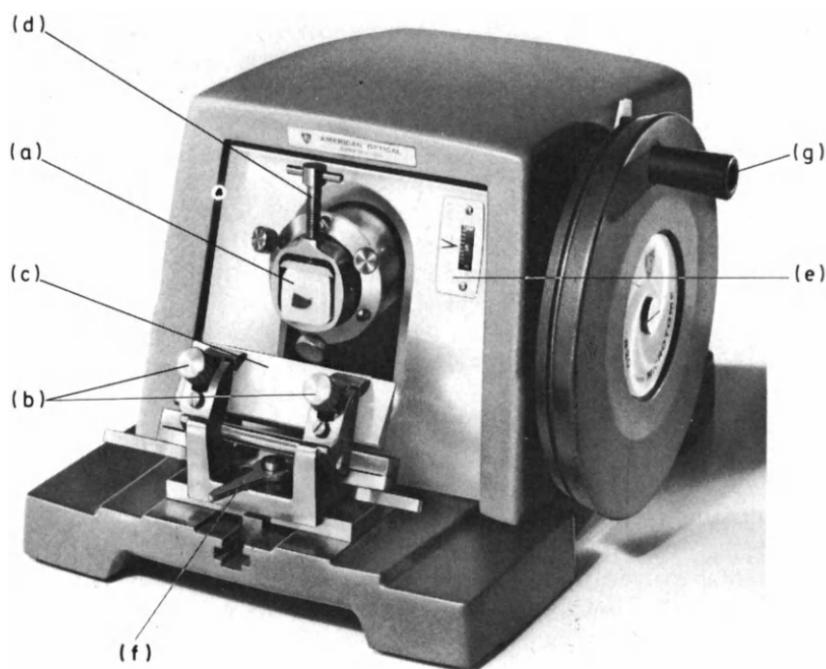


Figure 16.2. Rotary type microtome: (a) block-holder; (b) knife clamp screws; (c) knife clamps; (d) block adjustment clamp; (e) thickness gauge; (f) angle of tilt adjustment; (g) operating handle. (Reproduced by courtesy of American Optical Corporation)

microtome knife in all planes. The knife holder is movable and the knife clamps may be adjusted to vary the angle of tilt.

The base sledge microtome

The base sledge microtome is a rigidly constructed machine readily adaptable for sectioning specimens embedded in all forms of media. It is excellent for cutting sections from blocks of tough tissue, especially if the blocks are large and offer marked resistance to the knife. The sections may be cut with the knife at an angle to the face of the block or parallel to it. Larger sections can more easily be cut with the knife set at an angle, less resistance being offered by the block.

The microtome consists essentially of a heavy base and two

movable pillars which hold the adjustable knife clamps. Two accurately machined metal guides traverse the length of the base and carry the movable carriage. The hand-propelled movement of this carriage is checked by a buffer stop.

Movement of the operating handle on the carriage causes a pawl to become engaged in the upper of three ratchet gear wheels, turning it and actuating the two companion wheels and the micrometer screw. The movement of the micrometer screw raises the block-holder which is connected to it by means of a split nut clasp, the use of which adjusts the height of the block in relation to the knife.

The thrust exerted by the feed mechanism is determined by the setting on the thickness gauge, which is graduated in divisions of $1\text{ }\mu\text{m}$ up to $20\text{ }\mu\text{m}$. This gauge limits the movement of the operating handle which must be fully turned while the carriage

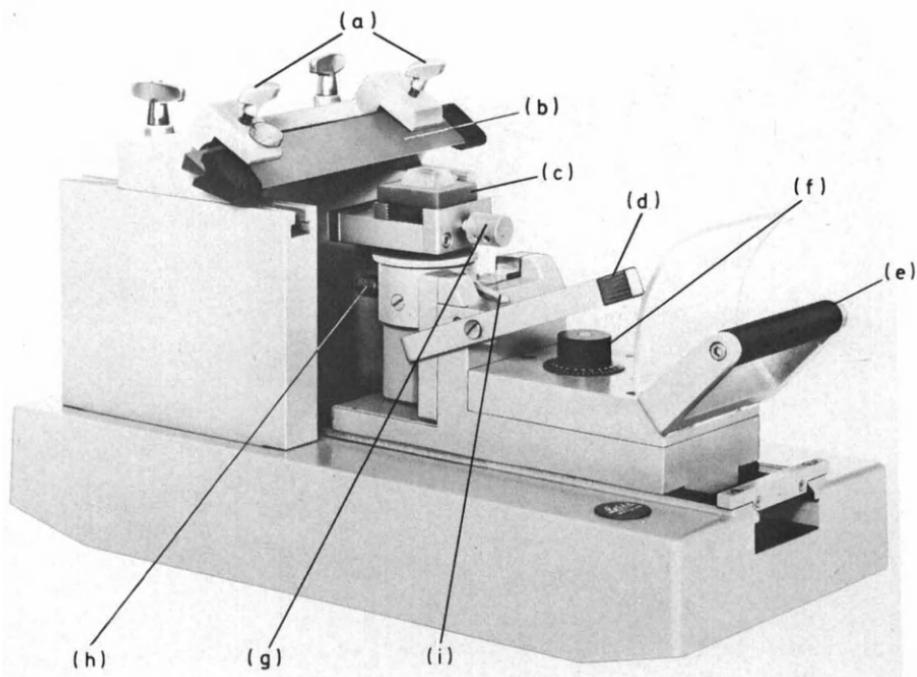


Figure 16.3. Sledge type microtome: (a) angle of tilt adjustment; (b) knife clamps; (c) block-holder; (d) coarse feed adjustment; (e) operating handle; (f) thickness gauge; (g) adjustment locking nut; (h) block adjustment screw; (i) split nut clasp.
(Reproduced by courtesy of Leitz)

is in the position shown to ensure that the sections are cut at the correct thickness. For obvious reasons the operating handle must not be turned while the block is on the opposite side of the knife to that illustrated in *Figure 16.3*.

This microtome may be adapted for frozen section cutting by replacement of the paraffin wax object holder with either a CO₂ freezing stage or a thermomodule (q.v.).

Sliding microtome

The fundamental difference between the sliding microtome and those models described earlier is that with this instrument the block remains stationary while the microtome knife moves during the process of sectioning. The main value of the sliding microtome is the ease with which it cuts sections from tissue embedded in celloidin. A number of instruments of varying design are produced commercially, one of the most popular being that illustrated in *Figure 16.4*.

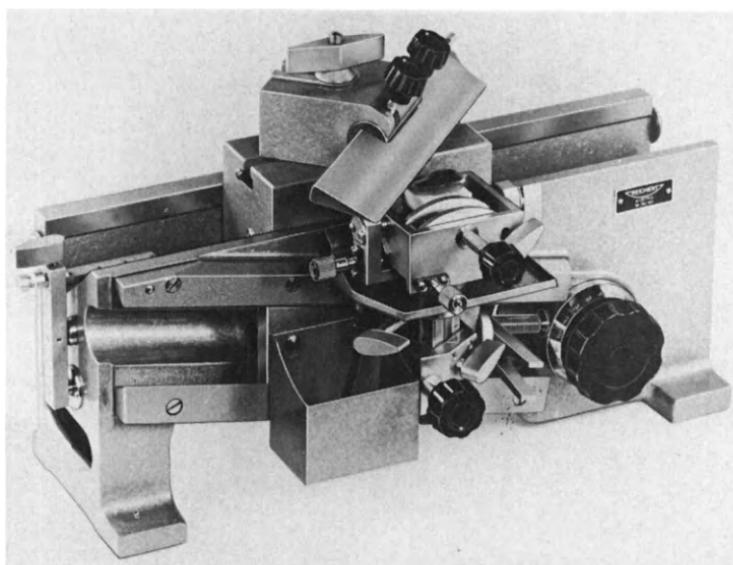


Figure 16.4. The Reichert Universal sliding microtome. This instrument is equipped with an automatic feed mechanism, which can be adjusted in steps of 1 µm, to allow sections to be cut up to 30 µm in thickness. Facilities for adjusting the slant of the knife and the clearance angle are provided and the object clamp can be orientated in two directions to adjust the block to the knife edge. (Reproduced by courtesy of Reichert Ltd)

The freezing microtome

The freezing microtome is used for cutting sections when (a) speed is of the utmost importance, (b) when it is required to demonstrate fat histologically, and (c) when certain neurological structures are to be studied.

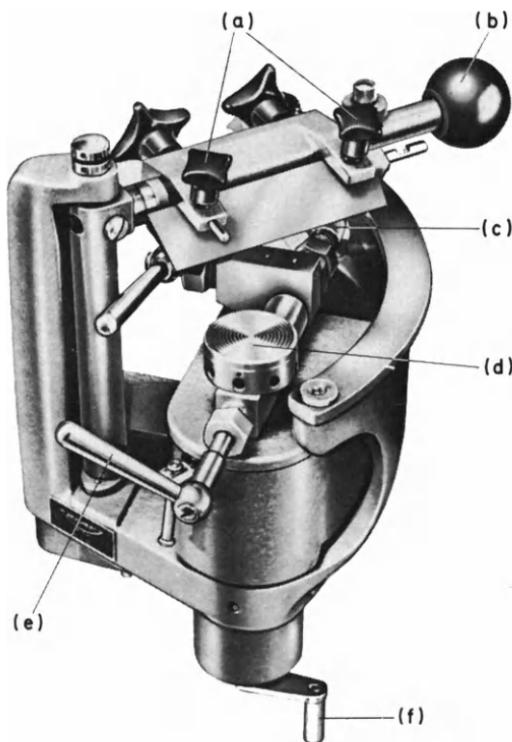


Figure 16.5. A type of freezing microtome: (a) knife clamps; (b) operating handle; (c) thickness gauge; (d) stage; (e) stage valve; (f) coarse adjustment. (Reproduced by courtesy of Reichert Ltd)

Several types of freezing microtomes are available but those most widely used take the form of the one illustrated in Figure 16.5. The freezing microtome differs markedly from those machines used for the preparation of paraffin wax sections. The stage of the freezing microtome, to which the CO₂ cylinder is connected by means of a reinforced flexible lead, is hollow and per-

forated around the perimeter. These perforations are an essential part of the cooling, allowing the gas to flow and freely escape, thereby producing even freezing of the tissue. A second cooling device for lowering the knife temperature to facilitate sectioning is also incorporated in most modern machines.

As the operating handle is moved back the knife edge clears the tissue. Continuation of the movement causes a pawl to engage with a ratchet wheel and turn it according to the predetermined thickness. Rotation of this wheel turns the micrometer screw which raises the block-holder. By pulling the operating handle forwards a section is cut as the knife edge slices through the raised tissue. The thickness at which the sections are cut is variable in units of 5 µm. The number of units by which the feed mechanism is turned is determined by the position of the knife stop on the graduated runner. To ensure that the section thickness is correct the operating handle must be pushed back along the runner until checked by the knife stop.

Thermoelectric cooling units may be used in place of CO₂ gas to freeze the tissue and cool the knife. These units, referred to as thermomodules, have a considerable refrigeration capacity and function by a phenomenon known as the 'Peltier' effect. When a direct current is passed across the junction of two dissimilar metals, heat is emitted or absorbed, according to the direction of the current. A flow of cold water maintained through the cooling unit ensures that the heat from the hot face is absorbed. The cooling produced by the thermoelectric unit is dependent upon the flow of the direct current and this may be regulated by means of power packs. The stage temperature can be reduced from ambient to - 36 °C in 60 s, but the optimum cutting temperature for the tissue is usually about - 20 °C.

These units are produced commercially and are designed to fit a large number of microtomes.

CRYOSTAT

The best method of preparing sections from unfixed tissue is by use of a cryostat. This consists essentially of a microtome housed in a deep freeze cabinet, maintained at a temperature of approximately - 15 °C to - 30 °C. Sections from fresh tissue can be cut on standard freezing microtomes, but they cannot be handled satisfactorily. This applies particularly when fluorescent antibody staining techniques or certain histochemical enzyme methods are

to be applied. To obtain satisfactory sections which can be transferred directly from the microtome knife to a slide or coverglass, the tissue, microtome knife and surrounding atmosphere must all be at a low temperature. These conditions are achieved by the use of a cryostat (*Figure 16.6*).

There is a variety of cryostats manufactured, the major fundamental difference between them being the type of microtome employed. The earliest models manufactured in Great Britain in-

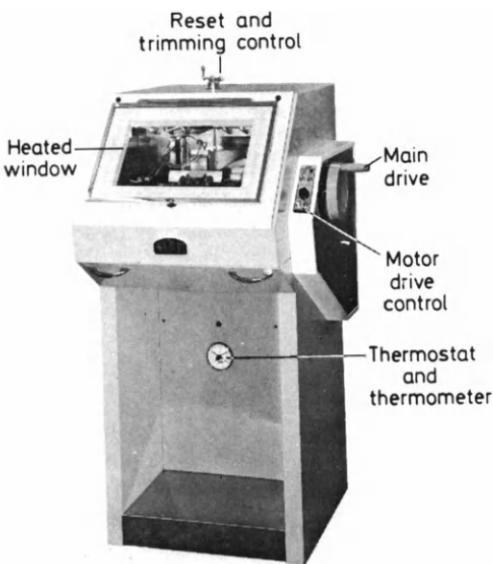


Figure 16.6. Photograph of the SLEE cryostat, showing the location of the controls and basic parts of the instrument. (Reproduced by courtesy of South London Electrical Equipment Ltd)

corporated the Cambridge rocking microtome. This instrument, which remains popular with many workers, has the advantages of being inexpensive and possessing few moving parts, an important factor when used continuously at low temperatures. Cryostats equipped with purpose-built rotary microtomes are now widely used, and models fitted with sledge microtomes, suitable for cutting larger and tougher tissue blocks, are also available.

In order to avoid the formation of large disruptive ice crystals when freezing fresh tissue, rapid freezing (quenching) is neces-

sary. Cryostats are usually provided with a rapid freezing attachment for this purpose and for attaching blocks of tissue to the block-holder. This latter refinement is extremely valuable when the instrument is to be used for preparing urgent sections from biopsies. Blocks of fresh tissue not to be sectioned immediately should be quenched and stored at a temperature of -20°C in air-tight containers or aluminium foil.

MICROTOME KNIVES

These are classified according to their cross-section (profile) as follows:

- (a) Planoconcave. Hollow ground on one side.
- (b) Wedge-shaped. Plane on both sides.
- (c) Biconcave. Hollow ground on both sides.
- (d) Tool-edge. Plane on both sides with a steep cutting edge.

Each of the profiles was originally introduced on knives designed for a specific purpose. In practice, however, there is considerable latitude in the utilization of each type and, provided that a knife is sharp and will fit the microtome, it may well be used effectively for cutting most types of tissue and embedding materials. Planoconcave knives are obtainable with profiles of greater or lesser degrees of concavity and are usually recommended for celloidin or wax-embedded tissues. The sturdy wedge-shaped knife is used for cutting frozen and paraffin sections and hard objects embedded in celloidin. Biconcave knives are used mainly for wax-embedded tissues. The popular Heiffor knife has a biconcave profile. This knife with its distinctive integral handle was designed for use with the Cambridge rocking microtome. The tool-edge knife is used in conjunction with a heavy, robust microtome for cutting extra hard materials such as undecalcified bone. With the exception of the Heiffor knife most knives have detachable handles fitted at one end.

THE CUTTING FACET (bevel)

In cross-section all microtome knives are basically wedge-shaped but the cutting edge is not the extension of the two converging sides of a wedge to form a point. Such an edge emanating from a relatively narrow base would be fragile, and subject to considerable vibration during section cutting. A more obtuse angle is therefore ground onto the tapering sides of the knife to form the actual

cutting edge. This angle is referred to as the facet angle and the sides that enclose it as the cutting facets or bevel (*Figure 16.7*). In order to manually sharpen microtome knives with one or more plane surfaces, it is necessary for them to be fitted with a special

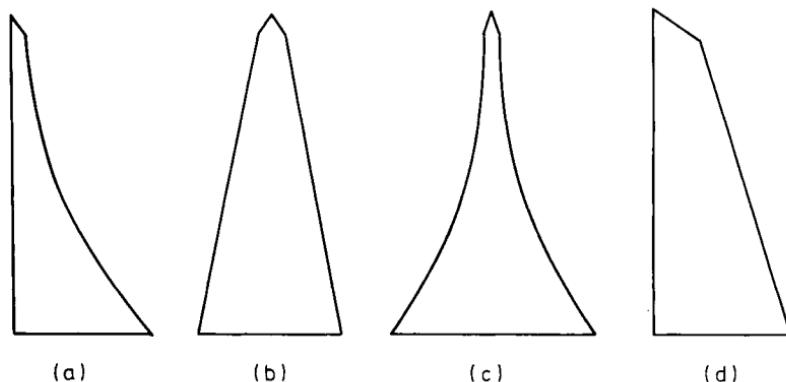


Figure 16.7. Microtome knives: (a) plano-concave; (b) semi-plano-concave; (c) plane-wedge; (d) bi-concave

device to produce and maintain the cutting facets. This is a spring-loaded semicircular metal sheath which is slipped onto the back of the knife and is known as a tubular knife back, or stropping device. Each knife should have its own back which should be marked to ensure that it is always fitted to the knife in the same way.

SHARPENING OF MICROTOME KNIVES

A microtome knife requires to be sharpened whenever its cutting edge becomes blunt or damaged. The process of sharpening is divided into two stages, honing and stropping, and each of these operations may be performed either by hand or by means of automatic knife-sharpening machines. Honing entails the grinding of metal from the knife edge with an abrasive substance until all nicks have been removed and the edge is sharp and straight; stropping is cleaning or polishing the knife edge on a softer material, usually leather.

Manual honing

The hone is a rectangular block of natural or synthetic stone, graded coarse, medium or fine according to the degree of its abrasiveness. A widely used natural stone of medium grade is the Belgian yellow stone, which gives good results at reasonable speed.

The size of the hone used is dependent upon that of the knife; the length should always be sufficient to permit the whole of the knife edge to be sharpened in a single stroke, while the width should provide sufficient support to prevent any rocking of the knife. Before honing, the knife should be cleaned with xylene, and where necessary, fitted with its handle and tubular back.

The hone is positioned on a bench of suitable height in front of the operator, who should be comfortably seated. A damp cloth placed beneath the stone will prevent its movement during use. The surface of the stone should be cleaned to remove any grit or dust and then lubricated with either a fine oil or soapy water.

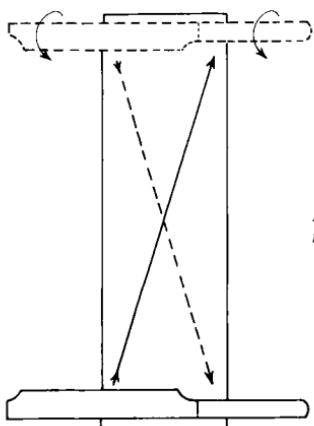


Figure 16.8. Diagram to illustrate the honing of a microtome knife

The knife is placed at one end of the hone (Figure 16.8) and is pushed diagonally forward with cutting edge leading, so that the whole edge is equally ground. Just before it reaches the end of the stone the knife is turned over *on its back*, and with the cutting edge leading again, it is steadily pulled back along the hone towards the operator. Pressure on the knife needs to be just sufficient to maintain its edge in contact with the surface of the hone. The number of strokes required depends on the condition of the knife, but honing is complete when all large nicks have been removed and the edge is straight and sharp. When viewed under a low-power microscope the edge will be seen to be finely but regularly serrated. These serrations are due to the abrasiveness of the stone and are removed by the polishing action of subsequent stropping.

Other stones in common use include:

Arkansas A natural stone of clear white to pale yellow colour.

It is less abrasive than the Belgian stone and is consequently slower in action.

Aloxite A series of composite stones ranging in abrasiveness from coarse to superfine. Only the fine and superfine grades are suitable for microtome knife sharpening.

Plate glass may be used as a hone (lapping plate) in conjunction with an abrasive such as aluminium oxide. This abrasive, available in a range of particle sizes, is suspended in oil or water and applied to the surface of the glass plate, which is then used in the same way as an ordinary hone. The advantage of this method is that by varying the grade of abrasive, all types of honing can be carried out. When a satisfactory edge has been obtained by either the stone or plate glass methods, the knife should be thoroughly cleaned and dried before stropping is attempted.

Stropping

This is performed in a manner similar to honing except that the knife is reversed and lightly stroked back and forth over a leather surface with its cutting edge *trailing* (Figure 16.9). Strops may be either flexible (hanging) or rigid. A good quality leather such as

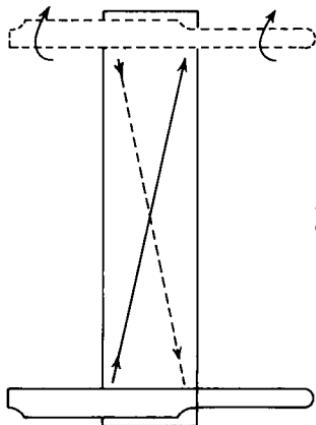


Figure 16.9. Diagram to illustrate the stropping of a microtome knife

horsehide, noted for its durability and effectiveness, is recommended. It should be kept supple by the occasional application of a little vegetable oil to its undersurface. Strop-dressings containing mild abrasives or polishing agents such as jeweller's rouge may be used. These are sparingly applied to the leather surface.

It is essential that hanging type strops are pulled as taut as possible during use to prevent rounding the cutting edge of the knife,

which will occur if sufficient tension is not maintained. The prepared canvas back fitted to most of these strops serves not only for support but also as a preliminary stropping surface before using the leather. Some hanging strops are available with two leather sides, one side impregnated with a fine abrasive paste, the other with a polishing agent.

The rigid type is essentially a leather strop stretched over a solid wooden block. This type is preferred by many workers because it gives firm support to the knife during stropping, thereby reducing the possibility of producing a rounded cutting edge.

Some authorities regard the practice of knife-stropping to be completely unnecessary, especially if a knife has been honed correctly, using a superfine abrasive or stone. It cannot be denied, however, that very sharp edges are obtained, and maintained for a long time, on microtome knives which are stropped not only after honing, but also after each section cutting session.

Knife sharpening machines

These machines, which have been used for many years in the USA are rapidly becoming more popular in Britain. Their high cost is offset by the tremendous saving in time and the ease with which relatively inexperienced personnel can produce well-sharpened knives with a uniform bevel.

Some automatic machines are available which require hand feeding of the knife against revolving glass or metal wheels. Sharpening is undertaken with the aid of lapping compounds composed of suspensions of alumina or diamond grit which are continuously recirculated by means of a built-in pump. The grade of lapping compound selected is dependent upon the condition of the knife edge.

The great drawback to using machines of this nature is that the knife is fed across the revolving wheel by hand. Uneven pressure and variation in the speed with which the knife is sharpened can result in an irregular knife edge. To hesitate too long with the knife in contact with the revolving wheels can cause considerable damage. A semi-automatic machine with facilities for both honing and stropping, and which is completely dry in operation, is currently marketed.

Fully automatic knife sharpeners at prices which are economically practicable are now manufactured. One model which is widely used is shown in *Figure 16.10*. The knife is held in a holder attached to the main spindle, in such a way that the cutting edge is in contact with a glass or metal plate. A mechanism is provided



Figure 16.10. A fully automatic knife-sharpening machine. (Reproduced by courtesy of Shandon-Elliott Ltd)

for adjusting the height of the glass plate to agree with the bevel of the knife. To ensure that the abrasive is spread evenly over the surface of the plate and that no uneven wear occurs, a combined oscillatory and rotary motion is incorporated. A mechanism incorporating a damping device automatically turns the knife over at suitable intervals to ensure that each facet is sharpened equally and a choice of speeds is provided, a slower speed being used for knives in poor condition.

TECHNIQUE OF SECTION CUTTING

Preparation of paraffin sections

TRIMMING THE BLOCK

If several pieces of tissue are embedded in the same mould, it must be divided into individual blocks. This may be done by cutting a V-shaped groove in the intervening wax, and breaking it along this line. If the pieces of tissue are embedded too closely to permit this, the mould should be divided with a fine fret saw.

The individual paraffin blocks should then be trimmed with a

hand razor to within $\frac{1}{8}$ -in of the tissue, taking care that the sides of the block are parallel. Excess wax on the face of the block should also be pared off. The shavings produced may be returned to the wax oven and used again. The trimmed blocks should be stored in cardboard boxes, each with its accompanying ticket.

ATTACHING THE BLOCK TO THE HOLDER

Heat a wooden-handled spatula over a bunsen burner, and hold it on the surface of the block-holder. Place the paraffin block on the spatula. The hot spatula melts the wax on the block-holder and the base of the block. After a few seconds the spatula is withdrawn and the paraffin block pressed down firmly on the holder. The junction between the two is now sealed by re-heating the spatula and running it round the base of the paraffin block.

ORIENTATION OF THE BLOCK ON THE MICROTOME

1. Fix the block-holder in position on the microtome.
2. Turn back the feed mechanism. On the rotary and base sledge microtomes a special 'split nut clasp' is provided for this purpose.
3. Insert a suitable knife in the microtome, and secure it with the tightening screws.
4. Move the block-holder forward until the paraffin block is almost touching the knife edge.
5. If the microtome has no centring screws, such as in the case of the rocker type microtome, the block must be accurately trimmed before it is attached to the block-holder. When adjusting screws are present they are used to orientate the block-holder so that the surface to be cut and the lower edge of the block are parallel to the knife edge at the moment of impact. In the case of the base sledge microtome it is often advantageous for the leading edge of the block to be set at an angle to the knife.
6. Check all tightening screws on the microtome.
7. The gauge controlling section-thickness is set to 15 μm , and the extreme end of the knife is used to trim the block until the whole surface is being cut. The block is now ready for sectioning.

CUTTING THE SECTIONS

Set the gauge to the required thickness and position the knife so that the centre of the blade is positioned for cutting. Screw back the feed mechanism slightly.

Note—This precaution should be taken whenever the knife is moved, for the slightest discrepancy in the knife may cause the cutting edge to dig into the paraffin block when the next section is cut.

Operate the microtome until complete sections are again being cut and then maintain a regular cutting rhythm. The cutting rate varies with the nature of the tissue, the size of the block, and the pattern of the microtome. The optimum cutting speed is determined empirically for each individual block. If the block face and upper and lower edges are parallel to the knife, the sections will form a ribbon. This ribboning is due to the slight heat generated between the block and the knife edge. Continue cutting until the ribbon produced is about 6 in long, supporting its free end all the while with a moistened camel-hair brush. Moisten a second camel-hair brush and gently raise the last section cut, thereby freeing the ribbon which is placed, matt surface uppermost, onto a section board or a sheet of black paper. When serial sections are being prepared, the section board is essential, for the glass cover protects the sections from draught and dust.

If the knife of the base sledge microtome is set at an angle, the sections should be removed individually, for ribboning does not usually occur.

If instructions for trimming the block and orientating it on the microtome have been correctly observed, poor results are usually due to one of the following causes.

INADEQUATE IMPREGNATION

If dehydration, clearing or wax-impregnation are inadequate, crumbling of sections may occur. This fault is easily detected, as the block usually smells of the clearing agent. The block should be trimmed down as near to the tissue as possible, returned to the embedding oven to melt the remaining wax, and then transferred to the clearing agent. If dehydration is suspected as being at fault, the tissue should be taken back to absolute alcohol (74 OP spirit).

IMPERFECT KNIFE EDGE

More failures can be attributed to badly prepared knives than to any other single factor. Nicks in the knife edge frequently result in scoring of the sections with vertical lines. To overcome this, re-sharpen the knife or move it along in the holder. If the knife is blunt, the sections may cut alternately thick and thin. This may be remedied by re-sharpening the knife.

INCORRECT SETTING OF THE KNIFE

The knife is set at a tilt on the microtome to allow a clearance angle between the cutting facet and the block of tissue (*Figure 16.11*). Clearance angles between 1 and 6 degrees have proved to

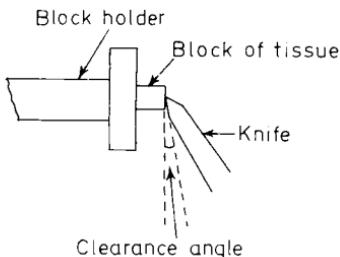


Figure 16.11. This diagram illustrates the angle of clearance of a microtome knife

be most satisfactory. Biconcave knives require a smaller clearance angle than wedge-type knives. The tilt of the knife may be adjusted by special attachments on the knife-holder of the microtome.

Faults due to an incorrect tilt are as follows.

Chattering

This term is used to describe horizontal lines or furrows across the section. The knife makes a hard metallic scraping sound as the sections are cut. This may be remedied by reducing the tilt of the knife.

Intermittent cutting

If the angle of tilt of the knife is too small, the block is compressed by the cutting facet and a section is not cut. The degree of compression increases until the tissue expands suddenly, and results in the cutting of a thick section. This may be remedied by increasing the angle of tilt of the knife.

BRITTLE AND TOUGH TISSUE

When possible the block should be cut on the base sledge microtome with the knife positioned to form an angle to the leading edge of the block, to decrease the resistance. The angle so formed is known as the angle of slant. The block should be carefully orientated so that the sections are cut along the line of least resistance.

DIRT

When dirt is present in the paraffin wax, re-embedding of the tissue in freshly filtered wax is necessary.

MINUTE PARTICLES OF CALCIUM IN THE TISSUE

Once the tissue has been embedded in paraffin wax, decalcification is impracticable but the base sledge microtome, using a wedge knife set at an angle, will often allow the cutting of good sections.

Table 16.1 SOME FAULTS ENCOUNTERED IN CUTTING PARAFFIN SECTIONS

Fault	Probable cause	Remedy
Sections scored or split vertically	(a) Knife edge is damaged (b) Embedding medium contains dirt (c) Knife edge is dirty	(a) Sharpen the knife (b) Re-embed the tissue in filtered wax (c) Clean the knife edge with xylene
Sections and block have parallel lines across them (Chatters)	(a) Tissue is too hard (b) Tilt of knife is too great	(a) Treat the tissue with Mollifex or take a fresh block and treat with phenol during processing (b) Decrease the tilt of the knife
Sections cut alternately thick and thin	(a) Knife is blunt (b) Tilt of knife is too great (c) Knife or block-holder is loose	(a) Sharpen the knife (b) Decrease the tilt of the knife (c) Tighten all locking and adjusting screws
Sections roll up on cutting	(a) Knife is blunt (b) Tilt of knife is too great	(a) Sharpen the knife (b) Decrease the tilt of the knife
Sections are squashed, the width of each section being less than that of the block	(a) The bevel on the knife has been lost due to incorrect sharpening	(a) Re-sharpen the knife using the correct knife back or setting on the automatic knife sharpener, until the bevel is restored
Sections crumble on cutting	(a) Ante-medium or alcohol is not properly removed (b) Wax is too soft (c) Knife is blunt	(a) Return to ante-medium or alcohol as described on p. 373 (b) Apply ice to the cutting surface or re-embed in harder wax (c) Sharpen the knife
Sections form a curved ribbon	(a) Knife is sharp and blunt in patches (b) Horizontal edges of block are not parallel (c) Horizontal edges of block are not parallel to the knife edge	(a) Sharpen the knife (b) Retrim block (c) Adjust block until it is parallel to knife edge
Sections fail to ribbon	(a) Horizontal edges of blocks are not parallel to each other (b) Wax is too hard	(a) Retrim block (b) Coat horizontal edges of block with wax of lower melting point

Attaching sections to slides

As sections tend to crease slightly on cutting they must be flattened by gentle heat before being attached to slides. Two methods in common use are described below. The glass slides should be thoroughly cleaned and be inscribed by diamond with appropriate name or identification number before use.

THE WATER BATH METHOD

A cut section or short ribbon of sections is gently lowered, by means of a camel-hair brush or fine forceps, onto the surface of warm water in a water bath. The temperature of the water should be approximately 10 °C below the melting point of the wax. When the section is flat and fully expanded, a prepared clean, grease-free slide is dipped obliquely into the water as close to the section as possible. Slowly withdraw the slide, allowing its surface to touch the edge of the section. Completely remove slide with attached section from the water. Adjust the section to a suitable position on the slide with a mounted needle. Drain off the excess water and transfer the slide to an incubator or hot plate (45 °C to 50 °C) for at least 1 h to ensure that the section is thoroughly dried before being stained.

THE HOT PLATE METHOD

A clean grease-free slide is placed on a warm hot plate and flooded with distilled water. A section or short ribbon is laid on the surface of the water and any major creases removed by stretching the surrounding wax carefully with mounted needles. As the water warms up the section will flatten out, and when it is fully extended remove the slide from the hot plate and drain off any excess fluid. Orientate and dry the section as in the previous method.

USE OF SECTION ADHESIVES

The attachment of sections to slides by either of the foregoing methods usually results in a firm bond so that the sections are capable of withstanding the several washings and manipulations of most of the common staining techniques. The two most important conditions governing their attachment are that the sections must be of good quality and the slides must be completely grease-free. Under certain circumstances however, and in spite of the correct attachment technique, sections will become partly or completely detached from slides during staining. Some of the causes

of section detachment are: prolonged immersion of sections in alkaline solutions, the fixation of tissues in powerful protein-coagulant fluids, e.g. Bouin's fixative; tissues containing blood clot or bone. The use of a section adhesive is advised in such cases and may be used routinely for all sections to ensure their attachment to slides. A well-established adhesive is Mayer's glycerol-albumen mixture:

Combine equal parts of glycerol and white of egg. Mix well, filter through muslin and add a small crystal of thymol as a preservative.

This mixture is lightly smeared over the surface of a clean slide before attachment of the section by the water bath or hot plate methods. Fresh serum may be used as an alternative to glycerol-albumen.

Starch paste is an adhesive of exceptional quality but because of its carbohydrate nature cannot be used for sections to be stained by the PAS technique. It is prepared as follows:

Powdered starch	3 g
Cold distilled water	30 ml

Mix to a paste and add to

Boiling distilled water	60 ml
Concentrated hydrochloric acid	0.5 ml

Boil for 5 min. Cool and add 0.1 g thymol. Use in the same way as Mayer's albumen mixture.

The addition of an adhesive to the warm water used for flattening sections is both simple and effective. The following combination is recommended:

Potassium dichromate	1 per cent in distilled water
Gelatin	1 per cent in distilled water

These are kept as separate stock solutions and are added to the water bath to give a concentration of 0.002 per cent of each.

Preparation of frozen sections

METHOD OF CUTTING FROZEN SECTIONS

1. Clamp the microtome to the bench and connect the CO₂ cylinder by means of the flexible lead provided. If an ordinary CO₂ cylinder is used it must be positioned in such a

- way that the cylinder valve is the lowest point of the cylinder. Special cylinders containing a central tube may be supported in a floor stand with the valve uppermost.
2. Close the release valve on the microtome and open the valve on the cylinder. Watch carefully to see that the connection between the lead and the cylinder does not leak and then open the release valve on the microtome. Allow a short burst of CO_2 gas to escape, 1 to 2 s, to ensure that the connection to the microtome does not leak and that there is gas in the cylinder.
 3. Slip the blade of the microtome knife into the knife clamps and secure it by tightening the locking screws.
 4. Place a piece of filter paper soaked in gum syrup on the stage of the microtome, lift the stage release valve and allow a short burst of CO_2 gas to escape, freezing the filter paper to the stage.
 5. With the microtome knife well clear, position the selected block of tissue on the stage and apply a few drops of gum syrup from a pasteur pipette. The tissue should be approximately 3–5 mm in thickness. Open the stage release valve and give several short bursts of gas, each of 1–2 s duration at intervals of 5 s. When the gum is frozen apply more and again freeze with short bursts of CO_2 gas. Continue to build up the gum in this way until the tissue is supported to a height of about 3 mm.
 6. Rack up the stage by means of the coarse feed adjustment at the bottom of the microtome until the surface of the tissue is just about level with the edge of the knife.
 7. Rub the top surface of the tissue with the ball of the finger until the tissue and supporting gum has a rubbery consistency. Take great care to see that the microtome knife is well clear while doing this. Trim down the block either by setting the feed mechanism to 20 μm or if more experienced by turning the coarse feed adjustment. Trim the block until complete sections are being cut.
 8. Set the thickness gauge to the required thickness (usually 10 μm) and quickly cut the sections, maintaining a steady rhythm on the microtome. The sections usually collapse on the microtome knife and require removing with a camel-hair brush, from which they are transferred to a dish of water. Some workers use a jerking movement to cut the sections, projecting them forward and catching them in a dish of water, but the technique demands that the cutting tempera-

ture of the tissue is correct, an assessment which requires considerable practice. Intermittent cooling of the knife while cutting the sections often helps to produce better results.

If the tissue is frozen too much, the sections will splinter and crumble and the knife edge may be damaged. Soften the surface of the block with the ball of the finger or thumb. If the tissue is not frozen enough, any attempt at section cutting will produce only a streak of useless slush on the edge of the knife. To remedy this give more bursts of CO₂ gas.

A thermomodule (p. 364) operated in conjunction with a suitable power control unit, may be used to freeze the tissue instead of the conventional freezing stage and CO₂ gas. The apparatus is quiet in operation and allows more accurate control of section cutting temperatures.

The thermomodule, suitably connected to a supply of cold running water and power unit, replaces the freezing stage of the microtome. A block of tissue is placed on the platform of the thermomodule together with a drop of water or gum syrup. The current is switched to maximum and the tissue commences to freeze. When the block is completely frozen the current supply is reduced until the required degree of freezing is obtained. This temperature can be maintained for several hours. The knife is inserted in the microtome, the tissue is trimmed, and section cutting and collection is performed as in the CO₂ method.

HANDLING FROZEN SECTIONS

Frozen sections may be handled loose by the careful use of a brush, mounted needle, seeker or glass hockey stick. They can also be attached to a slide and stained in a similar manner to paraffin wax sections. The latter is usually the method of choice when preparing sections in the theatre from biopsies on which an urgent diagnosis is required. Sections which are to be stained for fat or structural elements of the central nervous system are usually 'floated through' the various stains and reagents and mounted when finished. Slides prepared for the demonstration of fat must be mounted in an aqueous mounting medium.

FLOATING OUT FROZEN SECTIONS

Oriентate the section in a deep, glass dish of distilled water. The dish should preferably be placed on a sheet of black paper in order to give a dark background. Insert a slide beneath the section and

slowly withdraw the slide and attached section from the water. Drain the excess water from the slide and remove any creases which may be present in the section by returning the slide to the water in such a way that only half of the section is submerged or floating. The creases will be removed easily by a little careful manipulation. Remove the slide from the water, drain and if necessary insert the opposite half of the section into the water. Drain the slide carefully again and continue according to the method being used for attaching the section permanently to the slide.

ATTACHING FROZEN SECTIONS TO SLIDES

Frozen sections may be attached to slides by celloidinization, albumen or starch adhesive or by being floated onto gelatinized slides.

Celloidinization

Float the section onto a clean, grease-free slide, drain off excess water and blot gently but firmly with a Whatman No. 1 filter paper. The blotting should be done with a rolling action using the fleshy part of the hand. Blot the section a second time with fresh filter paper which has been soaked with absolute alcohol and then coat the slide with 1 per cent celloidin in ether-alcohol. The slide is coated by standing it in a Coplin jar of the celloidin for 1 min or longer, the back is then wiped and the slide transferred to a dish of 70 per cent alcohol for a few minutes to harden the celloidin. When the celloidin has hardened the section can be stained in the normal manner. Following staining the celloidin should be removed by immersion in equal parts of alcohol and ether. Once the celloidin is removed the slides must be handled carefully to prevent the sections from becoming detached.

Albumenized or starched slides

Float the section onto a slide smeared with albumen or starch paste (*see p. 377*) and drain off the excess water. Blot the section carefully with a Whatman No. 1 filter paper and cover with a few drops of a mixture of equal parts of clove and aniline oil. Allow the mixture to coagulate the adhesive for 3 min and then remove it from the slide with xylene followed by absolute alcohol. The section can now be stained in the normal manner. As an additional safeguard the sections may also be coated with celloidin following immersion in absolute alcohol.

Gelatinized slides

Float the section onto a slide which has been coated with 0.2 per

cent gelatin and allowed to dry. Drain off the excess water and transfer the slide to a dish of formalin vapour, the action of which converts the gelatin to an irreversible gel and holds the section in place. Remove the slide after several minutes' exposure and wash in running tap water. The section can now be stained in the normal manner.

Preparation of cryostat sections

MOUNTING TISSUES

The tissue to be sectioned is placed in a drop of water in the centre of a previously cooled block-holder. The holders are usually stored in the cryostat and are ready for use when required. Rapid freezing by conduction through the metal block-holder is then carried out. This is effected either by standing the block-holder in a bath of alcohol or acetone containing dry ice, or by placing the block-holder in the special freezing attachment and exposing the tissue to carbon dioxide gas. When the tissue is frozen, the holder is positioned in the microtome.

SECTION CUTTING

The tissue should be adjusted to the microtome knife and trimmed in the normal manner, using the remote controls situated outside the freezing chamber. The microtome knife should be placed in position at least 15–30 min before sectioning commences in order to ensure that it is cooled to the correct temperature. The cutting temperature is determined empirically for each piece of tissue, but as a general rule a chamber temperature of –20 °C is satisfactory. To obtain the best results the quenched tissue should be left in the cryostat for 15–30 min prior to trimming and sectioning. This does not apply when sections are required urgently for diagnostic purposes.

Sectioning at the predetermined thickness, usually between 5 and 10 µm, is performed at a slow rate, care being taken to ensure that a steady stroke is maintained during the period that the tissue is in contact with the knife edge. To produce flat sections an anti-roll plate is used. This device consists of either a piece of PTFE sheet or, more usually, a piece of a glass microscope slide with two narrow strips of Sellotape attached to its vertical edges. The plate is carried in a holder which is fitted to the microtome so that the strips of Sellotape, resting on the knife face, act as spacers between plate and knife. The gap between the plate and knife is approximately 70 µm, which is sufficient to prevent the sections from curling as they are cut. After each section is cut, the anti-

roll plate is slowly moved back and the section removed from the knife.

SECTION HANDLING

The sections may be attached directly to slides or coverglasses kept at room temperature, merely by touching the glass surface against the section. This is facilitated by using a special holder fitted with a suction cup. When mounted, the sections may be air-dried, fixed in an appropriate fixative (this step may be omitted) and stained by the chosen technique.

Preparation of celloidin sections

The microtome most suited for sectioning celloidin embedded tissue is the sliding type (*Figure 16.4*). The base sledge microtome may also be used for this purpose and special attachments designed to hold the knife at an oblique angle are manufactured for some rotary microtomes to allow celloidin sections to be cut. To avoid dehydration and shrinkage, the sections are usually cut by the 'wet method', the sections and block being kept wet at all times with 70 per cent alcohol. While actually cutting, the knife, sections and block are kept wet by applying 70 per cent alcohol to the knife by means of a large camel-hair brush. When cut, the sections and block are stored in a similar solution in jars with tightly fitting lids.

TECHNIQUE OF SECTION CUTTING

Clamp the vulcanite block securely into the object holder and turn back the feed mechanism. Secure a planoconcave knife in the knife-holders, adjust the angle of slant to about 40° and reduce the tilt to a minimum. Raise the block with the feed mechanism until it is almost touching the knife edge and with the adjustment screws provided, orientate until the surface to be cut and the edge of the knife are parallel. Set the automatic thickness gauge to about 15 µm, flood the knife and block with 70 per cent alcohol and trim until complete sections are being cut. Reset the automatic feed mechanism to the required thickness, reflood with 70 per cent alcohol and continue to cut the sections.

The method of cutting varies according to personal preference. Some workers favour a smooth cutting action, the section being kept flat on the knife by means of the camel-hair brush; others prefer to cut by using a jerking action, the section again being kept flat with the camel-hair brush; a third method used is to cut the

section quickly, thereby causing it to roll up and necessitating flattening out when transferred to a dish of 70 per cent alcohol. With this method the sections frequently leave the knife.

When using either of the first two methods, the sections are removed from the knife with forceps. The rate of cutting can be increased if the sections are slid along the knife as they are cut, five or six sections being accumulated before being removed. A small piece of wire twisted around the handle of the brush serves as a mounted needle and may be used to move the section along the knife.

When serial sections are required, a small piece of paper is placed over each section. The section and paper are then removed and stored in piles in suitable containers, being kept saturated in alcohol. Each piece of paper should preferably be numbered, but for speed, many workers only number every tenth piece.

The sliding microtome is the most dangerous type of microtome to use owing to the exposed knife-blade being movable. Great care should be taken when using this instrument.

17

Biological Staining

Cellular elements often have different refractive indices which can be utilized to permit partial identification by means of various forms of microscopy. For detailed study, however, and to prepare permanent preparations, staining procedures are invariably employed.

Biological stains are prepared from dyes which have been manufactured to rigid specifications for this purpose or have been subjected to rigid quality assurance procedures to ensure that they are suitable for the specialized purpose for which they are to be used. These dyes are classified into two groups, synthetic, which are by far the larger group, and natural. Some of the more important biological stains such as haematoxylin and carmine do, however, belong to the natural group. Dyes can be further subdivided into acid and basic groups, a combination of which can produce a neutral stain (p. 388).

One of the problems confronting biologists is the precise identification of dyes used in staining procedures owing to the enormous number of synonyms employed. The standard reference work used for the identification of dyes is the Colour Index, which first appeared in 1923. The Colour Index is published jointly by the Society of Dyers and Colourists and the American Association of Textile Chemists and Colorists.

The 3rd edition published in 1971 consists of five volumes. Dyes are grouped under generic names according to colour and usage. A constitution number (CI No.) is allocated when the chemical constitution of a dye is known and dyes of similar constitution but different trade names receive the same CI No.

The quality assurance procedure used by reputable manufacturers and suppliers of biological stains to test the efficiency of the products they sell are based upon the procedures advocated by the Biological Stain Commission of the USA: These tests comprise spectrophotometry, titaneous chloride precipitation and biological staining procedures. Minimum standards and dye content have been defined for 57 of the dyes more widely used in biological staining procedures.

NATURAL DYES

Haematoxylin

Haematoxylin is a dye derived by ether extraction from the wood of the Mexican tree *Haematoxylon campechianum*. Haematoxylin has poor staining properties and is normally used in conjunction with a mordant (see p. 402).

Haematoxylin must be 'ripened' by exposure to air and sunlight, which oxidize the haematoxylin to form the essential staining element haematein. This is a slow process, but it can be hastened by the addition of a little neutral solution of hydrogen peroxide or other powerful oxidizing agent.

Mordants have been described as substances which combine with the tissue and the stain, linking the two, and causing a staining reaction between them. Those commonly used in histology are compounds of aluminium and iron, but chromium and copper are also used. Aluminium compounds are suitable for the progressive staining (see p. 390) of tissues in bulk, but iron compounds may be used only for sections which permit differentiation. Mordants need not necessarily be included in the staining solution; some fixatives are used partly for their mordanting qualities. When myelinated fibres of the central nervous system are to be demonstrated the tissue is usually fixed in 10 per cent formol saline and, prior to embedding, is mordanted in Weigert's primary mordant, which is a mixture of potassium dichromate and fluorchrome.

Cochineal and its derivatives

Cochineal is one of the older histological dyes. It is extracted from the bodies of female cochineal bugs. The dye obtained is treated with alum, yielding a product relatively free of extraneous matter, and known as *carmine*. It is extensively used for staining zoological specimens and when combined with picric acid (picrocarmine) it is extremely useful in neuropathology. It is a powerful nuclear stain and it is used for the demonstration of glycogen (Best's Carmine) in permanent preparations.

Orcein

Orcein is a vegetable dye extracted from certain lichens by the action of ammonia and air. It has a violet colour, and is a weak acid, soluble in alkalis. Its main use in histology is the demonstration of elastic fibres (Tänzer-Unna orcein stain).

Litmus

Litmus is also obtained from lichens but they are treated with lime and potash or soda, in addition to exposure to ammonia and air. Litmus is a poor dye and is not used as a histological stain. It was widely used as an indicator but has been largely superseded by synthetic dyes.

Saffron

Saffron, a natural pigment extracted from the stigmata of *Crocus sativus*, is not widely used in histology, but has been incorporated by Masson into a connective tissue stain.

SYNTHETIC DYES

Synthetic dyes are sometimes referred to as 'coal tar dyes', since they are manufactured from substances which, until recently, were only obtained from coal tar. All these compounds are derivatives of the hydrocarbon benzene (C_6H_6), which consists of 6 carbon

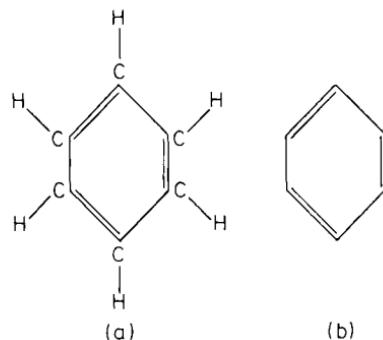


Figure 17.1. Hydrocarbon benzene: (a) showing hydrogen atom attached to each free valency; (b) as usually illustrated

atoms at the corners of an equal-sided hexagon, with a hydrogen atom attached to each carbon atom (see Figure 17.1a). For simplicity, benzene may be drawn with the C and H atoms omitted (Figure 17.1b).

Simple benzene compounds have absorption bands in the ultra-violet range of the spectrum. Certain substances ('chromophores') are capable of moving this absorption band into the visible portion of the spectrum, thereby producing visible colour. Benzene compounds containing chromophores are known as 'chromogens'.

The nitre group (NO_2) is a chromophore. If three of these groups displace three hydrogen atoms of benzene the compound trinitrobenzene is formed (*Figure 17.2a*).

The chromogen trinitrobenzene is coloured but it is not a dye. Chromogens differ from dyes in that any colouring they impart to tissue is easily removable. Before a substance can be called a dye, it must be capable of retention by tissue. A chromogen becomes a dye after the addition of another radicle, known as an 'auxochrome'. If a further hydrogen atom is replaced by a

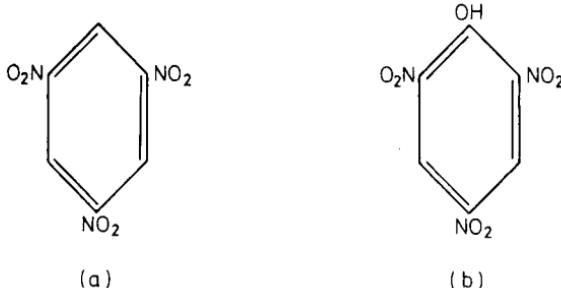


Figure 17.2. (a) Trinitrobenzene; (b) picric acid

hydroxyl group, which is an auxochrome, the compound results as shown in *Figure 17.2b*.

This compound, picric acid, is a dye by virtue of its capacity to form salts with alkalis. Picric acid is unique in that it is the only substance which may be used as a fixative, a differentiator and a stain. A synthetic dye may be described as a benzene derivative, to which a chromophore and an auxochrome have been added.

Care must be taken when handling dyes, both in powder and solution form, especially in cases of prolonged exposure. Inhalation of the powder should be avoided and dyes coming in contact with the skin should be cleaned off immediately to prevent absorption.

BASIC, ACID AND NEUTRAL DYES

The nature of the auxochrome ordinarily determines whether the resulting dye is acid or basic in character.

Basic dyes

In basic dyes, such as methylene blue, the colouring substance is contained in the basic part of the compound. The colourless acid radicle is usually derived from hydrochloric, sulphuric or acetic acid.

Acid dyes

In these dyes, for example eosin, the colouring substance is contained in the acid component, and the base is usually sodium.

Neutral dyes

These are obtained by combining aqueous solutions of basic and acid dyes. The resultant precipitates are usually insoluble in water, but soluble in alcohol, for example Leishman's stain.

Nuclei are usually stained by basic dyes, and cytoplasm by acid dyes. Neutral dyes stain both nuclei and cytoplasm.

Table 17.1 CI NUMBER AND SOLUBILITY TABLE

Solubilities, expressed as g per 100 ml of solvent, of the more common histological stains in water and ethyl alcohol at room temperature.

Stain	CI No.	Water	Alcohol
Acid fuchsin	42685	18.0	0.3
Alcian blue	74249	9.5	6.0
Alizarin	58000	nil	0.125
Alizarin red S	58005	5.3	0.15
Auramine O	41000	0.35	7.0
Aurantia	10360	1.3	0.3
Basic fuchsin	42510	0.4	7.6
Bismarck brown Y	21000	1.2	1.1
Brilliant crystal scarlet	16250	1.75	0.8
Brilliant green	42040	3.0	3.3
Carminic acid	75470	8.3	0.2
Celestine blue	51050	2.0	1.5
Chlorantine Fast Red 5B	28160	1.0	0.45
Congo red	22120	4.5	0.8
Crystal violet	42555	1.5	7.0
Eosin	45380	40.5	3.5
Eosin—alcohol soluble	45386	nil	0.45
Haematoxylin	75290	1.75	60.0
Janus Green B	11045	5.3	1.1
Light green	42095	18.5	0.85
Martius yellow	10315	4.7	0.16
Methyl green	42585	9.2	3.0
Methyl blue (Aniline blue)	42755	10.4	nil
Methyl violet 6B	42555	4.2	6.2

Stain	CI No.	Water	Alcohol
Methylene blue	52015	2.5	1.5
Nile blue	51180	1.0	1.0
Neutral red	50040	3.2	2.0
Oil Red O*	26125	nil	0.5
Orange G	16230	7.1	0.3
Phloxine	45410	36.4	8.0
Picric acid		1.1	8.5
Pyronin B	45010	10.0	0.5
Pyronin Y	45005	11.0	0.5
Purpurin	58205	nil	0.76
Safranin O	50240	6.0	2.5
Scarlet R	26105	nil	0.2
Soluble blue	42755	40.0	nil
Sudan black B	26150	nil	0.23
Sudan II	12140	nil	0.3
Sudan III	26100	nil	0.15
Sudan IV	19140	nil	0.08
Tartrazine†		11.0	0.13
Thionin	52000	0.22	0.23
Toluidine blue	52040	3.1	0.5
Trypan blue	23850	10.4	0.02
Victoria blue 4R	42563	2.0	18.4

* Oil red O: 0.1 per cent in isopropyl alcohol.

† Tartrazine: 2.3 per cent in Cellosolve.

The above figures can only be a guide since batches of stain vary slightly in solubility; room temperature, which varies between 19 °C and 25 °C, also affects the solubility.

THE STAINING PROPERTIES OF DYES

Dyes may be considered as having micro-anatomical or cytological staining properties, as already mentioned in Chapter 13.

Micro-anatomical stains are used for demonstrating the general relationship of tissues to each other. Nuclei and cytoplasm are differentiated but their included structures are not necessarily emphasized.

Cytological stains demonstrate the minute structures in the nucleus and cytoplasm of cells without necessarily aiding in the general differentiation of the various tissue types.

Staining brought about by the aid of a mordant is called *indirect staining*, for example haematoxylin. Conversely, where a mordant is unnecessary, as in the majority of the aqueous or alcoholic aniline stains, the term *direct staining* is used. *Mordants* are metallic substances which act as a link between the stain and the tissue to be stained. They may be used in three ways:

1. Before application of the stain (pre-mordanting), e.g. Weigert's iron haematoxylin.
2. In conjunction with the stain (metachrome staining), e.g. Ehrlich's acid alum haematoxylin.
3. After the application of the stain (post-mordanting), e.g. Gram's stain.

Substances which, when incorporated into a staining solution, increase the staining power of that solution without acting as a mordant, are termed *accelerators*.

Stains which colour the tissue elements in a definite order are termed *progressive stains*. Those which colour all the tissue elements at the same time, and necessitate 'washing out' (differentiating), before the individual elements can be studied are termed *regressive stains*.

The staining of inclusions in live cells is referred to as *vital staining*. Living cells may be stained after removal from the organism (supravital staining), or while still part of the body (intravital staining). *Specific stains* act only on certain constituents of cells and tissues, and have little or no effect upon the remaining elements. The specificity, however, is usually dependent upon the use of a definite procedure. Most dyes stain the tissue in various shades of their own fundamental colour. Some tissues, however, assume a different colour from that of the solution in which they are immersed. This is known as *metachromatic staining*, a phenomenon seen only with the basic aniline dyes, such as methyl violet, which is frequently used in pathology to demonstrate amyloid infiltration.

Negative staining is used for the examination of bacterial morphology. The organisms and substance of choice are mixed on a slide. When the slide is examined microscopically, the unstained organisms are sharply contrasted against a black background.

Certain tissue constituents and some organisms are demonstrated by a procedure known as *impregnation*. The solutions used in the impregnation techniques are not stains, but are solutions of metallic salts. They differ from stains in being colourless. Opaque chemicals are precipitated on the surface of the tissue or bacteria. A stain is absorbed by the tissue, but an impregnating agent is deposited on its surface. This makes certain organisms (such as Spirochaetes) appear larger than they actually are.

THEORY OF STAINING

A great deal of research has been undertaken in an attempt to solve the complex problem of why certain stains have an affinity for certain tissue structures and inclusions. A number of theories have been advanced to explain this phenomenon but at the present time no single explanation has been accepted as being entirely satisfactory. The practice of supporting either a purely physical or chemical theory has been decreasing in recent years and modern workers tend to base their observations upon all of the individual factors, both physical and chemical, which, separately or jointly, are thought to play a part.

The physical factors which are considered as significant are (i) osmosis and capillarity, (ii) absorption, and (iii) selective adsorption. Osmosis and capillarity are simple physical forces which are considered by some workers as being at least partly responsible for the penetration of stains into porous tissues. The absorption factor is also very simple and is demonstrated in the presence of certain mineral salts, by the action of certain stains on certain tissues. The third physical factor, adsorption, is thought by many authorities to provide an acceptable explanation of a great many staining reactions. Selective adsorption, which is a principle well known to physical chemists, is the property possessed by certain substances to adsorb certain ions from a solution more readily than others. It is also known that the presence of other ions in the solution can have a marked effect upon the ratio of adsorption, and it would appear, therefore, that the action is controlled in part by the concentration of the hydroxyl and hydrogen ions present in the solution.

The main factor in the chemical theory is the assumption that certain parts of biological tissue are acid in character, for example the nuclei of cells, while others such as the cytoplasm have a basic reaction. As has been shown earlier, the colouring substance in basic dyes is contained in the basic part of the compound, the acid radicle being colourless; conversely, with acid dyes, the colouring substance is contained in the acid component while the basic is colourless. It has been suggested that differential staining reactions could well be due to chemical combinations being formed between the tissue and stain, the actual reaction depending upon the character of the tissue and stains involved. Thus, acid tissue elements such as nuclei will have an affinity for a basic stain whilst cytoplasm, which is basic in character, will have an affinity for acid stains.

Bacteria which are rich in ribonucleic acid, therefore, have an affinity for basic stains and tend to be unaffected by acid stains. In bacteriology the latter are used mainly in negative staining techniques in which the bacteria are seen unstained against a stained background.

18

Staining Procedures

STAINING EQUIPMENT

There are three methods of staining slides in common use: (1) using staining dishes; (2) using a staining rack; and (3) using a staining machine.

Staining dishes

A variety of these dishes is available. Small jars are used for staining single slides; Coplin jars hold 5–10 slides. Large staining troughs with separate baskets enable up to 20 slides to be stained at the same time.

Staining racks

Staining racks are often used in medical laboratories. Two glass rods, 2 in apart, are fixed across the sink. The slides are laid across these rods and the solutions poured onto the slides, using drop bottles.

Staining machines

These are used for staining large numbers of slides by a routine staining procedure. They have a greater application in cytology and haematology for staining smears than for staining sections. Machines are designed with up to 23 stations to accommodate various staining procedures. Details are given in Chapter 17.

Other apparatus

Other apparatus required for staining includes the following.

1. A bunsen burner for heating stains.
2. A hot plate (thermostatically controlled) for hardening mounting media.
3. A microscope and lamp for controlling the degree of staining.

PROCEDURE FOR STAINING PARAFFIN WAX SECTIONS

Before sections prepared by the paraffin wax technique can be stained, the surrounding wax must be removed and the section transferred, through graded alcohols, to distilled water. To avoid undue repetition in the following staining procedures, the phrase 'de-wax and hydrate' will be used to indicate that the following procedure should be adopted.

1. Free the section from paraffin wax by immersing the slide in xylene for 2 or 3 min. This process may be speeded up by first gently warming the section over a bunsen burner until the paraffin wax just begins to melt.
2. Transfer the slide to absolute alcohol for 30 s to remove the xylene. Blunt-nosed forceps should be used for transferring the slides from one reagent to another.
3. Transfer the slide to a second dish of absolute alcohol for a further 30 s to ensure that all the xylene is removed and not carried over into the lower-grade alcohols.
4. Transfer the slide to 90 per cent alcohol for 30 s.
5. Transfer the slide to 70 per cent alcohol for 30 s.
6. Wash the slide thoroughly in distilled water.

The section is now ready for staining by the appropriate technique.

After staining, the section is passed back through the graded alcohols (that is, 70 per cent, 90 per cent and two changes of absolute alcohol), washing very thoroughly in the absolute alcohol.

The section is next cleared in two changes of xylene. The object of this is two-fold. First, the section, after having been immersed in xylene, is miscible with the xylene–balsam (or DPX). Secondly, the refractive index of the tissue is raised so that it is approximately the same as that of the glass slide to which it is attached. This is an important factor, as the refraction of the light is reduced to a minimum when the section is examined under the microscope.

Staining frozen sections

Frozen sections may be attached to slides before staining (p. 380) or may be stained separately. Some sections in which fat is to be demonstrated are normally stained by being passed through small quantities of staining solutions by means of a tapered glass rod, the end of which is bent to form an angle of 60° (hockey stick).

After staining, the sections are floated out in a dish of water, picked up on a slide and mounted in an aqueous mounting medium.

CONTROL AND TEST SLIDES

Known positive slides should be used as controls with all specialized staining procedures. New batches of stain should also be tested with control slides before being used for routine staining purposes.

PIGMENTS

When stained sections are examined microscopically, a deposit or pigment is frequently observed. This may be either artificial or natural in origin.

Artificial pigments

There are two artificial pigments commonly encountered, both being formed by the action of certain fixatives. They are: (1) mercuric chloride deposit; and (2) formaldehyde (post-mortem) precipitate.

MERCURIC CHLORIDE DEPOSIT

The exact nature of this deposit is not known. It is found in all tissue which has been preserved in a fixative containing mercuric chloride, and appears in the form of black clumps, differing from the fine brown deposit of the formaldehyde precipitate. It may be removed from sections by treatment with iodine as described on p. 400.

An alternative method is to add a few drops of a saturated solution of alcoholic iodine to each of the dehydrating alcohols, when dehydrating the bulk tissue. The disadvantage with this method is that the iodine tends to make the tissue rather brittle for sectioning.

FORMALDEHYDE POST-MORTEM PRECIPITATE

This pigment frequently occurs in post-mortem tissue if removed 24 h or more after death has occurred. It is believed to be a breakdown product of haemoglobin, and occurs chiefly in blood-forming organs, such as the liver and spleen. It does not occur when the formaldehyde solution has been neutralized or buffered to a

reaction of pH 7 and for this reason is referred to also as the acid formalin pigment. It is readily soluble in saturated alcoholic picric acid or in alkaline solutions, and is easily removed by one of the methods described on p. 401.

Natural pigments

Natural pigments are divided into two classes, exogenous and endogenous.

EXOGENOUS PIGMENTS

These pigments consist of foreign matter absorbed by the body during life. The most commonly encountered is carbon, which occurs as a jet-black pigment in sections of the lung and bronchial glands. It is impossible to remove the carbon pigment from sections. Another example of an exogenous pigment is tattooing ink.

ENDOGENOUS PIGMENTS

These are produced within the organism. There is a variety of pigments in this class which may be encountered when studying sections. Haemosiderin (free iron) is the one which occurs most commonly, and this can readily be demonstrated histochemically by the Prussian blue reaction (p. 422), or if required to be removed, it is soluble in acid. Other true pigments which fall into this class are melanin and calcium. Endogenous pigments can be identified easily by means of chemical tests.

MOUNTING OF SECTIONS

After the section has been stained it must be prepared as a permanent preparation for microscopic examination. This is accomplished by mounting the section in a suitable medium under a glass coverslip.

The mountants most commonly used for mounting stained sections are Canada balsam, which may be either acid or neutral, and DPX. The choice of medium depends entirely upon the staining procedure used.

Method of mounting sections

1. Clean a coverslip of the appropriate size, and place it on the bench on a sheet of Whatman's No. 1 filter paper.
2. Wipe off the excess xylene from the slide with a dust-free cloth.

3. Lay the slide on the filter paper in front of the coverslip.
4. Gently blot the section with a folded sheet of Whatman's No. 1 filter paper.
5. Place the necessary amount of mounting medium on the section.
6. Quickly invert the slide and lower it on to the coverslip, applying gentle pressure until the mounting medium flows evenly to the edge of the coverslip.
7. Turn the slide over and, if necessary, square up the coverslip by means of a mounted needle.
8. After the sections have been mounted, the slides should be transferred to the 37°C incubator for 12–24 h in order to harden the mounting medium. Slides which are examined before the mounting medium has hardened frequently have their coverslip moved, resulting in damage to the section. If the slides are required for examination quickly, they can be placed on a hot plate to dry.

Note—1. The object of blotting the section is to remove all *excess* xylene. If this is not done, the xylene will mix with the mounting medium and form air-bubbles which become trapped beneath the coverslip. On no account should the section be blotted so hard that it becomes dry, as shrinkage and cracking will result. 2. Excess balsam may be removed by wiping with a clean duster, dipped in xylene. 3. If DPX is used as the mounting medium, an excess amount should be placed on the slide, the surplus being stripped off 24 h later when it has hardened. This is necessary in order to counteract the shrinkage which it undergoes on drying.

Coverslips for mounting stained preparations should be kept stored in absolute alcohol on the staining bench. The coverslips most commonly used are approximately $\frac{1}{8}$ mm in thickness. For use with 3 in by 1 in slides the sizes range from 22 mm square to 50 mm by 22 mm.

MOUNTING MEDIA

Media for the mounting of microscopical preparations may be divided into two main groups: (1) aqueous media; (2) resinous media. The media in group (1) are designed to make either temporary or permanent mounts of water-miscible preparations, e.g. frozen sections stained for fat. Their formulas consist of a solidifying agent such as gelatine or gum arabic to which is added glycerol to prevent drying and cracking, various sugars to bring about an increase in the refractive index and a preservative.

The second group may be divided into natural and synthetic

media. The most important of the natural resins is Canada balsam; a large number of synthetic resins are available commercially.

Aqueous mountants

KARO CORN SYRUP (refractive index 1.47)

Formula

Karo corn syrup (clear)	1 volume
Distilled water	2 volumes
Thymol, as preservative	1 crystal

Mode of preparation

Dilute the corn syrup with the distilled water and add the thymol. Mix well, label and store in a refrigerator at 4°C.

LAEVULOSE (FRUCTOSE) SYRUP (refractive index 1.47)

Formula

Laevulose (fructose)	70 g
Distilled water	20 ml

Mode of preparation

Dissolve the laevulose in the distilled water by heating at 37°C for 24 h. Mix well and label.

FARRANT'S MEDIUM (refractive index 1.43)

Formula

Gum arabic	50 g
Distilled water	50 ml
Glycerol	50 ml
Arsenic trioxide, as preservative	1 g

Mode of preparation

Dissolve the gum arabic in the distilled water with the aid of gentle heat, add the glycerol and arsenic trioxide. Mix well and label.

Notes—(a) The addition of 50 g of potassium acetate to the above solution will give a neutral medium (pH 7.2) instead of an acid one (pH 4.4) and raises the refractive index to 1.44. (b) Sodium merthiolate (0.025 per cent) may be substituted with advantage for the arsenic trioxide as a preservative.

GLYCERINE JELLY (refractive index 1.47)*Formula*

Gelatin	10 g
Glycerol	70 ml
Distilled water	60 ml
Phenol crystals, as preservative	0.25 g

Mode of preparation

Weigh the gelatin into the distilled water and incubate in a water bath at 60 °C until solution is effected. Add the glycerol and then the phenol crystals. Mix well, label and store in a refrigerator at 4 °C.

Preparation for use

Melt the glycerine jelly by heating in a water bath or incubator at 60 °C.

Note—To avoid the formation of air-bubbles in the mounted specimen do not shake or stir the melted medium prior to use.

Resinous mountants**NEUTRAL BALSAM (refractive index 1.52)***Mode of preparation*

Dissolve Canada balsam in xylene to form a fairly thin solution (approximately 40–50 per cent). Add calcium carbonate to excess and stir thoroughly. Allow the mixture to settle, decant the supernatant fluid into a stock bottle and discard the residue. Record date and label.

Notes—(a) The Canada balsam dissolves more readily in the xylene when placed in an incubator at 37 °C or a paraffin wax oven at 58 °C. (b) Toluene may be used as a solvent in place of xylene. (c) Canada balsam is a natural resin obtained from *Abies balsamea*. Mountants prepared from this resin can only be neutralized temporarily, becoming acid and brown on storage.

ACID BALSAM (refractive index 1.52)*Mode of preparation*

Dissolve Canada balsam in xylene to form a fairly thin solution (approximately 40–50 per cent). Add salicylic acid to excess and stir thoroughly. Allow the mixture to settle, decant the supernatant fluid into a stock bottle and discard the residue. Record date and label.

Note—See Notes under Neutral Balsam above.

XYLENE DAMAR (refractive index 1.53)*Mode of preparation*

Prepare a thin solution of the gum damar by dissolving in chloroform. Filter through paper in a Buchner funnel, using negative pressure, and evaporate the filtrate until all traces of the chloroform are removed. Dissolve the purified gum in xylene until a suitable solution results (approximately 60 per cent). Record date and label.

Notes—(a) Gum damar is a natural resin obtained from the East Indian tree *Shorea wiesneri*. The commercial product invariably contains solid impurities and should always be purified before use. (b) Unlike Canada balsam gum damar does not become brown on keeping.

SYNTHETIC RESINS

A wide range of mountants prepared from synthetic resins is available commercially. The formula of most commercial synthetic mountants is kept secret but in general they are prepared by dissolving a polystyrene in an aromatic hydrocarbon solvent, and adding a plasticizer such as dibutylphthalate or tricresyl phosphate to prevent the formation of air spaces on drying.

DPX MOUNTANT (refractive index 1.52)

Distrene '80'	10 g
Dibutylphthalate	5 ml
Xylene	35 ml

Mode of preparation

Combine the dibutylphthalate with the xylene, mix and dissolve the distrene '80'. Record date and label.

Notes—(a) To remove the coverglasses from preparations mounted with DPX immerse in trichlorethylene. (b) Preparations mounted in DPX should be cleared in xylene free from paraffin wax.

PROCEDURES FOR THE REMOVAL OF PIGMENTS**MERCURIC CHLORIDE PRECIPITATE**

The removal of the mercuric chloride precipitate.

Lugol's iodine

<i>Solution 1.</i>	Potassium iodide	2 g
	Iodine	1 g
	Distilled water	100 ml
<i>Solution 2.</i>	Sodium thiosulphate	5 g
	Distilled water	100 ml

Mode of preparation

Solution 1. Dissolve the potassium iodide in the distilled water and then add the iodine. Record date and label.

Solution 2. Dissolve the sodium thiosulphate in the distilled water, mix and label.

Procedure

1. Bring sections to water.
2. Immerse in solution 1 for 10 min.
3. Rinse in water.
4. Bleach in solution 2 for 3–5 min.
5. Wash thoroughly in water.
6. Continue with required staining procedure.

SCHRIDDE'S METHOD

The removal of the formalin post-mortem pigment.

Formula

Ammonia (sp. gr. 0.880)	1 ml
Ethyl alcohol, 75 per cent	200 ml

Procedure

1. Bring sections to 70 per cent alcohol.
2. Treat with ammoniacal alcohol for 30 min.
3. Wash thoroughly in tap water.
4. Continue with required staining procedure.

VEROCAY'S METHOD

The removal of the formalin post-mortem pigment.

Formula

Potassium hydroxide, 1 per cent aqueous solution	1 ml
Ethyl alcohol, 80 per cent	100 ml

Procedure

1. Bring sections to 80 per cent alcohol.
2. Treat with alcoholic hydroxide solution for 10 min.
3. Wash with two changes of water.
4. Transfer to 80 per cent alcohol for 5 min.
5. Wash in water.
6. Continue with required staining procedure.

BARRETT'S ALCOHOLIC PICRIC ACID

The removal of the formalin post-mortem pigment.

1. Deparaffinize with xylene and wash thoroughly in absolute alcohol.
2. Immerse in saturated alcoholic picric acid (approximately 8.5 per cent) for 30 min or more.
3. Wash in absolute alcohol to remove the picric acid.
4. Bring the section to water and continue staining in the normal way.

HAEMATOXYLIN STAINING SOLUTIONS FOR CELL NUCLEI

The mordants used in conjunction with haematoxylin for demonstrating the nucleus and the cytoplasmic contents are alum and iron. Of these, the solutions containing alum stain the nucleus a light transparent blue which rapidly turns red in the presence of acid. The solutions containing iron as a mordant stain the nucleus a more intense grey-black colour and are less susceptible to acid. Iron-haematoxylin is of particular value when the counterstain to be employed is of a strongly acidic nature. It is also used when fine structural details of the nucleus and cytoplasm are required.

ALUM-HAEMATOXYLIN SOLUTIONS

A large number of haematoxylin solutions which contain alum as their mordant have been devised, but only three are commonly employed. Alum-haematoxylin is used as a routine stain in conjunction with eosin, for demonstrating the general structure of tissue.

IRON-HAEMATOXYLIN SOLUTIONS

There are two main iron-haematoxylin solutions employed for routine work in the laboratory, Heidenhain's and Weigert's.

Of these two solutions, that of Weigert has the advantage of being a much more rapid stain, and is of particular value in pathological work where time is an important factor. It is commonly used to stain the nuclei of sections which are to be counterstained with Van Gieson stain, to demonstrate collagen fibres. The Heidenhain's solution on the other hand, has the advantage of giving very precise staining of both nuclei and cytoplasmic inclusions. It is also used as a routine stain for demonstrating the striations in muscle fibres, and for staining fungi in sections.

TECHNIQUES AND RESULTS

MAYER'S ACID-ALUM-HAEMATOXYLIN AND EOSIN A general-purpose staining procedure.

Mayer's acid-alum-haematoxylin

Solution 1. Ammonium alum	50g
Chloral hydrate	50g
Haematoxylin (CI No. 75290)	1g
Citric acid	1g
Sodium iodate	0.2g
Distilled water	1000 ml

Dissolve the haematoxylin in the water with the aid of gentle heat and add the sodium iodate and alum, shaking at intervals to effect solution of the alum. Dissolve the citric acid and chloral hydrate, record date and label. The haematoxylin solution, which turns reddish-violet in colour, is ready for immediate use, no further ripening being necessary owing to the inclusion of the sodium iodate. The solution remains stable for several months.

Acid-alcohol

Solution 2. Hydrochloric acid (sp. gr. 1.19)	1 ml
Ethyl alcohol, 70 per cent	99 ml

Scott's tap water substitute—see Note (c) below

Solution 3. Sodium bicarbonate	3.5 g
Magnesium sulphate	20 g
Tap water	1000 ml
Thymol, as preservative	1 crystal

Solution 4. Eosin, w/s yellowish (CI No. 45380) 1 g

Distilled water 100 ml

Thymol, as preservative 1 crystal

Procedure

1. De-wax and hydrate.
2. Stain in solution 1 for 10–30 min.
3. Wash thoroughly in running tap water.
4. Differentiate in solution 2 until only the cell nuclei retain the stain.
5. Blue in running tap water or solution 3 for 5–10 min.
6. Counterstain in solution 4 for 1–2 min.
7. Wash with running water until the excess eosin is removed.
8. Dehydrate, clear and mount in neutral balsam or DPX.

Results

Cell nuclei, blue; red blood corpuscles, red; muscle, connective tissue and cell cytoplasm, varying shades of pink.

Notes—(a) Alum-haematoxylin can be used progressively, the optimum staining period being determined for each new batch of stain by staining a control section. For routine work the common practice is to use it as a regressive stain, owing to factors such as fixation and the type of tissue which influence the staining time. (b) When alum-haematoxylins which are ripened spontaneously are used for routine work, the laboratory requirements should be calculated some months ahead. (c) Sections require thorough washing in tap water after staining with acid-alum-haematoxylin in order to remove all traces of the acid and to bring out the required colour. In districts where the tap water is alkaline in reaction satisfactory blueing will be obtained without recourse to the use of solution 3. (d) 20 ml of glacial acetic acid may be substituted for the citric acid in the preparation of solution 1. (e) The addition of up to 1 per cent acetic acid to solution 4 is preferred by some workers. (f) The use of 4–5 drops of concentrated formaldehyde solution (40 per cent) may be used as a preservative in place of thymol in solution 4.

EHRLICH'S HAEMATOXYLIN

A general-purpose nuclear stain.

Formula

Ammonium or potassium alum	3 g
----------------------------	-----

Haematoxylin (CI No. 75290)	2 g
-----------------------------	-----

Ethyl alcohol, 95 per cent	100 ml
----------------------------	--------

Glycerol	100 ml
----------	--------

Distilled water	100 ml
Acetic acid, glacial	10 ml

Mode of preparation

Dissolve the haematoxylin in the alcohol, in a 1 litre flask and then add the water. Add the alum and shake until solution is effected. Incorporate the remaining ingredients. Plug the flask lightly with cotton wool and oxidize by exposing to the air and sunlight for 2 weeks or more, shaking daily. Transfer to a suitable storage bottle, record date, label and store in a warm place for 3 or 4 weeks. Repeat the shaking at intervals. The solution remains stable for several years.

Procedure

Proceed as for Mayer's acid-alum-haematoxylin (p. 403), using the above solution in place of solution 1.

Results

Cell nuclei, blue; other constituents according to counterstain.

Notes—(a) The addition of 0.4 g of sodium iodate will produce instant oxidation, but the stability of the solution will be affected and the period of optimal activity will be reduced. When iodate is employed, it must be added prior to the addition of the acetic acid. (b) The inclusion of the glycerol reduces evaporation and retards the staining rate of the solution.

HARRIS ALUM-HAEMATOXYLIN

A general-purpose nuclear stain of exceptional value in exfoliative cytology.

Formula

Ammonium or potassium alum	20 g
Haematoxylin (CI No. 75290)	1 g
Mercuric oxide	0.5 g
Distilled water	200 ml
Ethyl alcohol, absolute	10 ml

Dissolve the haematoxylin in the alcohol and the alum in the distilled water with the aid of gentle heat. Combine the two solutions in a 500 ml boiling flask and bring rapidly to the boil. Add the mercuric oxide and then cool immediately by immersing the flask in cold water. The solution should assume a dark purple colour on the addition of the mercuric oxide. Transfer to a suitable storage bottle, record date and label. The solution remains stable for years.

Procedure

Proceed as for Mayer's acid-alum-haematoxylin (p. 403), using the above solution in place of solution 1. See also Note (b) below.

Results

Cell nuclei, blue; other constituents according to counterstain.

Notes—(a) The addition of 4 per cent acetic acid gives more precise nuclear staining and should be used for cytology. (b) The usual staining time for smears is 4 min.

COLE'S HAEMATOXYLIN

A general-purpose nuclear stain.

Formula

Saturated aqueous aluminium sulphate	750 ml
Haematoxylin (CI No. 75290)	1.0 g
1 per cent iodine in 70 per cent alcohol	50 ml
Distilled water	250 ml

Using a 2 litre flask dissolve the haematoxylin in the distilled water with the aid of gentle heat.

Add the iodine solution and the aluminium ammonium sulphate solution and mix well. Bring rapidly to the boil and then cool.

Transfer to a suitable dark storage bottle, record date, label and store in a cool place. The solution remains stable for several months.

Procedure

Proceed as for Mayer's acid-alum-haematoxylin (p. 403), using the above solution in place of solution 1.

Results

Cell nuclei, blue; other constituents according to counterstain.

CELESTIN BLUE

A stain which, when used in conjunction with Mayer's acid-alum-haematoxylin (p. 403), is resistant to strong acid dyes and gives good nuclear definition.

<i>Solution 1.</i>	Iron alum	25 g
	Celestin blue R (CI No. 900)	1.25 g
	Glycerol	35 ml
	Distilled water	250 ml

Dissolve the alum in the distilled water. Solution is generally effected by allowing the flask to remain at room temperature overnight. Add the celestin blue and boil for 3 min. Cool, filter and add the glycerol. Record date and label. The solution remains stable for 6–12 months.

Solution 2. Mayer's acid-alum-haematoxylin (p. 403) or Cole's haematoxylin (p. 406).

Procedure

1. De-wax and hydrate.
2. Stain in solution 1 for 10–20 min.
3. Rinse in tap water.
4. Stain in solution 2 for 5–10 min.
5. Rinse in water.
6. Blue in running tap water.
7. Counterstain as required.
8. Dehydrate, clear and mount.

Results

Cell nuclei, blue; other constituents according to counterstain.

Note—Celestin blue is frequently used in conjunction with Mayer's acid-alum-haematoxylin in place of the older iron-haematoxylin solutions, the combined result being resistant to acid dyes.

WEIGERT'S IRON-HAEMATOXYLIN AND VAN GIESON

A widely used stain for differentiating muscle fibres and connective tissue.

Haematoxylin solution A

Haematoxylin (CI No. 75290)	1 g
Ethyl alcohol, 95 per cent	100 ml

Iron chloride solution B

Iron chloride (ferric), 29 per cent aqueous solution	4 ml
Hydrochloric acid (sp. gr. 1.19)	1 ml
Distilled water	95 ml

The working solution

Solution 1. Haematoxylin solution (see above)	1 volume
Iron chloride solution (see above)	1 volume

Combine the solutions and mix well. The mixture, which is deep purple in colour, is best prepared for use as required. The working solution will remain active for 1–2 days.

Solution 2. Acid-alcohol 1 per cent, p. 403.

Van Gieson's stain

Solution 3.

Picric acid, saturated aqueous solution (approx.

1 per cent)

100 ml

Acid fuchsin (CI No. 42685), 1 per cent

aqueous solution

5–10 ml

Procedure

1. De-wax and hydrate.
2. Stain in solution 1 for 20 min.
3. Wash in tap water.
4. Differentiate in solution 2, controlling the degree of differentiation microscopically, until the nuclei are just over-stained.
5. Wash in tap water.
6. Counterstain in solution 3 for 3–5 min.
7. Blot lightly; do not wash in water.
8. Dehydrate rapidly with 90 per cent and absolute alcohol, clear and mount in acid balsam or DPX.

Results

Cell nuclei, black; collagen, red; muscle fibres, cell cytoplasm and red blood corpuscles, yellow.

Note—The addition of a few drops of saturated alcoholic picric acid to the dehydrating alcohols is to be recommended.

HEIDENHAIN'S IRON-HAEMATOXYLIN

A precise cytological stain which may be used for demonstrating both nuclear and cytoplasmic inclusions. Striations in voluntary muscle are also well stained.

Solution 1. Iron alum 2.5 g
 Distilled water 100 ml

Solution 2. Haematoxylin (CI No. 75290) 0.5 g
 Ethyl alcohol, 95 per cent 10 ml
 Distilled water 90 ml

Dissolve the haematoxylin in the alcohol and add the water. Bottle, stopper with a cotton-wool plug and allow the solution to ripen for 4–5 weeks. Record date and label. The solution remains stable indefinitely.

Procedure

1. De-wax and hydrate.
2. Mordant in solution 1 for 3 h or longer.
3. Rinse in water.
4. Stain in solution 2 for a period equal to that for which the sections were mordanted in solution 1.
5. Rinse in distilled water.
6. Differentiate in solution 1, controlling the degree of differentiation microscopically.
7. Wash thoroughly in running tap water for 5–10 min to remove all traces of the iron alum.
8. Counterstain as required.
9. Dehydrate, clear and mount.

Results

Cell nuclei, cytoplasmic inclusions and muscle striations, black; other constituents according to counterstain.

Notes—(a) The addition of 0.1 g of sodium iodate will render the haematoxylin solution ready for use immediately. (b) Solution 2 may be diluted 1 : 1 with distilled water to give greater control during differentiation. (c) By heating solutions 1 and 2 to 45 °C the period of staining may be reduced to 45 min.

VERHOEFF'S ELASTIC FIBRE STAIN

The demonstration of elastic fibres.

Alcoholic haematoxylin

Haematoxylin (CI No. 75290)	5 g
Ethyl alcohol, absolute	100 ml

Ferric chloride solution

Ferric chloride	10 g
Distilled water	100 ml

Lugol's iodine solution (see p. 401)

Solution 1. Alcoholic haematoxylin	20 ml
Ferric chloride solution	8 ml
Lugol's iodine solution	8 ml

Add the ferric chloride and Lugol's iodine to the alcoholic haematoxylin.

<i>Solution 2.</i>	Ferric chloride solution	20 ml
	Distilled water	80 ml

Solution 3. Van Gieson's stain (p. 408).

Procedure

1. De-wax and hydrate.
2. Stain in solution 1 for 15–60 min.
3. Rinse in water.
4. Differentiate in solution 2, controlling the degree of differentiation microscopically.
5. Transfer to 95 per cent alcohol.
6. Wash in water.
7. Counterstain with solution 3.
8. Dehydrate, clear and mount.

Results

Elastic fibres, black; nuclei, blue to black; collagen, red; muscle fibres, yellow; red blood cells, yellow.

Notes—(a) Solution 1 will only remain active for 24–48 h. (b) To decrease nuclei staining double the quantity of Lugol's iodine solution. (c) Sections which have been over-differentiated may be returned to solution 1 and re-stained, providing that they have not been in contact with 95 per cent ethyl alcohol.

RESORCIN FUCHSIN

The demonstration of elastic fibres.

Solution 1. Basic fuchsin (CI No.

42510)	2 g
Resorcinol	4 g
Ferric chloride, 29 per cent aqueous solution	25 ml
Distilled water	200 ml
Ethyl alcohol, 95 per cent	(approx) 205 ml
Hydrochloric acid (sp. gr. 1.18)	1 ml

Measure the distilled water into an enamel dish and dissolve the basic fuchsin and resorcinol. Bring to the boil and add the ferric chloride solution. Boil for 3–5 min, stirring constantly, when a thick precipitate should form. Cool and filter. Discard the filtrate and allow the filter paper and enamel dish to dry. Place the filter paper in the enamel dish and dissolve the precipitate in 200 ml of alcohol by heating carefully with constant stirring. Remove the filter paper, cool and filter. Make up the volume to 200 ml with the alcohol and add the hydrochloric acid. Record date and label. The solution remains stable for 3–4 months.

Solution 2. Acid-alcohol 1 per cent, p. 403.

Procedure

1. De-wax and bring sections to 95 per cent alcohol.
2. Stain in solution 1 for 20 min or longer.
3. Rinse in 95 per cent alcohol.
4. Differentiate in solution 2.
5. Wash thoroughly in water.
6. Counterstain as desired.
7. Dehydrate, clear and mount.

Results

Elastic fibres, dark blue to black; other tissues according to counterstain used.

MARTIUS SCARLET BLUE (MSB) (LENDRUM *et al.*) FOR THE DEMONSTRATION OF FIBRIN

Solution 1. Celestin blue (*see* p. 406).

Solution 2. Mayer's haemalum (*see* p. 403).

Solution 3. 0.25 per cent hydrochloric acid in 70 per cent ethyl alcohol.

Solution 4. 0.5 per cent martius yellow (CI No. 10315) in 95 per cent ethyl alcohol containing 2 per cent phosphotungstic acid.

Solution 5. 1 per cent Brilliant Crystal scarlet (CI No. 16250) in 2.5 per cent aqueous acetic acid.

Solution 6. 1 per cent phosphotungstic acid.

Solution 7. 0.5 per cent soluble blue (CI No. 42755) in 1 per cent aqueous acetic acid.

Procedure

1. De-wax and hydrate.
2. Stain in solution 1 for 5 min.
3. Rinse in tap water.
4. Stain in solution 2 for 5 min.
5. Rinse in tap water.
6. Differentiate in solution 3.
7. Wash thoroughly in tap water.
8. Rinse in 95 per cent ethanol.
9. Stain in solution 4 for 2 min.
10. Rinse in water.
11. Stain in solution 5 for 10 min.
12. Rinse in water.
13. Transfer to solution 6 for 5 min.
14. Rinse in water.
15. Stain in solution 7 for 10 min.
16. Dehydrate, clear and mount.

Results

Cell nuclei blue; fibrin, red; collagen blue; red blood cells yellow.

Note—The procedure may be shortened and differentiation eliminated by staining in a single solution of the following for 8 min in place of steps 9–15. Solution 4, 3 vol., solution 5, 2 vol., solution 7, 3 vol. Rinse in water before dehydration.

GORDON AND SWEETS' RETICULIC STAIN

A routine method which demonstrates reticulin fibres but does not stain the cells.

Solution 1.

Potassium permanganate, 0.5 per cent aqueous solution	95 ml
Sulphuric acid, 3 per cent aqueous solution	5 ml

Solution 2.

Oxalic acid	1 g
Distilled water	100 ml

Solution 3.

Iron alum	2.5 g
Distilled water	100 ml

Solution 4.

Silver nitrate, 10.2 per cent aqueous solution	5 ml
Sodium hydroxide, 3.1 per cent aqueous solution	5 ml

Ammonia (sp. gr. 0.880)	as required
Glass-distilled water	as required

Measure the silver nitrate solution into a chemically clean 100 ml graduated cylinder and add ammonia water, drop by drop with constant shaking until the precipitate formed is redissolved. Add the sodium hydroxide solution and again dissolve the precipitate by adding ammonia water drop by drop. The precipitate should be only just dissolved and the final solution should be slightly turbid. Make up the volume to 50 ml with glass-distilled water. The solution must be prepared at the time of use.

Solution 5. Formalin, 10 per cent aqueous solution (see p. 318).

Solution 6. Gold chloride, 0.2 per cent aqueous solution.

Solution 7. Sodium thiosulphate, 5 per cent aqueous solution.

Solution 8. Saffranine O, 5 per cent aqueous solution.

Procedure

1. Bring sections to water.
2. Oxidize in solution 1 for 1–5 min.
3. Wash in water.
4. Bleach in solution 2 for 3–5 min.
5. Wash thoroughly in tap water and several changes of distilled water.
6. Mordant in solution 3 for between 10 min and 2 h (10 min will usually suffice).
7. Wash in several changes of distilled water.
8. Impregnate with solution 4 for 30 s.
9. Wash in several changes of distilled water.
10. Reduce in solution 5 for 1 min.
11. Wash in tap water and then distilled water.
12. Tone in solution 6 for 10–15 min.
13. Rinse in distilled water.
14. Fix in solution 7 for 5 min.
15. Wash in water for 1–2 min.
16. Counterstain nuclei with solution 8.
17. Dehydrate, clear and mount.

Note—It is dangerous to store the ammoniacal silver solution as gas is produced and this may result in an explosion.

SOUTHGATE'S MUCICARMINE

The demonstration of mucin secreted by epithelial cells.

Solution 1. Mayer's acid-alum-haematoxylin (p. 403).

Solution 2. Carmine (CI No. 75470)	1 g
Aluminium hydroxide	1 g
Aluminium chloride, anhydrous	0.5 g
Ethyl alcohol, 50 per cent	100 ml

Weigh the carmine and aluminium hydroxide into a 500 ml flask and then add the alcohol. Shake well and while still shaking add the aluminium chloride. Place in a boiling water bath for 2.5 min exactly. Cool, filter, record date and label.

Procedure

1. De-wax and hydrate.
 2. Stain with solution 1.
 3. Stain with solution 2 diluted 1 : 5 with distilled water for 30–45 min.
 4. Rinse in distilled water.
 5. Dehydrate, clear and mount.

Results

Cell nuclei, blue; mucin, red.

Note—More precise staining may be achieved by diluting solution 2 1:10 with distilled water and prolonging the staining period.

PERIODIC ACID SCHIFF METHOD

The Periodic Acid Schiff reaction (PAS) is widely used in histopathology. The reaction is due to 1;2-glycol groups in tissues that consist of or contain carbohydrates, being converted to aldehydes by oxidation with periodic acid. The aldehydes become coloured when treated with Schiff's reagent. It is particularly valuable for demonstrating glycogen, fungi and mucin.

Solution 1. Periodic acid 1 g
 Distilled water 100 ml

Solution 2. Schiff reagent
Basic fuchsin (CI No. 42510) 1 g

Distilled water	200 ml
N hydrochloric acid (98.3 ml HCl, sp. gr. 1.16 made up to 1 litre with distilled water)	20 ml
Sodium bisulphite (anhydrous)	1 g
Activated charcoal	0.5 g

Measure the distilled water into a 500 ml flask, bring to the boil and dissolve the basic fuchsin. Cool to 50 °C, filter and add the hydrochloric acid. Cool to 25 °C and add the sodium bisulphite. Store in the dark for 24–48 h, during which time the solution becomes straw-coloured. Shake up with the charcoal, filter immediately, transfer to a brown bottle and label. Store in a refrigerator.

Solution 3. Sulphurous acid rinse

Sodium metabisulphite, 10 per cent	6 ml
N hydrochloric acid	5 ml
Distilled water	100 ml

Solution 4. Harris haematoxylin (see p. 405).

Procedure

1. De-wax and hydrate.
2. Place in solution 1 for 5 min.
3. Rinse in tap water.
4. Rinse in distilled water.
5. Place in Schiff reagent (solution 2) for 5 min.
6. Place in 3 baths of solution 3 for 2 min in each bath.
7. Rinse in tap water.
8. Stain with solution 4 for 30 s.
9. Blue in tap water.
10. Dehydrate, clear and mount.

Note—Step 6 can be omitted and the section washed in running tap water for 10 min.

Results

Cell nuclei, blue; mucin and glycogen, purple; basement membrane of kidney and skin reddish-purple.

Control tests

A control test slide containing known positive material should always be stained to test the efficiency of the solutions.

THE FUELGEN REACTION

The demonstration of nucleoproteins

Solution 1. Hydrochloric acid, 1N solution (*see Appendix*)

Solution 2. Schiff's reagent (*see p. 414*).

Solution 3. Sulphite rinse

Potassium metabisulphite, 10 per cent aqueous solution	5 ml
Hydrochloric acid, 1N solution	5 ml
Distilled water	90 ml

Add the potassium metabisulphite solution and hydrochloric acid to the distilled water. Prepare immediately before use.

Procedure

1. De-wax and hydrate.
2. Rinse briefly in cold solution 1.
3. Transfer to solution 1, pre-heated to 60 C. The optimum period of hydrolysis varies according to the fixative used, but is usually 5–25 min.
4. Rinse briefly in cold solution 1.
5. Rinse in distilled water.
6. Transfer to solution 2 (Schiff's reagent) for 30–90 min.
7. Drain and transfer to 3 baths of solution 3 for 1, 2 and 2 min in each respectively.
8. Rinse well in distilled water.
9. Counterstain as desired.
10. Dehydrate, clear and mount.

Results

Desoxyribonucleic acid, reddish-purple; other tissues according to counterstain.

Note—It is advisable to stain several slides, varying the period of hydrolysis.

GOMORI'S ALDEHYDE FUCHSIN

The demonstration of cells of the islets of Langerhans and elastic fibres.

Solution 1. Lugol's iodine, p. 401.

Solution 2. Sodium thiosulphate, 5 per cent aqueous solution

Solution 3. Aldehyde-fuchsin

Basic fuchsin (CI No.	
42510)	1 g
Ethyl alcohol, 70 per cent	200 ml
Hydrochloric acid (sp. gr.	
1.18)	2 ml
Paraldehyde	2 ml

Solution 4. Light Green 0.2 per cent

Dissolve the basic fuchsin in the alcohol and then add the hydrochloric acid and paraldehyde. Shake the mixture well and stand at room temperature until deep purple in colour (24–48 h). Record date and label.

Procedure

1. De-wax and hydrate.
2. Mordant in solution 1 for 10–60 min.
3. Rinse in water.
4. Bleach in solution 2 for 5 min.
5. Wash in running tap water for 3 min.
6. Rinse in 90 per cent alcohol.
7. Stain in solution 3 for 5–10 min.
8. Rinse in 90 per cent alcohol.
9. Counterstain as desired.
10. Dehydrate, clear and mount.

Results

Elastic fibres, purple; other tissues according to counterstain used.

TRICROME-PAS METHOD (PEARSE)

A useful staining procedure for demonstrating the alpha and beta cells in the anterior lobe of the pituitary gland, following fixation in formol saline or Helly's fluid.

Solution 1. 1 per cent aqueous periodic acid (p. 414).

Solution 2. Schiff's reagent (p. 414).

Solution 3. 0.5 per cent sodium metabisulphite (p. 415).

Solution 4. Celestin blue (p. 406).

Solution 5. Mayer's haemalum (p. 403).

Solution 6. 1 per cent HCl in 70 per cent ethyl alcohol.

Solution 7. 2 per cent Orange G in 5 per cent phosphotungstic acid

Procedure

1. De-wax and hydrate.
2. Oxidize in solution 1.
3. Wash in distilled water.
4. Transfer to Schiff's reagent (solution 2) for 20 min.
5. Rinse quickly in 3 changes of reducing bath (solution 3).
6. Wash in water for 10 min.
7. Stain in celestin blue (solution 4) for 1-3 min.
8. Stain in Mayer's haemalum (solution 5) for 1-3 min.
9. Differentiate rapidly in solution 6.
10. Wash in running tap water for 5 min.
11. Stain in solution 7 for 10-20 s.
12. Differentiate in water until a yellow tinge is just visible, usually 1 min.
13. Dehydrate, clear and mount in DPX.

Results

Cell nuclei, blue-black; beta cell granules, red; alpha cell granules and other acidophilic substances, varying shades of yellow.

ALCIAN BLUE-PERIODIC ACID SCHIFF METHOD

The demonstration of acidic groups and 1:2 glycols of carbohydrates.

Solution 1. Alcian blue (CI No. 74240), 1 per cent in 3 per cent acetic acid.

Solution 2. Periodic acid, 1 per cent aqueous solution.

Solution 3. Schiff's reagent (p. 414).

Procedure

1. De-wax and hydrate.
2. Stain with solution 1 for 5 min.
3. Wash in running tap water for 2 min.
4. Rinse in distilled water.
5. Oxidize in solution 2 for 2 min.
6. Wash in running tap water for 5 min.
7. Rinse in distilled water.
8. Treat with solution 3 for 8 min.
9. Wash in running tap water for 10 min.
10. Dehydrate, clear and mount.

Results

Acid mucins, blue; neutral mucins, magenta; mixtures of acid and neutral mucins, purple.

Note—Cell nuclei may be stained with Cole's haematoxylin (p. 406) after step 9 if desired.

ALCIAN BLUE-CHLORANTINE FAST RED

The demonstration of mucin and connective tissue.

Solution 1. Ehrlich's haematoxylin (p. 404).

Solution 2. Alcian blue 8 G (CI No.

74240)	0.5 g
Distilled water	100 ml
Acetic acid, glacial	0.5 ml
Thymol, as preservative	10–20 mg

Dissolve the alcian blue in 50 ml of the distilled water and add the acetic acid to the remainder. Combine the two solutions, filter and add the thymol. Record date and label.

Solution 3. Phosphomolybdic acid, 1 per cent aqueous solution.

Solution 4. Chlorantine fast red 5 B

(CI No. 28160)	0.5 g
Distilled water	100 ml

Procedure

1. De-wax and hydrate.
2. Stain with solution 1 for 10–15 min.
3. Blue in tap water.
4. Stain with solution 2 for 10 min.
5. Rinse in distilled water.
6. Mordant in solution 3 for 10 min.
7. Rinse in distilled water.
8. Stain with solution 4 for 10–15 min.
9. Rinse in distilled water.
10. Dehydrate, clear and mount.

Results

Cell nuclei, purplish-blue; mucin, ground substance of cartilage, certain connective tissue fibres and mast cell granules, bluish-

green; cell cytoplasm and muscle fibres, pale yellow; collagen fibres, red.

GRAM'S STAIN (after Hucker and Conn) FOR BACTERIA

Ammonium oxalate-crystal violet solution

Solution 1. Crystal violet (CI No. 42555),

10 per cent alcoholic

solution

2 ml

Distilled water

18 ml

Ammonium oxalate, 1 per cent

aqueous solution

80 ml

Dissolve the potassium iodide in the distilled water and then add the iodine. Record date and label.

Gram's iodine

Solution 2. Potassium iodide

2 g

Iodine

1 g

Distilled water

100 ml

Decolorizer

Solution 3. Ethyl alcohol, 95 per cent.

Counterstain

Solution 4. 1 per cent neutral red (CI

No. 50040)

15 parts

Carbol fuchsin (p. 421)

1 part

Procedure

1. De-wax and hydrate.

2. Apply solution 1 for 1 min.

3. Rinse with water.

4. Apply solution 2 for 1 min.

5. Rinse with water.

6. Apply several changes of solution 3 until no more colour appears to flow from the preparation.

7. Wash with water.

8. Apply solution 4 for 10 s.

9. Dehydrate, clear and mount.

Results

Gram-positive organisms, blue-black; Gram-negative organisms, red.

**ZIEHL-NEELSEN'S STAIN FOR THE STAINING OF ACID-FAST
BACTERIA**

Solution 1. Ziehl-Neelsen carbol fuchsin.

Basic fuchsin (CI No. 42510)	1 g
Absolute ethyl alcohol	10 ml
5 per cent phenol in distilled water	100 ml

Dissolve the basic fuchsin in the ethyl alcohol. Combine with the phenol solution, record date and label.

Solution 2. Acid-alcohol, 1 per cent (p. 403).

Solution 3. Methylene blue, 0.2 per cent aqueous solution.

Procedure

1. De-wax and hydrate.
2. Flood the slide with solution 1. Heat by flaming until stain rises and re-heat at intervals. Stain for 10-15 min.
3. Wash with running tap water.
4. Differentiate with solution 2 until only the red blood corpuscles retain the stain when examined under the staining microscope with the 10 \times objective.
5. Wash with running tap water for a minimum of 10 min.
6. Counterstain with solution 3 for 2 min.
7. Wash with running tap water.
8. Dehydrate rapidly, clear and mount in neutral balsam or DPX.

Results

Acid-fast bacilli, red; cell nuclei, blue; red blood corpuscles, pink.

CARBOL-FUCHSIN-TERGITOL METHOD

Solution 1. Kinyoun's carbol fuchsin.

Basic fuchsin (CI No. 42510)	40 g
Phenol, cryst.	80 g
Ethyl alcohol, absolute	200 ml
Distilled water	1000 ml

Dissolve the basic fuchsin in the alcohol and the phenol in the distilled water. Combine the two solutions, mix and allow to

remain at room temperature overnight. Filter through wet paper and label.

Solution 2. Tergitol 7.

Solution 3. Acid-alcohol (p. 403).

Solution 4. Methylene blue (CI No. 52015), 3 per cent aqueous solution.

Procedure

1. De-wax and hydrate.
2. Flood the slide with solution 1.
3. Add one drop of solution 2 to the slide and stain for 1 min at room temperature.
4. Wash in water.
5. Differentiate in solution 3 for 10–15 s until the tissue appears clear.
6. Counterstain with solution 4 for 1 min.
7. Wash in water.
8. Dehydrate in 95 per cent alcohol and acetone.
9. Clear in xylene and mount in balsam.

Results

Acid-fast organisms, bright red; background, blue.

Notes—(a) Tergitol, wetting agent 7, is a 25 per cent aqueous solution of the sodium sulphate derivative of 3,9-diethyltridecanol-6 obtainable from BDH. (b) Solution 4 is a saturated solution and should be filtered before use. Any methylene blue solution will suffice providing that it does not contain potassium hydroxide, as this gives a blurred counterstain.

PERLS' METHOD FOR FERRIC IRON

The demonstration of ferric salts in tissue.

Solution 1. Potassium ferrocyanide, 2

per cent aqueous solution 1 volume

Hydrochloric acid, 2 per

cent aqueous solution 1 volume

Solution 2. Neutral red (CI No. 50040), 1 per cent aqueous solution.

Procedure

1. De-wax and hydrate.

2. Transfer to freshly prepared solution 1 for 30–60 min.

3. Wash in distilled water.
4. Stain with solution 2 for 3 min.
5. Wash in distilled water.
6. Dehydrate, clear and mount.

Results

Cell nuclei, red; ferric iron, blue.

VON KÓSSA'S METHOD FOR CALCIUM

The demonstration of calcium.

<i>Solution 1.</i> Silver nitrate	5 g
Glass distilled water	100 ml

Dissolve the silver nitrate in the distilled water. Store in a brown reagent bottle and label.

Solution 2. Sodium thiosulphate, 5 per cent aqueous solution.

Solution 3. Kirkpatrick's carmalum.

Note—(a) Solution 1 should be colourless. If tinged with blue it must be discarded. The distilled water must be iron free. (b) Fixatives containing an acid or potassium dichromate should be avoided.

Procedure

1. De-wax and hydrate.
2. Immerse in solution 1 for 5 min or longer, exposing to bright daylight.
3. Wash in distilled water.
4. Fix in solution 2 for 5 min.
5. Wash in distilled water.
6. Stain with solution 3 for 3–5 min.
7. Dehydrate, clear and mount.

Results

Calcium phosphate and carbonate, black; cell nuclei, red.

OIL RED O IN ISOPROPANOL (Lillie and Ashburn)

The demonstration of neutral fats.

<i>Stock solution.</i>	Oil red O (CI No. 26125) Isopropyl alcohol, 99 per cent (isopropanol)	0.5 g 100 ml
----------------------------	---	-----------------

<i>Solution 1.</i> Stock solution	6 volumes
Distilled water	4 volumes

Dilute the stock solution with the distilled water. Allow the diluted stain to stand for 10 min and filter through a Whatman's No. 2 filter paper. The filtrate will remain stable for several hours.

Solution 2. Mayer's acid-alum-haematoxylin (p. 403).

Procedure

1. Stain formalin-fixed frozen sections in solution 1 for 10 min.
2. Wash in distilled water.
3. Stain lightly in solution 2 for 1-2 min.
4. Blue in tap water.
5. Float out onto a slide.
6. Mount in glycerine jelly.

Results

Lipids, red; cell nuclei, blue.

Note—Sudan black B (CI No. 26150) may be substituted for oil red O.

PROPYLENE GLYCOL SUDAN METHOD (Chiffelle and Putt)

The demonstration of neutral fats.

<i>Solution 1.</i> Sudan IV	0.7 g
Propylene glycol	100 ml

Dissolve the sudan IV in the propylene glycol by heating to 100 °C for a few minutes. Filter the solution while still hot through a Whatman's No. 2 filter paper. Cool the filtrate and refilter through glass wool or a coarse sintered glass funnel using negative pressure. Record date and label.

Solution 2. Propylene glycol, 85 per cent.

Solution 3. Mayer's acid-alum-haematoxylin (p. 403).

Procedure

1. Wash formalin-fixed frozen sections in several changes of distilled water.
2. Dehydrate in pure propylene glycol for 3-5 min.
3. Stain with solution 1 for 5-7 min, periodically agitating the sections.
4. Differentiate in solution 2 for 2-3 min.
5. Wash in distilled water.
6. Counterstain lightly in solution 3 for 1-2 min.

7. Wash in distilled water.
8. Blue in tap water.
9. Float onto a slide.
10. Mount in glycerine jelly.

Results

Neutral fats, myelin, mitochondria and other lipids, orange-red; cell nuclei, blue.

19

Cytological Techniques

Exfoliative cytology entails the microscopical examination and interpretation of cells which are shed (exfoliated) spontaneously from epithelial surfaces of the body, or which may be removed from such surfaces or membranes by physical means.

Spontaneous exfoliation is a characteristic of normal epithelial surfaces which, because of constant growth, continue to shed cells from their superficial layers as they become replaced by new cells, but cells of malignant tumours exfoliate more readily than those from normal tissue even though the lesion may be so small as to escape clinical detection.

The diagnosis of cancer from smear preparations has been carried out in pathology laboratories for many years, but considerable impetus was given to diagnostic cytology by the work of Papanicolaou and the introduction of his colourful staining procedures, which led to a greater knowledge of cell morphology and its alteration in disease. Papanicolaou employed alcoholic fixing and staining solutions, thus giving increased transparency to stained preparations and allowing overlapping cells to be more readily seen and identified.

Exfoliated cells can be found in smears taken directly from epithelial membranes or certain accessible cavities of the body where they may accumulate, for example vagina, buccal mucosa; or from a variety of body fluids and effusions including sputum, urine, pleural fluid and gastric juice.

Cytology has become an established aid for the diagnosis of malignancy in various organs, particularly those of the respiratory, urinary and female genital tracts. The collection of material for vaginal cytology for example is readily available with minimal discomfort to the patient; this offers a relatively simple means of 'screening' for the detection of asymptomatic cancer in women.

Assessment of hormone activity in the female, which is of value in some cases of sterility and certain endocrine disorders, also can be established from vaginal smear study.

It has been found that the majority of nuclei belonging to the female sex show a conglomeration of chromatin which is thought

to represent the X-X chromosome; this observation is used to determine genetic sex. Scrapings from the oral (buccal) mucosa provide suitable material for this purpose.

Unlike histopathology, in which diagnosis of malignancy is formed often on the general behaviour and arrangement of cell aggregates, cytological diagnosis is based on the appearances of individual cells or small groups of cells. Most of the information in this respect is obtained from study of the nuclei, whereas the cytoplasm may assist to identify cell type. Nuclear abnormalities associated with cancer include the following:

1. Nuclear enlargement without an increase in the overall size of cell, giving a decreased cytoplasm/nucleus ratio.
2. Irregularity of nuclear outline, and variation in size and shape.
3. Hyperchromasia. Due to increased amounts of deoxyribonucleic acid (DNA), the nuclei of malignant cells often stain more deeply with basic dyes.
4. Multinucleation, resulting from abnormal cell division.
5. Uneven distribution and variation in size of chromatin particles.
6. Increase in size and number of nucleoli.

FIXATION

To prevent cellular distortion it is essential that all smear preparations for cytological study be fixed immediately before drying of the material occurs.

The fixative should be capable of penetrating rapidly, with good preservation of cell morphology. The fixing fluid advocated by Papanicolaou, consisting of equal volumes of ether and 95 per cent ethyl alcohol, is commonly used, but a mixture containing tertiary butyl alcohol, 3 volumes; 95 per cent ethyl alcohol, 1 volume, is equally effective and does not evaporate so readily. Smears should remain in fixative for a minimum of 15 min prior to staining, although prolonged fixation of several days or weeks is not harmful. The fixative supplied by the Department of Health and Social Security free of charge, has been used successfully for the fixation of all types of cytological smears. It requires dilution with 95 per cent alcohol before use.

A useful container and carrier for smear fixatives is the polythene screw-capped Coplin jar grooved to take up to 10 slides.

It is suitable for the transportation of smears from wards and clinics to the laboratory, but it should not be used for postal transmission because of the inflammable nature of the contained fixing fluids.

Smears which require to be sent by post to a cytology laboratory may be air-dried after fixation is complete and placed in suitable wooden, cardboard or plastic slide mailers. Alternatively, aerosol sprays are obtainable which offer an effective and convenient form of fixation and, with certain exceptions, are relatively cheap. They consist usually of polyethylene glycols, isopropyl alcohol and propellants. The alcohol fixes the smear and the wax sets to form a water-soluble protective coating. Some workers advise coating pre-fixed smears with glycerin or water-soluble wax before mailing; alternatively, they may be fixed and despatched in jars containing a non-combustible glycol fixing mixture.

On collection, each slide should be clearly marked with the patient's name or number; this is a simple matter if the smears are made on slides having a frosted patch at one end. The relevant information is written on the patch with a graphite pencil and this will survive normal handling of the slide throughout fixation and subsequent staining.

PREPARATION OF SMEARS

The successful evaluation of cytological material depends, to a great extent, on the technical quality of the preparations. The material often contains only scanty diagnostic evidence, and this may be unrecognizable at screening unless care is taken during the preparation of smears. The smears must be spread evenly, and be free from lumps. Areas containing unresolved lumps cannot be stained accurately and are usually too thick for critical microscopical study.

It is desirable that smears are made and fixed by a member of the cytology laboratory staff in order to obtain some uniformity of spread, and specimens such as sputum, bronchial aspirations, urine, pleural and abdominal fluids should be dealt with invariably by laboratory personnel.

Smears from the vagina, cervix, breast, etc., are prepared at the side of the patient and sent to the laboratory in containers of fixative, a supply of which should be maintained regularly in participating clinics and wards.

VAGINAL SMEARS

Material is usually obtained by aspiration of the posterior vaginal fornix with a stout-walled, slightly curved, glass pipette fitted with a rubber bulb (*Figure 19.1*). The aspirate should be spread rapidly and evenly onto pre-labelled clean glass slides which are put with-

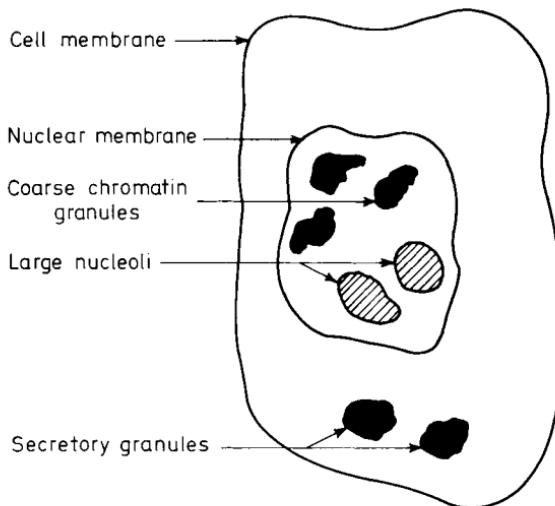


Figure 19.1. Diagrammatic representation of a malignant cell. Compare with Figure 12.1. Note the enlarged nucleus with consequent alteration of nucleus:cytoplasm ratio

out delay into a jar of fixative before drying occurs. Smears prepared by this method may contain not only vaginal cells but also cells exfoliated from other parts of the genital tract, for example endometrium and cervix. Another method, whereby material is gently scraped from the lateral wall of the vagina with a wooden spatula, is reliable only for hormonal studies.

CERVICAL SMEARS

To assist in the collection of material for this type of smear the clinician employs an instrument known as a speculum. The appliance is inserted into the vagina and allows the uterine cervix to be directly observed. A spatula or cotton-wool tipped applicator is introduced via the speculum and the cervical surfaces are gently swabbed or scraped. The Ayre spatula (*Figure 19.2*), made of wood or plastics material, is specially designed for this purpose, one end being shaped to fit the contours of the cervix. On with-

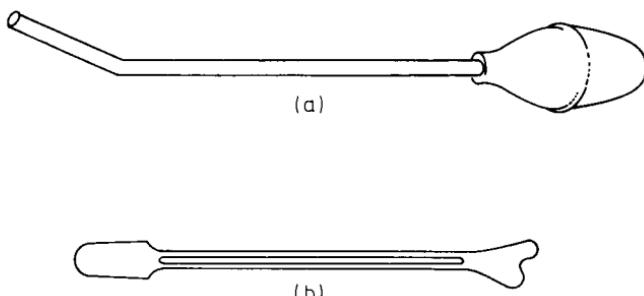


Figure 19.2. (a) Bulb and pipette for aspiration of posterior fornix; (b) Ayre spatula

drawal, the material contained on the swab or spatula is spread onto clean slides and fixed immediately.

SPUTUM SMEARS

Early morning specimens are recommended and should be produced by a deep cough. Several consecutive daily samples are advised ranging from 3 to 9 days. Suitable materials on subsequent microscopical examination should contain histiocytes and if these are not present the specimen may be discarded.

The sputum is poured from its container into a petri dish and examined microscopically against a black and white background for the presence of blood-flecked or white solid particles. If present, portions of these areas are removed and spread on a slide with a wire loop or spatula, the solid particles being crushed by means of a second slide. If no particles are found, smears should be made so as to include several representative portions of the specimen. The ideal smear should be somewhat thicker than one prepared for tubercle bacilli examination and should show a variation of thickness along its length. The prepared slides are transferred directly to fixative for 1–2 h.

SMEARS FROM URINE, PLEURAL AND ASCITIC FLUIDS AND GASTRIC WASHINGS

It is of the utmost importance that smears from fluid specimens are prepared and fixed as soon as possible after collection, otherwise much cellular detail will be lost. Cells of gastric fluids in particular undergo rapid degeneration and digestion at room temperature. Refrigeration will arrest these destructive processes for a short time only.

On receipt, the specimens are placed in 50 ml tubes, carefully balanced, and centrifuged at 2000 rpm for 20 min. The supernat-

ant fluid is decanted and the sediment spread evenly on to slides with a wire loop. The prepared slides are placed immediately in fixative in the same manner as other smears.

Sediments of fluids containing little or no protein, for example urine and gastric washings, tend to wash off the slides during fixation and staining. Adhesion of these sediments is improved if the slides are lightly coated with Mayer's albumen before spreading.

It must be remembered that many of the specimens sent for cytological investigation may be infected. Aseptic precautions should be observed during the handling of *all* specimens received for cytology, and specimens, glassware and other materials used in the preparation of smears must be autoclaved or placed in anti-septic solution before cleaning or discarding.

MEMBRANE FILTERS

Cellulose acetate membrane filters of graded pore size are useful for the concentration of cells from most body fluids. They are particularly useful where only a few cells are present because the total cellular content of a fluid may be collected onto a single membrane. A variety of pore sizes are available, the most useful being one with a mean flow pore size of 5 µm.

The specimen is filtered through a membrane attached to a special funnel-type holder using controlled negative pressure. Immediately following filtration the cells are fixed by placing the membrane in 96 per cent alcohol. It is then clipped to a slide, stained by the Papanicolaou method, dehydrated and cleared. After clearing the membrane filter pad may be cut into several strips and mounted onto microscope slides for examination. Special mounting media may be required for certain types of membrane filters.

Fixation of cells in fluid specimens may be carried out by adding an equal volume of formal saline prior to filtration, but generally the cells adhere to the membrane more readily and securely if fixation follows filtration.

CYTOLOGICAL STAINING TECHNIQUES

Papanicolaou method

The Papanicolaou method is designed to give sharp nuclear staining, transparency of cytoplasm and good differential colouring of acidophilic and basophilic cells.

The solutions required are Harris's alum haematoxylin, Orange G (OG 6), and a triple-dye mixture designated EA 36 or EA 50. All these solutions may be purchased commercially and give consistently good results. The formulas are as follows:

HARRIS'S ALUM HAEMATOXYLIN (see p. 405)

ORANGE G SOLUTION (OG 6)

0.5 per cent Orange G (CI No.	
16230) in 95 per cent alcohol	100 ml
dodeca-Tungstophosphoric acid	0.015 g

EA 36 OR EA 50

0.5 per cent light green SF (yellowish) (CI No. 42095) in 95 per cent alcohol	45 ml
0.5 per cent Bismarck brown (CI No. 21000) in 95 per cent alcohol	10 ml
0.5 per cent Eosin Y (CI No. 45380) in 95 per cent alcohol	45 ml
Phosphotungstic acid	0.2 g
Saturated aqueous lithium carbonate	1 drop

Mix well and store in tightly capped, brown bottles. A variation known as EA 65, requiring 0.25 per cent light green, is recommended for sputum staining.

Procedure

1. Remove smears from fixative and rinse in descending grades of alcohol (80, 70, 50 per cent), for 8–10 s each.
2. Stain in Harris's haematoxylin for 4 min.
3. Wash in tap water for 1–2 min.
4. Differentiate in 0.5 per cent hydrochloric acid until only the nuclei are stained.
5. Wash and 'blue' in tap water for 6–10 min.
6. Transfer to 70 per cent alcohol followed by two changes of 90 per cent alcohol for a few seconds each.
7. Stain in OG 6 for 2 min.
8. Rinse in three changes of 95 per cent alcohol.
9. Stain in EA solution for 2–4 min.
10. Rinse in three changes of 95 per cent alcohol.
11. Complete dehydration in absolute alcohol and clear in xylene.
12. Mount in DPX.

Result

Nuclei	Blue
Acidophilic cells	Red
Basophilic cells	Blue-green
Erythrocytes	Orange-red

Hormone assessment

Vaginal smears stained by the Papanicolaou technique may be used for the evaluation of hormonal (oestrogen) activity. Two of the methods commonly employed are the cornification index (CI) and the more accurate maturation index (MI). The cornification index, also known as the Karyopyknotic index (KPI) is calculated by the counting of at least 200 squamous cells and expressing as a percentage those that exhibit condensed, deeply stained, structureless (pyknotic) nuclei, with pink or red stained cytoplasmas. These are the superficial cornified squamous epithelial cells. Smears should be taken regularly, e.g. every 3 days, throughout the menstrual cycle, thereby obtaining a simple but useful assessment of oestrogenic influence. The maturation index is an extension of the above method and requires a differential count of at least 200 squamous cells. The degree of cell maturation is determined by means of their morphology and staining reactions and classified as being of superficial intermediate or parabasal type. The result is expressed as a percentage. High or low oestrogenic activity is indicated by the preponderance of superficial or parabasal cells respectively.

Additional methods

Following fixation in ether-alcohol, smears may be treated as blood-films and stained by any of the Romanowsky stains (p. 585), or they may be brought to water via descending grades of alcohol and stained with haematoxylin and eosin as for sections (p. 403). Good results are obtained by these methods although they lack the transparency of cytoplasm found in Papanicolaou preparations.

Schaudinn's fluid (p. 325) is often used as the fixative for smears prior to staining with haematoxylin and eosin.

SHORR STAINING METHOD

Suitable for hormonal studies in vaginal smears. The method requires a single differential staining mixture.

Staining solution

50 per cent ethyl alcohol	100 ml
Biebrich scarlet (CI No. 26905), water-soluble	0.5 g
Orange G (CI No. 12630)	0.25 g
Fast green FCF (CI No. 42053)	0.075 g
dodeca-Tungstophosphoric acid	0.5 g
dodeca-Molybdophosphoric acid	0.5 g
Glacial acetic acid	1 ml

Procedure

1. Fix smears while moist in equal parts of ether and alcohol. 1-2 min is adequate.
2. Stain for 1-2 min in Shorr's stain.
3. Rinse in 70 per cent alcohol to remove excess stain.
4. Transfer to 95 per cent alcohol, followed by absolute alcohol for a few seconds each.
5. Clear in xylene and mount in DPX.

Result

Nuclei	Red
Superficial cornified cells	Brilliant orange-red
Non-cornified cells	Green-blue

METHYLENE BLUE

A single-stain rapid method may be employed in the cytological examination of fresh sputum for malignant cells.

Staining solution

Methylene blue	1 g
Distilled water	100 ml

Procedure

Purulent or blood-flecked particles are selected from the sputum with a wire loop. These are transferred to a clean slide and mixed thoroughly with one drop of the stain. The stained mixture is then covered with a large coverslip and spread by gentle pressure. The prepared slide can be examined immediately.

The preparations are not permanent and any suspicious areas detected on microscopical examination should be recorded photographically. Alternatively, the coverslip may be removed and the smear placed in ether-alcohol to fix. It can then be stained with haematoxylin and eosin for confirmation.

Fluorescence methods have been applied to the differentiation of malignant cells from benign cells. These are based on the principle that certain substances emit visible light when excited by ultraviolet or blue light, usually of 350–400 nm wavelength.

The fluorescent dye acridine orange is capable of combination with both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). When excited by ultraviolet light, the DNA emits a green or greenish-yellow fluorescence and the RNA an orange-red fluorescence. Malignant cells have an increased RNA content in their cytoplasm and will appear brilliant red.

Techniques based on this knowledge, and which permit the rapid scanning of smear preparations, have been devised. One such technique is as follows:

ACRIDINE ORANGE TECHNIQUE (BERTALANFFY)

Solutions required:

(a) *M/15 Potassium dihydrogen orthophosphate*. Dissolve 9.072 g potassium dihydrogen orthophosphate (KH_2PO_4) in 1000 ml distilled water.

(b) *M/15 Disodium hydrogen orthophosphate*. Dissolve 9.465 g disodium hydrogen orthophosphate anhydrous (Na_2HPO_4) in 1000 ml distilled water).

Phosphate buffer (pH 6). 87.8 ml of solution (a) are mixed with 12.2 ml of solution (b).

Acridine orange stock solution

Acridine orange (CI No. 46005)	0.1 g
Distilled water	100 ml
Dissolve and store in a dark bottle in a refrigerator.	

Acridine orange staining solution

Acridine orange stock solution	10 ml
Phosphate buffer (pH 6)	90 ml

0.1 M Calcium chloride differentiator

Calcium chloride (CaCl_2)	11.099 g
Distilled water	1000 ml

Procedure

1. Fix smears in ether/alcohol (1:1) mixture for 15 min.
2. Hydrate in descending grades of alcohol (80, 70 and 50 per cent) and distilled water.
3. Rinse rapidly in 1 per cent acetic acid and wash in distilled water.
4. Stain with acridine orange staining solution for 3 min.
5. Wash with phosphate buffer for 1 min.
6. Differentiate with 0.1 M calcium chloride solution until nuclei are clearly defined.
7. Wash thoroughly with phosphate buffer.
8. Mount with a coverslip using phosphate buffer as the mounting and examine by fluorescent microscopy.

It should be noted that orange-red fluorescence is not specific for malignant cells. Certain normal cells, micro-organisms and trichomonads also exhibit varying degrees of red or orange-red fluorescence. The examiner must be experienced, therefore, in the identification of cells and cell structures.

Sex chromatin (Barr bodies)

Twenty to thirty per cent of cells from the female show a mass of chromatin beneath the nuclear membrane which is not evident in the male. Material for examination is most conveniently obtained from scrapings of the buccal mucosa where Barr bodies are usually seen attached to the membrane of the interphase nuclei of epithelial cells. The 'drumstick' form attached to the lobed nuclei of polymorphs in blood films has the same significance.

A method for the demonstration of sex chromatin in buccal smears and which requires no differentiation is both simple and effective.

Staining solution

Cresyl fast violet acetate	0.5 g
Distilled water	100 ml

Procedure

1. Fix smears before drying occurs in 95 per cent alcohol for 30 min.
2. Transfer to 50 per cent alcohol for a few seconds and then to distilled water.
3. Stain with Cresyl fast violet acetate solution for 5 min.

4. Rinse quickly in tap water.
5. Dehydrate with 95 per cent alcohol followed by absolute alcohol.
6. Clear in two changes of xylene and mount in DPX.

20

Introduction to Microbiology

HISTORICAL SURVEY

In 1675 Antony van Leeuwenhoek (1632–1723), a draper living in Delft, Holland, described ‘little animals’ he found when examining stagnant rainwater under his home-made microscope. The making of lenses was a hobby, yet the scrupulous way in which he recorded and illustrated his experiments would have done credit to any present-day scientist. Many of the first ‘animalcules’, as he called them, were protozoa, but later experiments yielded the first recorded account of micro-organisms.

After his death, very little progress was made in determining the relation between bacteria and disease, until towards the end of the eighteenth century. It was then that Dr Edward Jenner (1749–1825) substantiated the belief that cow-pox gave protection to people against small-pox. He introduced the term vaccine (from the Latin *vaccina*—cow) and established the idea of immunity.

The quality of microscopes was rapidly improving and many more micro-organisms were being discovered, but it was still not generally accepted that they were the cause of disease.

Barri in 1836 helped to establish that micro-organisms could cause disease when, using a heat-sterilized pin, he transmitted a disease from a silkworm infected with a fungus to a healthy silkworm.

Even after evidence such as this, the real science of bacteriology did not begin until the middle of the nineteenth century.

Much credit must be given to Louis Pasteur (1822–1895), a French chemist. It was through his work on the sterilization of liquids that today we have the autoclave. His work on fermentation proved that the breakdown of sugar to alcohol was the result of the activity of micro-organisms. He learned how to isolate and cultivate bacteria and how to study their effect on animals. In 1878 he read a paper on the germ theory of disease which helped to establish that specific organisms can give rise to specific diseases.

During Pasteur’s imaginative studies, Robert Koch (1843–

1910) was making enormous contributions to bacteriology in a practical way.

He developed methods of fixing and staining bacteria using aniline dyes, he discovered the tubercle bacillus, he isolated the anthrax bacillus in pure culture, he discovered the cause of cholera, and in 1881 he published a method of producing pure cultures of bacteria by growing them on the surface of a solid medium. The medium he devised was a meat infusion broth solidified with gelatin, and poured onto a glass plate. This was the beginning of our present-day culture media. Agar soon superseded gelatin and later Petri introduced his masterpiece—the Petri dish.

Many others, such as Lister, with his introduction of antiseptic and aseptic techniques, contributed to the vast amount of knowledge which has developed into the science of bacteriology. Today, with our ever-increasing knowledge of bacteria, fungi and yeasts, rickettsia, viruses, and protozoa, the more appropriate term Microbiology (from the Greek *micros*—small, *bios*—life) has come into general use.

A CLASSIFICATION OF MICRO-ORGANISMS

Although we are mainly concerned with bacteria, a brief description of other micro-organisms will be helpful. The size of bacteria is measured by the use of a graduated eyepiece calibrated by a micrometre slide, and the unit of measurement is the *micro-metre*, written μm (Greek *mu*). The micrometre is $1/1000$ of a millimetre (0.001 mm) or $1/25000$ of an inch. Viruses, being smaller than bacteria, are generally measured in millimicrometres ($1/1000$ of a micrometre, or $0.001\text{ }\mu\text{m}$ or $1.0\text{ m}\mu\text{m}$), now correctly called nanometres (nm).

Protozoa

These are small, single-cell animals belonging to the lowest division of the animal kingdom. They consist of protoplasm, which is differentiated into nucleus and cytoplasm, and they are non-photosynthetic. There are four classes of protozoa:

1. Class I RHIZOPODA move by means of protoplasmic projections called pseudopodia. *Entamoeba histolytica*, which causes amoebic dysentery, is an example.
2. Class II MASTIGOPHORA move by means of undulating mem-

brates or flagella. *Trichomonas vaginalis*, which causes a vaginal discharge, is a member of this class.

3. Class III CILIATA move by the beating of numbers of cilia, one member is *Balantidium coli*, which causes balantidial dysentery, a condition similar to amoebic dysentery.
4. Class IV SPOROZOA are non-motile organisms that live parasitically within the cells of host animals. *Plasmodium vivax*, the causal organism of malaria, belongs to this class.

Many protozoa when placed under unfavourable conditions pass into a resting phase, often with the formation of a distinctive cyst which can be used in identification.

Fungi

Like protozoa, fungi are non-photosynthetic organisms. They grow either as single cells, e.g. yeasts, or as colonies of multicellular filaments (hyphae), i.e. moulds. They reproduce by means of spores and the recognition of these spores is often an aid to identification. Some species cause disease in man and animals. For example, *Candida albicans*, a type of yeast, causes thrush, and *Microsporum canis*, a mould, causes ringworm.

Viruses and rickettsiae

These are minute organisms which can only multiply within living cells.

Viruses consist in their simplest form of an outer coat of protein and an inner core of nucleic acid which may be either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) (no virus has been shown to contain both).

Rickettsiae are small micro-organisms which are in some ways intermediate between viruses and bacteria. They are similar to bacteria in that they contain both RNA and DNA, possess metabolic enzymes and reproduce by binary fission; they resemble viruses by being able to multiply only within living cells.

Bacteria

Bacteria are microscopic unicellular organisms, the smallest having a diameter of about $0.5\text{ }\mu\text{m}$. *Figure 20.1* is a diagrammatic representation of a bacterial cell showing some of the essential constituents. The cell wall is a complicated lattice structure of lipoprotein, lipopolysaccharide and peptidoglycan, which gives the bacterial cell its shape and also protects the cytoplasmic membrane.

The cell wall of certain bacteria is covered with a capsule, which is usually a loosely attached slime layer consisting of polymerized sugars and amino sugars that are secreted by the organism. In some bacteria, notably *Bacillus* spp., the capsular material is polypeptide, e.g. polyglutamic acid in *B. anthracis*. In many cases, possession of a capsule correlates with virulence. The cytoplasmic membrane consists of a layer of lipoprotein and is 5–10 nm thick.

The cytoplasmic membrane consists of a layer of lipoprotein and is 5–10 nm thick. It encloses the cytoplasm, which contains soluble metabolites and precursors of macromolecules together

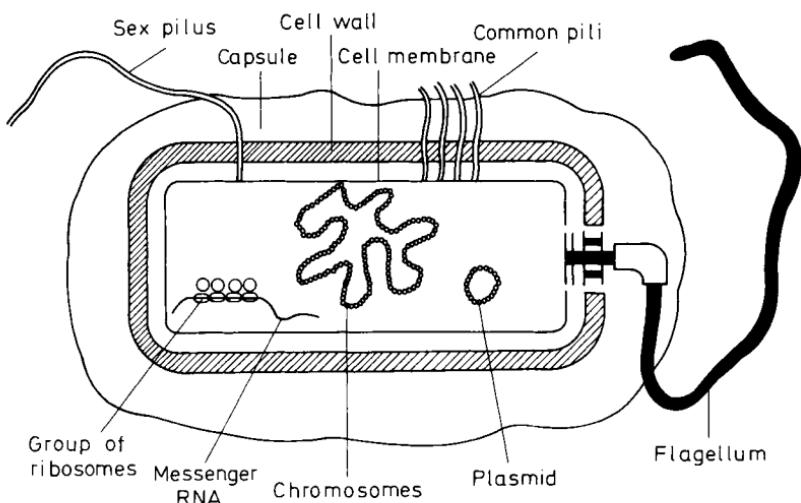


Figure 20.1. Diagram of a *bacillus* showing essential constituents

with organelles such as ribosomes in a proteinaceous gel. Lying within the cytoplasm is the bacterial *chromosome*—usually a single closed ring of double-stranded DNA. The information for making all of the cell's proteins is encoded in the DNA, and the assembly of these proteins is carried out on the ribosomes—which are made of RNA and protein. At binary fission a duplicate copy of the chromosome passes to the new cell, thereby ensuring uniformity among the descendants of a single cell (clone).

Recently, it has been found that many bacteria contain smaller circles of DNA, called plasmids, which often carry genes that confer antibiotic resistance on the cells carrying them. Of greater current interest is the fact that these plasmids may be transferred between cells of different type (e.g. non-pathogen to pathogen)

by a sort of mating process (*conjugation*) that involves the *sex pili* (see Figure 20.1).

Smaller pili (common pili or *fimbriae*) are often found on bacterial cells. These may be important in the attachment of pathogens to host tissue cells. The occurrence of antigenically similar

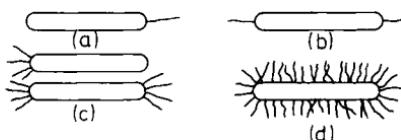


Figure 20.2. The flagella, which enable bacilli to move, may be arranged in one of four ways. (a) monotrichate—one flagellum at one pole; (b) amphitrichate—one flagellum at each pole; (c) lophotrichate—tuft of flagella at one or both poles; (d) peritrichate—flagella completely surrounding the bacterial body

pili on different species of bacteria can be a problem for the diagnostic bacteriologist.

Motile organisms possess flagella, which are thread-like appendages composed of protein called flagellin, and are about 20 nm thick. Their rotation enables bacteria to travel at speeds of up to 50 μm per s. Some organisms possess one flagellum, others more than one. The arrangement of the flagella may be as in Figure 20.2.

MORPHOLOGY

Bacteria can be classified into the following types of cell: the ovoid or spheroid, called *coccus*; the rod or cylindrical *bacillus*; the curved *vibrio*, the spiral-shaped *spirillum* and coil-shaped *spirochaetes*.

The *coccus* (plural *cocci*): size 0.5–1.0 μm in diameter. Coccii generally have one axis approximately equal to any other axis. Sometimes the cell is flattened (giving rise to a kidney-shaped cell) or distorted in some way as to depart from the spherical shape, e.g. in streptococci (see below).

If, after binary fission, the daughter cell remains attached to the parent cell, but separates before fission occurs again, these pairs of cocci are called *diplococci*. If fission continues whilst they remain attached, forming chains, they are termed *streptococci*, but if the division is not in one plane and random clumps of cocci occur, they are called *staphylococci*. Sometimes the cocci remain in pairs for one further division and a regular aggregate of four cocci is formed—these are called *tetracocci*, and if remaining for

one further division at right angles to the former, thereby giving rise to a cubical packet of eight cocci, they are called *sarcinae* (Latin = packets).

The terms *Staphylococcus*, *Streptococcus* and *Sarcina* are used as generic names (note the capital initials).

The *bacillus* (plural *bacilli*): size 1–10 µm in length, 0.3–1.0 µm in width. The bacilli or rods do not form as many groupings as the cocci, only forming *diplobacilli* or *streptobacilli* (pairs and chains). After fission, some rods form certain positions—the daughter cell, for example, remains attached to the parent cell, but swings away at varying angles, giving the appearance of Chinese lettering: a formation characteristic of the genus *Corynebacterium*. Sometimes a cuneiform bundle is the characteristic form, e.g. *Mycobacterium*.

Some bacteria, under unfavourable conditions, undergo changes resulting in the formation of intracellular spores. There is a localized concentration of nuclear material in the cell, with the subsequent development of a membrane around it. This is the resting stage of the bacillus, and germination does not take place until more favourable conditions arise. The mature membrane has a high resistance to ordinary staining, sunlight and heat. The spore often retains its capacity to germinate (generally the enlargement of the spore into bacillary form with subsequent shedding of the spore membrane) for many years.

The situation of the spore is an aid in the morphological diagnosis of the organism. Some occur at one end of the bacillus, with or without distension of the cell, others in the centre or towards one end.

The *Spirillum* (plural *spirilla*): Size variable—approximately 4 × 0.2 µm. Spirilla are rigid rods with helical (corkscrew) shape. They are motile by means of a tuft of flagella and are generally Gram-negative.

The *Vibrio* (plural *vibrios*): Size 4 × 0.5 µm. Vibrios are short, curved, rigid rods shaped rather like a comma. They are motile usually by means of a single flagellum and are generally Gram-negative.

Spirochaetes are also motile, and possess an axial fibre around which the body is twisted in a helical manner. Their length is usually 10–20 µm and thickness 0.2–0.4 µm. The number of spirals varies with the species. They are not easily stained with aniline dyes, and for the best results the silver impregnation methods (e.g. Levaditi) are used.

ELEMENTARY BIOLOGY OF BACTERIA

Bacterial metabolism

Basically, the properties and processes of life are essentially the same in all living things—whatever their size and whether they are plants or animals.

If an individual organism is to survive, it must be able to react to changes in its environment—it must be able to feed and respire and it must be able to reproduce.

To obtain optimal bacterial growth in laboratory-prepared media it is necessary to understand the metabolic role of nutrients. Metabolism can be considered as an interacting set of chemical reactions of which very few occur spontaneously and most have to be catalysed by specific proteins: *the enzymes*. There are two main types of reaction: those resulting in the breakdown of molecules (catabolic reaction) and those resulting in the synthesis of molecules (anabolic reaction). The energy needed to drive the synthetic reactions comes from the breakdown reactions, and the enzymes, which may number about 1000 in a single cell, are involved in its transfer.

The action of enzymes on their specific substrates is often used in the identification of bacteria. For example, the enzyme *urease* breaks down urea, $(\text{NH}_2)_2\text{CO}$ into ammonia (NH_3) and carbon dioxide (CO_2). The ammonium carbonate so formed can easily be detected in the growth medium by virtue of its alkalinity (pH indicator). The most obvious effect of oxygen on the growth of bacterial cells depends on whether it is used as the final hydrogen acceptor in its respiratory process, i.e. *aerobic respiration*. Bacteria which can grow only in the absence of free oxygen are termed *anaerobes*, and bacteria which can switch to alternative respiratory or energy-yielding pathways and therefore grow with or without oxygen are termed *facultative anaerobes*. Yet another group of bacteria grow best at reduced oxygen levels and these are called *microaerophilic*. All bacteria seem to need some CO_2 in the atmosphere and most grow more readily when a relatively high concentration (5–10 per cent) is supplied, irrespective of their requirement for oxygen.

Bacteria can be divided into groups based on their nutritional requirements in two different ways, *viz*—

1. on how they obtain their energy, and
2. on how they obtain the carbon needed for synthesis of all organic molecules.

1. ENERGY SOURCES

Some bacteria, found in water and soils, obtain energy from sunlight through the agency of pigments. These are called *phototrophs*. Other bacteria obtain energy for growth from the oxidation of inorganic compounds (*chemolithotrophs*) or from the oxidation, or fermentation, of organic compounds (*chemorganotrophs*). All bacteria of medical importance fall into this last category or, possibly, into the even more extreme one, the *paratrophs*. Paratrophs obtain their energy from the metabolism of the host cell and include viruses, probably rickettsiae, and possibly some bacteria.

2. CARBON SOURCES

Some bacteria, notably the phototrophs and chemolithotrophs, are able to grow with CO₂ as the sole carbon source. These are known as *autotrophs*. Most bacteria, however, require to be supplied with organic carbon molecules (e.g. sugars) and these are called *heterotrophs*. The range of possible carbon compounds that can be used for energy production or as carbon sources by different bacteria forms the basis of the 'sugar' fermentation reactions commonly employed in diagnostic bacteriology. Again, the most extreme group are the *hypotrophs*; these organisms rely on the enzymatic apparatus of the host cell for replication, e.g. viruses.

All bacteria must be supplied with water, inorganic salts—notably phosphates, a source of sulphur and a supply of nitrogen (necessary for both proteins and nucleic acids). Most, but not all, bacteria of medical importance will accept nitrogen only in organic form (e.g. peptones).

The range of temperatures over which different bacteria show optimal growth gives rise to three main groups:

The *thermophiles*, which have an optimal temperature of 55–75 °C,

the *mesophiles*, optimal temperature 30–45 °C, and the *psychrophiles*, optimum between 15–18 °C.

Most medically important bacteria are mesophiles.

Bacterial variation

Most of the genetic information of the bacterial cell is contained in the chromosomal DNA. This information is encoded in permutations of the four nucleotide bases: *thymine*, *adenine*, *cytosine* and *guanine*. The code is triplet, non-overlapping, and read sequentially, each set of three bases (Codon) coding for one

amino acid, e.g. *adenine-guanine-adenine* codes for *arginine*. The DNA nucleotide sequence is transcribed into a complementary sequence in messenger ribonucleic acid (mRNA). The mRNA is read on the ribosomes in conjunction with transfer RNA (tRNA) to link together amino acids to form polypeptides of the 'correct' sequences needed by the cell for its structural and functional (enzyme) proteins. It follows that if the bacterial cell is to continue to produce progeny identical to itself the information contained in the DNA must be capable of accurate duplication and transmission during cell division. Genetic changes may result from alterations that affect the sequence of bases. Therefore, while the maintenance of identity through many generations depends on accuracy of nucleic acid replication, transcription and translation, bacterial variability and adaptability depends on 'inaccuracies' occurring in one or other of the processes.

Several factors operate on bacteria which permit the exploitation of the inherent potential for variation to the advantage of the survival of the line of bacteria. Such factors include the rapid division times of bacteria growing optimally (e.g. 20 min for *Esch. coli*), thereby presenting many opportunities for genetic changes to occur; the essentially haploid nature of the bacterial genome, thereby allowing rapid expression of the results of genetic alterations; and the extremely important selection pressure exerted by the environment (outside or inside the laboratory). Most genetic alterations (mutations) are lethal to the cells receiving them but, because of the vast number of cells produced by optimally dividing bacteria, even low mutation rates (e.g. 10^{-6}) may produce at least one cell better able to cope should the environment, itself, alter.

This combination of events permits, in the short term, survival of the line during adverse conditions, e.g. during antibiotic treatment of a patient or an antibiotic-containing media in the laboratory. The long-term implication of the same processes is evolutionary exploitation of the many diverse environments in which we find bacteria today.

Bacterial associations

An organism living and multiplying within the living human body is termed a *parasite*, the body in this instance being a host. When harmless to the host, the parasite is termed a *commensal*, when harmful, a *pathogen*. Under certain conditions, commensals may become pathogens, and pathogens may assume a commensal role. Organisms living on dead matter are termed *saprophytes*. When both host and parasite mutually benefit the association is often

called *symbiosis*. This same term is used by some authorities irrespective of whether benefit occurs to either partner, but satellitism is the more correct term in these instances.

TOXINS

Certain substances produced by bacteria are injurious to the tissues and are capable of producing disease. These substances are termed *toxins*, and those produced and secreted into the environment whilst the organism is living are termed *exotoxins*; those that are liberated into the environment only on lysis of the organism are termed *endotoxins*. Exotoxins are produced mainly by Gram-positive bacteria, and are often relatively heat labile (destroyed at 60 °C). Endotoxins are often cell wall components of Gram-negative bacteria and are often relatively heat stable (withstanding 100 °C).

Exotoxins may be rendered non-toxic by the addition of chemicals such as formalin, or by heat treatment. When this conversion does not significantly impair the immunological properties, they can be used to produce active immunity in man and animals against the toxin and are called *toxoids*.

21

Microscopic Examination of Bacteria

Both shape and motility of bacteria can be studied by the microscopic examination of unstained preparations suspended in a fluid (the 'hanging drop' method), but to render the structures of cells visible, staining techniques must be used. These will only differentiate relatively gross individual structures, however, and to reveal those not shown by staining, more complex techniques, such as electron microscopy, are needed.

Apart from differentiating and rendering visible the constituents of a cell, staining will help to identify organisms and place them in their own particular group by their individual reactions to certain stains. An example is the Gram-positive or Gram-negative reaction to Gram's stain.

MAKING OF LOOPS

Wire loops or straight wires are necessary for making smears. They may be made of platinum or nicrome wire. Platinum by itself is too soft for making loops, and platinum wire is generally a mixture of platinum (90 per cent) and iridium (10 per cent). Nicrome wire is cheaper, more elastic and cools faster than platinum. It has to be renewed more frequently, however, as it burns.

Loops are usually circles of approximately 1.5 mm and 3 mm in

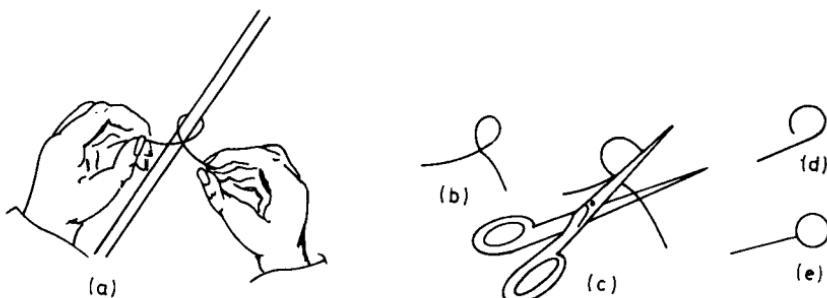


Figure 21.1. This illustrates the construction of wire loops necessary for making smears. For explanation see text

diameter. The thickness of the wire is SWG (Standard Wire Gauge) 26 or 27.

1. Wind the wire once round a metal rod of appropriate diameter (*Figure 21.1a and b*).
2. With a pair of old scissors, cut one arm of the wire at junction (*Figure 21.1c and d*).
3. Bend back the loop to centre it (*Figure 21.1e*).
4. Insert into a metal wire-holder.

Wire loops and disposable loops are available commercially.

MAKING OF SMEARS

GENERAL NOTES

1. Use clean slides free from grease.
2. Mark the slide with a glass writing diamond—grease pencil is easily rubbed away.
3. From liquid cultures make fairly heavy smears.
4. From cultures on solid media make thin smears.
5. Do not use water taken from rubber tubing attached to taps for making smears, as organisms may be transferred from the rubber.
6. When making films to demonstrate acid-fast organisms, use only one smear per slide and keep the slides apart when staining on a staining rack. This prevents any possibility of transference of acid-fast organisms onto another slide. Throw the slides away after use.
7. Never use Coplin or other staining jars for acid-fast material for the same reason as above.
8. When blotting slides, use a fresh portion of paper for each slide, to prevent transference of material.

From liquid media

1. Sterilize loop in bunsen flame.
2. Using aseptic precautions, withdraw 1 loopful of culture.
3. Transfer this to a clean slide and spread it with the loop, to form a thick film of liquid. Sterilize the loop.
4. Allow the film to dry without heating and then rapidly pass the slide 3 times through the bunsen flame. This kills the bacteria and fixes them to the slide.
5. Allow the slide to cool, and then stain the film by the requisite method.

From solid media

Aseptic precautions must be observed during the manipulation of culture tubes or plates.

1. Sterilize loop in bunsen flame.
2. Place 1 drop of distilled water on a clean slide, and resterilize loop.
3. With the loop or preferably a straight wire transfer to the slide a small portion of the growth to be examined and emulsify it in the drop of water until a thin homogeneous film is produced. Sterilize the loop.
4. Allow to dry, fix and stain.

MAKING OF HANGING DROP PREPARATIONS

When suspended in a fluid and examined microscopically many bacteria are seen to be motile, that is, they move from one position to another. True motility must not be confused with Brownian movement (vibration caused by molecular bombardment) or convection currents. A motile organism is one which *actively* changes its position relative to other organisms present.

1. Clean a slide and a 22 mm square coverslip.
2. On the slide make a ring of plasticine or Vaseline 2 cm in diameter. Alternatively a 'well slide' with a depression in the centre can be used.
3. Transfer a loopful of culture to the centre of the coverslip.
4. Gently press the ring of Vaseline or plasticine on to the coverslip, ensuring that the 'drop' of culture is in the centre of the circle, and does not come in contact with the slide. It is important that the slide and coverslip be completely sealed, otherwise 'draughts' can cause pseudo-motility. If a 'well slide' is used, seal the coverslip with Vaseline or nail varnish.

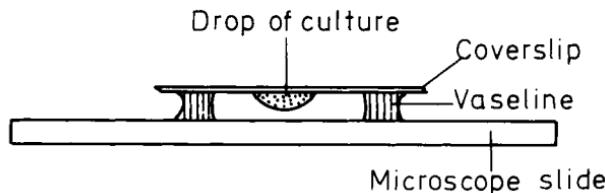


Figure 21.2. Hanging drop preparations

5. With a quick movement, invert the slide, so that the coverslip is uppermost (*Figure 21.2*).
6. Examine under the microscope, focusing first onto the edge of the 'drop', with the 16 mm objective (using a small cone of light), and when in focus swing round to the 4 mm objective to investigate motility.
7. Discard the whole hanging drop preparation into a jar containing a disinfectant, taking care that the disinfectant penetrates into the 'ring' and kills the culture.

MAKING OF WET PREPARATIONS

1. On a microscope slide, emulsify the specimen (such as faeces) in a small drop of saline, iodine or the required stain.
2. Carefully place a coverslip on to the suspension taking care that no fluid extrudes beyond the edges of the coverslip.
3. Paint the edges of the coverslip with nail varnish, paraffin wax or Vaseline. This effectively seals the preparations and prevents evaporation. Examine microscopically as for 'hanging drop'.

STAINING OF SMEARS

Gram's stain

In 1884 Gram described this method, which is the most important stain in routine bacteriology. It divides bacteria into two categories, depending on whether they can be decolorized with acetone, alcohol or aniline oil after staining with one of the rosaniline dyes such as crystal violet, methyl violet or gentian violet, and treating with iodine. Those that resist decolorization remain blue or violet in colour, and are designated Gram-positive, those that are decolorized and take up the red counterstain, such as neutral red, safranin or dilute carbol-fuchsin, are termed Gram-negative.

Although many investigators have tried to uncover the mechanism of the Gram reaction no universal answer has yet been found and it is possible that more than one mechanism exists.

Solution 1. Methyl violet 6 B (CI No. 42555) 0.5 g
Distilled water 100 ml

Dissolve the methyl violet in the distilled water and filter. Record date and label.

Lugol's iodine

Solution 2.	Iodine	10 g
	Potassium iodide	20 g
	Distilled water	1000 ml

Dissolve the potassium iodide in about 50 ml of the water, add the iodine, dissolve by shaking and make up to the final volume. Record date, label and store in a tightly stoppered bottle.

Decolorizer

Solution 3. Absolute ethyl alcohol or acetone.

Counterstain

Solution 4. Neutral red, safranin or dilute carbol-fuchsin.

PROCEDURE

1. Prepare a smear, allow to dry and fix with gentle heat.
2. Apply solution 1 for 30 s.
3. Replace solution 1 with solution 2 and allow to act for 30 s to 1 min.
4. Rinse with solution 3 and continue application until no more colour appears to flow from the preparation.
5. Wash with water.
6. Apply solution 4 for 3 min. (If dilute fuchsin is used, stain for 30 s.)
7. Rinse with water, blot carefully and dry with gentle heat.

Safranin

A counterstain for use with Gram's stain.

Safranin O (CI No. 50240)	5 g
Distilled water	1000 ml

Dissolve the dye in the distilled water and filter. Record date and label.

PROCEDURE

Apply the stain for 3 min, wash with water, blot carefully and dry with gentle heat.

Note—Some workers prefer to dissolve the safranin in ethyl alcohol and to dilute 10 ml of a saturated solution to 100 ml with distilled water.

Neutral red

A counterstain for use with Gram's stain.

Neutral red (CI No. 50040)	1 g
Acetic acid, 1 per cent	2 ml
Distilled water	1000 ml

Dissolve the neutral red in the distilled water and add the acetic acid. Filter, record date and label.

PROCEDURE

Apply the stain for 3–5 min, wash with water, blot carefully and dry with gentle heat.

Dilute carbol-fuchsin

A counterstain for use with Gram's stain.

Ziehl–Neelsen carbol-fuchsin	50–100 ml
Distilled water to	1000 ml

Add the carbon–fuchsin to distilled water and make up the volume to 1000 ml. Filter, record date and label.

PROCEDURE

Apply the stain for 10–20 s, wash with water, blot carefully and dry with gentle heat.

Note—While this stain may be of value as a counterstain for routine work it is not the stain of choice for the demonstration of *Neisseria gonorrhoeae* or other intracellular Gram-negative bacteria. Instead use neutral red.

Table 21.1 REACTION OF SOME ORGANISMS TO GRAM'S STAIN

<i>Gram-positive</i>	<i>Gram-negative</i>
Staphylococci	Coliforms
Streptococci	<i>Neisseriae</i>
Pneumococci	Vibrios
Corynebacteria	Spirochaetes
Clostridia	<i>Salmonellae</i>
Mycobacteria	<i>Shigellae</i>
Bacillus group	<i>Haemophilus</i> group

STAINS FOR ACID-FAST BACILLI**Ziehl-Neelsen stain**

The staining of *M. tuberculosis* and other acid-fast organisms.

Carbol-fuchsin

Solution 1. Basic fuchsin (CI No. 42510)	10 g
Phenol crystals	45 g
Ethyl alcohol, absolute	100 ml
Distilled water	900 ml

(a) Dissolve the phenol in the distilled water. Dissolve the fuchsin in the alcohol with the aid of gentle heat. Combine the two solutions, mix and allow to remain at room temperature overnight. Filter through wet paper and label.

or (b) Weigh the fuchsin and the phenol into a 2 litre flask and dissolve by heating over a boiling water bath. Shake the contents occasionally until solution is effected. Add the alcohol, mix thoroughly and add the distilled water. Allow to remain at room temperature overnight and filter through wet paper. Label.

Acid-alcohol

Solution 2. Hydrochloric acid (sp. gr. 1.19)	30 ml
Ethyl alcohol, absolute	970 ml

Counterstain

Solution 3. Methylene blue (CI No. 52015) or malachite green (CI No. 42000).

PROCEDURE

1. Prepare a smear, allow to dry and fix with gentle heat.
2. Apply solution 1 and heat until steam rises. Keep the stain hot for 5 min. (Do not allow the stain to boil or to dry on the slide.)
3. Wash with water.
4. Apply several changes of solution 2 until the preparation is colourless or a faint pink.
5. Wash with water.
6. Apply solution 3 for 20–30 s.
7. Wash with water, blot carefully and dry with gentle heat.

Auramine stain

The detection of acid-fast bacilli in sputum by fluorescence microscopy.

Staining solution

<i>Solution 1.</i>	Auramine O (CI No. 41000)	0.3 g
	Phenol crystals	3.0 g
	Distilled water	97.0 ml

Dissolve the phenol in the water with the aid of gentle heat. Add the auramine gradually and shake vigorously to effect solution. Filter, label and store in a dark stoppered bottle, when the solution will keep for about 3 weeks.

Decolorizer

<i>Solution 2.</i>	Sodium chloride	0.5 g
	Hydrochloric acid (sp. gr. 1.19)	0.5 ml
	Ethyl alcohol, 75 per cent	100.0 ml

Combine the alcohol with the acid and dissolve the sodium chloride. Label.

Counterstain

Solution 3. Potassium permanganate, 0.1 per cent solution.

PROCEDURE

1. Prepare a thin smear of sputum, allow to dry and fix with gentle heat.
2. Apply solution 1 for 10 min.
3. Rinse in tap water.
4. Apply solution 2 for 5 min.
5. Wash well in tap water.
6. Apply solution 3 for 30 s.
7. Wash well in tap water and drain dry. Do not blot.
8. Examine by fluorescence microscopy.

RESULT

Acid-fast bacilli appear as bright, luminous yellow rods against a dark background.

SIMPLE STAINS AND COUNTERSTAINS**Methylene blue**

A simple stain for routine use and as a counterstain with Ziehl-Neelsen stain.

Methylene blue (CI No. 52015) saturated alco-	
holic solution (approx. 1.5 per cent)	50 ml
Distilled water	950 ml

Combine the ingredients and filter. Record date and label.

PROCEDURE

Apply the stain for 30 s, wash with water, blot carefully and dry with gentle heat.

Malachite green

A counterstain for use with Ziehl-Neelsen stain.

Malachite green (CI No. 42000)	1 g
Distilled water	1000 ml

Dissolve the dyes in the alcohol and add the water and acetic acid. Record date and label.

PROCEDURE

Apply the stain for 20–30 s, wash with water, blot carefully and dry with gentle heat.

STAINS FOR CORYNEBACTERIA

Albert's stain

The routine staining of *Corynebacterium diphtheriae*.

Solution 1. Toluidine blue (CI No. 52040)	1.5 g
Malachite green (CI No. 42000)	2.0 g
Acetic acid, glacial	10.0 ml
Ethyl alcohol, 95 per cent	20.0 ml
Distilled water	1000.0 ml

Dissolve the dyes in the alcohol and add the water and acetic acid. Allow the solution to stand at room temperature for 24 h and filter. Label.

Solution 2. Iodine	6 g
Potassium iodide	9 g
Distilled water	900 ml

Dissolve the potassium iodide in about 50 ml of the water, add the iodine, dissolve by shaking and make up to the final volume.

PROCEDURE

1. Prepare a smear from an 18–24 h Loeffler serum culture of the test organism, allow to dry and fix with gentle heat.
2. Apply solution 1 for 3–5 min.
3. Wash with water and blot carefully.
4. Apply solution 2 for 1 min.
5. Wash with water, blot carefully and dry with gentle heat.

RESULT

Granules: bluish-black, remainder of organism green, other organisms usually pale green.

Pugh's stain

The routine staining of *Corynebacterium diphtheriae*.

Toluidine blue (CI No. 52040)	1 g
Ethyl alcohol, absolute	20 ml
Acetic acid, glacial	50 ml
Distilled water	950 ml

Combine the acetic acid with the distilled water and add the dye dissolved in the alcohol. Filter, record date and label.

PROCEDURE

1. Prepare a smear from an 18–24 h Loeffler serum culture of the test organism, allow to dry and fix with gentle heat.
2. Apply the stain for 2–3 min.
3. Wash with water, blot carefully and dry with gentle heat.

RESULT

Granules reddish-purple, remainder of organism light blue.

SPORE STAINS

The spore wall is relatively impermeable to stains, but spores can be stained by heating the preparations. The spore wall resists decolorization by such alcohol treatment as would be sufficient to decolorize vegetative organisms.

Fuchsin-methylene blue spore stain

- Solution 1.* Ziehl-Neelsen carbol-fuchsin.
- Solution 2.* Ferric chloride, 30 per cent aqueous solution.
- Solution 3.* Sodium sulphite, 5 per cent aqueous solution.
- Solution 4.* Methylene blue (CI No. 52015), 1 per cent aqueous solution.

PROCEDURE

1. Prepare a thin smear, allow to dry and fix with the minimum amount of heat.
2. Apply solution 1 for 3-5 min, heating the preparation until steam rises.
3. Wash in water.
4. Apply solution 2 for 1-2 min.
5. Replace solution 2 with solution 3 and allow to act for 30 s.
6. Wash in water.
7. Apply solution 4 for 1 min.
8. Wash in water, blot carefully and dry with the minimum amount of heat.

RESULT

Spores: bright red, remainder of organism blue.

Fuchsin-nigrosin spore stain (Fleming)

- Solution 1.* Ziehl-Neelsen carbol-fuchsin.

Decolorizer

- Solution 2.* (A) Nigrosin, 1 per cent solution (CI No. 50420)
or
(B) Sodium sulphite, 5 per cent solution.

- Solution 3.* Nigrosin 10 per cent solution.

PROCEDURE

1. Prepare a thin smear, allow to dry and fix with the minimum amount of heat.
2. Apply solution 1 for 5 min, heating the preparation until steam rises.
3. Wash with water.
4. Apply solution 2 (A) for 5-10 min or (B) for 5-30 s.
5. Wash in water, blot carefully and dry with the minimum amount of heat.
6. Place a small drop of solution 3 at one end of the slide and

spread in an even layer over the stained preparation with the edge of another slide.

7. Allow to dry and examine.

RESULT

Spore: bright red, remainder of organism unstained against a dark grey background of nigrosin.

CAPSULE STAINING

By ordinary staining methods, carbohydrate capsules are unstained, but are often seen as a clear zone around a stained organism. To demonstrate capsules either a direct staining or a negative staining technique is used.

Crystal violet capsule stain

Solution 1. Crystal violet (CI No. 42555), 1 per cent aqueous solution.

Solution 2. Copper sulphate, 20 per cent aqueous solution.

PROCEDURE

1. Prepare a thin smear, dry in air without fixation.
2. Apply solution 1 for 2 min without heating.
3. Wash with solution 2.
4. Blot carefully, dry in air and examine.

RESULT

Capsule: pale violet. Bacterial cell: deep violet.

Nigrosin-methylene blue capsule stain

<i>Solution 1.</i> Nigrosin (CI No. 50420)	5–10 g
Distilled water	100 ml
Formalin, as preservative	0.5 ml

Dissolve the nigrosin in warm distilled water, add the formalin and filter. Label.

Solution 2. Loeffler's alkaline methylene blue.

PROCEDURE

1. To one loopful of culture on a clean slide add one loopful of freshly filtered solution 1. Mix, allow to dry in air and fix with gentle heat.

2. Apply solution 2 for 30 s.
3. Rinse rapidly in water, blot carefully and dry with gentle heat.

RESULT

Bacterial cell: blue, capsule unstained against a dark grey background of nigrosin.

Note—Safranin may be employed in place of the methylene blue in solution 2.

India ink preparation

The demonstration of bacterial capsules in wet films. (Negative staining.)

PROCEDURE

1. Place one loopful of India ink on a perfectly clean glass slide.
2. Emulsify a small portion of solid bacterial culture in the drop of ink, or mix in a loopful of liquid culture.
3. Cover the mixture with a clean coverglass and press the latter down firmly, to form a very thin ink film. Seal the edges of the cover glass with paraffin wax or other suitable medium, and examine using oil-immersion objective.

RESULT

Bacteria: highly refractile, surrounded by a clear zone against a dark grey background of ink particles. Non-capsulated bacteria do not show this clear zone.

Simple stains

Most of the counterstains used in Gram's and Ziehl-Neelsen's method can be used as simple stains to show the morphology of organisms. In addition to these, the following stain can be used.

Loeffler's alkaline methylene blue

A basic dye for routine use in studying the morphology of micro-organisms in smears from cultures. The stain is more intense than neutral solutions of methylene blue and may show some degree of polychromatic staining.

Methylene blue (CI No. 52015), saturated alcoholic solution (approx. 1.5 g per 100 ml of 95 per cent alcohol)	300 ml
--	--------

Potassium hydroxide, 1 per cent aqueous solution	10 ml
Distilled water	990 ml

Measure the potassium hydroxide solution into the water and combine with the methylene blue solution. Mix thoroughly and filter. Record date and label.

PROCEDURE

Apply the stain for 30 s, wash with water, blot carefully and dry with gentle heat.

Polychrome methylene blue

This stain has a similar application to that of Loeffler's alkaline methylene blue and is of special value in McFadyean's reaction for demonstrating anthrax bacilli in blood.

PREPARATION FOR USE

Proceed as for the preparation of Loeffler's alkaline methylene blue and distribute the stain into bottles. Half fill the bottles and shake at intervals to thoroughly aerate the contents. Record date and label. The process of ripening may take several months but may be accelerated by chemical treatment.

PROCEDURE

See Loeffler's alkaline methylene blue.

22

Sterilization

The term *sterilization* strictly means the killing of all forms of life that may be present in a specimen or an environment. In bacteriology it is used to describe a variety of procedures directed to achieving this objective; examples are the destruction of bacteria in a contaminated sample and the active exclusion of unwanted bacteria from culture media by means of filtration techniques. The methods of sterilization that are used in laboratories may be conveniently divided into physical, chemical and mechanical methods.

PHYSICAL METHODS

Radiation

The commonest forms of electromagnetic radiation used in microbiology are ultraviolet light (uv) and the much more energetic gamma (γ) rays. It has long been known that exposure to direct sunlight slowly kills bacteria and that this is due to the uv rays which occur at the extreme limit of the visible spectrum. In the laboratory uv light is generated usually by means of a hot-cathode/low-pressure mercury vapour lamp and its bactericidal effect is maximal at wavelengths between 2500 and 2600 Å (Å = Angstrom unit = $1/10\,000 \mu\text{m}$). Ultraviolet light is absorbed by certain types of molecule found in living cells, notably nucleotides, and their electrons thereby gain extra energy. This is often sufficient to disrupt weak intramolecular bonds—such as the hydrogen bonds binding together the double helix of DNA. This, in turn, can cause intramolecular changes that are lethal to the cell. Some genes are more sensitive than others to this damage, for example those of which a cell has multiple copies will require a higher dosage to achieve the same effect as that observed when a single vital gene is inactivated.

Laboratory use of ultraviolet light is limited by very poor penetrating power. Even a thin glass coverslip is sufficient to protect bacteria on its undersurface completely, and drops of moisture in

aerosols may protect bacteria borne within them. The chief application of uv light is sterilization of the (still) air inoculating cabinets and 'sterile rooms', where the light may be left on for long periods between operations.

Gamma rays are an example of ionizing radiation, i.e. their energy is sufficient to knock peripheral electrons out of their orbits around the atomic nucleus and so produce ion pairs. These highly reactive ions (H^+ , OH^-) may be produced in the extracellular or intracellular water and interact with vital molecules in the cell. Additionally, such ionization may be produced in the DNA itself and cause irreparable damage.

The very high penetrating power of γ -rays makes them ideally suited to the sterilization of pre-packed disposable plastics, e.g. syringes. They are, however, useless for the sterilization of foods and pharmaceuticals because of chemical alterations produced in the products themselves. Special equipment is necessary for carrying out this procedure, in order to guard against irradiation of the operators (which could be fatal). This equipment is both heavy and costly to install and is therefore more likely to be used by manufacturers than by pathological laboratories.

Dry heat

Dry heat at high temperatures causes destruction of living cells and tissues by oxidation of their components. Its extreme form is simply the incineration of (inflammable and disposable) articles and their contaminating micro-organisms, e.g. the carcasses of infected animals. Less extreme applications are the raising of inoculating loops to red heat and the burning of alcohol on forceps, thereby incinerating the micro-organisms on their surfaces.

The commonest application of dry heat at moderate temperatures is in the use of the *hot-air oven*. This is used for materials that are unaffected by temperatures of 160–180 °C and for which autoclaving is unsuitable, e.g. dry glassware and unwettable materials such as powders, oils and waxes.

Air is a poor conductor of heat and the oven must not be packed so tightly as to impede circulation or to trap air pockets; a fan should always be fitted to aid convection. The poor heat conduction must be borne in mind when calculating exposure times. Powder contained in a 4 oz jar may take 45 min to reach the operating temperature of 160 °C and the sterilization time must therefore be increased by this period.

The sterilization periods commonly used are 160 °C for 1 h or 180 °C for 30 min. It is important to allow the oven to cool before

removing the contents lest, owing to rapid contraction of the air within petri dishes, unsterile air be sucked in. Occasionally, even modern glassware may be damaged by too rapid cooling.

Moist heat

BOILING WATER

A temperature of 100 °C will kill all non-sporing organisms within 10 min. Most spores will be killed in 30 min at this temperature, but some spores will resist boiling for several hours. The addition of 2 per cent sodium carbonate increases the bactericidal effect of boiling water, and spores that resist boiling water for 10 h have been killed in 30 min by this addition. This method is suitable for infected instruments (such as at animal autopsy) if they are to be used immediately, particularly as the sodium carbonate prevents rusting of the instruments. It is unsuitable if instruments are to be stored in a sterile condition.

STEAM AT 100 °C

Steam at 100 °C is used mainly to sterilize certain complex media, where the constituents might be 'broken down' (hydrolysed) at temperatures above 100 °C, e.g. sugars, gelatin, etc. Such media are sterilized by a form of intermittent steaming called 'Tyndallization'. This is steaming on three consecutive days. The medium is steamed for 30 min on the first day, incubated at room temperature overnight, steamed for a further 30 min on the second day, reincubated, and steamed again for 30 min on the third day. The first day's exposure kills non-sporing and vegetative organisms; the incubation period, provided the medium is favourable, allows germination of most spores, and the second steaming kills these. The repeated process usually ensures germination and subsequent killing of any spores remaining after the first and second exposures. It will be seen, therefore, that Tyndallization is only effective when the medium to be sterilized is favourable for the germination of spores: it is useless for non-nutritive fluids and may not kill anaerobic spore-bearers, unless the incubation is carried out anaerobically, and it will not kill thermophilic organisms.

STEAM UNDER PRESSURE

The sterilizing efficiency of steam under pressure is due to its temperature (> 100 °C), and its ability to condense on cooler, wettable objects, thereby rapidly transferring its *latent heat of vaporization* and raising their temperature. The change of volume caused by the condensation aids penetration of the steam and the moist

environment allows rapid heat coagulation of proteins—a feature that accounts for the ability of the process to achieve sterilization at temperatures much lower than those required by dry (oxidation) methods.

It should be noted that the part played by the *pressure* of the steam is solely the production of moist heat at temperatures above 100 C.

The simplest laboratory autoclaves are merely versions of the domestic pressure cooker. Steam is generated by applying heat (gas or electricity) to a small volume of water contained within the sealed body of the apparatus. All air *must* be displaced from the autoclave before bringing it up to sterilization pressure because it is the pressure, read from a gauge or pre-set by means of special valves, that is used as a guide to the steam temperature in those simple models. This relation (5 psi 110 C; 10 psi = 115 C, 15 psi 121 C, 20 psi 126 C) is true only if the atmosphere within the autoclave is pure steam. If, for instance, the autoclave contains half air, half steam 15 psi produces a temperature of 112 C. Under these conditions, sterilization might take as long as 12 h in contrast to the 15 psi (121 C) for 20 to 30 min routinely employed.

Another reason for ensuring that all air is displaced is that air pockets trapped between articles can behave only as 'hot air' at relatively low temperature and so fail to sterilize. This is true also if the steam becomes too dry (as may happen in steam-jacketed autoclaves when there is a higher pressure in the jacket than in the vessel); such steam is called *superheated* and behaves like 'hot air'.

Larger laboratory autoclaves are usually fed with steam from an external supply. The steam is usually admitted to the pressure vessel through a valve and baffle plate. The steam outlet valve is at the bottom and a thermocouple is inserted at this point to allow direct measurement of the temperature during various stages of the process. At the top of the vessel are mounted a pressure gauge and safety valve. Comparison of the pressure and temperature readings gives valuable information on the functioning of the apparatus.

TESTING OF AUTOCLAVES

Whether material is adequately sterilized may be determined by several methods. A method that is of particular value when the time factor for using the material is important is the use of Browne's sterilizer control tubes. These are tubes containing an

indicator liquid, which can be purchased for steamer, hot-air oven or autoclave. The liquid will change from red to green if the correct temperature-time combination has been employed; if not, they turn a reddish-brown colour.

Another method is by impregnating filter paper strips with a sporing organism, such as *Bacillus stearothermophilus*. The strip is enclosed in an envelope, and placed in a convenient part of the material undergoing sterilization. It must be remembered that the strip or Browne's tubes must be placed in a position where steam is least likely to penetrate. On removal, the spore strip is cultured in a suitable medium and incubated for 7 days at 55 °C. This method is excellent when time is not too important.

Commerically prepared strips of dried *B. stearothermophilus* are available and are used as described.

It is, however, recommended that whenever possible the working of an autoclave should be monitored by means of a series of thermocouples placed within the contents and calibrated to indicate the temperatures actually attained.

Low-temperature sterilization

Biological fluids may be sterilized by heating them in a water bath at 56 °C for periods of 1 h daily as long as may be necessary. The principle is the same as Tyndallization, but the lower temperature may necessitate more than three exposures to heat. If the temperature of 56 °C is exceeded, the fluids may be coagulated. This method of sterilization can be used only when the fluid does not contain resistant spores, or very thermoduric organisms.

Vaccines may be sterilized by placing them in a water bath at a temperature of 60 °C for 1 h. This is usually adequate, as vaccines are prepared under aseptic conditions and spores are not normally present. Temperatures higher than 60 °C may diminish the immunizing power of the vaccine.

CHEMICAL AGENTS

Many chemical agents are referred to as *disinfectants*, a term that is applied to substances which destroy micro-organisms on inanimate objects. Other terms with a similar meaning are *germicide* and *bactericide*. A disinfectant, which is non-injurious to human tissue, is called an *antiseptic* and chemicals which are used to prevent organisms growing in a sterile medium, but do not kill them, are called *bacteriostats*. The action of a disinfectant is modi-

fied by several factors. Some disinfectants are very efficient in the absence of organic matter, but are less effective in its presence.

Chemical agents function as sterilizing agents by the following lethal mechanisms:

1. Interfering with the enzymatic system of the organism (enzyme poisons).
2. Disruption of the cell membrane.
3. Coagulation of protein.
4. Oxidation.

Very many different compounds have been used as disinfectants and only a few commonly used examples are given below.

Alcohol (ethanol)

Absolute alcohol is not a very effective sterilizing agent, as at this concentration its power of penetration is very poor. When diluted with distilled water to a concentration of 70 per cent, however, it becomes effective as a skin sterilizer and is used prior to inoculations or venepunctures.

Chloroform

Chloroform is sometimes used to maintain the sterility of serum. When the serum is required for use it is placed in a 56 C water bath for a short while in order to evaporate the chloroform. To be effective, chloroform must be present in a concentration of 0.25 per cent.

Chlorine

Chlorine and its derivatives are used extensively in microbiological laboratories, especially in virology departments, where it is the disinfectant of choice. However, its activity is poor in the presence of organic matter and can be completely lost by combination with thiosulphate, sulphides and ferrous salts. It is necessary to guard against accidental mixture with such inhibitors.

Glycerol

Glycerol in 50 per cent solution will kill contaminating organisms. It is used for the preservation of certain viruses which are not affected by the glycerol.

Phenol and cresols

Phenol and cresols are powerful antiseptics. They are used mainly for discarded cultures, infected pipettes, and other infected

material. A 5 per cent solution is generally used: stronger solutions may be less effective owing to protection of organisms in the middle of a cluster of which the outer members have been coagulated into a shield.

Quaternary ammonium compounds

These are cationic detergents which are used mainly for skin disinfection and in the food industry. They are ineffective against *Mycobacterium tuberculosis*, bacterial spores and *Pseudomonas aeruginosa*. A form that is much used in hospitals is cetyl-trimethyl-ammonium bromide ('Cetrimide').

STERILIZATION BY FILTRATION

Several types of material have found use as microbial filters, such as diatomaceous earth, porcelain, asbestos, sintered glass and cellulose ester membranes.

Diatomaceous earth filters

Filter candles made of diatomaceous earth include the German 'Berkefeld' and the American 'Mandler' types. Berkefeld filters consist of Kieselguhr-asbestos and organic matter. They are made in three grades, V—viel (coarse), N—normal, and W—wenig (fine). The grade is assessed by the rate of passage of water through the filter at a standard pressure. V-grade filters are used mainly for 'clarifying' liquids, but they are not fine enough to sterilize liquids by holding back bacteria. The American Mandler filter candles are made from Kieselguhr-asbestos and plaster of paris. The three grades are determined by the amount of air pressure they will stand without bubbles being produced when the filter is immersed in water. This is known as the bubble pressure test, for example a filter with an average pore size of 2.3 µm will produce bubbles at 18 psi.

The diatomaceous earth filters are usually made in the form of hollow candles open at one end. A metal nozzle is inserted and cemented in the open end. These instruments may be used to filter liquid poured into the candles, or alternatively for the passage of fluid into the candle from without (*Figure 22.1*).

If necessary, these filters are autoclaved after use. They should then be well washed with soap and water, and scrubbed with a test-tube brush. A 2 per cent sodium hydroxide solution should then be passed through the filter in the opposite direction to that

in which it was last used. This is followed by passing N/1 HCl until the filtrate has a pH of approximately 7.4; washing through with distilled water; draining and drying. The instrument is finally wrapped in Kraft paper, and sterilized in the autoclave.

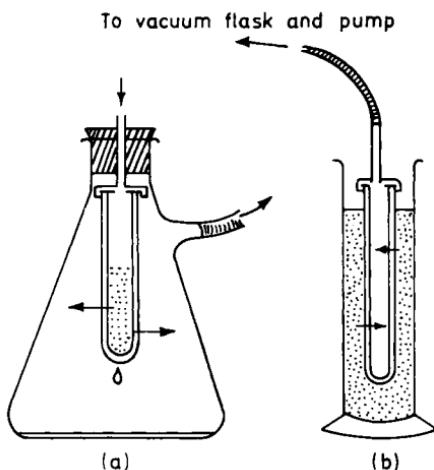


Figure 22.1. Two methods of filtration, using a Berkefeld filter candle

Porcelain filters

The most common type of porcelain filter is the Chamberland, a French filter candle made of kaolin and sand, and the English Doulton filter candle. The Chamberland filters are made in various porosities, which are graded L1, L1a, L2, L3, L5, L7, L9 and L11. L1 is for clarifying, and is equivalent to the Berkefeld V; the others are for sterilizing.

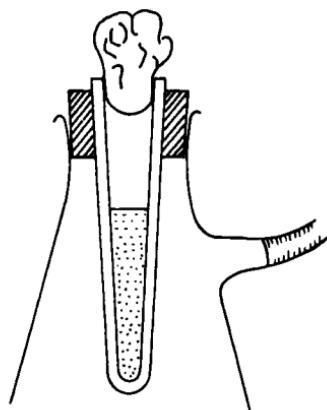


Figure 22.2. Filtration, using a porcelain filter candle

The Doulton filter candles are P2, P5 and P11: the last two being sterilizing filters. For use, the candle is mounted in a rubber bung, and filtration is effected from within outwards (*Figure 22.2*).

CLEANING AND STERILIZING

This may be done by the method described for Berkefeld and Mandler filters. A high-temperature muffle furnace may be used to remove all organic material, but this treatment eventually affects the porosity of the candle.

Seitz-type filters

The asbestos-disc or Seitz filter is probably the most satisfactory one for general purposes (*Figure 22.3*), and consists of an asbestos disc supported, rough side uppermost, in a metal mount.

The Seitz filter is attached to a vacuum flask through a silicone rubber bung. Red rubber bungs tend to vulcanize after heating, but the silicone rubber withstands repeated autoclaving.

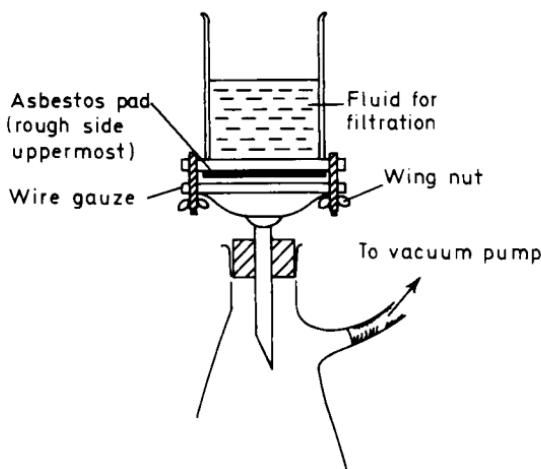


Figure 22.3. Filtration, using a Seitz-type filter

The side arm of the flask is plugged with non-absorbent wool and the filter unit wrapped in Kraft paper and sterilized at 15 psi for 20 min.

After use, sterilize if necessary, then discard the asbestos disc, clean the metal mount, insert a fresh disc, and sterilize by autoclaving. The asbestos discs are supplied in varying grades, the more important being as follows in Table 22.1. For small amounts a 'Hemming's filter' can be used. This consists of a filter pad fitted

between two bijou bottles. The bottles are centrifuged, and the liquid to be filtered is thereby forced through the pad from one bijou to the other.

Table 22.1 CARLSON-FORD FILTER SHEETS FOR STERILIZATION

HP PYR	For the removal of pyrogens.
HP EKS2	For removal of minute organisms from heavily infected liquids.
HP EKS	For absolute sterility using controlled standard conditions.
HP EK	For sterility down to <i>Serratia marcescens</i> (<i>C. prodigiosum</i>).

Notes—Never filter material containing solid matter if the latter can be removed by other methods, e.g. by centrifugation.

Test sterilizing filter candles periodically by filtering a culture of *Serratia marcescens* (*C. prodigiosum*) and testing the filtrate for sterility. Filtration should not be effected too rapidly; 5–10 drops per min is a suitable rate. When negative pressure is used this should not exceed 200 mmHg.

Sintered glass filters

Sintered glass filters are made of finely ground glass which is fused to make the particles adhere. They are available in 5 grades:

Grade 1	A coarse filter
Grade 2	A medium filter
Grade 3	For fine precipitations
Grade 4	For very fine precipitations
Grade 5	For bacteria

The grade 5 filter is generally supported by a disc of the grade 3 porosity and is called a 5/3 sintered glass filter.

To clean, first disinfect in a suitable disinfectant, run water through the filter in the reverse direction and then pass through warm sulphuric acid. Chromic acid should NOT be used, as this may have an adverse effect on the filter. The glass particles may become positively charged, causing possible adsorption of negatively charged material in the liquid being filtered. Wash well in tap water and distilled water, and dry in a warm—not hot—oven. Sterilize by steam under pressure.

Membrane filters

In recent years, membrane filters have replaced other types for many applications. These are now made of cellulose esters (cellulose nitrate or cellulose acetate) supported in a matrix of regenerated cellulose. Their advantages include:

1. ease of handling—they are relatively tough,

2. they are sufficiently inexpensive to be used once only,
3. electrical neutrality—charged molecules are not taken out of solution as readily as with other types,
4. minimal retention of solute,
5. accurate grading of pore sizes over a wide range.

Such membranes can also be used for bacterial counts in, for example, the examination of water supplies. A known volume of water is passed through a membrane filter: commercially available apparatus may pump many gallons through a single membrane. The membrane is then removed and placed on a pad moistened with an appropriate liquid culture medium (which may be selective, e.g. for pathogenic bacteria) and incubated. The colonies which develop can be counted to allow estimation of the numbers of viable cells in the original specimen. If these membranes are treated with microscopic immersion oil, they become transparent so that they can be examined by direct microscopy after staining the bacteria trapped on their surface.

23

The Use of Culture Media

ESSENTIAL REQUIREMENTS OF CULTURE MEDIA

To isolate, identify and study the characteristics of micro-organisms, it is essential to grow them on artificial media. As the basic requirements for bacterial nutrition are moisture, carbon and nitrogen, it is necessary for an artificial medium to provide these three essentials and for many pathogenic bacteria other components as well.

Moisture plays an important part in the nutrition of bacteria; in the absence of water bacteria cannot grow. (This fact is used in preservation of foodstuffs by drying, which although preventing bacteria from growing, will not necessarily kill them.)

Organisms cannot always obtain their nitrogenous and carbon requirements from complex proteins and these substances must be broken down into simpler compounds. This breaking down is performed by the organism's enzyme system. For their carbon requirements some bacteria can utilize the CO₂ in the atmosphere, whilst others have to decompose certain organic substances. The form in which nitrogen is added to the medium depends on the enzyme-reducing abilities of the organism. The simplest way of ensuring a supply of nitrogen is by the addition of peptone, as most organisms are capable of breaking down peptone into amino acids and using these.

In addition to the basic requirements of water, carbon and nitrogen, other chemical substances are necessary, such as sulphur, phosphorus, and very small traces of metal salts (referred to as *trace elements*) and in some cases certain vitamins and vitamin-like substances called '*essential metabolites*'.

With the addition of blood or serum, most common pathogenic bacteria can be cultivated.

Apart from these nutritional requirements, bacteria require certain other conditions before they will grow satisfactorily in or on artificial culture media. Oxygen must be present for the growth of some organisms, whilst others can live only in the absence of

free oxygen (Chapter 25). Some organisms will grow best in an alkaline medium, others in an acid environment, so that the accurate determination of the pH of the medium is essential (Chapters 24 and 27).

The temperature at which cultures are incubated must be observed, as the majority of organisms have an optimum temperature at which the most luxuriant growth is obtained. Organisms which grow in the human body are best cultivated artificially at approximately 37 °C (body temperature) and an incubator is used for this purpose. The medium must be sterile so that the organism inoculated in or on the medium is the only one cultivated, and external contamination must be prevented. Media are therefore prepared in tubes plugged with non-absorbent cotton wool, or in screw-capped bottles, and sterilized at temperatures which will not destroy the nutritional properties of the medium.

Liquid media

After introduction into a liquid medium, the organism takes a little time to adjust itself to its new environment—this is called the *lag phase*, but after this initial phase the organism commences to multiply by binary fission. This is called the *logarithmic phase*, as multiplication is by geometric progression. After a time, due to the exhaustion of the nutritional factors of the medium and the accumulation of waste products, some bacteria die, and there is a balance of dead and living bacteria. That is, the number of bacteria multiplying is equivalent to the number of dying. This is referred to as the *stationary phase*. After this short period of equilibrium the number dying is greater than the number multiplying and the *phase of decline* sets in.

As the organism grows in liquid media, it utilizes the components of the medium, and excretes by-products of bacterial metabolism into the medium. Provided the medium is originally free of these by-products, use can be made of their production to help identify the organism. For example, certain organisms produce a by-product called indole. By growing these organisms in a medium rich in the amino acid tryptophan and free from indole (e.g. peptone water) tests can be made on the culture to show whether the organism has, or has not, produced indole. Another use for liquid media is to demonstrate whether an organism has the power to ferment specific carbohydrates (sugars). To a sugar-free medium is added the specific carbohydrate, and the organism is then grown in the medium. An indicator included in the medium

shows whether acid has been formed due to the fermentation of the sugar, by the organism (*see p. 493*).

Solid media

In liquid media the bacteria are free to move about, but when grown in solid media they multiply at the site of inoculation and form colonies. The appearance of these colonies is often typical of the species. This makes possible the isolation of a single species of bacteria from a mixture. Liquid media are solidified by the addition of, for example, agar, a long-chain carbohydrate which does not affect the nutrient properties of the original medium.

PLATE CULTURAL METHODS

If colonial characteristics of an organism are to be examined, the petri dish is an excellent container for the medium. The shallowness of the dish and the large surface area render macroscopic examination of colonies easy, and, if necessary, microscopic examination is possible. The dish should be flat-bottomed, and either of heat-resistant soda-free glass or plastic. The most commonly used petri dishes are 90 mm diameter disposable plastic.

Glass petri dishes may be sterilized in copper tins which have a deep lid to prevent air penetration on cooling. They should be sterilized in a hot-air oven for 1 h at 160 °C and allowed to cool slowly in the oven.

Plate inoculation methods

To isolate single colonies, the medium in the petri dish should be inoculated as follows.

Using a sterile loop, smear a loopful of the specimen over area A (*Figure 23.1*). Sterilize the loop in the bunsen flame, and when

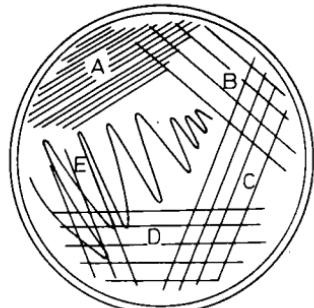


Figure 23.1. Plate inoculation method. (See text for explanation)

cool streak over area B. Repeat over area C, D and E. Incubate the plates at 37 °C. The maximum available area should be used but care must be taken not to cross a previously inoculated area.

An alternative method is to use a sterile spreader. This is a glass rod, 3 mm in diameter, bent at right angles and sterilized either by boiling, or by wrapping it in Kraft paper and placing in the hot-air oven at 160 °C for 1 h. A small amount of the specimen



Figure 23.2. Diagram illustrating a method of drying plates

is placed on the medium, and smeared over the whole surface, using a sterile spreader. With the same spreader, another petri dish is inoculated. Any of the specimen remaining from the first inoculation will be transferred to the second petri dish, and single colonies should be obtained. In both methods, it is essential that the medium surface is dry so that discrete colonies are obtained.

The drying of plates is performed by placing the flat surface of the lid onto an incubator shelf (at 37 °C) and angling the media-containing dish (media downward) either within or on the edge of the lid (Figure 23.2).

TUBE CULTURAL METHODS

Slope cultures

Many tests devised to differentiate organisms require solid cultures. It is not always necessary to grow an organism on a whole petri dish of medium, and slope cultures often suffice. 'Slopes'

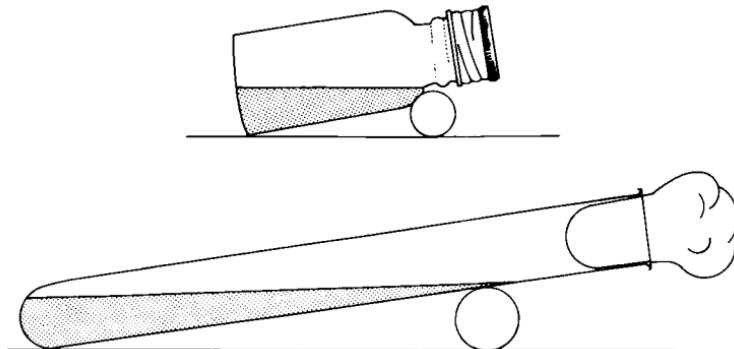


Figure 23.3. Slope cultures. A tube or a bottle containing a quantity of the medium to be solidified is slightly raised at one end

or 'slants' are tubes or bottles containing a small quantity of medium, that has been allowed to solidify with the bottles slightly raised at one end (*Figure 23.3*). Such slopes are used only for maintenance or biochemical tests *once the organism has been isolated in pure culture*.

Deep cultures

'Anaerobic' organisms (see p. 510) require an oxygen-free atmosphere. For cultivation of these organisms 'shake' or 'deep' cultures are sometimes made. The medium is distributed in 150 mm · 20 mm tubes to a depth of 6–7 cm and allowed to solidify. For use, the medium is melted, cooled to about 45 C, inoculated with the organism, and mixed by rotation between the palms of the hands. When it has solidified, the culture is incubated and the anaerobic organisms grow at the bottom of the tube. These shake, or deep, tubes can also be used for counts of viable organisms. In similar fashion, the medium is melted, cooled, inoculated with a known dilution of the organism and mixed. It is then poured into a sterile petri dish, and after incubation a count is made of colonies growing in and on the medium.

Roll tubes

The 'roll tube' method is also useful for counting viable organisms. The medium is distributed into $6 \times \frac{5}{8}$ in tubes, 1–2 ml per tube, and stored. For use, the medium is melted, cooled to approximately 50 C, and a known quantity of a known dilution of the test sample is added. The tube is then tilted and rolled between finger and thumb, allowing the medium to run all round the sides of the tube just below the half-way mark. This rolling is carried out under cold tap water. A thin film of agar solidifies around the sides of the tube, which is inverted for incubation. Colonies are counted on the following day. By varying the dilution of the bacterial inoculum and taking the mean of several readings, a fairly accurate count of viable organisms in a specimen can be obtained. Commercially made equipment is available for the rolling operation.

24

Preparation of Culture Media

Today many laboratories prepare excellent media from the dried or dehydrated products obtainable from commercial firms. However, it is still useful and necessary to know how to make media, and why certain reagents are used.

In routine bacteriology, the most important requirement of a culture medium is the ability to allow detectable growth from a minute inoculum, possibly a single organism, within the shortest period of incubation.

The medium which forms the basis of the majority of culture media is referred to as nutrient broth. It is designed to support the growth of a wide range of bacteria and consists in the main of meat extracts, peptone and mineral salts in clear solution at a pH of approximately 7.4. Meat extracts supply a wide range of growth factors including mineral salts and amino acids. Peptone is a source of nitrogen obtained by the peptic digestion of protein to give a heat-stable mixture of proteoses, peptones, polypeptides and amino acids. Several varieties of peptone are commercially available, two of the most widely used types being bacteriological and proteose peptones. The latter is especially rich in amino acids such as tryptophan, which is necessary for satisfactory indole production. The mineral salts essential to growth consist of sulphates, chlorides and phosphates of the acid radicals and calcium, phosphate and sodium among the bases.

Types of nutrient broth

INFUSION BROTH

This consists of a watery extract of meat prepared by infusing fat-free minced meat overnight at 4 °C. The extract is filtered, peptone and salt are added, and the mixture is adjusted to pH 8. After boiling to precipitate phosphates, the medium is filtered, re-adjusted to 7.4, bottled and sterilized.

MEAT EXTRACT BROTH

Commercial concentrated meat extracts are dissolved in tap water by gentle heat and peptone and salt are added. The mixture is

adjusted to pH 8.0, boiled, and filtered to remove precipitated phosphates. After readjusting to pH 7.4 the medium is sterilized in the autoclave.

DIGEST BROTH

Fresh lean fat-free minced meat is treated with sodium carbonate to neutralize sarcolactic acid present in the meat. The mixture is heated then cooled to 45°C. Trypsin is added and digestion takes place over a period of 4–6 h. To prevent bacteria multiplying under these conditions chloroform is added (being volatile it is easily driven off at a later stage). The trypsin in a warm alkaline medium will digest the protein to form peptone. A biuret test will indicate when the right stage of digestion has been reached. At this point concentrated hydrochloric acid is added to arrest digestion, and the medium is boiled and filtered. Sodium chloride is formed from the sodium carbonate and hydrochloric acid. The medium is adjusted to pH 7.4 and sterilized in the autoclave after removing the precipitated phosphates as previously described.

ENRICHED MEDIA

Many substances may be added to nutrient broth in the form of blood, serum, hydrocele fluid, glucose, etc., to support the growth of the more exacting bacteria.

SOLID MEDIA

Solidifying substances, referred to as gelling agents, may be added to nutrient broth: agar and gelatin are commonly used.

Agar is a carbohydrate derived from seaweed. The main supply these days comes in powder form as distinct from the earlier Japanese shred agar. A satisfactory gel is achieved at approximately 1 per cent concentration in nutrient broth. The *melting point* is 98°C and is cooled to 50°C before adding coagulable body fluids in the form of blood or serum. The *setting point* is 42°C.

Gelatin is a protein derived from the collagen of skin, hide, sinew and bone. It forms a satisfactory gel at a concentration of 12–15 per cent in nutrient broth. Prolonged exposure at temperatures above 100°C destroy its setting properties. The *melting point* is 24°C. Many bacteria produce gelatinase, and their ability to liquefy gelatin is used as a means of classifying them either by their remaining liquid after growth at 37°C or by showing liquefaction after growth at room temperature.

DIFFERENTIAL MEDIA

There are media containing substances or indicators which will differentiate one organism from another. MacConkey agar will distinguish lactose fermenting organisms from non-lactose fermenters. Blood agar will differentiate organisms by their ability to produce different types of haemolysis. These media are sometimes referred to as indicator media.

SELECTIVE MEDIA

These are *solid* media containing substances which inhibit the growth of most organisms other than those for which the media are devised; for example, tellurite media for the diphtheria bacillus and deoxycholate-citrate-agar for the *Salmonella* and *Shigella* groups.

ENRICHMENT MEDIA

These are *fluid* selective media which incorporate substances that inhibit the growth of organisms other than those for which the medium was devised; for example, Selenite F inhibits coliform bacilli, whilst allowing the typhoid-paratyphoid organisms to grow freely as an enriched culture.

Storage of culture media

Media may be dispensed in bottles with rubber-lined metal screw caps, polypropylene caps or tubes plugged with non-absorbent cotton wool. Small bottles may be sterilized with their caps screwed down firmly, but not packed tightly in the baskets. The larger bottles should have their caps loosened before heating and subsequently tightened for storage.

A useful method of distinguishing between types of media is the use of coloured non-absorbent cotton wool plugs in tubes or, in the case of bottles, coloured caps or beads may be used.

Cotton wool plugs must not be so tight that air is excluded. On the other hand, they should be firm enough to allow one to raise each tube by its plug.

Media for current use should always be stored in a dust-free cupboard or in a cool, moist atmosphere. For longer periods store at 4–6 °C. Each batch should be tested before use and labelled with a batch number. The shelf-life of media varies considerably. Poured plates will be used within a few days of preparation but agar slopes should be checked to ensure that moisture is still present. Supplies of the basal media should be such that each batch is renewed within 3 months of manufacture.

THE ADJUSTMENT OF pH

It is essential that all media are adjusted to the correct pH and great care must be taken to ensure that this is performed accurately. Methods 1 and 2 described below are those generally employed in small media preparation rooms, whilst others use a pH meter.

Method 1

This is a colorimetric method. An indicator is added to the medium and to a standard buffer solution. The medium is adjusted until the colours are matched.

APPARATUS REQUIRED

1. Comparator rack.
2. Set of standard pH tubes, that is, tubes containing buffer solution and indicator.
3. Comparator tubes, that is, glass tubes of same bore and wall-thickness as standard tubes.
4. Indicator solution. For media, phenol red is the indicator of choice.
5. Pipettes.
6. 10N, N and N/10 NaOH; 10N and N/10 HCl.
7. Micro-burettes.

METHOD

1. Measure 5 ml of medium into each of 3 comparator tubes and 5 ml of distilled water into another comparator tube.
2. To one of the comparator tubes of medium add the same amount of indicator as is present in the standard pH tube.
3. Place tubes in comparator rack as shown in *Figure 24.1*. The standard pH tubes used should be those above and below the required pH; for example, if pH 7.5 is the desired reaction of the medium, the two standard tubes to be used should be pH 7.4 and 7.6.

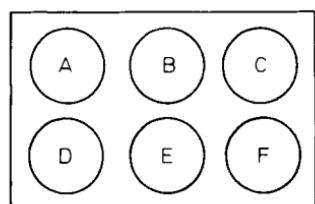


Figure 24.1. Illustrating position of tubes in a comparator rack. A and C: 5 ml of medium. B: distilled water. D and F: standard pH tubes. E: 5 ml of medium + indicator

4. If the medium is too alkaline, add sufficient N/10 HCl from a burette to alter the colour of the tube containing medium and indicator, to a tint midway between those of the two standard tubes. If the medium is too acid add N/10 NaOH instead of N/10 HCl.
5. Measure the volume of alkali or acid that was necessary to adjust the reaction of 5 ml of the medium.
6. Average two readings and calculate the amount of alkali or acid to add to the bulk of medium. Add the necessary alkali or acid in concentrated form.

EXAMPLE OF CALCULATION

If 0.5 ml of N/10 HCl is required to adjust the pH of 5 ml of medium; then 5 ml of N/10 HCl would be required for 50 ml of medium and 100 ml of N/10 HCl would be required for 1000 ml of medium, or 10 ml of N HCl, or 1 ml of 10N HCl, which is a suitable small quantity to add.

After the addition of alkali or acid to the bulk of the medium, mix well and check the pH using the same method.

Method 2

This method is also colorimetric and uses the Lovibond comparator. The sample of medium plus indicator is matched against permanent coloured-glass standards (*see Chapter 6*).

METHOD

1. Tubes A and B contain known volume of medium.
2. Add standard volume of indicator to tube B (right-hand tube).
3. Close comparator, and turn indicator disc to required pH reading.
4. Add alkali or acid to tube B, until colours are matched.
5. Calculate amount of acid or alkali to add to bulk of medium, as in Method 1.

Notes on estimating the pH of media using a Lovibond comparator

1. Readings must not be made until the medium is cool.
2. If agar is used, the tube containing agar plus indicator, and the tube containing agar alone, must be cooled until the agar has solidified.
3. All tubes must be of the same glass, bore and wall-thickness.

4. Tubes must be thoroughly washed and rinsed with neutral distilled water before they are used again.
5. Never match colours in direct sunlight; use a north light. A special viewing box is used for artificial light.
6. One's perception of the delicate colour tints is soon dulled by prolonged examination. It is advisable to glance at the tubes briefly but frequently, when matching colours.

Method 3: BDH capillitor outfit

This micro-technique is useful if small amounts of media are being prepared, and for the estimation of pH changes in bacteriological cultures. It is a modification of Method 1, using capillary tubes instead of test-tubes.

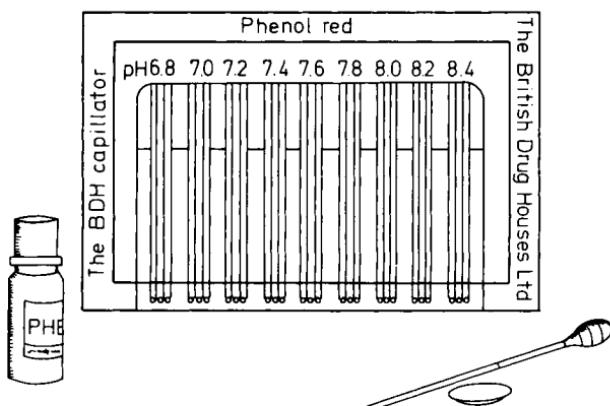


Figure 24.2. The British Drug Houses capillitor outfit

METHOD

1. Each standard consists of 3 capillary tubes containing buffer solution and indicator. To determine the pH of the medium, fill a fresh capillary tube up to the mark with the medium, and transfer to a watch glass.
2. Without preliminary rinsing, fill the same capillary tube with indicator and allow it to run on to the watch glass. Mix the medium and indicator on the glass.
3. Refill the capillary with the mixture in the watch glass, and compare it with the standard tubes on the card.
4. Add alkali or acid to small volumes of the bulk solution and test the pH after each addition, until the required reaction is attained.

Method 4: pH meter

This is the most accurate method of estimating hydrogen ion concentration. It involves the use of glass or hydrogen electrodes, but is not generally used in routine media preparation.

PREPARATION OF CULTURE MEDIA

1. At no stage of the preparation should a medium be overheated, for fear of destroying its nutritive qualities. Sterilization should be effected at the lowest temperature, and for the shortest time that will ensure complete sterility.
2. The adjustment of pH should be carried out accurately.
3. Utensils and glassware should be clean.
4. Fresh batches of peptone should be examined for solubility, colour (which should be pale), reaction (which should be neutral), absence of sugar and suitability for indole production.

To test for presence of sugar and suitability for indole production, prepare tubes of peptone water and of peptone water plus indicator. Inoculate them with a culture which is capable of fermenting most sugars (to produce acid) and of liberating indole; the most suitable organisms for this test are those of the *E. coli* group. Alternatively a chemical test for the presence of sugar may be performed.

5. Agar should be examined for cleanliness, absence of inhibitory growth factors, solidifying properties and clarity in solution.

Nutrient broth**INFUSION BROTH**

Minced ox heart (freed from fat)	500 g
Peptone	10 g
Sodium chloride	5 g
Tap water	1000 ml

1. Stir well and stand in a cool place overnight.
2. Steam for 2 h with occasional stirring.
3. Filter through muslin to remove coarse particles.
4. Filter through Chardin-type filter paper and adjust reaction to pH 8.0.
5. Bottle and sterilize at 10 psi for 15 min, label and store.
6. For use: filter, adjust reaction to pH 7.4.
7. Fill into tubes or bottles and sterilize at 10 psi for 15 min.

When broth of pH 8.0 is first autoclaved, phosphates present in the medium are precipitated, and require removal by filtration. Broth may be stored with phosphates present and filtered just prior to use. One heating is thereby avoided.

MEAT EXTRACT BROTH

Beef extract	10 g
Peptone	10 g
Sodium chloride	5 g
Water	1000 ml

1. Dissolve by heat, adjust reaction to pH 7.4.
2. Filter and bottle.
3. Sterilize by autoclaving.

DIGEST BROTH

A useful digest broth is as follows:

Minced ox heart or lean beef (freed from fat)	1800 g
Tap water	3000 ml

1. Heat to 80 °C, and add:
0.8 per cent anhydrous sodium
carbonate 3000 ml
2. Cool to 45 °C, and add:
Cole's pancreatic extract* or Liquor
Trypsin 60 ml
Chloroform 60 ml
3. Mix well, and incubate at 37 °C for 5–6 h, stirring every hour.
4. Toward the end of this period, perform the biuret test as follows: Add 1 ml of a saturated solution of potassium hydroxide and 0.1 ml of a 5 per cent solution of copper sulphate to a sample of broth. If a pink colour results, digestion is complete; if blue, digestion is incomplete.
5. When digestion is complete, add 480 ml of N/1 HCl and steam for 30 min, filter and adjust the pH to 8.0.
6. Bottle and sterilize at 10 psi pressure for 15 min.
7. Label and store.
8. For use: filter, adjust the reaction to pH 7.4, tube or bottle the broth, and sterilize at 10 psi for 15 min.

*** COLE AND ONSLOW'S PANCREATIC EXTRACT**

Cole's pancreatic extract is rich in the enzyme trypsin, which works better at an alkaline pH, hence the addition of sodium carbonate. The N/1 HCl is added to counteract the sodium carbonate and to stop digestion.

Fresh pig pancreas (minced and free from fat)	500 g
Distilled water	1500 ml
Absolute alcohol	500 ml

Mix well in a large stoppered bottle, and leave at room temperature for 3 days. Shake occasionally. Strain through muslin, then filter. Add 0.1 per cent pure concentrated HCl.

This extract will keep for several months.

Enriched media using nutrient broth**BLOOD BROTH**

Sterile nutrient broth plus 5 per cent sterile defibrinated or oxalated horse blood added aseptically.

SERUM BROTH

Sterile nutrient broth plus 5 per cent sterile serum added aseptically.

CHOCOLATE BROTH

Blood broth mixed and heated at 70–80 °C until a chocolate colour develops.

FILDES' BROTH

Sterile nutrient broth plus 5 per cent Fildes' medium added aseptically.

GLUCOSE BROTH

Sterile nutrient broth plus 0.25 per cent sterile glucose.

Nutrient agar

1. Adjust the reaction of broth to pH 7.6 and add the required amount of powdered agar (generally 1–1.3 per cent). As the quality of bacteriological agar is of a high standard, there will not be more than a 0.2 drop in pH.
2. Steam until the agar is dissolved.

3. Check the pH.
4. Bottle, and autoclave at 10 psi for 15 min.

FILTRATION OF AGAR

With the high-quality powdered agar in use today, filtration is seldom necessary. However, the following method may be used, if required:

1. Boil a quantity of paper pulp in distilled water.
2. Cover a large Buchner funnel with muslin. Add the pulp, and spread it evenly over the whole area of the filter.
3. Fold the sides of the muslin over the pulp, and press it down firmly. Insert the funnel into a large vacuum flask, and place

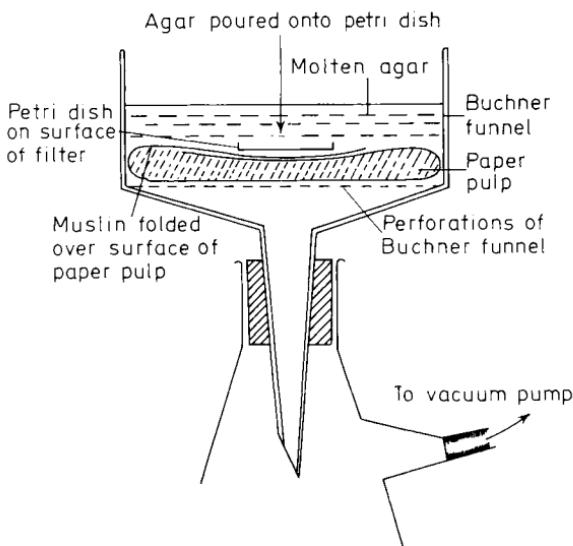


Figure 24.3. Illustrating the filtration of nutrient agar

- a petri dish lid on the centre of the muslin (Figure 24.3). Turn on the vacuum pump slowly, until water from the paper pulp begins to extrude, then pour hot water on to the petri dish. The dish serves to prevent destruction of the soft filter, by dispersing the water flow evenly.
4. Pour the agar onto the petri dish. When agar appears through the filter, empty the receiving flask.
 5. Add agar gradually, keeping the negative pressure as low as possible (3–4 litres of agar can be filtered without changing the paper pulp).

Some media made from nutrient agar

BLOOD AGAR SLOPES, SERUM AGAR SLOPES, CHOCOLATE AGAR SLOPES AND FILDES' AGAR SLOPES

These are prepared either from individual agar slopes which are melted down, cooled and the required enrichment added, or from a larger amount of agar, as for blood agar plates, which is distributed aseptically into sterile tubes or bottles which are then sloped.

Nutrient agar plates are used for the cultivation of many easily grown organisms (e.g. staphylococci, *E. coli*). Melt the nutrient agar by steaming, cool to 50 °C and pour 15–20 ml aseptically into clean sterile petri dishes.

Blood agar plates are used for the cultivation and differentiation of more delicate organisms (e.g. streptococci, gonococci).

Method 1

Melt the nutrient agar by steaming. Cool to 50 °C and add 5–10 per cent sterile defibrinated or oxalated horse blood. Pour 15–20 ml volumes aseptically into clean sterile petri dishes.

Method 2

Pour a thin layer of agar into sterile petri dishes, and when this has set, add the molten blood agar (7–10 per cent blood). It has been stated that this method is preferable to Method 1 in that haemolysis is more easily seen, the blood agar layer is more uniform in thickness and less horse blood is used. In practice, however, now that most petri dishes have flat bottoms, many laboratories use Method 1.

Chocolate agar plates may be used for the cultivation of certain organisms (e.g. *H. influenzae* and pneumococci). Add blood to nutrient agar, as when making blood agar plates. Heat at 70–80 °C for 10 min and pour aseptically into clean sterile petri dishes. *H. influenzae* requires two growth factors called X and V. Both of these are found in blood. The X factor is haematin, and the V factor nicotinamide adenine dinucleotide (NAD). Blood contains an enzyme NADase which progressively breaks down NAD leaving little available for the bacteria to utilize. The growth of *H. influenzae* is consequently poor on blood agar plates. If, however, the blood is heated as for chocolate agar, the enzyme NADase is inactivated and more NAD is available for the bacteria with the

result that colonies of *H. influenzae* on chocolate plates are greatly increased in size.

Fildes' agar plates are used for cultivation of *H. influenzae*.

Fildes' peptic digest broth

1. Mix together in a 250 ml ground glass stoppered bottle the following.

Saline (made with tap water)	150 ml
Pure hydrochloric acid	6 ml
Defibrinated sheep's blood	50 ml
Pepsin (BP granulated)	1 g
2. Shake well and place in a 55 °C water bath overnight.
3. Add 12 ml of 20 per cent NaOH.
4. Remove a small quantity of the mixture, dilute 20 times with distilled water, and test its reaction to cresol red and phenol red. Phenol red is just pink at the correct reaction of pH 7.6, but there is no colour change with cresol red. Correct adjustment of the pH is essential. If the addition of 12 ml of NaOH is insufficient, add more until the correct reaction is obtained. If 12 ml of NaOH was excessive, neutralize the excess with pure hydrochloric acid.
5. Preserve with 0.25 per cent chloroform, and store in a refrigerator.
6. For use: add to melted agar, as when making blood plates.

Glucose agar

This consists of 10 per cent solution of glucose in distilled water sterilized by Seitz filtration.

1. Taking aseptic precautions, add sufficient glucose solution to melted and cooled agar to give a final concentration of 0.5 per cent.
2. Distribute aseptically into required containers, that is, petri dishes, tubes or bottles.
3. If in tubes or bottles, steam for 20 min, and slope if necessary.

Nutrient gelatin

Nutrient broth	1000 ml
Powdered gelatin (Difco)	120–150 g

1. Dissolve in the steamer and check that pH is 7.4.
2. Tube or bottle in 10 ml quantities.
3. Sterilize by steaming for 20 min on 3 successive days.

A clear, satisfactory gel is obtained by this method, and clearing and filtering is seldom necessary. Should the medium need clearing, add the white of an egg, and steam for 30 min. Any particles present will adhere to the coagulated egg-white, and be removed by filtration.

The higher concentration of gelatin will be needed to produce a firm gel in countries with a warm climate.

CARBOHYDRATE MEDIA

The ability of different organisms to ferment certain carbohydrates is used in their identification and classification. It is essential that the medium used for this test shall be free from all carbohydrates except those specifically added. Nutrient broth is useless for this purpose, as it contains small amounts of 'muscle' sugar. An aqueous solution of suitable peptone and sodium chloride is prepared. The selected carbohydrate and indicator is

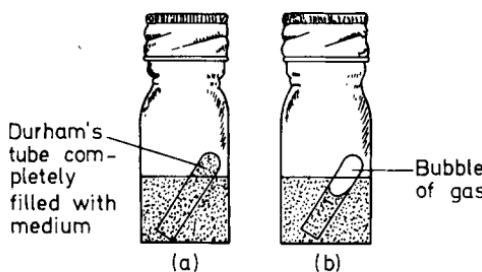


Figure 24.4. Production of gas in a Durham's tube

added to this solution, and it is dispensed in tubes or bottles containing a small Durham's tube. This must be inverted and completely filled with the medium. The indicator will reveal the production of acid and the inverted Durham's tube will trap any bubbles of gas that may be formed (Figure 24.4).

For the fermentation reactions of more delicate organisms such as streptococci, pneumococci and *C. diphtheriae*, the medium must be enriched with serum. Other organisms, such as gonococci and meningococci, grow better on a solid medium and for these agar is incorporated in the serum sugar media.

Peptone water

This is used for testing for indole production, for the preparation of sugar media, and, when alkaline, for the cultivation of *V. cholera*.

METHOD

Peptone	10 g
Sodium chloride	5 g
Distilled water	1000 ml

1. Dissolve in steamer.
2. Adjust reaction to pH 7.5.
3. Filter through Chardin-type filter paper.
4. Distribute in tubes or bottles.
5. Autoclave at 10 psi for 15 min.

Peptone water sugars**PREPARATION**

1. To sterile peptone water add 1 per cent Andrade's indicator and sufficient of a 10 per cent solution of the required sugar (sterilized by Seitz filtration) to give a final concentration of 0.5 per cent.
2. Distribute aseptically into sterile tubes or bottles containing inverted Durham fermentation tubes.
3. Steam for 30 min. If the medium is of the correct reaction and the indicator is satisfactory, the solution becomes pink during heating but returns to a straw colour on cooling.

PREPARATION OF ANDRADE'S INDICATOR

1. Dissolve 0.5 g of acid fuchsin (CI No. 42685) in 100 ml of distilled water.
2. Add 16 ml of N/1 NaOH and leave overnight.
3. The colour should change from pink to brownish-red and then to yellow.
4. If it is necessary to add more N/1 NaOH, small amounts only should be added, and 24 h allowed for any colour change.

Hiss' serum water sugars

These are used for fermentation reactions of *Corynebacterium* and other genera requiring serum for growth.

Ox serum	1 part
Distilled water	3 parts

1. Adjust reaction to pH 7.5 and add Andrade's indicator, 1 per cent, and sugar, 1 per cent.
2. Tube or bottle and steam for 20 min on 3 consecutive days.

Solid sugar medium

This is used for fermentation reactions of *Neisseria*, and other genera requiring serum for growth.

Peptone	20 g
Sodium chloride	5 g
Powdered agar	20 g
Nutrient broth	100 ml
Distilled water	900 ml

PREPARATION

1. Steam to dissolve and filter.
2. Adjust reaction to pH 7.6
3. Bottle in 100 ml amounts and sterilize at 10 psi for 15 min.
4. For use: Melt, cool to 55 °C and, using aseptic precautions, add to each 100 ml the following:

0.04 per cent phenol red solution	5 ml
Sterile rabbit serum	5 ml
10 per cent sterile sugar solution	10 ml

5. Distribute aseptically into tubes or bottles and allow to solidify in a sloping position.
- 6 Test for sterility by incubation.

Note—(1) It is essential to use rabbit serum (or human serum) in this medium, as horse, sheep or ox serum contains maltase, which may lead to a false reaction.

(2) the muscle sugar content, that may be present in the nutrient broth, is so diluted that erroneous results are not obtained.

Litmus milk

Litmus milk is used for the fermentation of its sugar, lactose and for the clotting and digestion of milk.

PREPARATION

1. Centrifuge fresh milk at high speed. The cream is freed from the milk, forming a surface layer.
2. Syphon off the milk and discard the cream.
3. Add sufficient of 10 per cent litmus solution to give the desired tint to the cream-free milk.
4. Tube or bottle and steam for 20 min on 3 consecutive days.

10 per cent litmus solution

Litmus	10 g
Distilled water	100 ml

Boil for 10 min. Allow to stand overnight. Filter and bottle.

MEDIA FOR SPECIAL PURPOSES**Media for the isolation and identification of Gram-negative intestinal bacilli**

Organisms isolated from faecal and urinary specimens may be lactose fermenters (generally non-pathogenic organisms) or non-lactose fermenters (generally pathogenic organisms). Special media are used for differentiating these organisms and for inhibiting the non-pathogens while allowing the pathogens to grow more freely.

MacConkey's agar

MacConkey's agar is used for differentiating intestinal organisms into lactose- and non-lactose-fermenting organisms. The peptone constitutes the nutrient base, solidified by the agar. Sodium taurocholate (bile salt) inhibits many Gram-positive organisms, and lactose and the indicator (neutral red) differentiate the lactose- and non-lactose-fermenting organisms. The lactose-fermenting organisms, by the fermentation of lactose produce acids which act upon the bile salt and absorb the neutral red, giving red colonies. The non-lactose-fermenting organisms give an alkaline reaction, do not absorb the neutral red, and produce colourless colonies.

PREPARATION

Peptone	20 g
Sodium chloride (optional)	5 g
Sodium taurocholate	5 g
Lactose	10 g
1 per cent aqueous neutral red (CI No. 50040) solution	5–7 ml
Agar	15 g
Distilled water	1000 ml

1. Dissolve the sodium taurocholate, peptone and salt in the distilled water by steaming.
2. Adjust the reaction to pH 7.8 and add the agar.
3. Autoclave at 10 psi for 20 min.

4. Filter and adjust the reaction to pH 7.5.
5. Add the lactose and sufficient neutral red to give a reddish-brown colour.
6. Bottle and autoclave at 10 psi for 20 min.

When pouring plates, cool the melted medium to 45–50 °C before pouring. This will prevent a 'scum' from appearing on the surface of the medium.

Deoxycholate-citrate agar

A selective medium for the isolation of the *Salmonellae* and the dysentery organisms.

PREPARATION

1. Dissolve 20 g of beef extract in 200 ml of water over a flame; make just alkaline to phenolphthalein with 50 per cent sodium hydroxide solution, boil and filter.
2. Adjust the reaction to pH 7.3, make up the volume to 200 ml and add 20 g of Difco proteose peptone.
3. Dissolve 90 g of agar in 3700 ml distilled water by steaming for 1 h.
4. Filter the agar and add the beef extract-peptone solution.
5. Add 10 ml of a 1 per cent solution of neutral red and 40 g of lactose.
6. Bottle in 100 ml amounts and sterilize by autoclaving at 10 psi pressure for 20 min.

Solution A

Sodium citrate (Analar. $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$)	17 g
Sodium thiosulphate (Analar. $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$)	17 g
Ferric citrate (scales)	2 g
Distilled water	100 ml

Dissolve by allowing to stand at room temperature for 2–3 days.

Solution B

10 per cent solution of sodium deoxycholate in distilled water. Sterilize solutions A and B in the 60 °C water-bath for 1 h.

7. For use: Melt 100 ml of the agar and add 5 ml of solution A. Mix thoroughly and add 5 ml of solution B. Mix and pour plates.

Sodium deoxycholate (bile salt) will inhibit the growth of many Gram-positive organisms, whilst favouring the growth of the in-

testinal Gram-negative organisms. The neutral red indicator is, however, toxic in the presence of sodium deoxycholate, and sodium citrate and sodium thiosulphate are also toxic for the coliforms and to a certain extent the *Salmonellae*. To neutralize this toxicity for the *Salmonellae*, ferric citrate is added, which does not interfere too greatly with the toxicity for the coliforms. Thus, whilst coliform organisms do grow on this medium, they do not grow as well as the non-lactose fermenters. The coliforms appear as pink colonies, with a precipitation of deoxycholate (due to acid production) surrounding the colony. *Proteus* species appear as colourless non-spreading colonies, generally with a black central dot, and with the characteristic fishy odour. Pathogens also appear as colourless, later pale pink, colonies sometimes with a black central dot but rarely after 24 h incubation. This black dot is due to H₂S production by the organisms, which combines with the ferric citrate, present in the medium, to form iron sulphide.

CLED medium (Mackey and Sandys)

The Cystine-Lactose-Electrolyte Deficient (CLED) medium is recommended for urinary bacteriology. Its electrolyte deficiency prevents the swarming of *Proteus* species and good colonial differentiation is obtained with most urinary pathogens.

Peptone	4 g
Beef extract	3 g
Tryptone	4 g
Lactose	10 g
L-cystine	0.128 g
Erom-thymol blue	0.02 g
Distilled water	1000 ml

Dissolve and adjust to pH 7.3. Add powdered agar 15 g.
Dissolve, distribute and autoclave at 15 psi for 15 min.

Growth characteristics are as follows:

<i>E. coli</i>	Yellow, opaque colonies with a slightly deeper coloured centre about 1.25 mm diameter. (Non-lactose-fermenting strains—blue colonies.)
<i>Klebsiella</i> spp.	Extremely mucoid colonies varying in colour from yellow to whitish-blue.
<i>Proteus</i> spp.	Translucent blue colonies usually smaller than <i>E. coli</i> .

<i>Salmonella</i> spp.	Flat blue colonies.
<i>Ps. pyocyanea</i>	Green colonies with typical matt surface and rough periphery.
<i>Strep. faecalis</i>	Yellow colonies about 0.5 mm diameter.
<i>Staph. aureus</i>	Deep yellow colonies about 0.75 mm diameter, uniform in colour.
Coagulase negative staphylococci	Pale yellow or white, more opaque than <i>Strep. faecalis</i> , often with paler periphery.
Diphtheroids	Very small grey colonies.
Lactobacilli	Similar to Diphtheroids but with a rougher surface.

Wilson and Blair's medium

Wilson and Blair's medium is prepared with the following solutions.

Solution A.

1. Bismuth ammonium-citrate scales	30 g
Distilled water	250 ml
Dissolve by boiling.	
2. Anhydrous sodium sulphite	100 g
Distilled water	500 ml
Dissolve by boiling.	

Mix 1 and 2, boil, and add 100 g of sodium phosphate crystals ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$). When cool add 250 ml of 20 per cent dextrose solution.

This constitutes the stock bismuth-sulphite-dextrose-phosphate mixture.

Solution B.

1 per cent solution iron citrate scales	200 ml
1 per cent brilliant green (CI No. 42040) in distilled water	25 ml

This constitutes the iron-citrate brilliant green mixture.

To prepare the medium, add 20 ml of solution A and 4.5 ml of solution B to 100 ml of nutrient 3 per cent agar (melted and cooled to 60 °C).

Mix and pour into petri dishes.

S. typhi reduces the sulphite to sulphide in the presence of dextrose and appears as black colonies.

Coliform organisms are inhibited by the brilliant green and bismuth sulphite in the presence of an excess of sodium sulphite.

Selenite F medium (modified)

Sodium acid selenite	4 g
Peptone	5 g
Mannitol	4 g
Disodium hydrogen phosphate (Na ₂ HPO ₄)	10 g
Distilled water	1000 ml

Distribute in 10 ml amounts into screw-capped bottle and steam for 30 min.

Sodium acid selenite has, at near neutral pH, a high toxicity for *E. coli*, but not for the salmonella organisms. As the pH increases, this toxicity decreases and the buffer salt is added to help maintain the near neutral pH. A fermentable carbohydrate is included to produce acid, thus neutralizing the alkali produced by the bacterial reduction of the selenite.

It is essential to use sodium acid selenite (sodium hydrogen selenite), as ordinary sodium selenite is very alkaline, but care must be taken when using this chemical as inhalation of the powder may be dangerous.

MEDIA FOR ISOLATION OF *CORYNEBACTERIUM* GROUP

Most media used for the isolation of *C. diphtheriae* from mixed cultures contain compounds of tellurium, which inhibit the growth of organisms not of the genus *Corynebacterium*. Once isolated, subcultures of corynebacteria can be maintained on serum media, such as Loeffler's medium.

Hoyle's medium

This is one of many media used for the isolation of Corynebacteria.

1. Beef extract	10 g
Difco proteose peptone	10 g
Sodium chloride	5 g
Agar	20 g
Water	1000 ml

Steam to dissolve. Adjust to pH 7.8. Autoclave at 10 psi for 15 min. Distribute in 200 ml quantities in screw-capped bottles.

2. Sterile laked horse blood.	
3. Potassium tellurite	0.7 g
Water	20 ml

Store, tightly stoppered in the dark.

To each 200 ml agar, melted and cooled to 55 C, add laked blood 10 ml, and potassium tellurite solution, 2 ml.

Pour into sterile petri dishes.

After 24–48 h incubation, colonies of the *gravis* type appear a slate-grey colour with a bluish tinge, the shape approximating to a daisy head. Colour and size of the *mitis* type colonies are similar to the *gravis* type, but appear more glistening, are convex and have a perfectly circular outline.

Intermediate type colonies are never larger than 2 mm. They are blacker than the other types and have a poached egg shape. Today the corynebacteria isolated do not always conform to the above description of the classical types.

Loeffler's serum slopes

These are used for the cultivation of organisms such as *C. diphtheriae*.

Ox serum, 3 parts
2 per cent glucose broth, 1 part

1. Sterilize the broth by autoclaving at 10 psi for 15 min.
2. Sterilize the serum by Seitz filtration.
3. Mix and distribute aseptically into sterile tubes or bottles.
4. Inspissate at 75 C until set.
5. Next day inspissate at 75 C for 1 h. It is important not to exceed 75 C.

EGG MEDIA

Egg media are generally used for growing the tubercle bacillus.

Lowenstein-Jensen medium is one of the most useful for the primary isolation of *M. tuberculosis*, the incorporated malachite green inhibiting the growth of many contaminants.

Glycerol egg

Glycerol egg is used for cultivation of human tubercle bacilli, which grow better in the presence of glycerol.

Egg	78 per cent
Nutrient broth	20 per cent
Glycerol	2 per cent

1. Sterilize the broth and glycerol in the autoclave at 10 psi for 15 min.
2. Break the eggs under aseptic conditions into a sterile flask containing glass beads.
3. Shake well and filter through sterile muslin into the sterile glycerol broth.
4. Distribute in sterile tubes or bottles under aseptic conditions and insipidate in a sloping position at 75–80 °C. Heat until solidified.

Dorset egg

This is used for cultivation of human and bovine tubercle bacilli.

Egg	80 per cent
Nutrient broth or saline	20 per cent

Proceed as for Glycerol egg medium.

Lowenstein-Jensen medium

This is used for the primary isolation of human and bovine tubercle bacilli.

Asparagin-mineral salt solution

Potassium dihydrogen phosphate (Analar)	0.4 per cent
Magnesium sulphate (Analar)	0.04 per cent
Magnesium citrate	0.1 per cent
Asparagin	0.6 per cent
Glycerol (Analar)	2.0 per cent

In distilled water.

Steam for 2 h and store in the refrigerator.

1. Add 6 g of potato flour (optional) to 150 ml of this solution, heat over a flame with constant stirring until a smooth mixture is obtained.

2. Sterilize in the autoclave at 10 psi for 20 min.
3. Break 5 eggs, under aseptic conditions, into a sterile flask containing glass beads. Shake well and filter through sterile muslin.
4. Mix the eggs with the cool asparagin-potato starch mixture and add 5 ml of 2 per cent malachite green (CI No. 42000) solution.
5. Distribute, under aseptic conditions, in sterile tubes or bottles and sterilize by inspissating at 75–80 °C until solidified.

The tubercle bacilli obtain their nitrogen from the asparagin, and their carbon from the glycerol. The malachite green helps to inhibit the growth of other organisms.

The human type of tubercle grows as heaped-up, dry, yellow colonies, and the bovine type as small, discrete, colourless colonies.

MEDIA FOR ISOLATION OF STAPHYLOCOCCI

Ludlam's medium

(Selective medium for isolation of *Staphylococcus aureus* from contaminated material.)

Beef extract	10 g
Peptone	10 g
Dipotassium hydrogen phosphate (anhydrous)	5 g
Lithium chloride	5 g
Mannitol	10 g
Distilled water	1000 ml

Heat to dissolve and adjust to pH 9.2.

Add agar powder, 10 g.

Dissolve and distribute in 100 ml amounts. Sterilize at 10 psi for 15 min.

For use: Melt and add 0.5 ml of sterile 0.25 per cent potassium tellurite solution.

After 48 hours' incubation:

Staphylococcus aureus—dark grey or black shiny colonies.

Staphylococcus epidermidis—either no growth or small pale colonies.

Mannitol salt agar

To nutrient agar add 7.5 per cent sodium chloride, 1 per cent mannitol and 0.3 per cent phenol red (0.4 per cent). Sterilize by autoclaving at 115 °C for 15 min.

This selective medium is useful when searching for carriers of staphylococci. Presumptive coagulase positive staphylococci produce colonies surrounded by bright orange-yellow zones. Coagulase negative staphylococci produce colonies surrounded with a reddish zone. For carrying out the coagulase test, colonies need to be sub-cultured onto a medium not containing an excess of salt.

MEDIA FOR ISOLATION OF *V. cholerae***Thiosulphate citrate bile salt agar (TCBS medium)**

Bacteriological peptone	5 g
Yeast extract powder	10 g
Sodium citrate (Analar) (Na ₃ C ₆ H ₅ O ₇ .2H ₂ O)	10 g
Sodium thiosulphate (Analar) (Na ₂ S ₂ O ₃ .5H ₂ O)	10 g
Sodium taurocholate	5 g
Feric citrate	1 g
Distilled water	1000 ml

1. Dissolve the above ingredients in the distilled water and adjust pH to 8.6.
2. Add 0.04 g thymol blue and 0.04 g bromothymol blue. Add 5 g agar and steam to dissolve.
3. Bottle and sterilize whilst still molten for 15 min at 15 psi.

For use: Steam medium to dissolve, cool to 50 °C, and pour plates. Colonies of *V. cholerae* and the El Tor biotype appear yellow on a bluish-green medium after 10–18 h incubation due to fermentation of sucrose.

MEDIA FOR ISOLATION OF *B. pertussis***Lacey's medium**

Agar base potato starch	15.0 g
Powdered agar	15.0 g
Tap water (cold)	600.0 ml

1. Steam to dissolve

Glycerol	5.0 ml
<i>dl</i> -alpha alanine	1.0 g
1-glutamic acid	3.7 g
Sodium fluoride	0.5 g
Sodium chloride	0.5 g
Potassium chloride	3.3 g
Tap water (cold)	100.0 ml

- Dissolve by heat and adjust to pH 7.0 with 10 per cent potassium hydroxide (Analar).
- Add to starch agar solution and add sufficient tap water to bring volume to 1070 ml. Adjust to pH 7.2, bottle in 100 ml amounts and autoclave at 5 psi for 10 min.

Solution 1. Cysteine-magnesium salt mixture

Magnesium lactate	7.6 g
Fumaric acid	3.3 g
Malonic acid	3.1 g
1-cysteine hydrochloride	2.0 g
Distilled water	90.0 ml

- Dissolve by boiling and cool to 80 °C.
- Add sufficient of a suspension of magnesium hydroxide until a slight excess remains after 3 min observation.
- Make up to 170 ml with distilled water.
- Filter through Whatman No. 1 filter paper and boil for 3 min.
- Transfer to a sterile screw-capped bottle and store in refrigerator.

Solution 2.

M & B 938 (4:4-diamido diphenylamine dihydrochloride)	100 mg
Sterile distilled water	10 ml

Store in refrigerator in sterile screw-capped bottle.

Solution 3.

Penicillin 50 iu per ml in 0.25 per cent sodium citrate (Analar)

- To 100 ml of agar base cooled to 50 °C add 8 ml solution.
- Mix well and add 1.5 ml of solution 2 diluted 1/10 with sterile distilled water.

3. Add 0.75 ml of solution 3. Mix well and add 60.0 ml of fresh defibrinated horse blood.
4. Mix well and pour plates. Use up to 10 days old.

Bordet-Gengou

Potato extract

Peeled potatoes (cleaned and cut into thin slices)	250 g
Tap water (cold)	500 ml
Sodium chloride	9 g

Boil until potato slices fall to pieces, filter through muslin and make up the filtrate to 500 ml. Adjust to pH 7.0.

Potato extract	500 ml
Agar	60 g
Proteose peptone	20 g
Glycerol	20 ml
Tap water (cold)	1500 ml

Steam to dissolve and distribute in bottles (25 ml or 50 ml amounts). Sterilize at 10 psi for 10 min.

For use, dissolve and cool to 50–60 °C. Add equal quantity of blood, heated to 40 °C. Pour plates and store in refrigerator.

MEDIA FOR CULTIVATION OF ANAEROBIC ORGANISMS

Thioglycollate broth (Brewer's broth modified)

Sodium thioglycollate	1 g
Glucose	10 g
Powdered agar	0.5 g
Methylene blue (CI No. 52015)	0.2 ml
Nutrient broth pH 7.4	1000 ml

Dissolve by steaming and distribute in 12 ml amounts in 150 mm × 15 mm test-tubes. Sterilize by autoclaving at 15 psi for 15 min. The glucose acts as a primary reducing agent, while the thioglycollate maintains the anaerobic conditions achieved after autoclaving; the sloppy agar prevents convection currents and the methylene blue acts as an indicator, remaining colourless except where oxygen is present at the surface of the medium.

Cooked meat medium

1. Boil 500g of minced ox heart in 500 ml of N/20 NaOH, to neutralize its lactic acid content.
2. Drain off fluid and partially dry the meat with a clean cloth.
3. Fill narrow-necked 1 oz bottles, with screw caps and rubber washers, to a depth of 2 in.
4. Add nutrient broth to 1 in above the level of the meat.
5. Sterilize at 15 psi for 20 min.

The meat particles contain reducing substances which maintain anaerobic conditions at the bottom of the tube, and also prevent convection currents.

TRANSPORT MEDIUM

Stuart's transport medium

A transport medium enables delicate pathogens such as *Neisseria gonorrhoeae* and *Bordetella pertussis* to survive on the swab until cultured. The medium is also of value as an aid in the recovery of *Shigella* species from rectal swabs.

Sodium thioglycollate	1.0 g
Sodium glycerophosphate	10.0 g
Calcium chloride	0.1 g
Powdered agar	2.0 g
Methylene blue (CI No. 52015), 1 per cent aqueous solution	2.0 ml
Glass-distilled water	1000.0 ml

PREPARATION FOR USE

The medium

Dissolve the ingredients (except for the methylene blue) in the 1000 ml of distilled water by heating in steam at 100 °C. Mix well and adjust the reaction to pH 7.4. Add the methylene blue, mix well and distribute into $\frac{1}{2}$ oz screw-capped bottles (bijou bottles) filling to capacity. Screw caps on securely, but not tightly. Sterilize at 10 psi for 20 min. Screw caps down tightly, record date, label and store after cooling. The final medium should be colourless.

Instructions sent with transport outfit

'Take the specimen and insert the swab or swabs into the upper third of the medium in the small bottle. Cut off the protruding

portion of the swab stick with scissors and screw the lid on the bottle, tightly. This usually forces the swab down slightly and centres it in the transport medium. Label the bottle and return it with swabs enclosed to the laboratory as soon as possible. Keep specimens in a refrigerator at 4 °C until ready for shipment.

The swabs are sterile. They have been treated with charcoal to improve the conditions for culture.'

Quality control of media

All fresh batches of bulk media should be tested for their quality, by comparison with either a previous batch and/or other media.

SELECTIVE MEDIA

Viable counts should be performed on plates poured from the bulk. Selected organisms are used. For example, with desoxycholate citrate media, *E. coli*, *Sal. typhimurium* and *Sh. sonnei* would be the organisms used, and viable counts compared with those performed on MacConkey or nutrient agar plates. The *E. coli* should be suppressed or grow very poorly on the DCA whilst the counts and size of the other organisms should be similar on both media.

ENRICHMENT MEDIA

These should be tested for their inhibitory and nutritional powers by inoculating with selected organisms.

For example, Selenite F medium would be inoculated with *E. coli* and *Sal. typhimurium*, incubated at 37 °C and then plated on to MacConkey agar. The *E. coli* should be inhibited, but the *Sal. typhimurium* should grow.

FERMENTATION MEDIA

These should be tested with selected organisms that will:

1. Ferment the sugar with gas production.
2. Ferment the sugar without gas production.
3. Not ferment the sugar.

Other media should be tested for their ability to support the growth of exacting and other organisms.

STERILITY TESTING

Media prepared from bulk should be tested for sterility by overnight incubation at 37 °C.

An exception to this rule may be made in the case of blood agar plates. Selected plates (the last one poured from each flask) are chosen for incubation to check the sterility of the blood, agar and glassware.

25

Methods of Anaerobic Cultivation of Bacteria

Oxygen is required for the growth of many, but not all, micro-organisms. Those that will grow only in the presence of free oxygen are called *obligatory* or *strict aerobes*, those that can grow only in the absence of free oxygen are called *oblige* or *strict anaerobes*, and those organisms that can grow in either state are termed *facultative anaerobes*. Most organisms of medical importance fall into this last group and generally grow more luxuriously under aerobic conditions. Those organisms which grow best in an atmosphere containing a reduced level of oxygen are termed microaerophilic.

Growth of the obligatory anaerobe depends on the state of oxidation or reduction in its environment. This oxidation-reduction (or redox) potential is a measure of the state of oxidation in a solution. It is determined by immersing an electrode in the solution and measuring the electrical potential set up between electrode and solution. This electrode potential, called Eh and measured in millivolts, is higher the more oxidized the system. Strict anaerobes are unable to grow in culture media unless the Eh is below a certain value. One explanation as to why anaerobic organisms do not grow in the presence of oxygen is that many organisms form hydrogen peroxide (H_2O_2) when incubated in the presence of oxygen. Most aerobic organisms produce an enzyme, called catalase, which catalyses $H_2O_2 \rightarrow H_2O + \frac{1}{2}O_2$. Anaerobes do not have this enzyme, and are therefore destroyed by the peroxide. When grown in the absence of oxygen, however, H_2O_2 is not produced.

Cultivation of organisms in the absence of oxygen can be performed in several ways as follows.

Growth in special media

GLUCOSE AGAR DEEPS

The organism is inoculated into the molten medium at 45°C . The culture is then allowed to solidify, and incubated at 37°C . Growth

of the anaerobic organisms takes place below the surface of the medium.

GLUCOSE BROTH

Long thin tubes are half-filled with this medium, boiled for 5 min to remove dissolved oxygen, and the surface of the medium is covered with sterile molten Vaseline. This seals the medium from the air, and inoculation is carried out through the layer of Vaseline by means of a pasteur pipette.

THIOGLYCOLLATE BROTH (*see p. 506*)

COOKED MEAT MEDIUM (*see p. 507*)

IRON STRIPS

Heated to redness, cooled, and added to liquid media, iron strips will oxidize in the medium, and produce anaerobic conditions.

SMITH-NOGUCHI METHOD

Rabbit kidneys are rich in catalase and can thus remove the peroxide formed by growing anaerobic organisms under aerobic conditions. The medium consists of fresh sterile rabbit kidney covered with a long column of ascitic fluid on which a layer of Vaseline is poured. The tubes are inoculated through the Vaseline surface by means of a pasteur pipette.

The last method is rarely used but is included here to illustrate the principles involved.

The anaerobic jar

Undoubtedly, the best general method of anaerobic cultivation is the use of the anaerobic jar. It is capable of producing conditions sufficiently anaerobic to grow the strictest anaerobes (e.g. *Clostridium tetani*) and allows specimens to be directly streaked out, in the normal way, on solid media.

One pattern of anaerobic jar commonly used is that of McIntosh and Fildes as modified by Baird and Tatlock (BTL Jar).

THE BTL ANAEROBIC JAR

A seamless metal or plastic vessel, sufficiently large to contain petri dishes and tubes, is fitted with a gas-tight lid. The lid has inlet and outlet valves and, on its inner surface, a room-temperature catalyst enclosed within a wire gauze envelope. The catalyst consists of alumina pellets coated with 0.5 per cent palladium

which catalyses the combination of oxygen in the jar with hydrogen to form water.

Method of use

1. Place cultures in the jar, taking care to loosen the tops of screw-capped bottles. Secure the lid.
2. Close the inlet valve, connect the outlet valve to a vacuum pump, and evacuate to approximately 10 cm of mercury. Close the outlet valve.
3. Connect the inlet valve to a supply of purified hydrogen gas (commercial cylinder gases are suitable). A convenient method is to supply the gas from a football bladder or anaesthetic apparatus bag that has been filled from a cylinder.
4. Open the inlet valve. The hydrogen will quickly enter the jar and the residual oxygen will be burnt to form water on the gauze cover of the catalyst.
5. After 3 min the reaction should be complete and the inlet valve may be closed, the bag disconnected and the jar placed in an ordinary incubator.

For optimum growth of strict anaerobes, 5–10 per cent CO₂ is often incorporated in the hydrogen supply.

One advantage of this type of jar is that the process of evacuation causes oxygen dissolved in liquid media (e.g. sugar fermentation tubes) to be displaced by the reduced pressure, thereby ensuring correct anaerobic conditions *within the tubes*.

THE 'GAS PAK' SYSTEM

Recently, another version of the anaerobic jar has come into common use, *viz* the self-contained CO₂-H₂ anaerobic system ('Gas Pak'). The principle employed here is the generation of both hydrogen and carbon dioxide by adding water to pre-packed chemicals, thereby eliminating the need for any external apparatus and manipulations.

Testing anaerobic jars

In order to test that anaerobic conditions have been achieved, jars are commonly fitted with side arms to which are attached tubes containing chemical redox indicators.

If anaerobic conditions have not been obtained in the anaerobic jar the reason may be that either the jar leaks, or the capsule is not working.

To test if the jar is leaking, place a piece of cotton wool soaked

in ether into the jar. Clamp down the lid and close all taps. Immerse in a bucket of warm water. This will cause the ether to expand in the jar, and force air out of any leak that has occurred, thus producing bubbles at the site of the leak. The capsule may need renovating and heating it will remove the moisture which can interfere with the catalyst activity.

INDICATORS

Alkaline methylene blue-glucose solution

Solution 1.	0.1N sodium hydroxide	6 ml
	Distilled water	94 ml
Solution 2.	0.5 per cent methylene blue (CI No. 52015)	3 ml
	Distilled water	97 ml
Solution 3.	Glucose	6 g
	Distilled water	100 ml

Add a small crystal of thymol as a preservative.

For use, mix equal volumes of solutions 1, 2 and 3 in a test-tube. Boil until colourless and place in the anaerobic jar. Clamp lid and proceed as previously described.

Alternatively, alkaline glucose broth (pH 8.5) with methylene blue added can be used in the same way.

Semi-solid indicator

Some anaerobic jars have a side arm to which an indicator tube is attached with a short length of rubber tubing. The following indicator is for use with such jars.

Sodium thioglycollate	0.1 per cent
Borax	1.0 per cent
Methylene blue (CI No. 52015)	0.02 per cent
Agar	0.5 per cent

Dissolve by boiling, cool to approximately 50 °C and dispense in 6.5 × 100 mm tubes (as used for freeze drying). Place on manifold of freeze-drying machine, evacuate over P₂O₅ and seal tubes with double-headed burner.

If a freeze-drying unit is not available, the tubes are boiled and sealed immediately the mixture is colourless.

Both these indicators will remain colourless in the absence of

free oxygen. If they have turned blue, anaerobic conditions have not been achieved.

Biological indicators

A plate inoculated with a strict anaerobe, e.g. *Clostridium tetani*, can be included in each jar and examined after incubation for growth.

However, the discovery on the following morning that the jar was not anaerobic is of little value and no comfort to the bacteriologist. It is therefore of more importance regularly to check the jar for air leaks and for catalyst activity.

The use of an anaerobic jar for CO₂ cultivation

It is often necessary to grow organisms in an atmosphere of CO₂. The simplest method is to place the cultures in a jar together with a lighted candle. Screw down the lid of the jar and incubate. The candle utilizes some of the oxygen present, giving an atmosphere of CO₂.

If an exact 10 per cent CO₂ is required the procedure is as follows:

1. Place the cultures in an anaerobic or similar jar, and screw down the lid.
2. Attach one outlet tap on the lid to a manometer and the other to a vacuum pump.
3. Evacuate air, until the manometer reads 100 mmHg.
4. Close the vacuum pump tap, and attach to a source of CO₂ (a rubber football bladder filled from a cylinder).
5. Allow CO₂ to enter the jar until the manometer reads 24 mmHg.
6. Close both taps, take the jar into the incubator, open the taps and allow the warm air to enter the jar.
7. Close the taps and leave to incubate.

Notes—If a complete vacuum was obtained in the jar the manometer would read 760 mmHg, therefore, 10 per cent of that atmosphere would be 76 mmHg. By evacuating to 100 mmHg and running in CO₂ until the manometer reads 24 mmHg, 10 per cent of that atmosphere has been replaced with CO₂.

The opening of the taps in the incubator warms the cultures and also mixes the CO₂ with the air present in the jar. Using this method, any amount of CO₂ can be introduced into the jar.

26

Antigen—Antibody Reactions

ANTIGENS AND ANTIBODIES

When certain foreign substances, usually proteins, are introduced into the animal bloodstream, they trigger a specific response by certain specialized lymphoid cells resulting in the production of blood proteins called *immunoglobulins*. Each such foreign protein is an *antigen*; a bacterial cell thus contains many different antigens. The specific immunoglobulin produced in response to each different antigen is called *antibody*, and a serum containing antibodies is an *antiserum*.

Antibody molecules combine specifically with their corresponding (homologous) antigens. *In vivo*, this constitutes an important defence mechanism against microbial infection. Motile bacteria are immobilized when their flagellar antigens combine with specific antibodies, and toxic substances—both diffusible toxins, e.g. diphtheria toxin and cell-bound virulence factors, e.g. streptococcal M-substance—can be neutralized by combination with antibodies. The blood also contains a series of proteins known collectively as *complement* (C'). Complement is bound non-specifically by antigen–antibody complexes and can then initiate lysis (if the ‘antigen’ is a bacterial or other cell) and promote phagocytosis of bacteria in which the antibody has been absorbed.

These same reactions can be exploited by the bacteriologist in the laboratory as aids to diagnosis and identification. The chief methods of doing so are:

1. Agglutination reactions, in which *particulate* ‘antigens’ (e.g. bacterial cells) are made to form clumps by binding together with antibody molecules.
2. Precipitin reactions, in which a *solution* (or a chemical extract) of an antigen combines with specific antibody to form a precipitate, e.g. Elek plate test for diphtheria toxin.
3. Toxin neutralization reactions, which may be carried out in laboratory animals or *in vitro* as, for example, in the specific neutralization of α -toxin in the Nagler reaction for *Cl. perfringens*.

4. Complement-fixation reactions, which are often employed to test for the presence of specific antibody in a patient's serum when other reactions are impossible (for example, viral particles are too small to show visible agglutination).
5. Other tests, such as immobilization reactions, capsule-swelling reactions, fluorescent antibody techniques, etc.

It is important to note that the bacteriologist may use a 'known' antiserum to detect specific antigens in an 'unknown' organism and, vice versa, a known antigen preparation to test for specific antibody in an 'unknown' serum. For example, in the diagnoses of typhoid fever, bacteria isolated from faecal specimens may be identified as *Salmonella typhi* by agglutination reactions using a variety of known antisera—some of which have been purified to contain antibodies against a single antigen ('single factor sera'). Conversely, a presumptive diagnosis of typhoid fever may be obtained by agglutination reactions carried out onto patient's serum against known suspensions of *Salmonella typhi* and the closely related bacteria of paratyphoid fever. (This is known as the Widal Reaction.) This approach is, in general, less satisfactory, since each bacterial suspension is necessarily a mixture of antigens, many of which are shared by related bacteria. Previous immunization may also confuse the issue.

H and O antigens

In 1903 Smith and Reagh discovered that the motile hog cholera bacillus had a non-motile variant. When agglutination tests were performed using antiserum produced by the motile organism, the non-motile organism gave a different agglutination reaction to the motile type. Whereas the motile organism gave a rapid fluffy type of agglutination, the non-motile organism gave a slower granular agglutination. A year later Beyer and Reagh heated the motile organisms to 70 °C for 15 min and showed that the heated motile organism agglutinated in the same way as the non-heated, non-motile organism did, because the flagella antigen had been destroyed. These workers proved that a motile organism has two antigens, one flagella antigen, the other the body antigen, whilst a non-motile organism only has the body antigen.

In 1917 Weil and Felix were performing agglutination tests with a flagellated motile organism that spread over the surface of an agar plate (*Proteus*). It gave an appearance resembling the mist caused by breathing on glass, and they named it the *Hauch* form—*Hauch* meaning breath. A non-flagellated, and therefore non-

motile, variant of the same organism gave discrete colonies on the same media and was named the *Ohne Hauch* form—the non-breath form. The two forms were symbolized as the H and O forms and this designation has been extended, and H is now used as a symbol for all flagella antigens irrespective of whether the organism spreads on agar, and O as the symbol for the body or somatic antigen. The antibodies against these antigens are called H and O antibodies, respectively.

Agglutination tests

METHOD

Apparatus required

Pipettes, test-tubes, agglutination tubes, grease pencil, racks for tubes, serum, saline, antigen and 50°C water bath.

1. Place 10 test-tubes in a rack.
2. Add 4 vol of saline to tube 1 and 1 vol to tubes 2 to 10.
3. Add 1 vol of serum to tube 1 and mix. This dilutes the serum 1 in 5, i.e. 1 vol of serum plus 4 vol of saline, giving 5 vol of solution of which 1 vol is serum.
4. Transfer 1 vol of serum saline solution to tube 2. This dilutes the serum 1 in 10.
5. Repeat the procedure up to and including tube 10. This gives serum dilutions of 1 in 5, 1 in 10, 1 in 20, 1 in 40, 1 in 80, 1 in 160, 1 in 320, 1 in 640, 1 in 1280 and 1 in 2560.
6. Using a fresh pipette and starting from the highest dilution, transfer 0.5 ml from each test-tube into a corresponding agglutination tube rack.
7. Add 0.5 ml of antigen to each tube. The addition of an equal quantity of antigen dilutes the serum again, the final serum dilution being 1 in 10 in the first tube, 1 in 20 in the second tube, and so on.
8. To another agglutination tube add 0.5 ml of saline and 0.5 ml of antigen. This tube serves as a control and shows if the antigen is salt-agglutinable.
9. Place the agglutination rack in the water bath and adjust the water level until it covers one-third of the tube.

Slide agglutination test

For rapid identification of colonies from an agar medium, slide agglutinations can be performed using the colony suspended in saline as the antigen and mixing with known serum. Only O agglutinations should be performed this way, as solid media are

not good for the formation of flagella and false negative slide H agglutinations may occur. Slide agglutinations should be confirmed by the tube technique.

METHOD

1. Place 1 drop of saline on a slide, and next to it, 1 drop of required serum.
2. Using a straight wire, transfer part of the colony to be tested to the saline and mix, making a smooth suspension.
3. If no autoagglutination has taken place, mix the serum with the smooth suspension.
4. Look for agglutination which should occur within 10–15 s.

Notes on agglutination tests

1. Agglutination will take place only in the presence of an electrolyte; therefore, 0.9 per cent sodium chloride should be used as a diluent.
2. Immunoglobulins are thermolabile, therefore temperatures in excess of 50 °C should not be used.
3. Flagella antigens agglutinate more quickly than the somatic antigens; they can be read after 2 h incubation at 50 °C. Somatic antigens are best incubated at 37 °C for 2 h followed by refrigeration at +4 °C overnight; the results are recorded after warming to 37 °C for 10 min.
4. During incubation the water level should be adjusted so that one-third of the tube is immersed. This allows the formation of convection currents which aid mixing and thereby speed the agglutination reaction.
5. Slide agglutination tests are most commonly used to identify unknown organisms. When performing such tests on live bacteria great care should be taken to avoid splashes which could easily infect the operator or his colleagues.

Dilutions

The preparation of 'dilutions' often causes a certain amount of trouble. The one cardinal rule to remember is that if one volume of a concentrated or neat solution is diluted with an equal volume of diluent (e.g. distilled water), then that solution has been diluted 1 in 2.

That is, 1 volume of solution + 1 volume of diluent = 1 in 2.

If this 1 in 2 solution is further diluted with an equal volume of diluent then the solution has been diluted 1 in 4.

That is, 1 volume of 1 in 2 solution + 1 volume of diluent = 1 in 4 or a $\frac{1}{2}$ -solution diluted $\frac{1}{2} \times \frac{1}{2} \times \frac{1}{2} = \frac{1}{4} = 1$ in 4.

If a neat solution is diluted with twice its volume of diluent, then the resultant dilution is 1 in 3.

That is, 1 volume of solution + 2 volumes of diluent = 1 in 3.

It will be seen, therefore, that if a 1 in 3 solution is diluted 1 in 4 the resultant dilution is 1 in 12.

That is, $\frac{1}{4} \times \frac{1}{3} = \frac{1}{12} = 1$ in 12.

If the above approach is taken, any dilution should be readily obtained.

The following equation is useful when the dilution of solutions of known strengths is required.

Where R = required concentration

V = total volume of solution required

O = original concentration

then $\frac{R \times V}{O}$ gives:

Volume of original solution to be diluted with distilled water to the final volume required.

Example

The original solution is 70 per cent; 45 ml of 30 per cent solution is required.

Using the equation

$$\frac{30 \times 45}{70} = 19.3$$

Therefore, 19.3 ml of 70 per cent solution must be diluted with 25.7 ml of distilled water to obtain 45 ml of a 30 per cent solution.

27

Routine Bacteriological Examination of Specimens

Many types of specimen are received daily in a bacteriology laboratory, both from in-patients and out-patients.

It is difficult to lay down hard and fast rules for the treatment of specimens. Many laboratories have individual methods for treating specimens. The student should be familiar with the methods in his own laboratory and make reference to more advanced textbooks on microbiology for further information on this subject. For this reason only a broad outline will be given for the examination of specimens.

GENERAL PROCEDURE

1. The specimen should be properly labelled with the patient's name, hospital number, ward and date of collection. This is essential in order to prevent confusion of specimens from patients of similar name.

The date of collection is required so that delays in reaching the laboratory are apparent, and misleading results avoided; for example, pathogens in urines may be overgrown by contaminants if there is a long delay in transit from ward to laboratory.

2. The request form should state the provisional diagnosis and the nature of examination required. This facilitates selection of techniques; for example, if a specimen of sputum is sent for detection of *Mycobacterium tuberculosis* (in a suspected case of pulmonary tuberculosis) detailed examination for other organisms is obviously not required.
3. Any information with regard to the chemotherapy should be noted. Certain precautions may be necessary; for example, specimens from patients receiving sulphonamides should be inoculated on to media containing *p*-aminobenzoic acid, which prevents the bacteriostatic action of sulphonamides

on the organisms. Similarly, the use of penicillinase may be required for the isolation of organisms from a patient receiving penicillin.

4. The correct container should be used. Most specimens for bacteriological examination should be received in a sterile container. For sputum, particularly for out-patients who send their specimens by post, urine and blood, 1 oz, wide-mouthed universal bottles are convenient. For specimens that are to be treated by a concentration method for *M. tuberculosis*: waxed or plastic cartons are suitable for sputa or faeces; narrow-mouthed universal bottles, containing 3.8 per cent sodium citrate for pleural fluids; and 1 oz wide-mouthed universal bottles with 15 per cent tri-sodium phosphate, for specimens of gastric lavage. It is obvious that a specimen received in a gastric lavage bottle would be useless for general investigation of organisms present. By the time it reached the laboratory, many of the bacteria would be destroyed by the tri-sodium phosphate.
5. With all specimens extreme care must be taken with all manipulations and they should be carried out under the protection of an exhaust inoculating cabinet.

THE INOCULATING CABINET (SAFETY HOOD)

All work involving the handling of such specimens and cultures should be performed under cover of an inoculating cabinet. The design of a cabinet should be such that air is drawn through the cabinet to a filter and then to an outlet funnel (by means of an extractor fan) at the rate of approximately 150 linear feet per min. If a filter is not used the outlet should lead to an exit-pipe opening to the atmosphere 6 feet above roof level.

The flow of air has the purpose of carrying infected particles and aerosols away from the environment of the operator to the outside atmosphere where they are at once diluted and destroyed by the ultraviolet light in the sun's rays.

The base of the cabinet should be such that swabbing with 1 per cent Hycolin, or other disinfectant, can be effected after each operation.

FURTHER PRECAUTIONS

Other precautions when dealing with all specimens or cultures are:

Never lay a culture tube on the bench; always place it in a rack or tin.

Label clearly *every* tube or plate with the specimen's number.

When finished always discard the cultures into an appropriate discard receptacle for sterilizing. Never remove the cultures once discarded until they have been sterilized.

Keep the working space on the bench clear so that if an accident occurs the minimum number of articles will be involved.

Handle all apparatus and materials carefully.

Do not smoke when working with specimens or cultures. Never lay a pipette mouthpiece on the bench.

When pipetting always use a teat, never pipette by mouth.

Do not lick gummed labels.

Report any accident, however trivial, to the senior person in the laboratory.

Always wash your hands with soap and water after handling cultures and specimens, and before going off duty. It is recommended that disposable paper or continuous roller towels be provided to minimize any possibility of cross infection.

POSTAGE OF PATHOLOGICAL SPECIMENS

Certain regulations are laid down by the Postmaster-General for the sending of specimens through the post. Universal containers wrapped in absorbent cotton wool and sent in thick cardboard boxes are permissible, providing that the label is clearly marked 'Pathological specimen' and 'Fragile with care'. These regulations may be obtained from the Post Office, and if the suitability of any box or container is in doubt, it should be submitted to the General Post Office, for confirmation of its suitability. Failure to do so may lead to the prosecution of the person sending the specimen.

Examination of specimens

A general plan for examining specimens is as follows.

MACROSCOPIC EXAMINATION

Note the following:

1. Colour, opacity, consistency.
2. Presence of blood, mucus or pus.
3. Presence of macroscopic bodies, such as parasites.

MICROSCOPIC EXAMINATION

1. Unstained film or negative staining, e.g. when looking for cells or casts in urine deposit.
2. Stained film by (a) Gram.
(b) Acid-fast bacilli stain.
(c) Special stains.

CULTURE

Blood agar incubated aerobically and anaerobically.

MacConkey agar.

Special media.

Sensitivity tests—direct where necessary.

SEROLOGY

Widal special agglutination tests or complement-fixation tests.

ANIMAL INOCULATION

Animal inoculation is carried out only if necessary.

EXAMINATION OF CULTURES

Keep extensive notes on the examination of the cultures set up as follows:

Plate cultures

Note types of colony seen and list as 1, 2, 3, and so on.

Note the shape, colour, size, consistency, haemolysis and Gram stain reaction.

Liquid cultures

Note nature of the medium such as colour, type of growth (granular, smooth, surface, etc.) or deposit.

Microscopic appearance of bacteria

Note shape, size, arrangement, motility, staining reaction, spores, capsules, pleomorphism.

For the final report, however, it is only necessary to report the organism or organisms seen in smear and isolated on culture together with the sensitivity pattern.

BLOOD CULTURES

In certain diseases, for example, septicaemia and typhoid fever, bacteria may be present in the blood, and the presence of the causative organisms may be detected by cultures. Blood is taken from the patient, and immediately inoculated into tubes or bottles of a suitable medium. These are incubated at 37°C. If possible a 'pour plate' should be made with the patient's blood and nutrient agar. This is a good guide to the number of organisms present

in the blood as well as hastening identification. From normal blood, the cultures are sterile.

The blood must be added to media that inhibit the natural antibacterial power of the blood and that prevent clotting. Polyanethyl sulphonate (Liquoid) not only prevents clotting but also neutralizes the bactericidal power of fresh blood when used at a concentration of 0.03–0.05 g per 100 ml.

The medium is placed in bottles which have perforated caps, thus enabling the syringe needle to be pushed through the rubber liner. This prevents the bottle being opened until required for sub-culturing. 5 ml of blood is added to 100 ml of glucose broth and, for anaerobic bacteria, 100 ml of thioglycollate broth. A diphasic medium (castaneda) may also be set up. This medium is made up of a slope of nutrient agar to which glucose broth has been added. The exposed surface of the slope is examined daily for growth and by tilting the blood broth over the agar slope each day, opening the bottle to sub-culture is avoided. The other bottles should be sub-cultured daily for 3 days and then after 1 week's incubation. Incubation should be continued for up to 3 weeks sub-culturing weekly. Sub-cultures are usually made into blood agar plates which are then incubated aerobically, anaerobically and in an atmosphere of 10 per cent CO₂.

There are many modifications to blood culture techniques. In cases such as bacterial endocarditis, a pour plate is of value using the patient's blood, as often identification of any organism present can be performed the next day. A Gram film from a spun deposit often gives an indication of the type of organism present. The use of Thiol broth (Difco) is also recommended for ANO₂ cultivation.

The following organisms are associated with septicaemia: *S. pneumoniae*, *S. pyogenes*, viridans streptococci, coliform bacilli, Brucellae, *S. typhi*, *S. aureus*, but *any organism* isolated must be investigated carefully and regarded as a pathogen unless it is established as a contaminant.

CEREBROSPINAL FLUID

It is very important that the clinician should be informed, as soon as possible, of any organisms found in a cerebrospinal fluid specimen (generally sent in a sterile universal bottle). In most cases this can be ascertained by examination of stained direct smears, but cultures should also be set up immediately. Normally cerebrospinal fluid is sterile, but contaminants may be introduced by careless technique, both in ward and laboratory.

METHOD

1. Note the appearance of the fluid—for example, whether clear or cloudy—and transfer it to a clean sterile centrifuge tube, taking a small quantity for cell count.
2. Centrifuge at 3000 rpm for 5 min.
3. Discard the supernatant fluid into a jar of disinfectant (unless required for biochemical examination or WR) and make two smears from the deposit.
4. Stain one smear by Gram and the other for acid-fast organisms. If *torulosis* is suspected prepare a 'negative stain' film using Nigrosin or Indian Ink.
5. Inoculate the following media from the deposit: One blood agar plate and incubate aerobically at 37°C; one chocolate agar plate and incubate in 10 per cent CO₂ at 37°C. Direct sensitivity tests may be performed.
The following day, any organisms grown should be identified and sensitivity tests performed.
6. If *tuberculous meningitis* is suspected, inoculate two Lowenstein-Jensen slopes (in addition to the plates), and incubate at 37°C for 8 weeks, examining the slopes at weekly intervals.

(In suspected meningococcal meningitis the cerebrospinal fluid may be incubated overnight at 37°C and then cultured as above.)

The following organisms are associated with meningitis: *Neisseria* species, *S. pneumoniae*, *H. influenzae*, *S. pyogenes*, *S. aureus*, *Listeria*, *Cryptococcus*, *M. tuberculosis*, but any organism isolated should be thoroughly investigated and regarded as a pathogen unless established as a contaminant.

Note—In cases of tuberculous meningitis a spider-clot is often noted on the fluid. The clot is carefully decanted into a watch glass. If a piece of lens paper is carefully laid on the clot, the clot will adhere to the paper. The clot is then 'blotted' on to a clean glass slide, fixed and stained by the Ziehl-Neelsen method.

FAECES

Faeces are sent to the laboratory in a suitable wide-mouth container with a screw-capped lid or in the form of a rectal swab. Usually the organisms to be isolated are pathogenic Gram-negative bacilli, *S. aureus*, tubercle bacilli, or parasites. The normal flora may include coliform bacilli, *S. faecalis*, *B. subtilis*, *C. perfringens* and *bacteroides*.

METHOD FOR GRAM-NEGATIVE BACILLI

1. Inoculate a large loopful of the faeces onto a deoxycholate citrate agar plate and into a tube of Selenite F.
2. Emulsify a further portion in peptone water; from the peptone water inoculate a MacConkey agar plate and a Wilson and Blair plate if enteric fever is suspected. Incubate at 37°C.
3. The following day examine the plates for non-lactose-fermenting colonies.
4. Pick off suspicious colonies and identify by biochemical reactions and agglutination tests.
5. Plate out the Selenite F medium onto a MacConkey agar plate. Incubate at 37°C overnight.
6. Examine and identify any non-lactose-fermenting colonies present.

If *Vibrio cholerae* is suspected, inoculate into alkaline peptone water and thiosulphate-citrate-bile salt-sucrose medium (TCBS medium). If *S. aureus* is suspected inoculate into salt cooked meat broth or on Ludam's medium and make a Gram film.

METHOD FOR ISOLATION OF *M. tuberculosis*: ETHER CONCENTRATION TEST

1. Make a thick saline suspension of faeces in a screw-capped bottle.
2. Add an equal volume of ether and shake well.
3. Centrifuge at 3000 rpm for 5 min.
4. Pipette off the supernatant ether into disinfectant and make a smear from the gelatinous layer.
5. Stain smear by Ziehl-Neelsen and examine.
6. If culture is required, remove gelatinous layer, treat as for sputum (*see below*), and inoculate two Lowenstein-Jensen slopes.
7. Incubate at 37°C for 8 weeks, examining weekly.

Alternatively, treat the faeces by a sputum concentration method.

FLUIDS**Pleural, peritoneal and other fluids**

The fluids are sent to the laboratory in a sterile narrow-mouthed 1 oz bottle containing 3 ml of 3.8 per cent sodium citrate. This prevents any clotting of the fluid. A similar procedure is adopted

as for cerebrospinal fluid. These fluids are commonly sterile, but contaminants may occur as in blood cultures.

METHOD

1. Note the appearance and quantity of fluid.
2. Transfer to a clean sterile centrifuge tube and centrifuge at 3000 rpm for 15 min.
3. Discard the supernatant fluid into disinfectant or keep for protein estimation. From the deposit inoculate two blood agar plates and make two smears.
4. Incubate the blood plates aerobically and anaerobically at 37 °C.
5. Stain the smears by Gram, Ziehl–Neelsen, auramine–phenol or Leishman and examine.
6. After incubation identify any organisms isolated.
7. If *M. tuberculosis* is suspected it is inoculated directly on to two Lowenstein–Jensen slopes. Then the deposit is treated as for sputum and two more Lowenstein–Jensen slopes inoculated.

Possible pathogens that might be isolated include *M. tuberculosis*, viridans, streptococci, haemolytic streptococci, *S. aureus*, *S. pneumoniae* and anaerobic streptococci.

PUS

The nature of the examination is again governed by the type of pus received, that is, whether from ear, wound or boil. It may be sent in a sterile container or on a swab or gauze. The contaminants found depend on the site of the pus. If from the skin, *S. epidermidis* diphtheroids and coliform bacilli may be present as commensals.

METHOD

1. Note the appearance. If actinomycosis is suspected, examine for sulphur granules.
2. Inoculate two blood agar plates and make two smears.
3. Stain the smears by Gram and for acid-fast bacilli.
4. Incubate the plates aerobically and anaerobically. If gonococci are suspected, incubate a third plate in CO₂.
5. If indicated by smears, a direct sensitivity test should be set up.

6. Next day, identify any organisms isolated. Re-incubate up to 7 days if actinomycosis is suspected.
7. If *M. tuberculosis* is suspected, the pus is inoculated onto two Lowenstein-Jensen slopes and then treated as for sputum.

Possible pathogens that might be isolated include *S. aureus*, haemolytic streptococci, *M. tuberculosis*, *C. perfringens*, *B. subtilis*, *P. vulgaris*, *A. israeli*, *Bacteroides* and *Haemophilus* species.

SEROLOGY

For serological examination, whole blood is sent to the laboratory in a sterile universal bottle. The blood is allowed to clot, then freed from the sides of the bottle with a firm straight wire and incubated at 37 °C for a short time to hasten clot retraction. The serum is removed with a sterile pasteur pipette into a centrifuge tube and spun down to remove any free red cells. For complement-fixation tests it is better to keep the whole blood specimen in the refrigerator overnight before removing the serum.

The serum is pipetted into a clean sterile bottle and clearly labelled with the patient's name, hospital number, ward, date and nature of specimen and kept in the freezing compartment of the refrigerator. The tests to be carried out depend on the provisional diagnosis. These may include: Widal tests in suspected *Salmonella* or dysentery infections; agglutination tests in suspected *Brucella* infections; VD serology in suspected syphilitic cases; Rose-Waaler test and anti-streptolysin titre in cases of rheumatoid arthritis, and virus serology in suspected virus diseases.

SPUTUM

Sputum examinations can be divided into two main groups. Examination may be for *M. tuberculosis* or for other organisms. The normal flora may include viridans and non-haemolytic streptococci, *Neisseria*, diphtheroids, fusiform bacilli and spirochaetes.

METHOD FOR ORGANISMS OTHER THAN *M. tuberculosis*

1. Note appearance of sputum, whether salivary, mucoid, purulent or blood-stained.

2. Homogenize the sputum either by shaking with sterile Ringer's* solution and glass beads, or by adding 1 per cent pancreatin† and incubating for 1 h. Culture onto blood agar and any other media routinely used and make smears.
3. Stain the smears by Gram and Ziehl-Neelsen or auramine-phenol.
4. Incubate the blood agar plate aerobically at 37 °C.
5. Examine the smears and next day identify any organisms isolated.

Possible pathogens that might be isolated from sputa include *H. influenzae*, haemolytic streptococci, *K. pneumoniae*, *S. pneumoniae*, *S. aureus* and *P. aeruginosa*.

*Ringer's solution

Sodium chloride	9.0 g
Potassium chloride	0.42 g
Calcium chloride	0.48 g
Sodium bicarbonate	0.2 g
Glass-distilled water	1000 ml

†1 per cent pancreatin solution for homogenizing sputum

Add 1 g of pancreatin to the following solution:

Sterile normal saline solution	100 ml
Buffer solution	7 ml

Buffer solution N/5 NaOH, 1 vol.
 M/5 KH₂PO₄ 1.2 vol.

Routine examination for *M. tuberculosis*

A smear from a specimen of sputum will demonstrate the presence or absence of acid-fast bacilli, but will not prove they are tubercle bacilli. Culture for *M. tuberculosis* must be performed together with subsequent tests if the culture yields growth. A concentration method is given which will kill most organisms other than mycobacteria.

PETROFF'S METHOD (MODIFIED)

1. Mix the sputum with 3 or 4 times its volume of 4 per cent sodium hydroxide in a sterile, 1 oz wide-mouth universal bottle.

2. Shake on a mechanical shaker (housed in an exhaust cabinet) for 20 min or shake by hand frequently during a 20 min period, again in an exhaust cabinet.
3. Centrifuge at 3000 rpm for 15 min.
4. Pour the supernatant fluid into disinfectant and re-suspend the deposit in 25 ml of sterile glass-distilled water containing 100 iu per ml of penicillin.
5. Spin at 3000 rpm for 15 min. Pour off supernatant fluid and film the deposit.
6. Inoculate two Lowenstein-Jensen slopes..
7. Incubate at 37 °C in a flat position for 24 h to allow the fluid to spread evenly over the surface of the medium. Then incubate in an upright position for up to 8 weeks.
8. Examine the cultures weekly.

SWABS

Throat and nasal swabs

These should be cultured immediately on arrival, as the material soon dries on the swabs. If delay in transit is anticipated the swab should be moistened with a little broth before inoculating media, or a suitable transport medium used. All media should be inoculated before films are made, owing to the scantiness of material on the swab. Reliance should not be placed on the film result alone; its main use is to exclude Vincent's infection, that is, the presence of spirochaetes and fusiform bacilli from throat swabs.

The normal flora may include *Neisseria*, viridans and non-haemolytic streptococci and diphtheroids.

METHOD

1. Inoculate two blood agar plates and incubate aerobically and anaerobically at 37 °C.
2. In suspected diphtheria cases, a Loeffler's serum slope and blood tellurite plates are also inoculated and incubated aerobically at 37 °C.
3. The next day identify any organism grown.

Pharyngeal swabs (post-nasal) and per-nasal swabs

These swabs are prepared from 150 mm lengths of SWG 18 copper wire, slightly bent 1 in from one end. Absorbent wool is wrapped around this end, which has been flattened. The swab is

sterilized in a 125 mm \times 12 mm tube by hot air. These swabs are received from cases of suspected whooping-cough and meningococcal carriers.

METHOD

1. In suspected whooping-cough cases inoculate Bordet-Gengou plates with 0.25 units of penicillin per ml and/or Lacey's DPF plates. Incubate at 37 °C for 2–4 days.
2. In suspected meningococcal cases inoculate chocolate agar and incubate in 10 per cent CO₂ at 37 °C for 1–2 days.

Genito-urinary swabs

These swabs are generally received from cases of suspected puerperal sepsis, gonorrhoea or trichomoniasis. Stuart's transport medium should be used if delay in sending to the laboratory is likely.

The normal flora may include staphylococci, diphtheroids, faecal streptococci, coliform bacilli, fusiform bacilli.

METHOD

1. Inoculate two blood agar plates and incubate one anaerobically and the other in 10 per cent CO₂ at 37 °C.
2. Make a smear, after inoculation of plates, and stain by Gram's method and a wet preparation for examination for *Trichomonas vaginalis*.
3. The next day, identify any organism grown on the plates.

Possible pathogens may include haemolytic streptococci, *N. gonorrhoeae*, *S. aureus*, *C. perfringens*, *C. albicans*.

Eye swabs

Swabs taken from eye infections should be cultured immediately they are taken, to prevent enzymatic action killing any organisms present.

METHOD

1. Inoculate two blood agar plates and incubate one aerobically and the other in 10 per cent CO₂ at 37 °C.
2. Next day identify any organisms grown on the plates.

Possible pathogens include *S. aureus*, pneumococci, *H. influenzae*, Koch-Weeks bacilli, *N. gonorrhoeae*, haemolytic streptococci and diphtheroids.

Laryngeal swabs (for *M. tuberculosis*)**METHOD 1: ABSORBENT WOOL SWABS**

1. Immerse the laryngeal swab in a few ml of 6 per cent sulphuric acid for 6 min.
2. Pour off acid and replace with 4 per cent NaOH. Leave for 20s and inoculate the swab on two Lowenstein-Jensen slopes.
3. Incubate slopes at 37 °C for 8 weeks, examining at weekly intervals.

METHOD 2: ALGINATE WOOL SWABS

1. Immerse the laryngeal swab in 5 ml of 15 per cent trisodium phosphate.
2. Agitate the swab until the wool has dissolved.
3. Centrifuge. Inoculate two Lowenstein-Jensen slopes from deposit.

Films should *not* be made owing to scanty amount of material on the swab.

GASTRIC LAVAGE

On the wards, the gastric washings are placed in a sterile 1 oz universal bottle containing 5 ml of 15 per cent trisodium phosphate, and sent to the laboratory. On receipt the specimen is centrifuged and the deposit treated as for concentration of sputum. By placing the gastric washings direct into trisodium phosphate, the acid washed from the stomach (which would be sufficient to kill the tubercle bacilli) is neutralized.

URINE

'Cleaned up' mid-stream specimens of urine should be sent to the laboratory in suitable sterile containers, with the minimum of delay. Catheter specimens should be avoided because of the high incidence of bladder infections after catheterization. A film of the specimen without centrifugation is made and stained. If organisms are seen, this (provided the specimen was freshly voided) indicates that the organisms are present in large enough numbers to cause infection. When culturing, it is suggested that some form of viable

count be performed. A standard loop is used which will take up a known amount of urine. Counting of the colonies, next day, on media inoculated this way, will give an approximate number of viable organisms per ml. It has been said that 100 000 organisms per ml is indicative of infection. It is imperative that the urine specimen be examined without delay as organisms will reproduce rapidly in urine.

If isolation of *M. tuberculosis* is requested three consecutive early morning specimens should be sent.

METHOD

1. Note appearance of urine.
2. Using a standard sterile loop*, insert vertically into the urine and inoculate MacConkey or CLED and blood agar plates. Make a film. Incubate at 37 °C and next day count and identify any organisms present. A colony count of 400 is indicative of infection.
3. Transfer to centrifuge tube and centrifuge at 3000 rpm for 15 min. Pour off the supernatant fluid into disinfectant.
4. From the deposit make a wet preparation.
5. Examine for cells, casts, crystals, organisms, etc.

Provided the specimen has been taken with adequate aseptic precautions the following organisms may be considered to be pathogenic: Coliform bacilli, *S. faecalis*, *S. aureus*, haemolytic streptococci, *Proteus*, *Shigella* and *Salmonella* species.

METHOD FOR *M. tuberculosis*

1. The specimen of urine is allowed to stand overnight in the refrigerator.
2. Discard the supernatant (under cover of inoculating cabinet) and transfer the sediment to universal containers.
3. Centrifuge the bottles at 3000 rpm for 20 min.
4. Discard the supernatant and treat each deposit as for sputum.
5. Inoculate six Lowenstein-Jensen slopes from each deposit.

* A standard loop containing $\frac{1}{250}$ ml of water can be made by using a metal rod, 3.26 mm diameter (30 Morse Gauge) and nichrome or platinum wire SWG 28. The content of the loop can be checked by weighing a bijou bottle of water, inserting the loop in a vertical position and spreading on a piece of blotting paper. After removal of 500 loopfuls the bottle and water are reweighed and the loopful content calculated.

**A GENERALIZED SCHEME FOR THE ISOLATION
AND IDENTIFICATION OF BACTERIA FROM
PATHOLOGICAL SPECIMENS**

On the day that a specimen arrives in the laboratory (i.e. as soon as possible after being taken) it is usually plated out onto a variety of enriched, selective or differential media. The choice of these and of the other conditions of incubation will be guided by the clinical information that should accompany all such specimens. These conditions of incubation include temperature—usually 37°C for pathogenic bacteria, and the choice of atmosphere—aerobic, anaerobic, microaerophilic, with or without 5–10 per cent CO₂. With certain specimens it is worthwhile to put up direct drug-sensitivity tests.

After overnight incubation the plates are scanned for growth and individual colonies examined with a $\times 8$ hand lens or a plate microscope. These examinations often yield much information. An experienced bacteriologist can bring together evidence from the colonial morphology, the nature of the media on which the colonies have or *have not* grown, and the effect of the organism's growth on the medium, e.g. haemolysis or pH change. These clues, when taken together with the clinical information, will often point strongly to the identity of the bacteria under examination but they can never be conclusive and confirmatory tests must always be carried out.

One vital procedure is to make films from individual colonies for examination of cellular morphology and arrangement, and staining reaction notably to Gram's method. In some cases it is advantageous to use special techniques, e.g. to demonstrate spores by specialized staining reactions, or motility by the use of a hanging drop preparation.

At this stage it is also possible to carry out certain 'instant' tests, such as the catalase and oxidase reactions and the slide test for cell-bound coagulase.

The bacteriologist is now usually ready to make a provisional report to the physician on the likely identity of the bacteria isolated (and, possibly, their drug sensitivity) so that treatment can be started or modified.

Confirmation usually requires more time-consuming biochemical tests and often the determination of the types of antigen on the bacterial surfaces by means of agglutination or precipitation methods (e.g. *Salmonella* and *Streptococcus*). Occasionally, the susceptibility of the bacteria to highly specific bacteriophages may

be useful confirmatory evidence (e.g. with *Brucella* spp. and *Bacillus anthracis*).

Toxigenic pathogens, e.g. *Corynebacterium diphtheriae*, may require an animal pathogenicity test for final confirmation.

It is important to realize that the confirmation of an organism as being 'X' may have serious consequences—both medical and legal—for the patient and others. 'Confirmation' based on insecure evidence must therefore be avoided, even when it means a longer wait for the 'Final Report'.

SOME TESTS USED FOR THE IDENTIFICATION OF ORGANISMS

Catalase activity

One use of this test is to differentiate staphylococci (catalase+) from streptococci (catalase-).

METHOD

To the organism growing on a suitable solid medium add a drop of 10 vol hydrogen peroxide. Examine immediately, and after a few minutes, for bubbles of gas which indicates catalase production.

Note—Blood-containing media are unsuitable for this test. Alternatively a small portion of the colony under test is placed in a drop of hydrogen peroxide on a microscope slide; catalase-positive strains cause effervescence in the drop.

Coagulase activity

A pathogenic staphylococcus, *Staph. aureus*, has the power of clotting or coagulating blood plasma. This is due to the production by the pathogenic staphylococci of the enzyme *coagulase*. Coagulase may be bound to the organism, in which case it is demonstrated by the slide test, or 'free', when the tube method is used. The vast majority of pathogenic staphylococci produce both forms. Occasionally, however, some strains only produce one or the other. It is necessary to perform a tube test on all 'slide negative' staphylococci.

METHOD 1: SLIDE TEST

1. Emulsify a colony of staphylococci in one drop of distilled water on a clean glass slide. The opacity should be such that

the hands of a watch can be seen through the suspension.

2. Add a small loopful of rabbit plasma and mix.
3. A positive coagulase test will show immediate clumping—a negative test will show no clumping.

A known positive staphylococcus should be tested at the same time, to check that the plasma is working properly.

METHOD 2: TUBE TEST

1. Dilute fresh rabbit plasma 1/10 with normal saline.
2. To 0.5 ml of this (in a $3 \times \frac{1}{2}$ in tube), add 5 drops of an overnight broth culture of the staphylococcus under test.
3. To another 0.5 ml add 5 drops of sterile broth (this acts as a negative control).
4. To another 0.5 ml add 5 drops of a known coagulase-positive, staphylococcus culture. This acts as a positive control.
5. Incubate at 37°C for up to 6 h. A positive coagulase test will show clotting usually within 1 h.

Test for indole production

REAGENT (KOVACS REAGENT)

<i>p</i> -Dimethylaminobenzaldehyde	5 g	(12.5)
Amyl alcohol	75 ml	(182.5)
Conc. HCl	25 ml	(62.5)

Dissolve the aldehyde in the alcohol by gently warming in a water bath (about $50\text{--}55^{\circ}\text{C}$). Cool and add the acid. Protect from light and store at 4°C .

METHOD

To a peptone water culture (24–48 h incubation) add 0.5 ml of reagent. Shake well and examine after 1 min.

A red colour indicates the presence of indole.

Optochin sensitivity (ethylhydrocuprein hydrochloride inhibition)

S. pneumoniae is sensitive to 'Optochin' but viridans streptococci and *S. faecalis* are resistant. This fact is used for the identification of *S. pneumoniae*.

METHOD

Place a disc impregnated with ethylhydrocuprein on the surface of a blood agar plate inoculated with the organism. Incubate and examine after 18 to 24 h. Sensitivity to the compound is shown by inhibition of bacterial growth around the disc.

PREPARATION OF DISCS

To a filter paper disc 0.5 cm diameter add 0.02 ml of a 1/5000 solution of ethylhydrocuprein hydrochloride. Dry at 37 °C or freeze-dry. Store in a closed container. These discs may be obtained commercially.

Oxidase test

This is used for distinguishing colonies of *Neisseria* from mixed cultures and *Pseudomonas aeruginosa* from enteric bacteria. Both *Neisseria* and *Pseudomonas aeruginosa* are oxidase-positive; *Neisseria* are strongly positive.

METHOD 1

Flood the colonies with a solution of 1 per cent aqueous tetramethyl-*p*-phenylenediamine hydrochloride solution. A positive oxidase reaction turns the colonies a purple colour; a strong reaction is almost black. It is important to sub-culture immediately after observing the reaction—the reagent is lethal to *Neisseria*.

METHOD 2

Place 2–3 drops of oxidase reagent on a piece of filter paper. Smear the colony under test across the paper. A positive reaction turns the paper a dark purple within 10 s.

SENSITIVITY TESTS

The sensitivity of organisms to antibacterial substances, e.g. antibiotics, is an important factor in the treatment of patients. There are two main methods of sensitivity testing, namely: *incorporation* methods and *diffusion* methods. Each of these may be carried out by a variety of techniques. Only a brief reference will be made to these methods, and for a more detailed account bacteriology textbooks should be consulted (*see* Bibliography).

Tube dilution (incorporation of drug in broth media)

A range of doubling dilutions of the drug is prepared (in duplicate) in glucose broth.

To one row of drug dilutions is added a standard drop of a standard suspension of the test organism.

To the other row (the control row) is added a standard drop of a standard suspension of a known sensitive organism. The tube with the lowest concentration of antibiotic showing no growth indicates the amount of antibiotic per ml to which the organism is sensitive (MIC = minimum inhibiting concentration).

Incorporation in solid media

The use of solid media avoids reporting strains as resistant when such resistance is due to very few mutant cells.

Disc method

Discs can be obtained commercially from many sources.

METHOD OF USE (STOKES METHOD)

1. Inoculate half of a blood agar plate with one specimen.
2. Inoculate the other half with a sensitive organism of the same species.
3. Place disc of one antibiotic 2 cm from side of plate and on the gap between the inoculated areas.
4. Place disc of other antibiotic on other side of plate.
5. Incubate overnight.

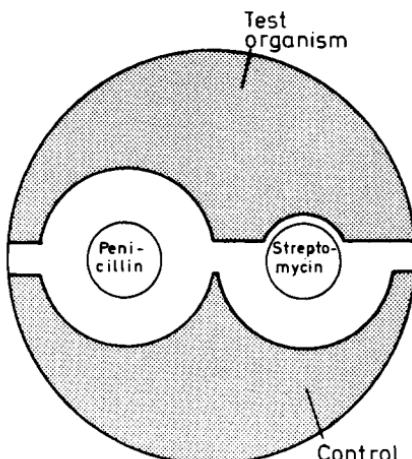


Figure 27.1. Disc sensitivity showing test organism sensitive to penicillin and resistant to streptomycin. The control shows sensitivity to both antibiotics

6. Compare zones of inhibition of test organism with that of known sensitive organism.
7. A zone comparable with or greater than that of the sensitive organism is reported as sensitive. Those with smaller zones are reported as resistant (*Figure 27.1*).

Gutter plate method

1. From a blood agar plate cut and remove a strip of the agar about 1 cm in width.
2. Fill the gutter with nutrient agar containing the required amount of antibiotic.

METHOD OF USE

1. Streak loopfuls of the organisms to be tested across the plate at right angles to the gutter.
2. Include on each plate a streak of a known sensitive organism.

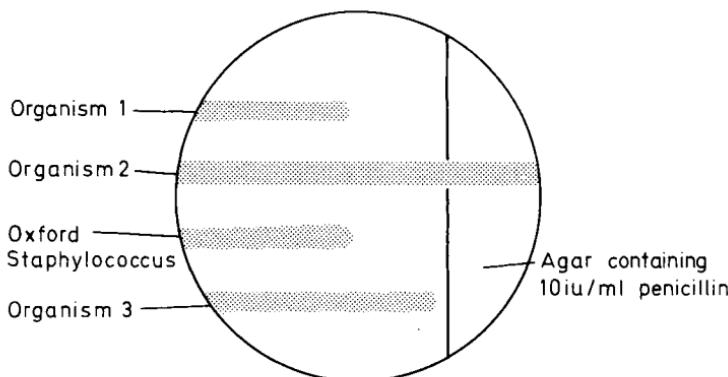


Figure 27.2. Gutter plate sensitivity test showing organism 1 sensitive to penicillin; and organisms 2 and 3 resistant to penicillin

3. Incubate for 18 to 24 h.
4. Compare and assess the sensitivity or resistance of the organism in terms of the known sensitive organism (*Figure 27.2*).

Several organisms can be tested on the same plate.

Sensitivity tests for *M. tuberculosis* and other mycobacteria are performed using Lowenstein-Jensen slopes in which doubling dilutions of the drug are incorporated. These drugs are added to the L-J medium before the slopes are inspissated. A series of four known sensitive strains is set up at the same time. After incubation for two weeks at 37 °C the MIC is determined, and the results

are expressed as a ratio of the MIC of the strain under test to that of the known sensitive strains. With a ratio of up to

2 : 1 the organism is considered sensitive,

4 : 1 probably resistant, and the test repeated. If the same result is obtained the organism is regarded as resistant.

8 : 1 and over the organism is resistant.

These sensitivity tests should be performed only in reference laboratories or laboratories where large numbers of sensitivity tests are routinely performed.

Table 27.1 FINAL STRENGTHS OF ANTIBIOTIC PER ML FOR GUTTER

Penicillin	10 iu per ml
Streptomycin	200 µg per ml
Tetracycline	25 µg per ml
Chloramphenicol	50 µg per ml
Terramycin	25 µg per ml
Erythromycin	50 µg per ml
Novobiocin	50 µg per ml
Ampicillin	10 µg per ml
Polymyxin	200 µg per ml
Neomycin	100 µg per ml
Furadantin	50 µg per ml
Sulphathiazole	100 µg per ml

Table 27.2

Antibiotic	Organism
Penicillins	Oxford staphylococcus, <i>M. lutea</i>
Streptomycin	Oxford staphylococcus, <i>K. pneumoniae</i> , <i>B. subtilis</i>
Gentamicin	Oxford staphylococcus, <i>K. pneumoniae</i> , <i>B. subtilis</i>
Kanamycin	Oxford staphylococcus, <i>K. pneumoniae</i> , <i>B. subtilis</i>
Viomycin	<i>B. subtilis</i>
Capreomycin	<i>B. subtilis</i>
Polymyxins	<i>B. bronchiseptica</i>
Tetracyclines	Oxford staphylococcus, <i>M. lutea</i>
Chloramphenicol	Oxford staphylococcus, <i>M. lutea</i>
Erythromycin	Oxford staphylococcus, <i>M. lutea</i>
Novobiocin	Oxford staphylococcus, <i>M. lutea</i>
Fucidin	Oxford staphylococcus, <i>M. lutea</i>

ASSAY METHODS

When patients are not responding to adequate dosages of antibiotics, it is often necessary to determine the level of antibiotic

in the blood or urine. Assay methods are then used, the technique being determined by the nature of the antibiotic, the specimen and the disease.

Either clotted blood or a specimen of urine is used, preferably at intervals after the last dosage of antibiotic. Ideally the specimens should be taken before dosage, and 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h thereafter. If this is not practicable two specimens, one before and the other 2 h after dosage, are often sufficient.

Although the Oxford staphylococcus is sensitive to most antibiotics, larger zones in the punch hole assay method may be obtained with other organisms.

Agar diffusion method

REQUIRED

Specimen under test.

Standard solution of antibiotic to be assayed.

18–24 h culture of Oxford staphylococcus or other sensitive organism.

Nutrient agar plates 8 mm deep.

Cork borer to punch holes in agar (8 mm internal diameter).

METHOD

1. Flood the agar plates with $\frac{1}{10}$ dilution of overnight culture of Oxford staphylococcus—pipette off excess fluid and dry plates for 1–2 h at 37 °C.
2. Punch 5 holes, equidistant from each other, around the plate.
3. Dilute the standard solution of antibiotic to give 3 suitable known dilutions and fill 3 of the holes with these solutions.
4. Add the specimen to the fourth hole and dilute the specimen accordingly before filling the fifth hole.

This dilution depends on the nature of the specimen and the probable amount of antibiotic in the specimen.

5. The plates are then placed in the incubator, for 18 to 24 h.
6. Measure the zones of inhibition and plot a graph showing relation of zone diameter to strength of antibiotic for the 3 controls.
7. Estimate strength of unknown from this graph.

Note—More refined versions for the assay of body fluids are available when greater precision is needed.

Table 27.3 gives a general guide to the isolation and identification of some common organisms. It must be stressed that this is only a general guide and more specialized books should be consulted for additional details.

Table 27.3 ISOLATION OF ORGANISMS FROM SPECIMENS

Organism	Specimen	Gram stain reaction	Suggested media	Remarks on isolation and identification
<i>Bordetella</i>	Per-nasal and pharyngeal swabs	Negative	Bordet-Gengou	Serology.
<i>Brucella</i>	Exudates, blood	Negative	Lacey DPF Liver agar Serum dextrose agar	10% CO ₂ cultivation. Phage. Serology—dye plates, H ₂ S production.
<i>Corynebacterium</i>	Nasopharynx wounds	Positive	Blood agar Tellurite agar Loeffler's	Aerobic cultivation. Toxin production, serum sugar reactions, virulence tests.
<i>Clostridium</i>	Wounds, pus, exudates, blood	Positive	Blood agar Cooked meat Thioglycollate	Anaerobic cultivation, sugar reactions, litmus milk. Animal inoculation.
<i>Coliforms</i>	Urine, exudates, blood, pus, CSF faeces, sputa	Negative	Blood agar MacConkey agar	Nagler plate, stormy clot. Aerobic cultivation. Biochemical tests including EMVIC reactions. Serology.
<i>Gonococcus</i>	Exudates from genitalia, eye, joints	Negative	Chocolate agar Nile blue sulphate	10% CO ₂ cultivation. Serum sugar reactions. Oxidase test.
<i>Haemophilus</i>	CSF, blood, sputum, exudates	Negative	Blood agar Chocolate agar	Aerobic cultivation. Serology. X and V factors. Satellitism.
<i>Klebsiella pneumoniae</i>	Sputum, blood, CSF, exudates	Negative	Blood agar Blood broth	Aerobic cultivation. Mouse inoculation. Serological typing.
<i>Mycobacterium tuberculosis</i>	Sputum, CSF exudates, urine, pus, faeces	Positive (not easily stained)	Lowenstein-Jensen	Aerobic cultivation. Concentration by alkali methods. Acid-fast stains.
<i>Meningococcus</i>	Blood, CSF, nasopharynx	Negative	Chocolate agar	Niacin and catalase peroxidase tests. 10% CO ₂ cultivation. Serum sugar reactions oxidase test.

<i>Pneumococcus</i>	Sputum, blood, CSF, pus, exudates	Positive	Blood agar	Aerobic cultivation. α -haemolysis. Bile or optochin sensitivity. Typing with specific antiserum.
<i>Proteus</i>	Urine, exudates, CSF, blood	Negative	High concentra- tion agar Salt-free agar Blood agar	Aerobic cultivation. Swarming. Splitting of urea, sugar reactions.
<i>Pseudomonas</i>	Urine, exudates, pus, CSF, blood	Negative	Blood agar	Aerobic cultivation. Pigmentation. Hugh and Liefson.
<i>Pasteurella</i>	Sputum, blood, exudates, pus	Negative	Blood agar	Aerobic cultivation. Growth on MacConkey, animal inoculation, sugar reactions. Motility, serology
<i>Staphylococcus</i>	Pus, exudates, blood, CSF faeces, sputum	Positive	Blood agar Salt medium	Aerobic cultivation. Coagulase, phage typing.
<i>Streptococcus</i>	Pus, exudates, blood, CSF, throat swabs	Positive	Blood agar	Aerobic or anaerobic cultivation. Haemolysis, soluble haemolysin, Lancefield group. Growth on MacConkey, heat resistance.
<i>Salmonella</i>	Faeces, blood, urine, exudates	Negative	MacConkey's agar Desoxycholate- citrate agar Selenite F Wilson and Blair's medium	Aerobic cultivation. Sugar reactions, indole, motility, serology.
<i>Shigella</i>	Faeces	Negative	MacConkey Deoxycholate- citrate media Selenite F	Aerobic cultivation. Sugar reactions. Indole, motility, serology.
<i>Yeasts and fungi</i>	Skin, nails, hair, exudates, pus, sputum, blood	—	Sabouraud's dextrose agar Penicillin and streptomycin Blood agar	Aerobic cultivation at 37 °C and 22 °C. Needle mount. Fluorescence of hair. Corn meal agar } yeasts. Sugar reactions } yeasts. Growth on rice grains.

SOME OF THE MORE COMMON ORGANISMS ISOLATED FROM CLINICAL SPECIMENS

Useful pointers to final confirmation are given in brackets.

Gram-positive cocci**STAPHYLOCOCCUS AUREUS**

Pathogen found in pyogenic infections and often in nose and on skin in health.

(Catalase- and coagulase-positive.)

STAPHYLOCOCCUS EPIDERMIDIS (ALBUS)

Commensal found in the nose and on the skin, but may be pathogenic under certain conditions.

(Catalase-positive and coagulase-negative.)

STREPTOCOCCUS PYOGENES

Pathogen found in tonsillitis, scarlet fever and pyogenic infections.
(Catalase-negative, identified by Lancefield's grouping.)

STREPTOCOCCUS FAECALIS

Pathogen found in urinary infections and in normal intestine.
(Catalase-negative and grows on bile salt media and is resistant to penicillin.)

STREPTOCOCCUS PNEUMONIAE

Pathogen found in respiratory infections and meningitis.
(Catalase-negative, and 'Optochin'-sensitive, unlike 'viridans streptococci'.)

VIRIDANS STREPTOCOCCI

Commensal found in mouth and throat, occasionally pathogenic.
(Catalase-negative, 'Optochin'-resistant).

Gram-negative cocci**NEISSERIA GONORRHOEAE**

Pathogen found in cases of gonorrhoea.
'Oxidase-positive, identified and confirmed by typical sugar reactions.)

NEISSERIA MENINGITIDIS

Pathogen found in cases of meningitis, rarely in healthy persons.

(Oxidase-positive, identified by typical sugar reactions and antigenic structure.)

NEISSERIA CATARRHALIS

Commensal found in throat and mouth, in health but especially in catarrhal secretions.

(Oxidase-positive, grows on nutrient agar, fermentation tests negative.)

NEISSERIA PHARYNGIS

Commensal found in throat and mouth.

(Oxidase-positive, ferments most sugars, grows on nutrient agar.)

Gram-positive bacilli

CLOSTRIDIUM SPP.

A group of sporing anaerobic organisms generally pathogenic when isolated from clinical material.

BACILLUS ANTHRACIS

Pathogen isolated from cases of anthrax.

BACILLUS SUBTILIS AND B. CEREUS

Saprophyte found in soil and dust, common laboratory contaminant.

CORYNEBACTERIUM DIPHTHERIAE

Pathogen found in cases of diphtheria.

(Catalase-positive, identified by sugar reactions and toxin production.)

CORYNEBACTERIUM HOFMANNII

Commensal found on skin and in the upper respiratory tract.

(No fermentation of sugars used for *C. diphtheriae*.)

MYCOBACTERIUM TUBERCULOSIS

Pathogen isolated from cases of tuberculosis.

(Acid-fast bacillus identified by special methods.)

Gram-negative bacilli

ESCHERICHIA COLI

Pathogen or commensal. Found in urinary tract infections and in normal intestine and sewage.

(Identified by indole production, sugar reactions, and other tests.)

KLEBSIELLA PNEUMONIAE

Pathogen found in respiratory infections.
(Identified by special biochemical tests.)

SALMONELLA SPP.

Pathogens found in typhoid and paratyphoid fevers and food poisoning.
(Over 1000 species identified by sugar reactions and antigenic structure.)

SHIGELLA SPP.

Pathogens found in bacillary dysentery.
(Identified by sugar reactions and antigenic structure.)

PROTEUS SPP.

Pathogens found mainly in urinary tract infections or commensals found in normal intestine and sewage.

PSEUDOMONAS AERUGINOSA

Pathogen found in wound and urinary infections.
(Special tests.)

PASTEURELLA MULTOCIDA

Pathogen found occasionally in respiratory infections and also infections from animal bites.
(Identified by indole production, failure to grow on bile salt media, and other tests.)

HAEMOPHILUS INFLUENZAE

Pathogen found in meningitis and bronchitis, but also in normal nasopharynx.
(Identified by X and V factor requirements.)

BORDETELLA PERTUSSIS

Pathogen found in cases of whooping cough.
(Special tests and antigenic structure).

BRUCELLA ABORTUS AND BRUCELLA MELITENSIS

Pathogens found in undulant fever.
(Identified by special tests and antigenic structure.)

28

Introduction to Haematology

Haematology is the study of blood, and in a routine hospital laboratory, is concerned largely with abnormalities of the blood. One of its primary functions is to detect anaemia, which means lack of haemoglobin, and it is the task of the laboratory to assist in the diagnosis of the exact type of anaemia to enable the right treatment to be given. Other important aspects of routine haematology are the investigation of coagulation defects and the control of treatment in such diseases as coronary thrombosis and leukaemia.

Essentially, blood consists of *plasma*, a fluid medium in which is suspended *erythrocytes* (red blood cells), *leucocytes* (white blood cells) and *thrombocytes* (platelets). Plasma is a complex solution of proteins, salts and numerous metabolic substances and acts as a transport medium carrying its constituents to specialized organs of the body. As blood passes through the intestinal circulation, nutrients are absorbed into the plasma and carried to the liver and other tissues. As the blood passes through the kidneys, waste products of metabolism are filtered off into the urine. Many of the plasma proteins such as the blood clotting factors, antibodies and enzymes have specialized functions.

The majority of cells suspended in the plasma are erythrocytes which comprise about 45 per cent of the total volume of the blood. They contain a high concentration of haemoglobin, the oxygen-carrying pigment, which gives blood its red colour. The leucocytes are very much fewer in number but several different forms exist, each having different functions. The blood platelets are small tissue particles which arise in the bone marrow by 'budding off' the cytoplasm of their mother cells, which are called megakaryocytes. They are intimately concerned with the blood-clotting process.

STRUCTURE AND FUNCTION OF BLOOD CELLS

Erythrocytes

Unstained, these cells are seen under the microscope as non-nucleated, pale greenish-yellow, biconcave discs. When stained by

Romanowsky stains they have an affinity for the eosin and therefore stain a pinkish colour.

The diameter of the red cell is between 6.7 and 7.7 μm with a normal average of 7.2 μm . The thickness of the cell is between 1.7 and 2.4 μm with an average normal of 2.1 μm .

There are approximately five million red cells in each cubic millimetre of blood. The average normal range, depending on the age and sex of the person, is from 4.5 to 6.5 million per mm^3 in men, and in women from 3.9 to 5.6 million per mm^3 .

The red cell normally survives in the blood stream for 110 days after which time it is removed by the phagocytic cells of the reticuloendothelial system, broken down and some of its constituents re-utilized for the formation of new cells.

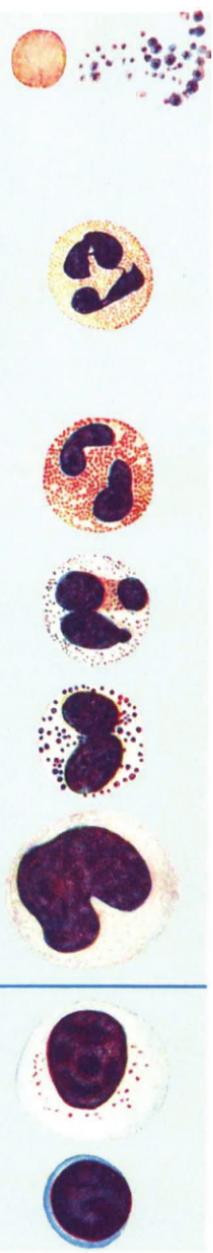
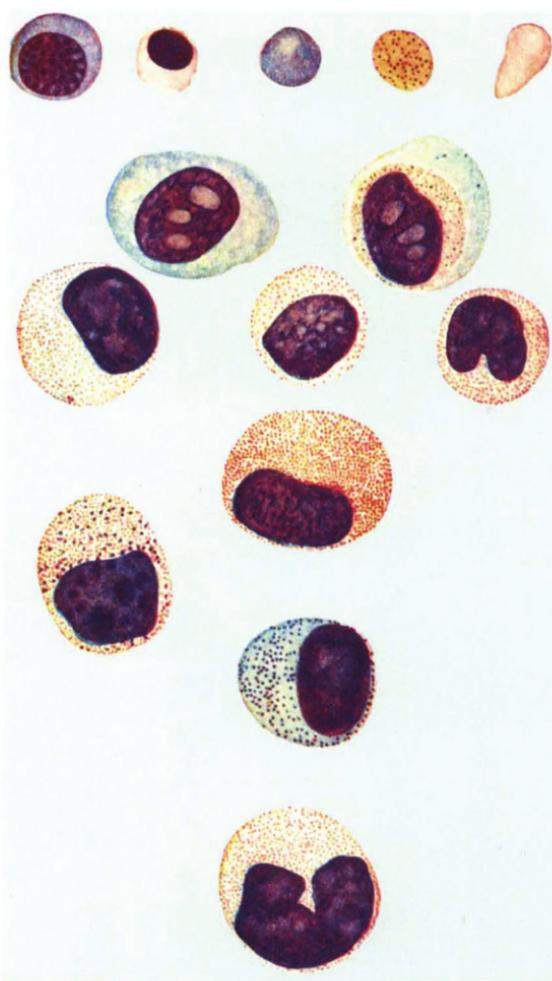
The primary function of the red cell is to transport oxygen from the lungs, via the heart, to the tissues. The cell contains a substance known as haemoglobin, which has the power of combining reversibly with oxygen. In the lungs the haemoglobin in the red cell combines with oxygen, and releases it to the tissues of the body during its circulation where it is utilized. Carbon dioxide, a waste product, is then absorbed by the red cell and transported to the lungs to be exhaled. A fresh inhalation provides further oxygen. If the amount of circulating haemoglobin decreases, thereby reducing the amount of oxygen to be supplied to the tissues a state of anaemia is said to exist.

Leucocytes

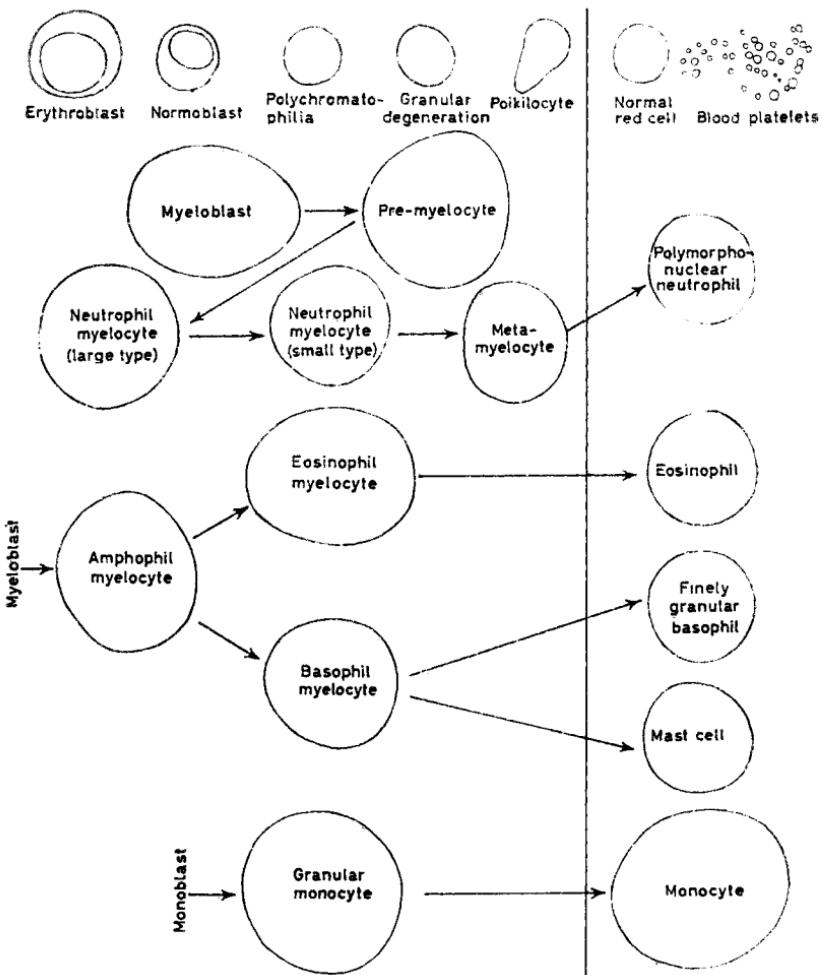
These are nucleated cells, some of which are capable of amoeboid movement. They are present in normal blood in smaller numbers than red cells, the normal adult range being between 4000 and 11 000 per mm^3 of blood. As they are capable of phagocytosis (ingestion of bacteria and other harmful particles) their main function is to act as one of the body's defences. Some of the white cells are also connected with antibody formation.

Certain conditions, such as acute bacterial infections, are capable of producing a variation in the white cell count. *Leucocytosis* is the term used to describe an increase in white cells, that is above 11 000 per ml. *Leucopenia* is the term used to describe a decrease in white cells, that is below 4000 per mm^3 .

Unstained leucocytes appear colourless, but a thin blood film, stained by the Romanowsky method, can be seen to contain white cells of three main types; the polymorphonuclear cells (granulocytes), lymphocytes and monocytes (*see Figure 28.1*).



Normal and abnormal blood cells.
(Leishman's Stain.)



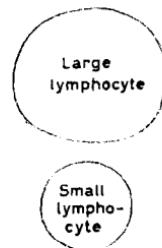
The cells on the right of the vertical line are those found in normal blood.

The cells on the left of the vertical line include the marrow prototypes of the normal cells, and may appear in the blood in disease.

Above the horizontal line are the myeloid cells, and below, the lymphoid cells.

The arrows indicate the probable cycle of cell development.

(From "Clinical Pathology" by Panton and Marrack.
By courtesy of J. & A. Churchill, Ltd.)



POLYMORPHONUCLEAR LEUCOCYTES

These cells have nuclei consisting of a number of lobes. The younger cells have 2 or 3 lobes, the older ones have 4 or 5 lobes. They are also called granulocytes as they contain small granules in their cytoplasm. There are three types of polymorphonuclear leucocytes which can be differentiated according to the staining reactions of these granules.

Polymorphonuclear neutrophil

Usually these cells are between 10 and 12 µm in diameter and are capable of amoeboid movement.

When stained by one of the Romanowsky stains they show a lobed nucleus, which stains a purple-violet colour. As the cell ages, so the number of lobes increases. Most of the cells will have 2 or 3 lobes, but it is possible to see as many as 7 (see Cooke-Arneth count, p. 594). The cytoplasm stains a light pink colour and contains small, violet or pinkish staining, dust-like granules. The term neutrophil, a relic of earlier staining methods, is perhaps a little misleading since the granules do not stain in a neutral manner, but rather in an acidic manner.

Neutrophils are increased in acute bacterial infections such as pneumonia and are the main constituents of pus. They are often seen in the urine of patients with urinary tract infections.

Polymorphonuclear eosinophil

These cells are the same size as a neutrophil, but usually have only 2 lobes to their nucleus, often in a 'spectacle' arrangement. The nucleus stains a little paler than the neutrophil and the cytoplasm contains many large, round, or oval, deep orange-pink granules.

The eosinophil is not as amoeboid as the neutrophil and its function is thought to be one of detoxication (the removal of foreign substances from the body).

Eosinophils are increased in allergic conditions and when intestinal parasites are present.

Polymorphonuclear basophil

These cells are about 8–10 µm in diameter and are not as active as the neutrophil or eosinophil. The nucleus is usually kidney-shaped and the cytoplasm contains a mass of large, deep purple staining granules which frequently obscure the nucleus.

The function of the basophil is still obscure although it is known that the granules contain heparin. Basophils are rarely increased

in the peripheral blood but usually are in granulocytic leukaemia.

One condition in which these cells are increased in the peripheral blood is granulocytic leukaemia.

Lymphocytes

These cells, which are concerned with antibody formation, exhibit two forms, the large lymphocyte and the small lymphocyte.

SMALL LYMPHOCYTES

These have a diameter of 7–10 µm and have a round, deep purple staining nucleus which occupies most of the cell so that the cytoplasm, which stains a pale blue colour, can be seen only as a rim around the nucleus.

LARGE LYMPHOCYTES

These are thought to be young lymphocytes. They are between 12 and 15 µm in diameter, the nucleus staining a little paler than the small lymphocytes. The cytoplasm is more plentiful, staining a pale blue colour.

The lymphocytes sometimes show a few reddish granules in the cytoplasm.

Lymphocytes may well be increased in infants and children who have bacterial infections. *Lymphocytosis*, an increase in lymphocytes, is a common feature of viral disease such as mumps and measles and very high counts are seen in cases of whooping-cough. Patients with chronic lymphatic leukaemia often show an extremely high lymphocytosis.

Monocytes

Monocytes are cells capable of ingesting bacteria and particulate matter, and act as 'scavenger cells' at the site of infections. They are larger than other leucocytes, measuring between 16 and 22 µm in diameter. Monocytes have one large nucleus, which is usually centrally placed within the cell and often kidney shaped (reniform). This nucleus has a stranded appearance, like a skein of wool, and when stained, is a pale violet colour. The copious cytoplasm, staining a pale greyish-blue, contains innumerable dust-like granules of reddish-blue. It may also possess vacuoles, which appear as clear spaces in the cytoplasm.

A moncytosis may occur in some bacterial infections and malaria. A marked increase will always be shown in monocytic leukaemia.

Normal values for the various white blood cells are as follows:

neutrophils, 2500–7500; eosinophils, 40–440; basophils, 0–100; lymphocytes, 1000–3000; monocytes, 400–1000.

Platelets (thrombocytes)

These cells appear in films stained by a Romanowsky technique as small non-nucleated oval or round cells, 2–3 µm in diameter, which stain blue with pinkish granules.

Platelets are capable of sticking together when touching a rough surface such as a cut blood vessel, thereby forming a physical barrier, preventing bleeding and subsequently releasing substances which hasten blood coagulation.

A decrease in platelets is called *thrombocytopenia* and may cause either an internal or external haemorrhage. An increase in platelets is called *thrombocythaemia* and may follow haemorrhage, surgery and fractures of bones.

BLOOD CELL MATURATION

In the adult, blood cells are produced in the reticuloendothelial tissue, mainly in the bone marrow. The cells normally found in the circulation develop from immature forms known as *blast* cells (*Figure 28.2*).

The development of a normal cell from the original blast cell is accompanied by a diminution in the size of the cell because of successive divisions of the cell. The nucleus also decreases in size, the nuclear chromatin condenses and the nucleoli are lost. In the case of the red cell precursors, haemoglobin appears and in the case of the polymorphonuclear precursors the characteristic granules appear.

RED CELL DEVELOPMENT

The earliest red cell precursor in the bone marrow is the *proerythroblast*, a large cell with a large nucleus and a thin rim of blue cytoplasm. As the cell divides haemoglobin synthesis starts in the cytoplasm, and with successive cell divisions the amount of haemoglobin increases. As the cell becomes smaller its cytoplasm when stained first becomes purple and then a pinkish-purple. These dividing cells are called *normoblasts* and are classified as early, intermediate, or late, according to (1) the amount of haemoglobin present, (2) the degree of contraction of the originally large nucleus and, (3) condensation of nuclear chromatin. When the cell is fully haemoglobinized the small dense nucleus remaining is

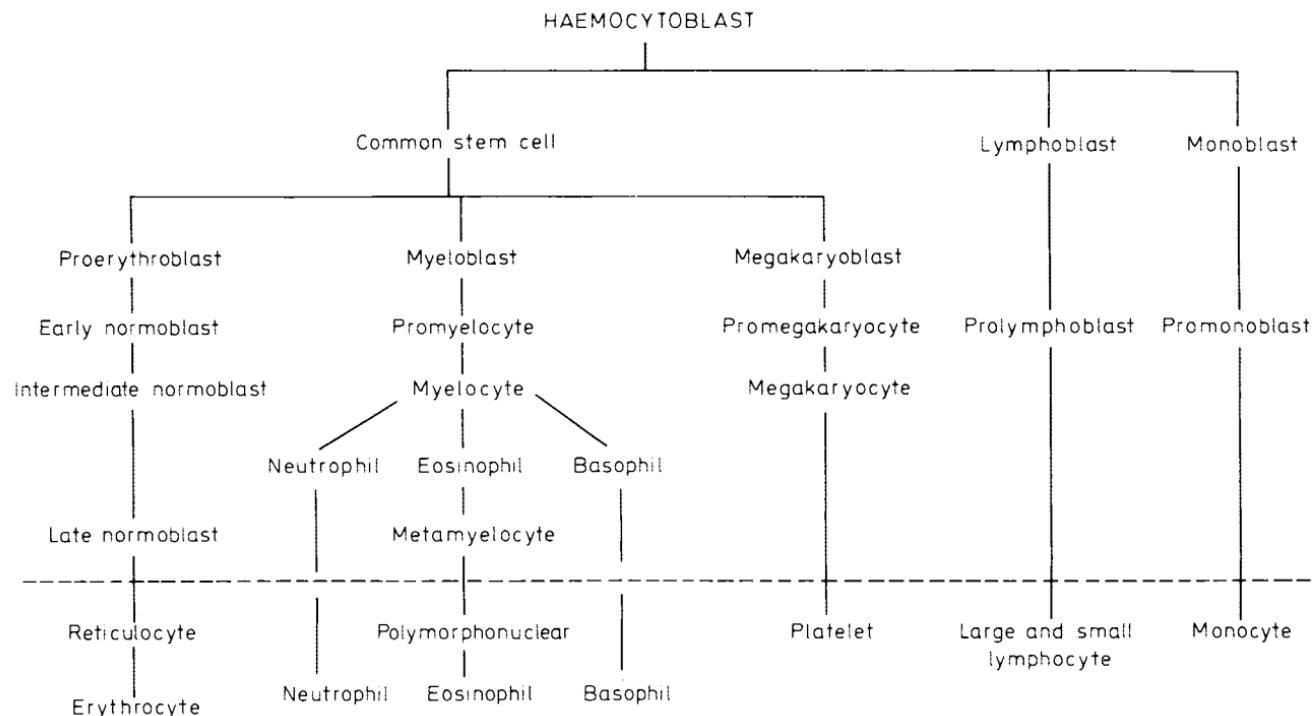


Figure 28.2. Only those cells below the dotted line normally appear in the circulation

extruded, leaving a young greyish-blue cell when stained, called a polychromatic cell. These cells are also called *reticulocytes* because a reticular structure is revealed in the cytoplasm by brilliant cresyl blue staining. After 24–48 h in the peripheral blood the reticulocyte matures to an adult red cell.

WHITE CELL DEVELOPMENT

The polymorphonuclear cells arise from primitive cells in the bone marrow of which the *myeloblast* is the earliest recognizable form. This is a large cell with a large nucleus and little cytoplasm. As development takes place through successive cell divisions the nucleus becomes smaller and loses its nucleoli, at the same time the characteristic granules begin to appear in the cytoplasm and the cell is then called a *myelocyte*. As the single nucleus becomes bean shaped, and then horse-shoe shaped, the cell is called a *meta-myelocyte*. When this horse-shoe nucleus forms separate lobes, the cell is a true polymorphonuclear.

Although *lymphoblasts* are found both in the bone marrow and lymphoid tissues, from which mature lymphocytes are derived, the full development potentiality of the lymphocyte is not fully understood. It is possible that the cells seen in the peripheral blood may undergo further transformations which have not yet been adequately demonstrated.

The monocyte develops from a primitive marrow cell known as a *monoblast*.

PLATELET DEVELOPMENT

A platelet is formed by the process of 'budding' or 'pinching off' of the cytoplasm of a giant multi-nucleated marrow cell known as a *megakaryocyte*.

Haemoglobin

Haemoglobin is a large complex molecule (molecular weight 68 000). It is synthesized in the developing red cells and consists of four polypeptide chains closely linked together (*globin*). An iron-containing complex called *haem* is attached to each polypeptide chain and it is this part of the molecule which is responsible for its oxygen-carrying properties. If the ferrous iron of haem is oxidized the oxygen-carrying capacity of the haemoglobin is lost.

Haemoglobin has the property of combining reversibly with oxygen. Oxygen is taken up by the red cell in the lungs and then given up to the tissue whilst circulating through the body. The oxygen is bound to the iron component of haemoglobin, forming

oxyhaemoglobin. When the oxygen has been given up to the tissues *reduced haemoglobin* is formed.

Haemoglobin also plays a part in the transport of carbon dioxide to the lungs, where it is exhaled. Carbon dioxide is not bound to the haemoglobin in the same way as oxygen, but is carried in the red cell in the form of bicarbonate. About 90 per cent of the carbon dioxide is removed from the tissues in this way, the remainder being carried away as bicarbonate in the plasma.

The haemoglobin value is usually recorded in g per 100 ml of blood. The present accepted standard is that 100 per cent is equivalent to 14.6 g haemoglobin per 100 ml of blood. With older types of apparatus other standards were used, for example Haldane chose 13.8 g haemoglobin per 100 ml as 100 per cent haemoglobin, and Sahli chose 17.2 g haemoglobin per 100 ml.

In women, the normal haemoglobin content per 100 ml of blood is 11.5–16.4 g, and in men it is between 13.5 and 18.0 g. The haemoglobin level of a newborn infant is very high, possibly as high as 19.0 g per 100 ml. This level falls quite steeply in the first few weeks to around 15.0 g per 100 ml and then falls slowly to about 11 g during the next year or so. This then climbs slowly to reach the adult level at about 15 years of age.

FATE OF HAEMOGLOBIN

When the red cell reaches the end of its lifespan after 110 days' circulation, it is removed by the cells of the reticuloendothelial system. The haemoglobin is then broken down into two components: (1) an iron-containing compound which is stored in the liver and spleen as *haemosiderin*, in order that it may be used again for haemoglobin production; (2) an iron-free compound called *bilirubin*, which is transported to the liver, where it enters the bile to form one of the bile pigments.

HAEMOGLOBIN PIGMENTS

In the circulation haemoglobin normally takes the form of oxyhaemoglobin, or reduced haemoglobin. Certain other forms can be produced if haemoglobin is acted upon by other chemicals. These haemoglobin pigments are:

Carboxyhaemoglobin

Found in the blood after carbon monoxide poisoning. It may also be found in the blood of heavy smokers.

Methaemoglobin

The iron contained in haemoglobin is normally in the ferrous state.

If it is oxidized to the ferric state, methaemoglobin results. Certain drugs are capable of converting haemoglobin into methaemoglobin.

Sulphaemoglobin

This can also be produced by certain drugs, such as the sulphonamides.

It is sometimes necessary to examine blood for these pigments, this is performed using a spectroscope (*see* Chapter 6). In this apparatus, white light is split into its component colours, forming a spectrum. When a specimen of haemoglobin is placed between the light source and the spectroscope, dark bands will be seen in the spectrum. The various haemoglobin pigments produce these absorption bands at different parts of the spectrum and can therefore be identified.

HAEMOGLOBIN A AND F

The haemoglobin present in normal adult blood is known as adult haemoglobin or haemoglobin A. The blood of the fetus and newborn infant contains a high proportion of another type of haemoglobin known as fetal haemoglobin or haemoglobin F. The high proportion of haemoglobin F present at birth (approximately 70–90 per cent of the total haemoglobin), falls during the first six months of life to about 1 per cent, remaining at this level throughout life. (In some diseases such as thalassaemia, haemoglobin F values may be increased.)

Haemoglobin F has two important characteristics, firstly it resists denaturation with alkalis more than haemoglobin A. Secondly it has the ability to combine more readily with oxygen than haemoglobin A, a property which allows the fetus to acquire as much oxygen as possible from the maternal circulation.

THE HAEMOGLOBINOPATHIES

These are inherited abnormalities of haemoglobin structure and are sometimes referred to as 'haemoglobin variants'. There are many of these variants now recognized and the abnormality in all these conditions lies in the globin fraction of the haemoglobin molecule, which is composed of two pairs of polypeptide chains. There are one pair of alpha chains and one pair of beta chains in the normal adult haemoglobin molecule. These chains are produced by the linking of amino acids, and each alpha chain has 141

amino acids and each beta chain 146 amino acids. The chains have a correct sequence of amino acids and any substitution of one amino acid for another may produce a haemoglobin variant which can cause anaemia, or in the very severe forms, death. The commonest form of haemoglobin variant which is easily recognizable is haemoglobin S, so called because the red cells take on the characteristic 'sickle' shape when subjected to reduced oxygen tension. This abnormality exists in two forms, depending on whether the gene determining haemoglobin S is passed from either one or both parents to the offspring. If only one parent passes on the S gene it is called *heterozygous* and the offspring will produce both normal haemoglobin (Hb A) and the sickle haemoglobin (Hb S), which is often termed 'sickle-cell trait' and is clinically harmless. If both parents pass on the S gene it is called *homozygous* and the offspring will only produce sickle haemoglobin (Hb SS); this condition is termed 'sickle-cell disease', which is often clinically very serious. This form of haemoglobin variant is found almost exclusively in Negroes. There are many other forms of haemoglobin variants each having a different amino acid substitution and also present genetically in the heterozygous and homozygous state. Letters of the alphabet are used to differentiate them and they are Hb C, Hb D, Hb G, Hb O, etc. Many of these may be differentiated by haemoglobin electrophoresis.

THALASSAEMIA (MEDITERRANEAN ANAEMIA)

As the name suggests this anaemia was first discovered in people living around the Mediterranean sea, but it is now known to be far more widespread and is found in India and the Far East and also in immigrants from these regions. No true haemoglobin variant is found in this disease but rather increased amounts of fetal haemoglobin (Hb F) and haemoglobin A2, both of which are present in small amounts in normal adults.

Thalassaemia presents in two forms which are genetically determined, the gene from both parents to an offspring giving rise to thalassaemia major, the severest form, and the expression of one gene to an offspring called thalassaemia minor. The disease is caused by diminished production of either the alpha chains or beta chains and therefore the total amount of globin available to link with the haem fraction is inadequate. Hence the rate of haemoglobin production is diminished.

29

Blood Coagulation

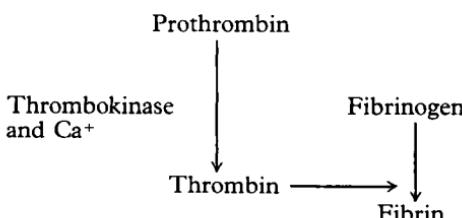
Blood does not normally clot in the body, but when it is shed it coagulates within 5–10 min.

MORAWITZ THEORY OF COAGULATION

Morawitz attempted to explain this phenomenon in 1905 in his *Classical Theory of Coagulation*. He stated that four substances present in normal blood were responsible for blood clotting:

1. Thrombokinase, which he said was liberated from platelets when they came into contact with a water-wettable surface.
2. Prothrombin—a proteinous substance formed in the liver and found in plasma.
3. Fibrinogen—a plasma protein produced by the liver, and also found in plasma.
4. Free calcium ions.

Morawitz stated that when blood was shed, thrombokinase was liberated from the platelet, and reacted with the prothrombin, in the presence of calcium ions, converting the prothrombin into a substance called *thrombin*. The fibrinogen in the presence of the thrombin was then converted into fibrin, forming a fine network of strands which trap the blood cells thus forming a typical clot.



This theory is still essentially correct, but it has become apparent that the reactions leading to the conversion of prothrombin are more complicated than those suggested by Morawitz. The latter part of the classical theory, however, that is the production of fibrin from fibrinogen in the presence of thrombin, is still

regarded as true, but modern workers have postulated a more complex theory termed the intrinsic and extrinsic mechanism of blood coagulation.

INTRINSIC-EXTRINSIC COAGULATION MECHANISM

Normal blood contains certain factors which enable it to clot within 5–10 min when collected in a glass tube. Tissue contains a substance which, when added to this blood, accelerates the clotting. This substance is commonly called thromboplastin, but a more exact term would be tissue extract.

The intrinsic-extrinsic theory of blood coagulation suggests that prothrombin can be activated to thrombin by one of two substances which are termed (a) extrinsic thromboplastin (extrinsic prothrombin activator), and (b) intrinsic thromboplastin (intrinsic prothrombin activator).

Extrinsic thromboplastin is produced when tissue extract is acted upon by certain factors present in normal blood. The tissue extract requires to be activated by factor V, factor VII, factor X and calcium, to form extrinsic thromboplastin (*see Figure 29.1*).

Intrinsic thromboplastin is produced entirely from substances present in the blood. These substances are factor V, factor VIII, factor IX, factor X, factor XI, factor XII, free calcium ions, and platelet lipoid factor but no tissue extract. It is thought that factor

Table 29.1

<i>International nomenclature</i>	<i>Synonyms</i>
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Thromboplastin
Factor IV	Calcium
Factor V	Proaccelerin, labile factor
Factor VI	Accelerin (no longer used)
Factor VII	Proconvertin, stable factor
Factor VIII	Antihæmophilic globulin (AHG) Antihæmophilic factor A (AHF)
Factor IX	Christmas factor, antihæmophilic factor B, plasma thromboplastin component (PTC)
Factor X	Stuart-Prower factor
Factor XI	Plasma thromboplastin antecedent (PTA)
Factor XII	Hageman factor

INTRINSIC SYSTEM

EXTRINSIC SYSTEM

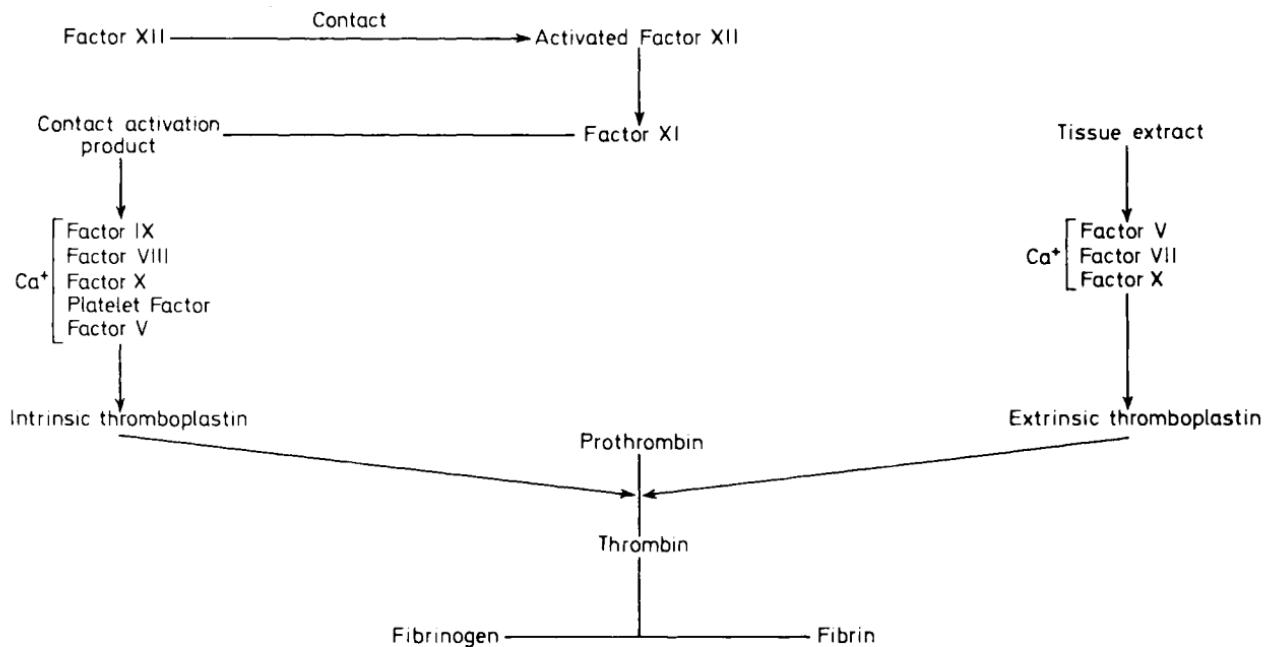


Figure 29.1. Chart showing intrinsic-extrinsic coagulation mechanism

XII, when coming into contact with a foreign surface, is activated to form activated factor XII. This then reacts with factor XI to form an activation product. The activation product then reacts with factor IX, factor VIII, factor X and factor V and the platelet lipid factor, in the presence of free calcium ions to form intrinsic thromboplastin.

Both the intrinsic and extrinsic thromboplastins are capable of converting prothrombin to thrombin, which in turn converts fibrinogen to fibrin. The coagulation mechanism is summarized in *Figure 29.1*.

Some confusion has developed over the different terminology used by various workers in the field of blood coagulation. International agreement has now been reached whereby Roman numerals are used to identify the factors. Table 29.1 lists the coagulation factors with their synonyms.

Anticoagulants

Samples of blood will clot if transferred to dry containers. While this is desirable for certain laboratory investigations (such as serological examinations), the examination of blood cells requires blood which has not been allowed to clot.

As has been shown above, certain steps are involved in blood coagulation, and if one of the factors is removed or inactivated, the coagulation reaction will not take place. The substance responsible for this removal or inactivation is called an *anticoagulant*. Un-coagulated blood is obtained by transferring blood samples immediately they are obtained into bottles containing a known amount of anticoagulant. Thorough mixing of the blood in the bottle is now necessary. There are several anticoagulants in general use, some of which are described.

OXALATES

Oxalates are often used for laboratory investigations of blood, but never in transfusion work, as they are poisonous. Sodium, potassium and ammonium oxalate are all used; they act by combining with the calcium in blood to form insoluble calcium oxalate. Once the calcium is so combined it cannot be utilized, and blood coagulation does not take place.

CITRATE

Sodium citrate is used as an anticoagulant in blood transfusion work, coagulation studies and the Westergren ESR. It combines

with calcium, thereby preventing the conversion of prothrombin to thrombin, and coagulation does not occur.

HEPARIN

This substance is believed to inactivate thrombin, preventing conversion of fibrinogen to fibrin. Red cells are unaltered by the action of heparin, and it is a useful, though expensive, anticoagulant to use.

Leucocytes are often clumped by heparin and should not be used when these cells are to be counted. The effect of heparin may be neutralized by the addition of protamine sulphate. Heparin is also given to patients when immediate total anticoagulation is required such as in coronary thrombosis, where oral anticoagulants will take 48 h to have any effect on the coagulation system.

SEQUESTRENE

This substance, which combines with calcium, is a chelating agent. It is in fact the disodium or dipotassium salt of sequestric acid, that is disodium or dipotassium ethylene diamine tetra-acetic acid (EDTA).

Sequestrene prevents the clumping of platelets and is therefore the anticoagulant of choice where total platelet counts or concentrated platelets for transfusion are required.

30

Enumeration of Blood Cells

COLLECTION OF BLOOD

For haematological investigations, capillary or venous blood may be used. It is essential that adequate mixing of the blood and anti-coagulant or diluting fluid is carried out prior to any investigation.



Figure 30.1. Rotating mixer. (Reproduced by courtesy of Denley Instruments Ltd)

For this purpose a rotating mixer (*Figure 30.1*) is required. The type of mixer illustrated has been proven to give excellent mixing and is standard equipment in many laboratories. When not available the specimen should be mixed by slow inversion.

Capillary blood

It must be remembered that capillary blood samples, although of great value in children and in adults with 'difficult' veins, are not only subject to sampling error but tests cannot be repeated in the laboratory, as the whole sample will have been used and further tests which may be required cannot be performed.

Select a suitable site for puncture, the ball of the finger, or the side of the thumb. Blood from a baby is best obtained from the

base of the heel. The area chosen must be vigorously cleaned with 75 per cent alcohol*, and allowed to dry. This sterilizes the skin, and promotes a free flow of blood. A quick stab is made, preferably with a pre-sterilized disposable blood lancet, the use of which reduces the hazard of cross-infection.

After the skin has been punctured, a little pressure is applied to ensure a free flow of blood. Undue squeezing must be avoided as this can cause lymph to dilute the blood, giving erroneous results. Undue or prolonged pressure can cause congestion and concentration of cells and haemoglobin. Wipe away the first few drops of blood, and then carefully draw blood into the appropriate pipette by means of gentle suction applied to the rubber tubing attached to the pipette. Make sure that there are no air-bubbles, and check that the blood level is exactly to the mark. Wipe the outside of the pipette, and either fill the bulb of the pipette with diluting fluid, or slowly blow the blood into a tube containing diluent (*see* below for details of cell counting and haemoglobin methods).

If blood films are required, gently touch a fresh drop of blood onto one end of a clean, grease-free slide or onto a coverslip as described under 'Differential White Cell Counting'.

Venous blood

If larger volumes are required, a venous sample of blood must be obtained. Using a dry, sterile syringe and needle, the blood is withdrawn from a suitable vein in the arm.

If serum is required, the needle of the syringe is removed, and the blood slowly ejected into a clean, dry, sterile bottle. If plasma is required, the blood is transferred to a bottle containing a suitable anticoagulant.

It is important to realize the difference between serum and plasma.

PLASMA

This is the fluid portion of the blood, so that when blood is maintained in a fluid state *in vitro*, by the addition of an anticoagulant, the fluid is referred to as plasma. This plasma contains all the coagulation factors except the one removed by the anticoagulant. If this substance is replaced in sufficient quantity the plasma will clot.

* Medical Research Council recommendation.

SERUM

This is the fluid which remains after blood has clotted. Some of the clotting factors are not present in serum, as these will have been used to produce the fibrin clot.

BLOOD COUNTS

Considerable clinical importance is placed on red and white blood cell counts. Accurate results are dependent upon careful manipulation of the apparatus, and attention to technical detail. Considerable practice is required before optimum results can be obtained.

The apparatus for counting blood cells is called a haemocytometer and consists of a counting chamber, a coverglass, pipettes for diluting blood, and a rubber tube with a plastic mouthpiece for drawing the fluid into a pipette. If a stained preparation is required, slides and a 'spreader' are also necessary.

Counting chamber and coverglass

This consists of a heavy glass slide, with four troughs or channels extending across the slide, set parallel to each other. The centre platform thus formed is set slightly lower than the two adjacent

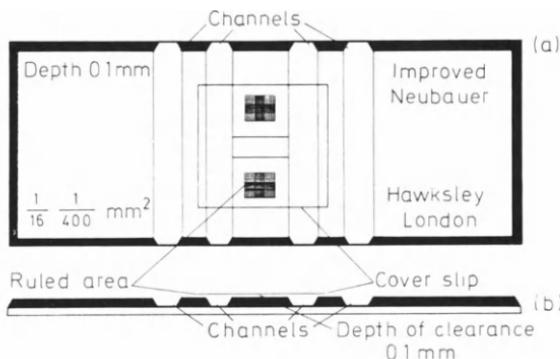
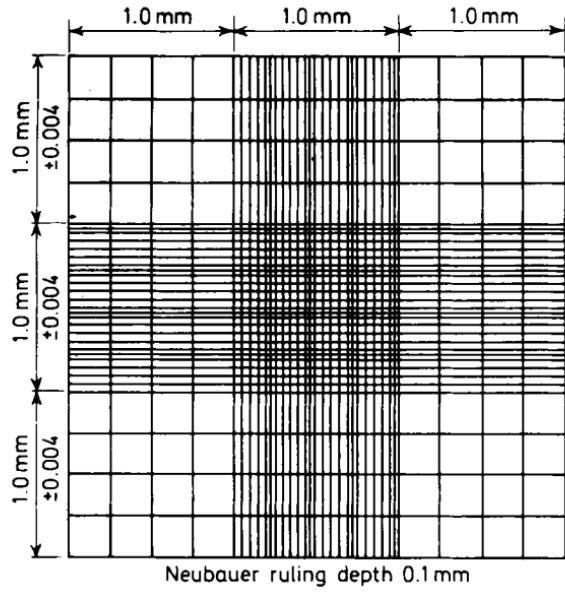
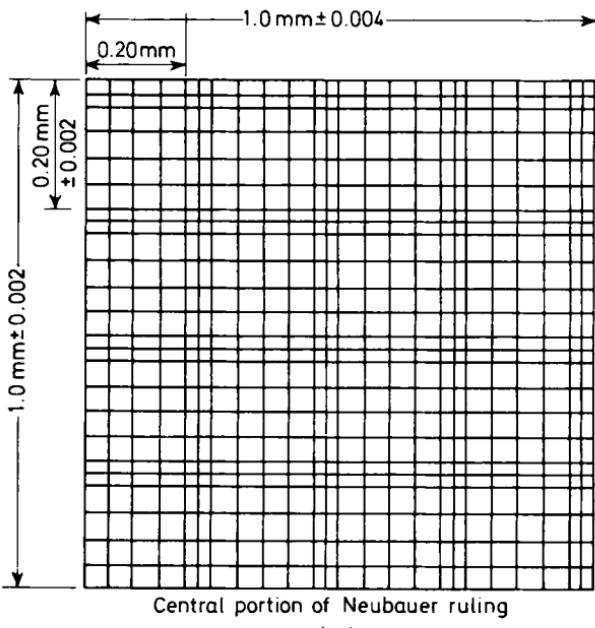


Figure 30.2. (a) Double-sided counting chamber; (b) side view of chamber

ones (Figure 30.2) and is engraved by one or more of several rulings (Figures 30.3 and 30.4). When placed in position the coverglass rests upon the two outer platforms, producing a clearance between itself and the rulings on the central platform. This clearance is referred to as the depth of the counting chamber.



(a)



(b)

Figure 30.3

ORDINARY NEUBAUER COUNTING CHAMBER

The central platform is set 0.1 mm below the level of the two side ones, giving the chamber a depth of 0.1 mm. The engraving covers an area of 9 mm^2 divided into 9 squares of 1 mm^2 each. The 4 corner squares are divided into 16 squares, each with an area of $1/16$ th of a mm^2 . The central *ruled area* of 1 mm^2 is divided into 16 *large squares* by sets of triple lines. These large squares are further subdivided into 16 *small squares* by single lines. It will be noticed from the diagram (*Figure 30.3*) that the width of the triple lines dividing the large squares is the same as the width of a small square. Two adjacent sides of the ruled area are bounded by triple lines, the other two by single lines. Each side is, therefore, divided into 20 equal divisions (the width of 16 small squares and 4 sets of triple lines). Each small square is, therefore, $1/20$ th of 1 mm squared, that is $1/400$ th of 1 mm^2 .

IMPROVED NEUBAUER COUNTING CHAMBER

This is similar to an ordinary Neubauer chamber, except that the triple lines dividing the central large squares are very much closer together (*Figure 30.4*). The central ruled area is divided into 25 large squares. These squares are subdivided to form 16 smaller squares each with an area of $1/400$ th of 1 mm^2 . The space occupied by the triple lines in the ordinary Neubauer chamber being used to produce extra large squares.

BURKER COUNTING CHAMBER

Like the Neubauer counting chamber, this has a ruled area of 9 mm^2 and a depth of 0.1 mm (*see Figure 30.5*).

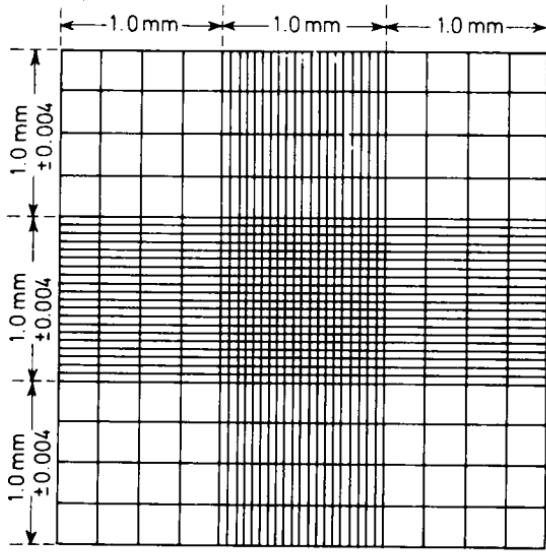
FUCHS-ROSENTHAL COUNTING CHAMBER

This chamber was originally designed for counting cells in cerebrospinal fluid, but as such a relatively large area is covered, it is preferred by some workers for counting leucocytes. The depth is 0.2 mm and the ruled area consists of 16 millimetre squares divided by triple lines. These squares are subdivided to form 16 smaller squares, each with an area of $1/16$ th of a mm^2 (*Figure 30.6*).

Another type of Fuchs-Rosenthal chamber is now available, which has the same depth as the one described, but is ruled over 9 mm^2 only.

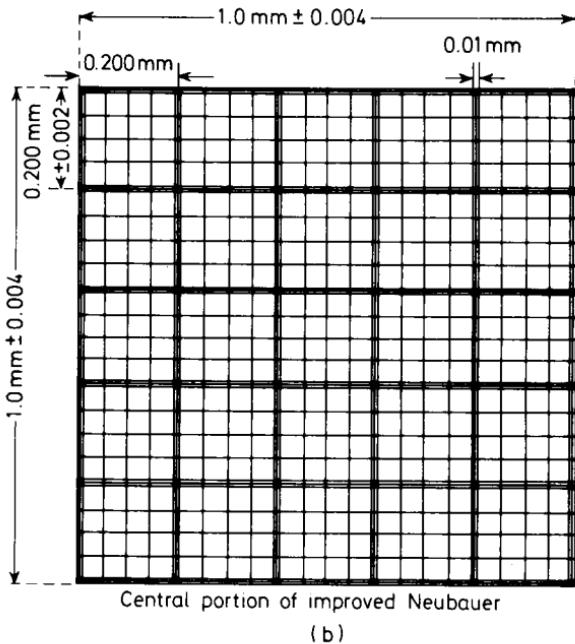
BLOOD DILUTION

The blood must be diluted if red blood cells are to be counted. This can be achieved using special diluting pipettes or by using a 'bulk' dilution method.



Improved Neubauer ruling depth 0.1 mm

(a)



Central portion of improved Neubauer

(b)

Figure 30.4

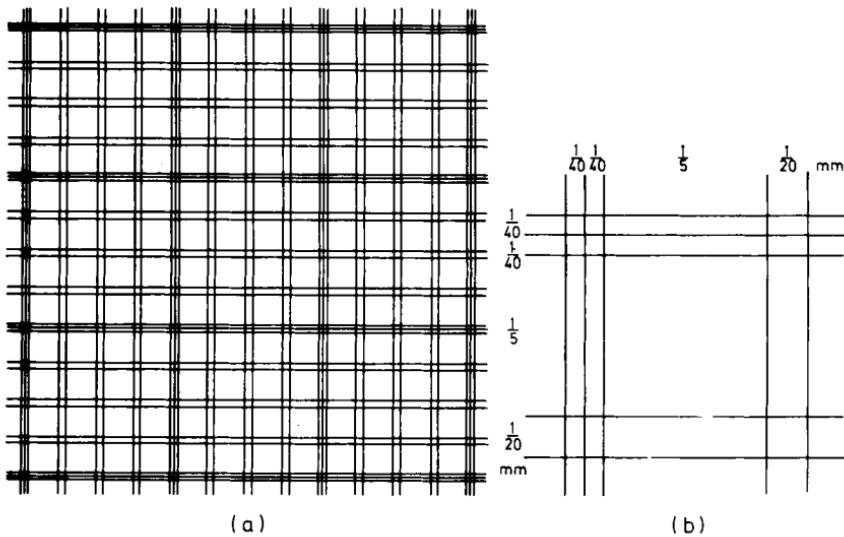


Figure 30.5. (a) The ruled area of the Burker counting chamber; (b) enlarged view showing actual measurements

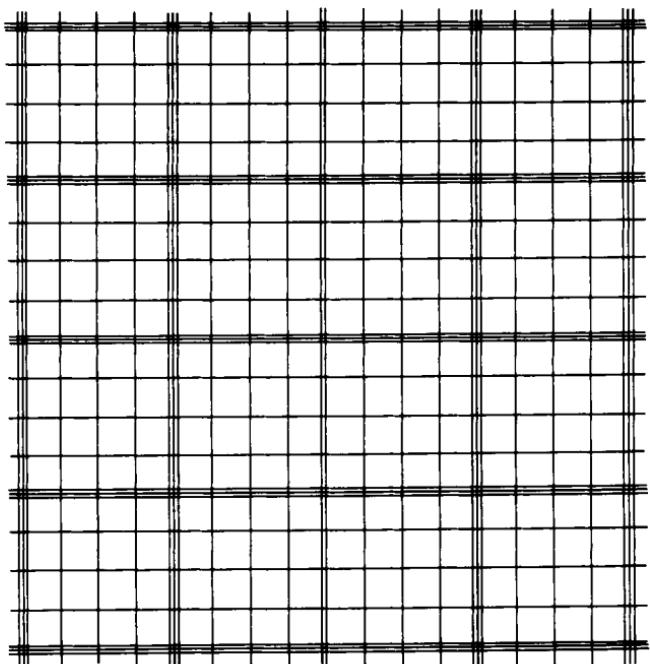


Figure 30.6. The ruled area of the Fuchs-Rosenthal counting chamber

Diluting pipettes

RED CELL PIPETTES

These are graduated to give a dilution of 1 in 100 or 1 in 200. The stem of the pipette is etched with two lines marked 0.5 and

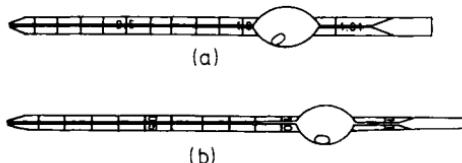


Figure 30.7. Pipettes: (a) Red cell diluting; (b) white cell diluting

1.0. Another line, immediately above the bulb, is marked 101 (*Figure 30.7a*).

In order to facilitate adequate mixing of blood and diluent, a bead is contained within the bulb. This bead is usually coloured red to indicate that the pipette is used for counting erythrocytes.

WHITE CELL PIPETTES

These pipettes are similar in design to the above, with the figures 0.5 and 1.0 engraved on the stem, but it has the figure 11 marked just above the bulb (*Figure 30.7b*). This gives a dilution of 1 in 10, or 1 in 20. The bulb contains a white bead, to distinguish it from the red cell pipette.

The red and white cell pipettes supplied for use with a haemocytometer are usually Thoma pipettes. Such pipettes conform to the British Standards Institution criteria.

The bulb capacity of the Thoma red cell pipettes is one hundred times the volume of fluid drawn up to the 1.0 mark on the stem of the pipette. When the diluent is drawn into the pipette, the blood held within the stem is drawn into the bulb, together with 99 times its own volume of diluting fluid. One volume of diluent remains within the stem of the pipette. When the blood diluent suspension is mixed within the bulb, a final dilution of 1 in 100 is obtained, the fluid held within the stem not entering into the dilution factor. If the blood is only drawn to the 0.5 mark, the dilution within the bulb will be 1 in 200. The same principle is applicable to the Thoma white cell pipette, the final dilution obtained being 1 in 10 or 1 in 20. Therefore all the fluid contained within the stem of the pipette must be expelled before charging the counting chamber.

HAEMOGLOBIN PIPETTES

As these pipettes do not contain the final dilution of blood and diluent they do not have a bulb for mixing purposes. They are graduated to contain 0.02 ml and 0.05 ml of blood (*Figure 30.8*). (Standardization of these pipettes, p. 577.)

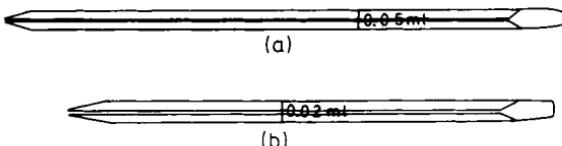


Figure 30.8. Haemoglobin pipettes: (a) 0.05 ml; (b) 0.02 ml

'Bulk' dilution method for RBC and WBC counting

When preparing dilutions it is always more accurate to use as large a volume of blood and diluent as possible. When preparing dilutions in diluting pipettes, these volumes are quite small and, furthermore, mixing of the contents can only be achieved by the small bead during shaking.

It is, therefore, preferable to prepare dilutions for red cell counting by taking 20 mm³ (0.02 ml) of blood in a haemoglobin pipette and washing it into 4 ml of diluting fluid contained in a bijou bottle, or other suitable container (this gives a 1 in 200 dilution). White cells can be similarly diluted by taking 50 mm³ (0.05 ml) of blood into 0.95 ml of white cell diluting fluid (this gives a 1 in 20 dilution). Mixing is facilitated in these procedures by the large bubble of air in the container, which is much more satisfactory. The container is placed on a rotating mixer until thoroughly mixed.

Diluting fluids

Fluids used as diluents must be isotonic, and have a high specific gravity which prevents the cells from settling too quickly. The following are commonly used.

RED CELL DILUTING FLUIDS

Hayem's fluid

Mercuric chloride	0.5 g
Sodium sulphate	5.0 g
Sodium chloride	1.0 g
Distilled water	200.0 ml

Toisson's fluid

Sodium chloride	1.0 g
Sodium sulphate	8.0 g
Glycerine	30.0 ml
Distilled water	160.0 ml

Formol citrate

Sodium citrate	3.0 g
Formaldehyde	1.0 ml
Distilled water	100.0 ml

WHITE CELL DILUTING FLUIDS

Türk's solution. 1 per cent glacial acetic acid, which destroys the erythrocytes, tinged with gentian violet, which stains the leucocytes.

Toisson's fluid. This solution, tinted with methyl violet to give a purple colour, may be used for leucocyte counts.

RED CELL COUNTING METHODS

Method 1

APPARATUS AND REAGENT

1. Improved Neubauer counting chamber.
2. Red cell pipette (bulb type).
3. Red cell diluting fluid.

TECHNIQUE

1. Draw blood from the chosen site of the patient, or from a sample of oxalated blood, into a red cell pipette, until it is level with the 0.5 mark. This will give a final dilution of 1 in 200. If the patient is very anaemic, it is advisable to draw the blood up to the 1 mark, to obtain a final dilution of 1 in 100. If blood is drawn above the chosen mark, the end of the pipette should be touched against the hand, to withdraw the excess.
2. Wipe the outside of the pipette with a piece of clean gauze and draw the diluting fluid up to the 101 mark, rotating the pipette during the process.
3. Withdraw the pipette from the diluting fluid and wipe the outside with a piece of clean gauze. Close the tip of the pipette with the thumb, remove the sucker, place the middle

finger over the top and mix well by shaking. Alternatively, prepare a 1 : 200 dilution of blood as described under the bulk dilution method.

4. Thoroughly clean the counting chamber and the coverglass, place on a flat horizontal surface and, using a firm pressure, slide the coverglass into position on the counting chamber, obtaining a rainbow effect on both sides (Newton's rings).
5. Mix the suspension well by shaking the pipette for 3–4 min and discard about a quarter of the mixture.
6. Fill the chamber by holding the pipette at an angle of 45 degrees and lightly touching the tip against the edge of the

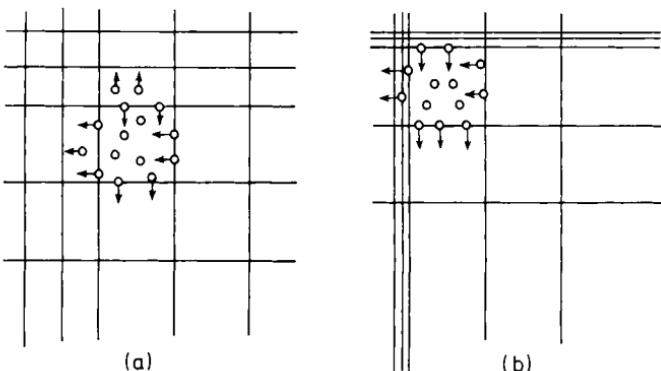


Figure 30.9. Diagram showing the cells to be counted within each small square: (a) ordinary Neubauer chamber; (b) improved Neubauer chamber

coverslip. It is important that the fluid is not allowed to overflow into the channels. Should this occur, the chamber should be cleaned and refilled. Too much fluid in the chamber may raise the coverglass, causing a variation in the depth, resulting in gross errors.

7. Place the chamber on the microscope stage, allow several minutes for the cells to settle. Using a 4 mm objective and $\times 10$ eyepiece, focus the objective onto the central square millimetre of the counting chamber and count all the cells contained within 80 of the 400 small squares (five groups of 16 small squares) (*Figure 30.3*). Cells touching the centre line bordering the top and right-hand side of each group of 16 squares should be included with the count. Those which touch the centre line on the left hand and lower border should be disregarded (*Figure 30.9*).

Using an ordinary Neubauer counting chamber, cells touching the inside line of the triple lines bordering the top and right-hand side of each large square should be included with the count; those touching the other two sides should be disregarded.

For the final result to be expressed as the number of cells per cubic millimetre, the following calculation is necessary.

CALCULATION

Let N = number of cells counted in 80 small squares.

The area of each small square is $\frac{1}{400} \text{ mm}^2$, and the depth of the chamber is $\frac{1}{10} \text{ mm}$.

The volume of fluid over small square is therefore

$$\frac{1}{400} \times \frac{1}{10} = \frac{1}{4000} \text{ mm}^3$$

If N cells are counted in $\frac{80}{4000} \text{ mm}^3$ of diluted blood.

1 mm^3 of diluted blood contains $N \times \frac{4000}{80}$ cells.

Since blood is diluted 1 in 200, 1 mm^3 of blood contains $N \times \frac{4000}{80} \times 200$ cells

$$= N \times 10000 \text{ cells.}$$

In practice, with a dilution of 1 in 200, and when cells in 80 small squares are counted, four zeros may be added to the number of cells counted.

Method 2

APPARATUS AND REAGENT

1. Burker counting chamber.
2. 0.02 ml haemoglobin pipette.
3. Red cell diluting fluid.
4. $3 \times \frac{1}{2}$ in tube with rubber bung.

TECHNIQUE

1. Prepare the counting chamber as for the previous method.
2. Draw blood up to the 0.02 ml (20 mm³) mark of a haemoglobin pipette, wipe the outside and slowly discharge into 4 ml of the diluting fluid in a $3 \times \frac{1}{2}$ in tube. Thoroughly rinse the pipette with the diluting fluid to ensure removal of all traces of blood. This gives a dilution of 1 in 200 (5 ml may be used, to give a dilution of 1 in 250). Stopper with a rubber bung and mix thoroughly by slow inversion.
3. Using a fine pasteur pipette, fill the counting chamber, taking care that no fluid flows into the surrounding channels.

4. Allow the cells to settle, and then using a 4 mm objective and a $\times 10$ eyepiece, count a minimum of 500 red cells. The cells in three, or if necessary six of the $3\text{ mm} \times 1/20\text{th mm}$ rectangular columns should be counted, as previously described (*Figure 30.9*).

CALCULATION

Let N = number of cells counted in three rectangular columns.

The area of each column is $\frac{3}{20}\text{ mm}^2$ and the depth of the chamber is $\frac{1}{10}\text{ mm}$.

The volume of fluid over each column is therefore

$$\frac{3}{20} \times \frac{1}{10} \text{ mm}^3 = \frac{3}{200} \text{ mm}^3$$

If N cells are counted in $\frac{3}{200} \text{ mm}^3$ of blood,

1 mm^3 of diluted blood contains $\frac{200}{3 \times 3}$ cells.

Since blood was diluted $\frac{1}{200}$,

1 mm^3 of blood contains $\frac{N \times 40\,000}{9}$.

EXAMPLE

Number of cells counted in 3 rectangular columns = 810

$$\therefore \text{erythrocyte count} = \frac{810 \times 40\,000}{9} = 3\,600\,000 \text{ RBC per mm}^3.$$

WHITE CELL COUNTING METHOD

Leucocytes may be counted in a similar manner to erythrocytes.

APPARATUS AND REAGENT

1. Improved Neubauer counting chamber.
2. White cell pipette (bulb type).
3. White cell diluting fluid.

TECHNIQUE

Draw blood to the 0.5 mark on the stem of a white cell pipette, and diluting fluid to the 11 mark immediately above the bulb. Alternatively prepare a $\frac{1}{20}$ dilution of blood as described under the bulk dilution method. Using the improved Neubauer chamber,

count the cells in the 4 corner square millimetres, and those in the central square millimetre of the ruled area. Apply the same margin rule as for the erythrocyte count.

CALCULATION

Let N = number of cells counted in 5 mm^2 .

Since the depth of the chamber is $\frac{1}{10}\text{ mm}$,

N cells are counted in $\frac{5}{10} = \frac{1}{2}\text{ mm}^3$ of diluted blood.

Therefore 1 mm^3 of diluted blood contains $N \times \frac{2}{1}$ cells. Since blood was diluted 1 in 20,

1 mm^3 of blood contains

$$N \times \frac{2}{1} \times 20 = 40N \text{ cells}$$

In practice, with a dilution of 1 in 20 and when 5 millimetre squares are counted, the number of white cells may be multiplied by 40. For example, number of white cells counted = 200. Multiplying this by $40 = 8000$ per mm^3 .

NOTES ON TECHNIQUE

- When using bulb type pipettes, be sure to expel all the fluid contained within the stem of the pipette before filling the counting chamber. The stem contains only diluting fluid, the blood diluent suspension being within the bulb of the pipette.
- All pipettes should be cleaned immediately after use. The counting chamber and coverglass must be cleaned both before and after use.
- Pipettes should be examined periodically to ensure that the tips are not chipped or damaged.
- After cleaning, new pipettes should be checked for accuracy. Haemoglobin pipettes should be filled to the mark with mercury which is then expelled into a vessel and carefully weighed (Chapter 1). 0.02 ml of mercury weighs 272 mg, and 0.05 ml of mercury weighs 680 mg. The graduation marks of bulb type pipettes are relevant to each other and their accuracy may be calculated accordingly.
- Alternatively pipettes may be standardized by performing replicate haemoglobin estimations or cell counts in comparison with pipettes where the accuracy has been previously established.

Total eosinophil count

Diluting fluid (Discombe's fluid)

1 per cent aqueous eosin Y (CI No. 45380)	5 ml
Acetone	5 ml
Distilled water	to 100 ml

METHOD

1. Take blood to the 0.5 mark of a white cell diluting pipette.
2. Take diluting fluid to the 11 mark (=dilution of 1 in 20).
3. Mix, blow out one-third of the content of the bulb and charge a Fuchs-Rosenthal chamber with the diluted blood.
4. Allow to stand for about 5 min for the cells to settle.
5. Examine under the 16 mm objective with the light as brilliant as possible.

The eosinophils appear as red staining particles and are counted over at least two ruled areas of the counting chamber (that is $2 \times 16 \text{ mm}^2$).

CALCULATION

Let N = number of cells counted in two ruled areas.

Since the ruled area is 16 mm^2 and 0.2 mm deep.

N cells were counted in $32 \times 0.2 \text{ mm}^3$ of diluted blood.

Since the blood was diluted 1 in 20,

$$1 \text{ mm}^3 \text{ of diluted blood contains } \frac{N}{32 \times 0.2} \times 20 = \frac{10N}{3.2}$$

Notes—This calculation would need modifying if the Fuchs-Rosenthal chamber was ruled over 9 mm^2 (p. 568). Some workers prepare a 1:10 dilution of blood by taking blood to the 1.0 mark and diluting to the 11 mark.

SOURCES OF ERROR IN COUNTING

Errors in counting may be due to any of the following factors.

Low counts

1. Squeezing the site of puncture when filling the pipette.
2. Insufficient blood being drawn into the pipette.
3. Too much diluent being drawn into the pipette.
4. Insufficient mixing.
5. Using the first fluid expelled from the pipette.

6. Insufficiently filling the counting chamber.
7. Undue delay in performing the count after filling the counting chamber.
8. Uneven distribution of the cells in the counting chamber.
9. Inaccurately calibrated pipettes.
10. Faulty counting technique.
11. Saliva in the mouthpiece increasing the dilution.
12. Errors in calculation.

High counts

1. Using the first drop of blood expelled from the site of the puncture.
2. Too much blood being drawn into the pipette.
3. Insufficient diluent being drawn into the pipette.
4. Insufficient mixing of the blood diluent suspension.
5. Overfilling the counting chamber.
6. Uneven distribution of the cells in the counting chamber.
7. The inclusion of yeasts and particles of dust in the count.
8. Inaccurately calibrated pipettes.
9. Faulty counting technique.
10. Errors in calculation.

Normal errors

If the erythrocytes in two groups of 80 small squares are counted from the same specimen of blood diluent suspension, it is most unlikely that identical counts will be obtained. The difference in the two results is caused by the random distribution or 'normal error'. When the counting chamber is charged, the erythrocytes take up their position 'at random' among the ruled squares of the chamber, resulting in a variation of the number of the cells which appear in each square. In order to minimize this error and increase the accuracy of the count, as many cells as possible should be counted. It is for this reason that the method is used whereby 500 cells are counted (*see* Method 2, p. 575). This number is considered as the absolute minimal count. The inherent error is much higher with the erythrocyte count than with the leucocyte count, as a larger number of cells is involved and there is a greater multiplication factor.

ELECTRONIC BLOOD COUNTING MACHINES

Electronic machines for counting red and white blood cells are now standard equipment in many routine clinical laboratories and

their speed, accuracy and reproducibility has raised the standard of routine haematological investigations. Some of the counters available in addition to red and white cell counts, perform haemoglobin estimation, packed cell volume (PCV) (either directly or indirectly) and calculate the mean cell haemoglobin concentration (MCHC), mean cell haemoglobin (MCH) and mean cell volume (MCV). Even more sophisticated counters perform the platelet

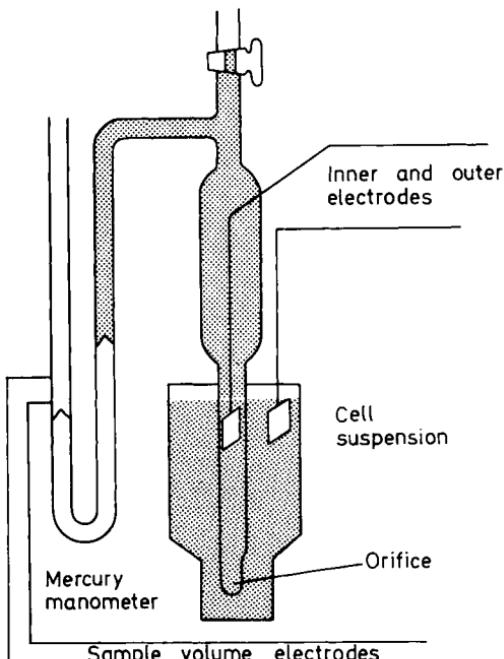


Figure 30.10. Principle of the Coulter counter

count and also the prothrombin time. Contrary to popular belief the use of these electronic counters is not just a case of 'pushing the button', but rigorous quality control procedures must be maintained to ensure accurate results. This is attained by using a standard cell suspension which is usually purchased from the manufacturer of the machine in conjunction with 'standards' prepared in the laboratory.

The Coulter* counters range from the 'simple' machine which will count the total red cells, white cells and platelets using pre-

* Coulter Electronics Limited.

diluted blood, to the complex electronics and automatic dilution systems incorporated in the Model S, which accepts whole blood and in 20 s produces a seven-parameter printed result (WBC, RBC, Hb, PCV, MCV, MCH and MCHC). The Coulter principle is based on an electric current path of small dimensions which is modulated by the momentary passage of each cell passing through an orifice one by one. Cells suspended in an electrolyte

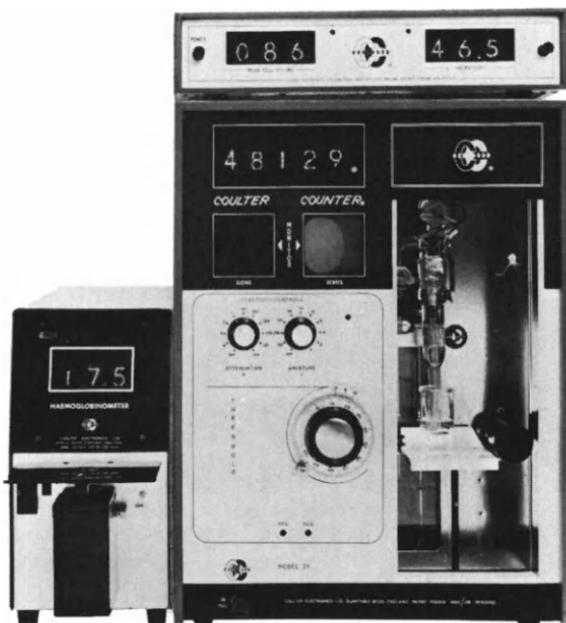


Figure 30.11. Coulter counter. (Reproduced by courtesy of Coulter Electronics Ltd)

are made to pass through a small aperture producing a pulse directly proportional to its volume. Each pulse which reaches or exceeds a preset threshold level will be counted. In the Model S both the red and white cells are counted in triplicate and the count automatically averaged. If one of the triplicate counts falls outside preset limits it is discarded and the result is taken as an average of the remaining two. If two of the counts disagree the count is stopped and no results are recorded.

The MCV is computed using the fact that the voltage pulse

signals are proportional to the volume of the individual cells which produce them. The pulse signal heights are added and one divides the sum by the number of pulses.

The PCV is obtained mathematically from the red cell count and the MCV, there are therefore no problems which sometimes arise from 'trapped' plasma in the centrifuge method. The remaining absolute values are calculated directly from the figures which have been obtained.

The whole blood platelet count may be obtained using the Coulter principle, which is a separate instrument called Thrombo-counter.

The Hemalog 8† uses the peristaltic pump for the sampling,

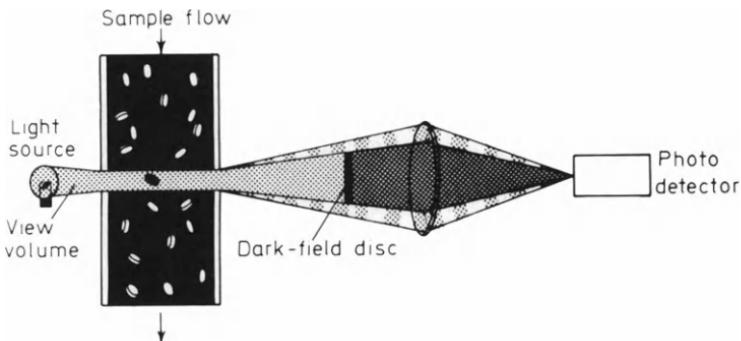


Figure 30.12. Dark-field optics. Schematic diagram of cell counting

dilution and feeding of the sample for the various counting procedures, and in addition to the seven basic parameters automatically performs a platelet count. The Hemalog system has three separate cell counters, flow cells and filtering diaphragms for platelets, red cells and white cells and each counter operates on the principle of counting by reverse dark-field microscopy. A beam of light focuses onto each stream, forming a precisely defined view volume in the flow cell. When no cells flow in the stream through the lighted view volume, the dark-field disc catches all light rays. When cells flow through their respective flow cells, each cell scatters light rays peripheral to the dark-field disc and is counted individually (*Figure 30.12*).

The estimation of the PCV in the Hemalog is unique in the field of electronic haematology machines, as this parameter is actually centrifuged automatically. A sample of whole blood is fed

† Technicon Instruments.

directly to the automated centrifuge, filling an injection chamber. The enclosed air in the chamber reaches a pressure sufficient to inject the sample into a wide-mouth 'J'-shaped tube (*Figure 30.13*). This tube is permanently embedded in a centrifuge head. The centrifuge spins continuously at a speed of 20 000 rpm with blood samples automatically loaded and PCVs read without ever slowing or stopping the centrifuge.

The absolute values MCV, MCH and MCHC are computed electronically from the figures obtained and in addition to a 'print-out' result a single-pen, multi-point recorder follows curves on

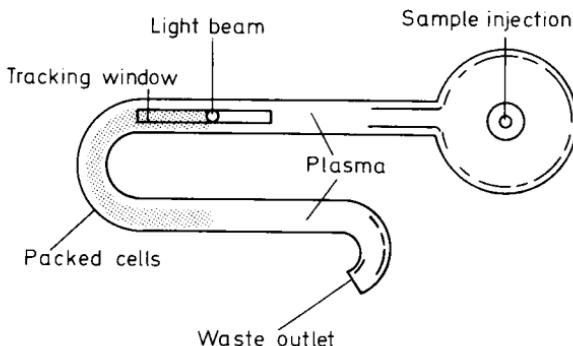


Figure 30.13. PCV 'J' tube

the red cells, white cells, platelets and PCV channels as they are generated.

There are other electronic cell counting machines available which vary in the method by which the cells are actually counted; the latest of these, the Hemac[‡], uses the laser beam, which is accurately focused on the stream of passing cells.

The advantage of using these highly sophisticated electronic particle counters is not only their phenomenal speed in comparison to chamber counters, but the huge numbers of cells which are counted, thereby considerably reducing the inherent error. The Coulter counters for example allow more than 5000 cells to be counted and sized every second!

DIFFERENTIAL LEUCOCYTE COUNT

A smear or film of blood must be prepared and stained. These can be prepared on slides or coverslips and stained by a Romanowsky method.

[‡] Ortho Diagnostics Instruments.

Slide film

Gently touch a fresh drop of blood onto one end of a clean grease-free slide. Using a bevelled piece of glass a little narrower than the slide, allow the drop to spread along it. Holding the slide and 'spreader' at a suitable angle (*see Figure 30.14*), push the spreader along the slide, drawing the blood behind it, until the whole of the drop has been smeared. Do not have too large a drop, or incline

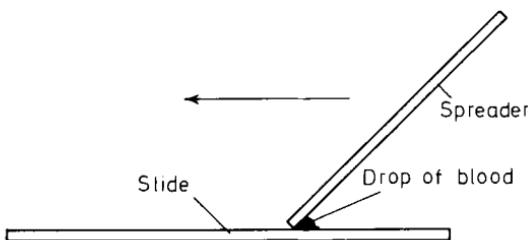


Figure 30.14. Making a blood smear

the spreader at too great an angle, as the film will be too thick for satisfactory microscopic examinations.

The thickness and the even distribution of the cells plays an important part in obtaining accurate results. The smear should be slightly thicker at the commencement than at the tail end. In badly prepared films, the polymorphonuclear neutrophils frequently concentrate at the edges of the preparation. The count is performed using either the battlement method or the longitudinal method.

BATTLEMENT METHOD

The film is examined systematically, by being traversed 3 fields along the edge, 2 fields up, 2 fields along and 2 fields down. This sequence is continued until a minimum of 200 cells has been enumerated.

LONGITUDINAL METHOD

The cells are counted in one complete longitudinal strip of the film. If less than 200 cells are counted, a second strip should be similarly enumerated.

COVERSILK FILMS

Many workers prefer to make blood films on coverslips (*Figure 30.15*). Although they are more difficult to handle during preparation and staining, this problem is largely overcome by the use of

automatic staining machines. Coverslip films are preferred since the distribution of cells is more even. Battlement counts are, therefore, not necessary. As coverslips are usually made from better quality glass than slides, cells do not deteriorate so badly, and when mounted on a slide for microscopic examination there is no mounting medium between the cell and the lens.

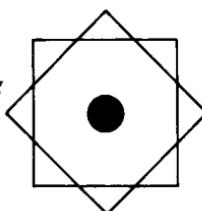


Figure 30.15. Diagram illustrating the technique of preparing coverglass films

Films are prepared by taking a small drop of blood into the centre of a clean, grease-free coverslip. A second coverslip is then dropped gently on top of the blood specimen diagonally. The blood is allowed to spread gently to the edges and the coverslips are then drawn apart by sliding one coverslip across the other. They can be stained by placing them film-side downwards in watchglass.

Romanowsky stains

These stains depend, for their staining properties, upon certain derivatives produced when alkaline methylene blue is combined with eosin. Methylene blue, when treated with alkali, forms a methylene azure. The various Romanowsky stains differ in the method of preparation of the methylene azure and in the proportion of eosin and methylene azure used to prepare the stain.

All the stains in the group are sensitive to changes in pH, the methylene blue component staining more intensely in an alkaline environment and the eosin derivative staining more intensely in an acid environment. When using these stains buffered distilled water is used throughout the staining procedure. The alcohol used to dissolve the stain must be free from acetone, acetic acid and water.

Romanowsky stains may be purchased in powder, tablet or liquid form. For the busy laboratory the stain already in solution is probably the most convenient, but each batch purchased or prepared should be tested for its optimum staining time.

Leishman's stain

A Romanowsky-type stain designed to differentiate leucocytes.

*Leishman's stain**Solution 1. Methylene blue (CI No.*

52015)	1 g
Sodium carbonate, 0.5 per cent aqueous solution	100 ml
Eosin BA (CI No. 45400) 0.1 per cent aqueous solution	100 ml
Methyl alcohol, absolute	to 100 ml

Dissolve the methylene blue in the sodium carbonate solution. Heat at 65 °C for 12 h, cool and allow the mixture to stand for 10 days. Add an equal volume of the eosin solution, mix well and allow the mixture to stand for 6–12 h. Filter and collect the precipitate. Wash the precipitate with several changes of distilled water until no more colour is extracted. Dry the precipitate in a 37 °C incubator and grind to a powder in a glass mortar. Weigh out 0.15 g of the powder and triturate in a mortar with methyl alcohol. Pour off the supernatant and add more methanol until all of the powder is dissolved. Make up the volume to 100 ml with methyl alcohol, store in a tightly stoppered dark bottle and label. Allow the stain to stand for 24 h prior to use.

*Buffer solution (pH 6.8)**Solution 2. Disodium hydrogen phosphate Na₂HPO₄ (An-*

hydrous), M/15 solution (9.47 g per litre)	49.6 ml
---	---------

Potassium dihydrogen phosphate KH ₂ PO ₄ (An-hydrous) M/15 solution (9.08 g per litre)	50.4 ml
---	---------

Dissolve the phosphates in the distilled water, check the reaction (pH 6.8) and label.

PROCEDURE

1. Prepare thin blood films and fix in solution 1 for 1–2 min.
2. Add to the undiluted stain on the slide twice its volume of solution 2 and mix by gentle rocking. Allow the diluted stain to act for 10 min.

3. Wash and differentiate with solution 2. When correctly differentiated the smear should be a salmon-pink colour.
4. Drain and dry in the air at room temperature. Clean the back of the slide and examine microscopically.

RESULTS

Nuclei of leucocytes, purple; eosinophilic granules, orange-red; basophilic granules, dark blue; lymphocytes, dark purple nuclei with pale blue cytoplasm; platelets, violet granules; Auer bodies, red; red blood corpuscles, salmon pink.

Notes—(a) Leishman's stain may be purchased commercially in the form of the precipitated powder or as a ready prepared solution. The mode of preparation of the commercially prepared powder is similar to that described above, 0.15 g of the powder being dissolved in 100 ml of methyl alcohol. Alternatively, place the dye and alcohol together in a flask, plug with cotton wool and warm for 15 min in a water bath, shaking at intervals.

(b) The use of a buffer solution for diluting the Romanowsky stains is recommended owing to the varying pH of tap water in different localities. The buffer solution should be added to the slide gently without displacing any of the stain. After staining, the dye should be washed from the slide in such a manner that the scum is removed first.

(c) When dry, the stained film can be mounted with a coverglass using a neutral medium as a mountant. Although optional, this step is preferred by some workers.

Wright's stain

A Romanowsky-type stain for the differentiation of leucocytes.

Solution 1. Methyl alcohol, absolute (Analar)

Wright's stain

Solution 2. Methylene blue (CI No.

52015) 1 g

Sodium carbonate, 0.5 per cent aqueous solution 100 ml

Eosin, w/s (yellowish) (CI No. 45380) 0.1 per cent aqueous solution 500 ml

Methyl alcohol, absolute 60 ml

Add the methylene blue to the sodium carbonate solution and grind in a mortar until the dye is in solution. Place the mixture in an Erlenmeyer flask, taking care that the fluid does not exceed

6 cm in depth. Place the flask in a steam sterilizer and heat at 100 °C for 60 min. Cool, filter (e.g. Whatman's No. 1 filter paper) and save the filtrate. Add the eosin solution slowly, with constant shaking, to the filtrate. The mixture becomes purplish and a finely granular black precipitate is formed. Filter (e.g. Whatman's No. 5 filter paper) and discard the filtrate. Dry the precipitate in an incubator at 37 °C and store in a glass-stoppered bottle. Dissolve 0.1 g of the dried powder in 60 ml of methyl alcohol, filter (e.g. Whatman's No. 1 filter paper), store the filtrate in a tightly stoppered dark bottle and label.

Buffer solution (pH 6.5)

Solution 3. Disodium hydrogen phosphate, Na_2HPO_4 , M/15 solution (9.47 g per litre)	31.8 ml
Potassium dihydrogen phosphate, KH_2PO_4 , M/15 solution (9.08 g per litre)	68.2 ml

Dissolve the phosphates in the distilled water, check the reaction (pH 6.5) and label.

PROCEDURE

1. Prepare thin blood films and fix in solution 1 for 3–5 min.
2. Dilute one volume of solution 2 with two volumes of solution 3, flood the slide and allow the stain to act for 5 min.
3. Wash and differentiate with solution 3.
4. Drain and dry in the air at room temperature. Clean the back of the slide and examine microscopically.

RESULTS

Nuclei of leucocytes, dark blue to purple; eosinophilic granules, red to orange; basophilic granules, dark purple to black; lymphocytes, dark purple nuclei with light blue cytoplasm; platelets, violet to purple granules; erythrocytes, yellowish-red.

Notes—(a) Wright's stain is seldom prepared in the laboratory but is usually purchased in powder or liquid form from a reliable commercial source. The commercial stain is prepared by dissolving 0.2–0.3 g of the dried powder in 100 ml of methyl alcohol.

(b) See Notes under Leishman's stain.

Giemsa stain

A Romanowsky-type stain designed to differentiate leucocytes.

*Solution 1. Methyl alcohol, absolute (Analar)**Giemsa stain*

<i>Solution 2. Azur 11-eosin</i>	3 g
Azur 11	0.8 g
Glycerol, pure	200 ml
Methyl alcohol, absolute (Analar)	300 ml

Grind the two dyes together in a clean mortar and combine the alcohol with the glycerol in a 1-litre flask. Sprinkle the mixed dye carefully over the surface of the alcohol-glycerol and allow the mixture to stand for 24 h. Stir the mixture at intervals after the 24 h has elapsed to ensure that all of the dye has passed into solution. Store in a tightly stoppered dark bottle and label.

Buffer solution (pH 7.0)

<i>Solution 3. Disodium hydrogen phosphate, Na_2HPO_4 (anhydrous), M/15 solution (9.47 g per litre)</i>	61.1 ml
Potassium dihydrogen phosphate, KH_2PO_4 (anhydrous), M/15 solution (9.08 g per litre)	38.9 ml

Dissolve the phosphates in the distilled water, check the reaction (pH 7.0) and label.

PROCEDURE

1. Prepare thin blood films and fix in solution 1 for 3 min.
2. Dilute one volume of solution 2 with nine volumes of solution 3, flood the slide and allow the stain to act for 1 h.
3. Wash and differentiate with solution 3, controlling the degree of differentiation microscopically.
4. Drain and dry in the air at room temperature.

RESULTS

Nuclei of leucocytes, reddish-purple; eosinophilic granules, red to orange; basophilic granules, blue; lymphocytes, dark purple

nuclei with light blue cytoplasm; platelets, violet to purple granules.

Notes—Giemsa's stain is available commercially either in liquid form ready for use or as a combined powder. The azur dyes frequently vary from batch to batch and for this reason most laboratories prefer to purchase the commercial product. The commercial powder is dissolved as follows: weigh out 1 g of the powder and place it in a 250 ml conical flask. Add 66 ml of pure glycerol and heat the mixture at 56 °C for 90–120 min. Add 66 ml of absolute methyl alcohol, mix thoroughly and allow the solution to stand for 7 days at room temperature. Filter, store in a tightly stoppered bottle and label. The solution should be diluted for use as in the above procedure.

Jenner's stain

A neutral stain for the differentiation of leucocytes.

Solution 1. Methyl alcohol, absolute (Analar)

Jenner's stain

Solution 2. Eosin, w/s yellowish, 1 per

cent aqueous solution 1 litre

Methylene blue, 1 per cent
aqueous solution 1 litre

Methyl alcohol, absolute
(Analar) 100 ml

Combine the two stains and allow the mixture to stand for several days. Filter (e.g. Whatman's No. 1 filter paper) and wash the sediment remaining on the filter paper with cold distilled water until no more colour is extracted. Dry the precipitate in an incubator at 37 °C and store the dried powder in a dark, glass-stoppered bottle. Weigh out 0.3 g of the dried powder and add slowly to the methyl alcohol, shaking vigorously between each addition. Filter, store in a glass-stoppered bottle and label.

Buffer solution (pH 6.8)

Solution 3. Disodium hydrogen phosphate, Na₂HPO₄, (anhydrous), M/15 solution (9.47 g per litre)

49.6 ml

Potassium dihydrogen phosphate, KH₂PO₄, (anhydrous), M/15 solution (9.08 g per litre)

50.4 ml

Buffer solution. Dissolve the phosphates in the distilled water, check the reaction (pH 6.8) and label.

PROCEDURE

1. Prepare thin blood films and fix in solution 1 for 5 min.
2. Dilute one vol of solution 2 with one vol of solution 3, flood the slide and allow the stain to act for 5 min.
3. Wash and differentiate with solution 3.
4. Drain and dry in the air at room temperature.

RESULTS

Nuclei of leucocytes, dark blue; eosinophilic granules, orange to red; basophilic granules, dark blue; lymphocytes, dark blue nuclei with pale blue cytoplasm; erythrocytes, yellowish-red.

Notes—(a) Jenner's stain is available commercially either in liquid form ready for use or as a combined powder. The commercial stain is prepared by dissolving 0.3 g of the combined powder in 100 ml of methyl alcohol.
(b) Jenner's stain is not a true Romanowsky stain.

Jenner-Giemsa stain

For morphological details in marrow cells.

Solution 1. Methyl alcohol, absolute (Analar)

Solution 2. Jenner stain

Solution 3. Giemsa stain

Solution 4. Buffer solution, pH 6.8

PROCEDURE

Smears

1. Prepare thin blood or marrow films and fix immediately in solution 1.
2. Flood the slide with solution 2 and leave for 3 min.
3. Dilute the stain with an equal volume of distilled water and allow the diluted stain to act for 1 min.
4. Drain without rinsing.
5. Dilute the Giemsa stain, 3 ml of stain to 2 ml of distilled water, and restain the smear for 12 min.
6. Wash and differentiate in distilled water, controlling the degree of differentiation microscopically.
7. Drain and dry in the air at room temperature.

RESULTS

Nuclei, red lavender; nucleoli, blue; eosinophilic granules, red; basophilic granules, deep blue; neutrophil granules, purple-pink; cytoplasm of lymphocytes and lymphoblasts, blue.

Staining machines

When staining large numbers of blood films it is an advantage to use a staining machine, as not only do they stain a large batch of films more quickly but they also give more uniform staining results. Machines have been designed to stain both slide and coverslip films. The principle of both types of machine is much the same and for simplicity the coverslip staining machine will be described here.

The machine incorporates a central shaft which has a plastic disc fitted at the top end. This disc is fitted with hooks at regular intervals. The coverslips to be stained are placed in stainless steel containers which are hung on these hooks. The beakers containing the reagents are placed at stations underneath the plastic discs in such a way that when the machine is at rest the hooks are directly over the reagent beakers. A timing mechanism similar to that on a tissue processing machine allows the central shaft to be raised at intervals. This lifts the coverslips clear of the beaker and transfers them to the next one. For the purpose of staining blood films the last two beakers are filled with buffered water. The surface of this buffer tends to become covered with a scum of stain precipitate, which deposits on the film when it is lifted clear of the fluid. For this reason air is bubbled through these two beakers in order to keep the fluid in motion. At the last station the coverslip holder is lowered onto a ramp so that it leans over slightly and is released from the hook. The ramp then revolves and removes the holder so that the next one is not lowered on top of it.

Staining machines may have 8, 12 or 23 stations. The coverslip technique described below is for a 12-station machine using, however, 10 stations only. The staining procedure is the Jenner-Giemsa technique.

1. The first two beakers contain absolute methyl alcohol.
2. The next three beakers contain Jenner stain diluted 1 : 1 with buffered water pH 6.8.
3. The next three beakers contain Giemsa stain diluted 1 : 10 with buffered water pH 6.8.

4. The last two beakers are filled with buffered distilled water pH 6.8 and the bubbling device fitted.

The timing device is cut so that the coverslips spend 2 min in each beaker. The film is therefore fixed for 4 min, stained with Jenner for 6 min, Giemsa for 6 min and then washed in two changes of buffer for 2 min each.

The stain should be replaced daily and the buffered water changed at least twice a day.

Differential count

All types of leucocytes encountered should be noted until a total of 200 has been examined. The result is then expressed as a percentage and in absolute numbers of the total leucocyte count (Table 30.1).

In cases where extreme accuracy is required a minimum of 400 cells should be counted. A multiple counting machine greatly facilitates this technique. The number of each individual type of cell counted is recorded as well as the gross total.

Table 30.1 NORMAL DIFFERENTIAL COUNT IN ADULTS

Type of cell	Total per mm ³	Percentage of total leucocytes
Neutrophils	2500–7500	40–75
Eosinophils	40– 440	1– 6
Basophils	0– 100	0– 1
Lymphocytes	1500–3500	20–45
Monocytes	200– 800	2–10

The absolute number of a particular type of leucocyte can be calculated from the results of the total white cell count and differential count.

Example

Total white cell count 8000 per mm³.

Differential count	Per cent
Neutrophils	65
Eosinophils	2
Basophils	1
Lymphocytes	25
Monocytes	7

Therefore for every 100 white cells per mm³ there are 65 neutrophils. For 8000 white cells there are therefore:

$$65 \times 80 = 5200$$

Similarly there are 160 eosinophils, 80 basophils, 2000 lymphocytes and 560 monocytes.

An increase in the number of circulating neutrophils is known as a *neutrophilia* and a decrease as a *neutropenia*.

An increase in the number of circulating lymphocytes is known as a *lymphocytosis* and a decrease as a *lymphopenia*.

An increase in the number of circulating eosinophils is known as an *eosinophilia*.

An increase in the number of circulating basophils is known as a *basophilia*.

An increase in the number of circulating monocytes is known as a *monocytosis*.

It is important to remember that an increased percentage of neutrophils does not necessarily indicate neutrophilia. For example, 90 per cent neutrophils in a total white cell count of 8000 WBC per mm³ is equal to 7200 neutrophils per mm³, that is a normal value.

Whilst performing differential white cell counts, it is essential to examine the red cells for any abnormality and also the platelets as to whether they are present in approximately normal numbers and morphology.

A deficiency of haemoglobin results in a pale, or unstained area, in the centre of the red cell. This is called *hypochromia*. A diffuse purplish-grey staining of the cell is called *polychromasia* and is a sign of cell-immaturity. These cells are usually seen to be reticulocytes when stained with a vital stain (cells stained whilst still viable and not fixed). The term *anisochromasia* is used to describe varied stain uptake by the cell. Abnormalities in red cell shape are referred to as *poikilocytosis*, the commonest abnormal shape being pear-shaped. Variation in cell size is termed *anisocytosis*. Occasionally some oval-shaped red cells are seen; this inherited characteristic is called *ovalocytosis*, or *elliptocytosis*.

Cooke-Arneth count

Arneth attempted to classify the polymorphonuclear neutrophils into groups according to the number of lobes in the nucleus and also according to the shape of the nucleus. The procedure was too cumbersome for routine use and was modified by Cooke, who

classified the neutrophils into five classes according to the number of lobes in the nucleus.

- Class I. No lobes, that is an early cell in which the nucleus has not started to lobulate.
- Class II. Two lobes.
- Class III. Three lobes.
- Class IV. Four lobes.
- Class V. Five or more lobes.

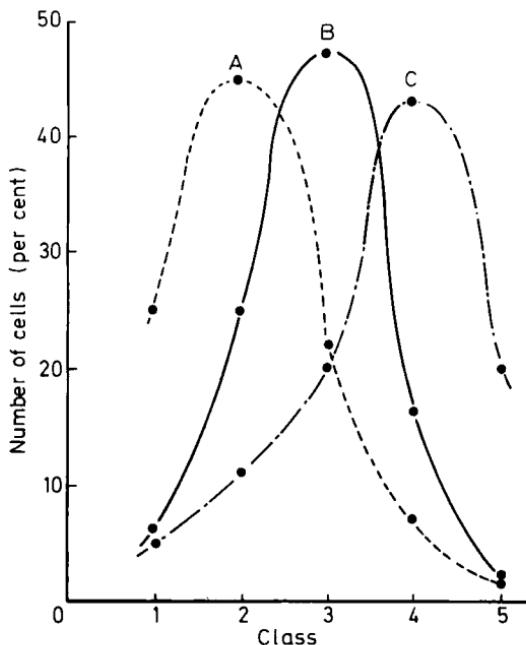


Figure 30.16. Cooke-Arneth count. (a) Showing a shift to the left, (b) normal curve, and (c) showing a shift to the right

The lobes cannot be said to be separated if the strand of chromatin joining them is too thick. The strand must be a very fine one. Some workers suggest that the strand must be less than one-quarter of the width of the widest part of the lobe.

The count is performed by examining 100 neutrophils and placing them in their correct class. The normal proportions are:

- Class I. 10 per cent.
- Class II. 25 per cent.
- Class III. 47 per cent.
- Class IV. 16 per cent.
- Class V. 2 per cent.

When the sum of Class I and Class II exceeds 45 per cent a 'shift to the left' in the Arneth count can be said to exist, that is if the figures were to be plotted on graph paper as in *Figure 30.16* the peak of the graph would move to the left-hand side of the normal curve. The shift to the left occurs in infections, since new cells are released into the circulation from the marrow.

A 'shift to the right' can also occur, that is the peak of the graph moves to the right-hand side of the normal graph. This occurs in certain conditions, notably pernicious anaemia, where the rate of polymorphonuclear production is slowed down.

RETICULOCYTE COUNT

Reticulocytes (immature erythrocytes) are slightly larger than normal red cells, and by using a supravital staining technique, basic dyes, such as brilliant cresyl blue, are precipitated into a meshwork (called reticulum) within the cell. This meshwork appears deep blue against a relatively unstained background. The reticulum is considered to be remnants of basophilic ribonucleoprotein (normally found in the cytoplasm), and the more mature the cell the less reticulum found.

APPARATUS AND REAGENT

Small glass tube (8 mm internal diameter), clean slides and spreader, Ehrlich's microscope eyepiece, 37 °C incubator, and brilliant cresyl blue.

Sodium citrate	0.6 g
Sodium chloride	0.7 g
Distilled water	100.0 ml
Dissolve and add	
Brilliant cresyl blue G (Gurr)	1.0 g
Dissolve and filter. The solution is now ready for use.	

TECHNIQUE

1. Place 2–3 drops of the dye in the glass tube.
2. Add 2–4 drops of the patient's blood and gently mix.
3. Incubate at 37 °C for 15–20 min.
4. Gently re-suspend the red cells.
5. Make blood films and allow them to dry quickly.
6. Using the Ehrlich's eyepiece* and the 2 mm oil objective,

* Ehrlich's eyepiece has a square diaphragm, inserted below the eye lens, which can be adjusted to give a square field of varying size.

examine the unfixed, uncounterstained film. Normal red cells appear greenish-blue, reticulocytes greenish-blue with deep blue precipitate network.

7. Count at least 1000 red cells and calculate the percentage of reticulocytes present.

Normal range—Adults 0.2–2 per cent; cord blood 2–6 per cent.

If a permanent preparation is desired, counterstain with a Romanowsky stain such as Leishman's stain, but fix the film in undiluted stain for 30 s only and then treat with diluted stain for a similar period of time, wash off and dry in the usual way. The fixation in methyl alcohol decolorizes the cresyl blue staining of the red cells but leaves the precipitate in the reticulum.

31

Haemoglobin Estimation

The object of estimating haemoglobin (Hb) is to determine the oxygen-carrying capacity of the blood. The results assist in detecting diseases which cause a deficiency or excess of haemoglobin, and in studying changes in the haemoglobin concentration before or after operations and blood transfusions.

Generally, haemoglobin estimations rely on a comparison of colours. Visual comparisons can lead to inaccuracies, and the use of photoelectric absorptiometers is to be preferred. Most large laboratories have these instruments, but as they are not universally used, four methods of haemoglobin determination will be described, one relying on visual matching (Sahli) and three using the absorptiometer (alkaline haematin, oxyhaemoglobin and cyanmethaemoglobin).

All these methods depend upon matching the colour produced by the test sample with the colour produced by a standard sample of known haemoglobin concentration. The haemoglobin concentration of the standard sample of blood must be very accurately measured. This can be achieved by one of two ways:

1. By measuring the amount of *oxygen* contained in the haemoglobin.

If a known volume of blood is taken and the haemoglobin it contains is saturated with oxygen, the oxygen can be driven off and measured in a Van Slyke apparatus. Then applying Hufner's factor the amount of haemoglobin in the known volume of blood can be calculated. Hufner's factor is that 1 g of haemoglobin can combine with 1.34 ml of oxygen. This method only estimates the haemoglobin capable of carrying oxygen. Inert forms such as methaemoglobin and sulphohaemoglobin are not measured. It is now realized that a more correct figure to use for Hufner's factor would be 1.36.

2. By measuring the amount of *iron* contained in the haemoglobin.

This second method measures the amount of iron contained in a known volume of blood. This can be performed

chemically using potassium thiocyanate. 100 g of haemoglobin are known to contain 347 mg of iron, so that having estimated the amount of iron contained in the blood sample the total haemoglobin concentration can be calculated. This is the more preferable procedure and ideally it would be better to apply this method to routine haemoglobin estimation. Unfortunately, it is too cumbersome and time-consuming.

Acid haematin method (Sahli)

Haemoglobin is converted to acid haematin by the action of hydrochloric acid.

APPARATUS AND REAGENT

1. Sahli graduated tube and standard.
2. N/10 HCl.
3. 0.02 ml pipette.

TECHNIQUE

1. Fill the graduated tube to the 20 mark with N/10 HCl.
2. Add 0.02 ml of blood, mix well and leave for 5–10 min.
3. Add N/10 HCl drop by drop, mixing between each addition, until the colour matches the standard.
4. Read the amount of solution in the graduated tube. The calibrations give the haemoglobin concentration as a percentage.
5. Calculate the haemoglobin content, in g per 100 ml of blood, as follows:

The standard tube will state the number of grams per 100 ml of haemoglobin that is equivalent to 100 per cent. Some of the older Sahli standards use 17.2 g per 100 ml as 100 per cent haemoglobin. Using this apparatus, if a haemoglobin value is 90 per cent the haemoglobin content in g per 100 ml is

$$\frac{90}{100} \times 17.2 = 15.48 \text{ g per 100 ml}$$

If a percentage haemoglobin is to be reported this 15.48 g per 100 ml would need to be converted to a percentage, regarding 14.6 g per 100 ml as being equivalent to 100 per cent.

The percentage haemoglobin equals

$$\frac{15.48}{14.6} \times 100 \text{ per cent} = 106 \text{ per cent}$$

Alkaline haematin method (Gibson and Harrison standard)

Haemoglobin is converted to alkaline haematin by the action of sodium hydroxide. The standard may be purchased ready made. It is equivalent to 16.0g haemoglobin per 100ml provided the blood is treated as described.

APPARATUS AND REAGENT

1. Photoelectric absorptiometer with a green filter.
2. Gibson and Harrison artificial haemoglobin standard.
3. N/10 NaOH.
4. 0.05 ml pipette.
5. Test-tubes.

TECHNIQUE

1. Add 0.05 ml of blood to 4.95 ml of N/10 NaOH.
2. Mix well and boil for 4 min. At the same time, boil 5 ml of standard solution. This is essential.
3. Cool both tubes quickly in cold water, and match the test against the standard, using the colorimeter and a green filter. If the tests give too high a reading add 5 ml of water to both test and standard and read again.
4. Calculate the haemoglobin content in g per 100 ml of blood.

Notes—(a) It is essential to heat the standard at the same time as the test solution; (b) matching must take place within 30 min after boiling; (c) the standard is equivalent to 16.0g haemoglobin per 100ml of blood.

CALCULATION

Let colorimeter reading of test = 21.

Let colorimeter reading of standard = 28.

As the standard is equivalent to 16g of haemoglobin per 100 ml
the test haemoglobin

$$= \frac{21}{28} \cdot 16 \text{ g per 100 ml} = 12 \text{ g per 100 ml}$$

As 14.6 g per 100 ml = 100 per cent, the percentage haemoglobin

$$= \frac{12}{14.6} \cdot 100 = 82 \text{ per cent}$$

Oxyhaemoglobin method

The haemoglobin is converted to oxyhaemoglobin by dilution with ammoniated water. The standard haemoglobin solution is prepared as directed by Thomson.

APPARATUS AND REAGENT

1. Photoelectric absorptiometer with a yellow/green filter (Ilford No. 625).
2. Standard haemoglobin solution.
3. 0.04 per cent ammonia in distilled water.
4. 0.02 ml pipette.
5. Test-tube with a rubber bung.

TECHNIQUE

1. Add 0.02 ml of blood to 4 ml of ammoniated water in a test-tube.
2. Mix well by inversion.
3. Read the standard solution, and then the test solution in the absorptiometer against distilled water.
4. Calculate the amount of haemoglobin in g per 100 ml of blood using the formula:

Reading of test haemoglobin content per 100 ml of standard
 Reading of standard

Cyanmethaemoglobin method

The haemoglobin is converted by the action of ferricyanide to methaemoglobin. This is then converted to cyanmethaemoglobin by the action of potassium cyanide.

DRABKINS SOLUTION

Potassium cyanide	KCN	0.2 g
Potassium ferricyanide	$K_3Fe(CN)_6$	0.2 g
Sodium bicarbonate	$NaHCO_3$	1.0 g
Distilled water up to		1000 ml

APPARATUS AND REAGENTS

1. Photoelectric absorptiometer with a green filter.
2. Drabkins solution.
3. 0.02 ml pipette.
4. Test-tubes.
5. Standard solution of cyanmethaemoglobin (BDH).

This is labelled with the concentration of haemoglobin in the actual solution. The dilution factor of blood and diluent used in the test must therefore be taken into consideration when calculating the result.

TECHNIQUE

1. Add 0.02 ml of blood to 5 ml of Drabkins solution (1 in 250 dilution). Mix well.
2. Allow to stand for 10 min for full colour development to take place.
3. Read in the absorptiometer using a tube of Drabkins solution as a blank. This compensates for the yellow colour of the solution.
4. Read the standard solution of cyanmethaemoglobin.
5. Calculate the concentration of haemoglobin as follows:

Colorimeter reading of test

Colorimeter reading of standard

$$\times \text{concentration of standard solution} \times \text{dilution} \left(\frac{1}{250} \right)$$

EXAMPLE

Standard solution = 50 mg haemoglobin per 100 ml. If the test reading was 28 and the standard reading was 35 haemoglobin concentration

$$\begin{aligned} &= \frac{28}{35} \times 50 \times 250 \text{ mg per 100 ml} \\ &= 10000 \text{ mg per 100 ml} \\ &= 10 \text{ g per 100 ml} \end{aligned}$$

Percentage haemoglobin when 14.6 g per 100 ml is equivalent to 100 per cent

$$= \frac{10}{14.6} \times 100 = 68 \text{ per cent}$$

CALIBRATION OF AN ABSORPTIOMETER FOR HAEMOGLOBIN MEASUREMENT

In routine practice it would be very time-consuming if standard solutions were to be tested with every batch of tests. Since any particular absorptiometer will usually give the same reading for

any haemoglobin concentration it is more usual to prepare a standard calibration graph.

Preparation of curve (using cyanmethaemoglobin method)

Choose a dilution of blood which gives a reasonable deflection in the absorptiometer to be used. In the EEL photoelectric absorptiometer a 1 in 400 dilution of blood is satisfactory for this technique. This dilution will produce a straight line graph. Stronger dilutions will sometimes produce a graph with a slight curve.

METHOD

1. Take a blood with a high haemoglobin value, for example 140 per cent. This can be prepared by removing some of the plasma from a normal blood.
2. Carefully measure the haemoglobin content of the blood. This can be conveniently performed by reference to a known standard, but a more accurate procedure is to measure the iron content of the blood.
3. Prepare a bulk 1 in 400 dilution of this blood by placing 0.5 ml into a 200 ml volumetric flask and fill up to the mark with Drabkins solution and mix thoroughly.
4. Take 10 test-tubes and prepare percentage solutions of the

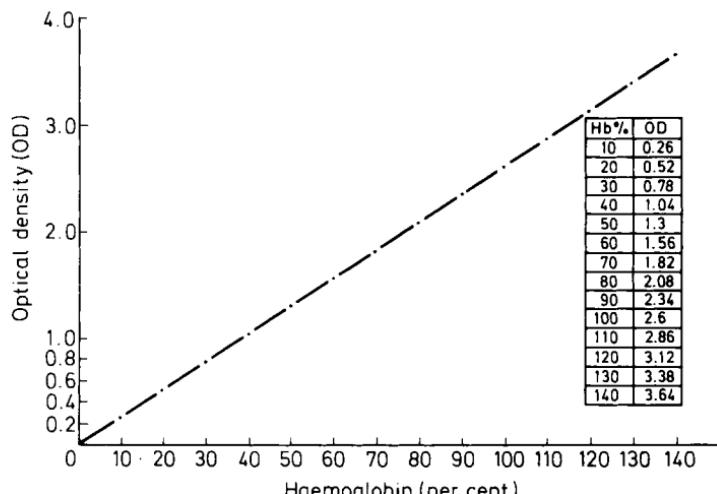


Figure 31.1. Calibration graph

1 in 400 bulk dilution. The percentage range is from 10 to 100 (see Table 31.1).

5. Read each of these 10 tubes in the absorptiometer using the diluent as a blank. Plot these readings on graph paper against the percentage haemoglobin (*Figure 31.1*).
6. From this graph prepare a table converting all the relevant absorptiometer readings into percentage haemoglobin and grams of haemoglobin per 100 ml.
7. Haemoglobin estimations can be performed using this dilution of blood (i.e. 20 mm³ of blood to 8 ml of diluent) and the absorptiometer readings converted into haemoglobin results very quickly.

This procedure can of course be modified for any particular haemoglobin method.

Note—The calibration curve should be checked twice daily using a known haemoglobin standard. Accurate high and low cyanmethaemoglobin standards are available commercially.

Table 31.1

Tube No.	1	2	3	4	5	6	7	8	9	10
ml of 1/400 dilution	1	2	3	4	5	6	7	8	9	10
ml of diluent	9	8	7	6	5	4	3	2	1	0
Per cent of 1/400 dilution	10	20	30	40	50	60	70	80	90	100
Percentage Hb concentration (original blood 140 per cent)	14	28	42	56	70	84	98	112	126	140

32

Tests for Erythrocyte Sedimentation and Fragility

ERYTHROCYTE SEDIMENTATION RATE (ESR)

If anticoagulated blood is allowed to stand undisturbed, the red cells will gradually settle to the bottom of the container, leaving a clear layer of plasma. This sedimentation occurs in three phases. First the cells tend to aggregate and form rouleaux and only fall slightly. In the second phase the speed of the fall is increased and as the cells pack this speed is decreased during the third phase.

Rouleaux formation is controlled by the concentration of fibrinogen and the amount of globulins present. The ESR is of great value to the clinician and is commonly used as a screening test at the initial examination of the patients. An increased rate of fall in chronic conditions such as rheumatoid arthritis and tuberculosis is usual, and the increase or decrease in ESR is used to monitor the progress of the disease. It is also elevated in acute and chronic infections and the malignant diseases where the plasma proteins are abnormal. A decreased rate of fall is often present in polycythaemic subjects and a reading of 0 mm is not unusual.

Certain physical conditions also affect the rate of sedimentation and stringent precautions are therefore necessary to bring about standard conditions for the test. These precautions are discussed later (*see p. 607*).

There are two main methods of performing the ESR, namely Wintrobe's and Westergren's method.

Wintrobe's method

APPARATUS AND REAGENT

1. Wintrobe haematocrit tube (*Figure 32.1*).

This is a tube 11 cm in length with an internal diameter of 2.5 mm. It is graduated from the bottom in millimetre divisions over a 10 cm scale. When filled the tube contains 0.7 ml of blood.

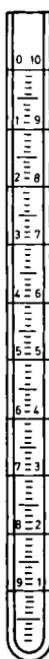


Figure 32.1. A Wintrobe haematocrit tube for sedimentation rate and packed cell volume determinations

2. Venous blood taken into sequestrene (potassium EDTA) (see p. 72) or alternatively blood taken into Wintrobe's mixture (p. 72) may be used.

TECHNIQUE

With a pasteur pipette, fill the haematocrit tube to the 10 mark with well-mixed blood. Fix the tube in the stand, ensuring that the tube is vertical and not in direct sunlight. Read the level of red cells after 1 h. (The calibrations used are those from 0 (at the top) to 10 (at the bottom) of the tube.)

Normal values are: men, 0–9 mm; and women, 0–20 mm.

Westergren method

APPARATUS AND REAGENTS

1. Westergren ESR tube. This tube looks rather like a 1 ml pipette. It is 300 mm long with an internal diameter of 2.5 mm. It is graduated from the bottom over a 200 mm scale in millimetre divisions.
2. Venous blood taken into sodium citrate. One part of 3.8 per cent sodium citrate to 4 parts of blood. Blood taken into

sequestrene may be used but must be similarly diluted with 3.8 per cent sodium citrate.

TECHNIQUE

The blood is well mixed and sucked into the tube to the top mark (0 mark). The tube is then stood vertically for 1 h. The level of the red cells is then read as the ESR. Normal values are: men, 3–5 mm; and women, 4–7 mm.

Precautions to be taken when performing ESR tests

1. As blood is taken by venepuncture prolonged venous congestion must be avoided.
2. The test must be set up within 3 h of blood collection or sedimentation will be retarded.

Some workers have shown that blood taken into sequestrene can be stored for up to 24 h at 4 °C and then diluted with 3.8 per cent citrate and a Westergren ESR performed.

3. Haemolysed blood must *not* be used.
4. Blood containing the slightest trace of a clot must be discarded.
5. Tests must *not* be performed in direct sunlight.
6. Tests should be performed between 18 °C and 22 °C as higher temperatures accelerate the sedimentation rate.
7. All the apparatus used must be scrupulously clean.
8. There must be no air-bubbles in the sedimentation tube.
9. The tube must be placed in an absolutely vertical position.

PACKED CELL VOLUME (PCV)

The estimation of the packed cell volume is often a valuable guide in diagnosing certain blood disorders. Wintrobe's tubes are used in this estimation and anticoagulated blood is spun at high speed. The height of the column of red cells is taken as the packed cell volume. The normal ranges are: men, 40–54 per cent; women, 36–47 per cent; cord blood, 44–62 per cent.

METHOD

1. Add venous blood to a bottle containing heparin 0.1 mg per ml of blood, Wintrobe's anticoagulant, or sequestrene (1–2 mg per ml of blood).
2. With a capillary pipette fill the haematocrit tube to 100 mm.
3. Centrifuge at 3000 rpm for 30 min.
4. Read height of red cells and express result as a percentage.

EXAMPLE

If the height of the column of red cells was 51 mm, then in the 100 mm column of venous blood 51 mm consisted of red cells. Therefore percentage = 51, that is, packed cell volume = 51 per cent.

The appearance of the centrifuged haematocrit tube should always be examined for any abnormalities. Above the packed red cells will be a white layer of leucocytes and immediately above this a creamy layer of platelets (these layers are called the buffy coat); any increase in these cells will be easily detectable after a little experience. The plasma which is usually straw-coloured will be bright yellow if the patient is jaundiced, colourless in iron-deficiency anaemia and red if haemolysis is present.

The packed cell volume is now often estimated by the micro-haematocrit method, instead of the Wintrobe technique. Using this method, the blood is spun at 10 000 rpm for 5 min in a special centrifuge which automatically attains the correct speed. This results in the elimination of all trapped plasma. It has been proved that blood samples spun by the conventional method and then corrected for trapped plasma, give the same results as by the micro-haematocrit method, which give results 1–2 per cent lower, due to complete packing of the red cells. The method has the advantages of speed and simplicity and is suitable for use with capillary blood when a venous sample is not available.

METHOD

1. Fill the special capillary tube either with well-mixed venous blood or directly from a freely flowing capillary puncture (in this case, use a capillary containing heparin).
2. Seal the end of the capillary tube in a bunsen flame or preferably use the special plasticized sealer which requires no heat and always gives a flat bottom.
3. Place the capillary in the micro-haematocrit centrifuge (*see Figure 32.2*), and screw on the safety cover.
4. Close the lid and spin at 10 000 rpm for 5 min (2 min is normally sufficient, but 5 min will also ensure that polycythaemic blood is fully packed).
5. Place the spun tube into the specially designed scale, and read off the PCV as a percentage.

When the micro-haematocrit method is used, owing to the elimination of trapped plasma, the apparent range of the mean cell haemoglobin concentration (MCHC) (p. 619) is extended.



Figure 32.2. Micro-haematocrit centrifuge (Reproduced by courtesy of Hawksley Ltd)

Normal range of the MCHC is 32–36 per cent. Using the micro-haematocrit technique this range may be 33–38 per cent.

RED CELL FRAGILITY TEST

Red blood cells, suspended in an isotonic solution of saline (0.85 per cent) remain intact. As the salt concentration is decreased (making a hypotonic solution), the cells disrupt, causing haemolysis. The red cell fragility test is performed to find the salt concentration at which lysis takes place.

Normal blood shows slight haemolysis at 0.45–0.39 per cent, complete haemolysis occurring at 0.33–0.30 per cent (Wintrobe). In some diseases the fragility of the cells is increased (hereditary spherocytosis) and in others the fragility is decreased (hypochromic anaemia).

As the differences in salt concentrations are extremely small, great care and accuracy must be exercised when performing this test.

APPARATUS AND REAGENTS

1. *Stock sodium chloride solution (pH 7.4).* Osmotically equivalent to 10 per cent sodium chloride. Sodium chloride 18 g, disodium hydrogen phosphate (Na_2HPO_4) 2.731 g, sodium

Table 32.1 SALINE SOLUTIONS FOR RED CELL FRAGILITY TEST

<i>Tubes</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>
ml of buffered saline	85	70	65	60	55	50	45	40	35	30	20	10
ml of distilled water	15	30	35	40	45	50	55	60	65	70	80	90
Final saline percentage (osmotic equivalent)	0.85	0.70	0.65	0.60	0.55	0.50	0.45	0.40	0.35	0.30	0.20	0.10

dihydrogen phosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) 0.486 g. Dissolve the salts in 200 ml of glass distilled water and store in a well-stoppered bottle.

2. *Test concentrations.* Dilute the stock sodium chloride solution 1 in 10 with distilled water to produce a solution with an osmotic equivalent of 1 per cent sodium chloride. From this solution prepare 100 ml amounts of the following dilutions: 0.85, 0.70, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20, 0.10 per cent. These solutions can be conveniently prepared by adding the stock saline solution from a burette into a 100 ml volumetric flask. The amounts are listed in Table 32.1. The volume is then made up to the 100 ml mark with glass-distilled water.
3. Venous blood (heparinized or defibrinated) from patient.
4. Normal venous blood (heparinized or defibrinated).
5. 0.05 ml disposable micropipettes.
6. Small test-tubes and racks.

TECHNIQUE

1. Set up two racks of 13 test-tubes and label 1-13.
2. Into tubes Nos. 1-12 measure 5 ml of each of the test concentration into tube 13, add 5 ml of distilled water.
3. Add 0.05 ml of well-mixed aerated blood to each tube, using the blood under test for one rack and the control specimen for the other. This gives a dilution of 1 in 100.
4. Invert to mix and allow to stand at room temperature for at least 30 min. Centrifuge the tubes gently and read the

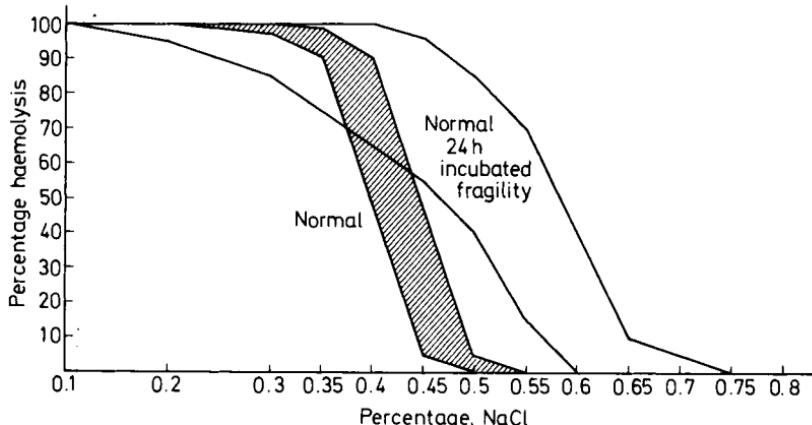


Figure 32.3. Osmotic fragility curve

- supernatant fluids in a colorimeter using a tube of buffered saline as a blank and a yellow-green filter (Ilford No. 625).
5. Lysis is recorded as a percentage (using Tube 13 as a 100 per cent lysis) and plotted against per cent sodium chloride to obtain a 'fragility curve' (*Figure 32.3*). The curve obtained with the patient's blood is compared against that of the normal control. It is also useful to record the concentration of saline causing 50 per cent lysis which is referred to as the Median Corpuscular Fragility (MCF).

Note—The use of oxalated or citrated blood is not recommended for this test owing to the additional salts added to the blood.

Before adding the blood to the tubes (step 3, above) it must be well aerated. This may be achieved by blowing air, using a pasteur pipette fitted to the positive pressure side of a vacuum pump, through the blood.

A direct visual examination of the tubes can be made and the findings reported as commencement of haemolysis and completion of haemolysis for both test and normal blood.

Table 32.2 NORMAL RANGE OF OSMOTIC FRAGILITY

% NaCl	Room temperature % lysis	After 24 h at 37 °C % lysis
0.10	100	100
0.20	100	95–100
0.30	97–100	85–100
0.35	90–99	75–100
0.40	50–95	65–100
0.45	5–45	55–95
0.50	0–6	40–85
0.55	0	15–70
0.60	0	0–40
0.65	0	0–10
0.70	0	0–5
0.85	0	0

Median corpuscular fragility 0.40–0.45 per cent NaCl; 0.47–0.60 per cent NaCl after 24 h at 37 °C.

Incubated osmotic fragility

Red cells from patients with hereditary spherocytosis have a greater increase in osmotic fragility, when their blood is incubated at 37 °C for 24 h. An incubated fragility is performed exactly as the technique described but the patient's blood and the control blood are incubated at 37 °C for 24 h prior to performing the test.

33

Tests for Blood Coagulation

In order to study patients thought to be suffering from blood clotting defects, various tests may be carried out. The simplest of these are the platelet count, bleeding time, whole blood clotting time and Quick's one-stage prothrombin time. It must be emphasized that normal results in these four tests do not exclude a haemorrhagic disorder. Further tests are necessary before the patient can be said to have a normal clotting mechanism.

Platelet count

Capillary blood can be used for this test. It is diluted 1 in 20 in a white cell diluting pipette, but in order that platelets are not destroyed by direct contact with the glass of the pipette, platelet diluting fluid is first taken up to the 0.5 mark of the pipette. Blood is then taken up to the 0.5 mark (that is the top of the column of diluting fluid is on the 1.0 mark). The dilution is then completed by diluting up to the 11 mark. Venous blood which has been anticoagulated with sequestrene is preferable for this test, in which case this precaution is unnecessary and the blood may be taken up to the 0.5 mark and then diluted in the usual way up to the 11 mark. An alternative method is to take 0.1 ml of blood into 1.9 ml of diluent.

The diluted blood is well mixed and a Neubauer counting chamber filled with the diluted blood, after blowing out at least a third of the contents of the bulb.

The chamber is then stood in a petri dish, containing a piece of moist filter paper, for 20 min to allow the cells to settle. The moist filter paper prevents evaporation during the long period of standing.

The cells are counted as for red cells (that is in 80 small squares). Some workers prefer to use phase contrast microscopy for counting platelets.

CALCULATION

This is the same as for red cell counting, except that as the dilution is only 1 in 20, the number of platelets per mm³ is a thousand

times the number counted in 80 small squares. Normal range—between 150 000 and 400 000 per mm³ with an average normal of 250 000 per mm³.

DILUTING FLUIDS

Baar's fluid. This fluid incorporates formalin as a platelet fixative so that they are not disintegrated by the Saponin used to haemolyse the erythrocytes.

Saponin BDH	0.25 g
Sodium citrate	3.5 g
Formalin	1.0 ml
Brilliant cresyl blue	0.1 g
Distilled water	to 100 ml

Filter before use. If brilliant cresyl blue is omitted filtration is not necessary and the platelets can easily be seen as small refractile unstained bodies.

Kristenson's fluid (Lempert's modification)

<i>Solution A.</i> Sodium citrate	1 g
Mercuric chloride	0.002 g
Brilliant cresyl blue	0.2 g
Distilled water	100 ml
(added at a temperature of 45 °C)	

Note—Store in the dark.

<i>Solution B.</i> Urea (Analar)	20 g
Buffered distilled water	
(pH 7.2)	100 ml

Note—It is vital that Analar grade urea be used or clumping of the platelets may occur.

Mix equal volumes of solutions *A* and *B* immediately before use.

Ammonium oxalate. One per cent ammonium oxalate in distilled water. Filter before use. 500 ml should be the maximum prepared on each occasion. Store at 4 °C.

Bleeding time

DUKE'S METHOD

- With the patient in a sitting position place a towel on the shoulder to prevent blood accidentally dropping onto the patient's clothing.

2. Sterilize the patient's ear lobe with alcohol and puncture to a depth of about 2 mm with a needle that has three cutting edges (Hagerdorn needle). Start a stop watch at the same time as the ear is punctured.
3. Touch the formed drops of blood with the edge of a filter paper every 30 s. Avoid touching the skin or the result may be affected.
4. Take the time when bleeding stops. If the bleeding time exceeds 15 min apply pressure to the puncture to stop the bleeding and report as 15 min plus.

Normal range 2–5 min.

IVY'S METHOD

1. Place a sphygmomanometer cuff around the patient's upper arm and raise the pressure in the cuff to 40 mmHg.
2. After sterilizing the skin make five punctures in the pronator surface of the forearm (the front of the forearm), avoiding any blood vessels or scar tissue. The punctures should preferably be made with a sterile Frank's automatic lancet to a depth of 3 mm.
3. Remove drops of blood (as in Duke's method) from the first three punctures that bleed and take the average of the times when bleeding stops.

The normal range for this technique is $1\frac{1}{2}$ –4 min.

Clotting time

LEE AND WHITE

1. Take four test-tubes with an internal diameter of 8 mm and place them in a 37 °C water bath.
2. Take venous blood with a clean dry syringe, taking care that no tissue juices enter the syringe. Start a stop watch immediately blood enters the syringe.
3. Deliver 1 ml amounts of the blood into each of the four warmed test-tubes. Examine them at 30 s intervals and observe for clotting by gently tilting the tube.
4. The clotting time is reported as the average of the times given by the four tubes.

Normal range 5–11 min (usually 6–9 min at 37 °C).

Quick's one-stage prothrombin time

This technique is so-called because it was designed to measure prothrombin on the basis of Morawitz's *Classical Theory of Coagulation*.

The test employs a tissue extract which is added to plasma in the presence of excess calcium. The time taken for the mixture to clot is recorded.

It is apparent from the extrinsic clotting system that deficiency of factor V, factor VII and factor X will give an abnormal result, as well as a deficiency of prothrombin and hypofibrinogenaemia.

REAGENTS

(a) *Dried brain extract (brain thromboplastin)*. This can be purchased commercially or prepared as follows:

1. Use a fresh human brain removed at post-mortem. It should be removed as soon as possible after death and certainly within 24 h.
2. Remove the superficial blood vessels and membrane. Cut the brain into small pieces with scissors and place in a large mortar.
3. Add a volume of acetone equal in volume to the brain and macerate the brain in the acetone.
4. Allow to settle and pour off the acetone. Repeat the process at least four times.
5. The material can be dried on a suction filter, or it can be placed between sheets of blotting paper and placed in the incubator, but it must not be left at 37°C for more than 15 min.
6. Material can then be stored in a vacuum desiccator over calcium chloride or phosphorus pentoxide at 4°C. Alternatively, it can be placed into ampoules in convenient amounts and sealed in an atmosphere of dried nitrogen.
7. For use, take 0.3 g of the powder and add 5 ml of phenol saline (0.5 per cent phenol in physiological saline). Place in the water bath at 37°C for 30 min, inverting periodically to mix.
8. Centrifuge lightly. Remove the cloudy suspension for use, discarding the large coarse particles in the bottom of the tube.

(b) *M/40 calcium chloride*. Dissolve 2.775 g of anhydrous calcium chloride in some distilled water and make up to 1000 ml.

(c) *Control and test plasma.* The blood is taken by venepuncture and 9 parts of blood are added to 1 part of 3.13 per cent sodium citrate solution.

Centrifuge as soon as possible after the blood has been taken and remove the plasma.

TEST

1. Using a 0.1 ml pipette with a rubber teat, place 0.1 ml of test plasma into each of three small test-tubes and place in a 37 °C water bath.
2. Add 0.1 ml of the brain suspension to each tube and allow the contents to reach 37 °C.
3. Add 0.1 ml of calcium chloride solution to the first tube, simultaneously starting a stop watch.
4. Mix and leave the tube in the bath for 9–10 s, then remove and examine for clot formation, stopping the watch at the first sign of a clot.
5. Note time taken to clot and repeat procedure with other two tubes.
6. Repeat the whole procedure using the Control normal plasma. In practice, it is advisable to perform this test first so that any possible errors in the test procedure will be recognized immediately. This normal plasma should clot between 11 and 14 s after the addition of the calcium chloride.

RECORDING OF RESULTS

1. These can be reported as the time taken in seconds for the test to clot, quoting also the clotting time of the control plasma, or
2. Can be expressed as a *prothrombin index*. This is calculated as follows:

$$\text{Prothrombin index} = \frac{\text{normal plasma clotting time} \times 100 \text{ per cent}}{\text{test plasma clotting time}}$$

Note—The percentage arrived at by this method has no relation to the percentage read off a dilution curve (*see* below), or

3. Can be expressed as a ratio:

$$\text{Prothrombin ratio} = \frac{\text{test plasma clotting time}}{\text{normal plasma clotting time}}$$

Note—This method is probably to be preferred since it is not ambiguous.

4. A dilution curve can be prepared from normal plasma. The normal plasma is diluted to give 10, 20, 30 per cent, etc., to 90 and 100 per cent dilution in normal saline, and the prothrombin time of each dilution is obtained. The clotting times are plotted on graph paper against the concentrations of prothrombin, assuming the normal plasma to contain 100 per cent prothrombin activity. The prothrombin times of the tests can then be read off this graph as per cent prothrombin activity.

Note—Unfortunately, dilution of the plasma with normal saline also dilutes other clotting factors. Some workers, therefore, prefer to use prothrombin-free normal plasma as a diluent instead of saline. This can be obtained by treating the plasma with aluminium hydroxide. The shape of the graph obtained will vary from one laboratory to another and also with the strength of the brain suspension used. It is, therefore, necessary to prepare a new dilution curve for every batch of thromboplastin used.

34

Haematological Indices

ABSOLUTE VALUES

These values are calculated from the red cell count, haemoglobin content and packed cell volume. The information obtained provides a valuable guide to the classification of anaemia and has made obsolete the 'colour index' which compared arbitrary normal figures with those actually calculated. The figures calculated are the average of the red cells present and may at times be within the normal range, but on examination of the stained blood film the red cells may show marked morphological changes.

As the results are dependent upon the accuracy of the various estimations, it must be remembered that the red cell count, which has the greatest potential error, must be performed with extreme care, preferably using an electronic counter.

Mean cell haemoglobin concentration (MCHC)

This refers to the percentage of haemoglobin in 100 ml of red blood cells, as opposed to the percentage of haemoglobin in 100 ml of whole blood, giving the concentration of haemoglobin in the cells.

The normal MCHC ranges from 32 to 36 per cent and is rarely diminished below 22 per cent.

CALCULATION

Divide the haemoglobin content in g per 100 ml by the PCV per 100 ml of blood, expressing the result as a percentage.

EXAMPLE

$$\begin{aligned}\text{Hb content} &= 15 \text{ g}/100 \text{ ml blood} \\ \text{PCV} &= 48 \text{ per cent}\end{aligned}$$

Therefore 100 ml of blood contains 15 g haemoglobin.

Therefore 48 ml of packed cells contains 15 g haemoglobin.

$$\text{Therefore } \text{MCHC} = \frac{15}{48} \times 100 \text{ per cent} = 31.25 \text{ per cent}$$

Mean cell volume (MCV)

This is the average volume of a single red cell expressed in cubic micrometres (μm^3).

The normal MCV ranges from 76 to 96 μm^3 , and is seldom lower than 50 μm^3 or higher than 150 μm^3 .

CALCULATION

Divide the packed cell volume (expressed as μm^3 per mm^3) by the red cell count per mm^3 .

EXAMPLE

$$\begin{aligned}\text{PCV} &= 40 \text{ per cent} \\ \text{RBC count} &= 5000000 \text{ per } \text{mm}^3\end{aligned}$$

There are $10^3 \mu\text{m}$ in 1 mm and $10^9 \mu\text{m}$ in 1 mm^3 .
As the PCV = 40 per cent, in 100 mm^3 of blood there are 40 mm^3 of packed cells.

$$\therefore \text{the volume of packed cells in } 1 \text{ mm}^3 = \frac{40}{100} \text{ mm}^3$$

There are 5 000 000 RBC in 1 mm^3 .

$$\therefore \text{volume of 1 cell} = \frac{40}{100} \div 5000000 \text{ mm}^3$$

$$\frac{40 \times 10^9}{100 \times 5000000} \mu\text{m}^3 = 80 \mu\text{m}^3$$

Therefore the MCV = 80 μm^3 .

In practice multiply the PCV by 10 and divide by the number of millions of red cells per mm^3 .

$$\text{That is } \frac{40 \times 10}{5} = 80 \mu\text{m}^3$$

Mean cell haemoglobin (MCH)

This expresses the average haemoglobin content (in picograms) of a single red blood cell. The normal MCH ranges from 27 to 32 pg and is seldom lower than 15 pg or higher than 50 pg.

CALCULATION

Express the haemoglobin content (in pg) per 1 mm^3 of blood and divide by the red cell count per 1 mm^3 .

EXAMPLE

$$\begin{aligned} \text{Hb} &= 14.5 \text{ g/100 ml of blood} \\ \text{RBC} &= 5000000 \text{ per mm}^3 \end{aligned}$$

There are 10^3 mm 3 in 1 ml and 10^{12} pg in 1 g. As the Hb content in 100 ml = 14.5 g

$$\therefore \text{the Hb content in 1 ml} = \frac{14.5}{100} \text{ g}$$

$$\text{and the Hb content in } 1 \text{ mm}^3 = \frac{14.5}{100 \cdot 1000} \text{ g}$$

$$\text{That is } \frac{14.5}{10^5} \text{ g or } \frac{14.5 \cdot 10^{12}}{10^5} \text{ pg}$$

There are $5000000 (5 \cdot 10^6)$ RBC per mm 3

$$\therefore \text{the Hb content of 1 cell} = \frac{14.5 \cdot 10^{12}}{10^5 \cdot 5 \cdot 10^6} \text{ pg}$$

That is 29 pg of Hb per single cell.

Therefore the MCH = 29 pg.

In practice, a convenient way of determining the MCH is to multiply the Hb content in g per 100 ml by 10 and divide by the number of millions of red cells per mm 3 .

$$\text{That is } \frac{14.5 \cdot 10}{5} = 29 \text{ pg}$$

Mean corpuscular diameter (MCD)

This can be measured by either direct measurement or by halometric techniques.

DIRECT MEASUREMENT

1. The red cells can be measured using a stage micrometer and micrometer eyepiece.
2. A less laborious process is to project the image of the red cells onto a screen using a known magnification. The diameter of the cells can then be measured using a suitable measuring device. The Price-Jones method measures the size of 500 cells this way and groups them in classes according to their size. The number of cells in each class are then plotted on a graph against the cell diameter and compared with a normal graph. Using statistical methods the MCD can then

be calculated. For further information on this subject reference should be made to the standard works.

Halometric methods

When a parallel beam of light is passed through a blood film, the film will act as a defraction grating, defracting the light at the edge of the red cells. The degree of defraction depends upon the distance between the defracting edges, that is the MCD. When a source of light is viewed through an evenly spread blood film a halo can be seen surrounding the light source. The size of this halo varies inversely with the diameter of the red cell.

EVE'S HALOMETER

In this portable apparatus a single light source is divided into two by use of a mirror. The two light beams are then viewed through the blood film, which results in the observer seeing two red rings. The distance between the film and the light source is adjusted until the edges of the red rings just touch. The instrument is then calibrated in relation to the angle formed between the film and the two light beams.

WATERFIELD HALOMETER

In this apparatus a monochromatic light source is viewed through a film of blood resulting in the formation of a halo. By means of a perforated screen a number of bright pinholes can be made to appear on the halo. By adjustment of the screen it is possible to make the pinholes appear exactly on the brightest central area of the halo. The instrument is so calibrated that the position of the perforated screen can be read directly on the scale as the MCD.

Mean corpuscular average thickness (MCAT)

The calculation of the MCAT assumes that the cell is in the form of a cylinder. As the cell is really a biconcave disc only an average thickness can be calculated. To obtain this average thickness the values of the Mean Cell Diameter (MCD) and the Mean Corpuscular Volume (MCV) are required.

The volume of a cylinder is obtained from the formula $\pi r^2 h$

where r the radius of the cylinder

h the height of the cylinder.

In the case of a red cell,

r half the mean cell diameter.

h mean corpuscular average thickness.

Therefore the mean volume of a red cell, using the formula $\pi r^2 h$ will be

$$\text{MCV} = \pi \left(\frac{\text{MCD}}{2} \right)^2 \times \text{MCAT}$$

From the above equation,

$$\text{MCAT} = \frac{\text{MCV}}{\pi \left(\frac{\text{MCD}}{2} \right)^2}$$

35

Introduction

Blood transfusions are now commonplace throughout the hospitals of the world and although apparently undertaken as routine procedure it should always be remembered that blood is a tissue, albeit in a fluid state, and every unit of blood given is in effect a tissue transplantation. The administration of blood to a patient is potentially a life-saving procedure, but if the grouping and crossmatching of both the recipient's and donor's blood is not undertaken with extreme care the reverse may be equally true.

During medieval days blood, usually of animal origin, was administered by drinking it. This produced little effect, with the possible exceptions of patients suffering from iron deficiency anaemia. It can certainly be said that no harm would come to the unfortunate recipients of such therapy. It was not until Christopher Wren, the architect of St Paul's Cathedral, developed a sort of intravenous needle from a quill that experiments of transfusions using dogs began. Many mishaps occurred when animal blood was given to patients and for about one hundred and fifty years no more blood transfusions were given. An obstetrician, James Blundell, was the first recorded doctor to transfuse blood successfully to a number of women after severe blood loss during childbirth. In subsequent years the transfusion of blood was considered technically far too difficult to be used with any frequency, and was only used in extreme cases, often with no success. It must be remembered that the prevention of coagulation had still not been solved and the infusion of even small quantities of blood would have been most frustrating if great haste had not been achieved. The breakthrough came in the year 1900, when Landsteiner demonstrated the ABO system of blood grouping.

The ABO groups

Landsteiner observed that the red cells of some individuals were agglutinated by the serum of other individuals. He demonstrated that these people could be classified into four groups according to which of two antigens were detectable, A, B, AB and if no agglutination occurred group O. He also showed that an indivi-

dual's serum does not contain the antibody for an antigen present in his own red cells. This can be summarized as follows.

<i>Antigen in red cells</i>	<i>Antibody in serum</i>
A	anti-B (β agglutinins)
B	anti-A (α agglutinins)
AB	none
O	anti-A + anti-B ($\alpha + \beta$ agglutinins)

It therefore follows that the serum of a group A individual will agglutinate the cells of a group B person, and the serum of a group B individual agglutinate the cells of a group A person and so on. This characteristic is of great value in the determination of an individual's ABO group.

It is of interest to note that in 1907 Jansky, a Czech, published a paper in a Bohemian journal where he names four blood groups using Roman numerals, and in 1910 Moss, an American, independently named the four groups, also using Roman numerals but in a different sequence. Occasionally individuals from outside the United Kingdom present blood group cards showing both the International nomenclature and a Roman numeral identification.

<i>International</i>	<i>Jansky</i>	<i>Moss</i>
AB	IV	I
A	II	II
B	III	III
O	I	IV

The sub-groups of A

In 1911 von Dungern and Hirschfeld described the sub-groups A₁, A₂, A₁B and A₂B. Nearly all anti-A sera (from group B persons) contain two anti-A antibodies, namely anti-A and anti-A₁ (α and α_1). Anti-A agglutinates the cells of groups A₁, A₂, A₁B and A₂B. Anti-A₁ agglutinates the cells of groups A₁ and A₁B only. It must be emphasized that a person whose blood group is simply written as A is synonymous with group A, and does not in any way indicate a further sub-group.

The A₂ antigen reacts more weakly than the A₁ antigen to anti-A serum because there are less antigen sites for the anti-A to attach itself to. The use of group O serum (anti-A - anti-B) often agglutinates group A₂ cells more strongly than anti-A alone; the reason for this is unknown.

The antibody anti- A_1 occurs 'naturally' in about 2 per cent of A_2 individuals and in about 25 per cent of A_2B individuals. This may seem confusing but it must be clearly understood that the A_2 antigen is not attacked in any way by the anti- A_1 , therefore no harm is done to the red cells in these persons who possess an anti- A_1 . The same 'naturally occurring' antibodies to the ABO system are still present in A_2 and A_2B individuals. The whole system can be summarized as follows:

<i>Antigen in red cells</i>	<i>Antibodies in serum</i>
A_1	anti-B
A_2	anti-B + anti- A_1 (2 per cent of subjects)
B	anti-A
A_1B	none
A_2B	anti- A_1 (25 per cent of subjects)
O	anti-A + anti-B

No specific antibody to the antigen A_2 has been described but the use of anti-H from serum or the use of lectins specific for the H antigen may be used (see p. 634). The H antigen is present in a much higher amount in A_2 cells than in A_1 cells.

ABO antigens

An antigen is any substance which when given parenterally to an animal which lacks that substance will give rise to an antibody to that substance. This antibody will react in some observable way, and in the subject of blood grouping is usually agglutination of red cells or haemolysis of red cells.

All individuals have antigens within the ABO blood group system (rare exceptions exist, see Bombay blood, p. 631). The specificity of these antigens is determined by a precursor substance which is acted upon by the H gene and converted to H substance. This is then acted upon by the various A, B or O genes and the terminal end sugar, which in H substance is fucose, adds further sugars depending upon the specific gene present. The A gene adds N-acetylgalactosamine and the B gene D-galactose. If the O gene is present the H chain is unaltered and the terminal end remains fucose. Conversion to A or B chains is never complete and all red cells, except the rare Bombay blood, have the H antigen with the groups O and A_2 having the largest amount.

The A and B antigens can be detected at an early stage in the fetus but are still not fully developed at birth. There is no real

problem in grouping cord blood cells with potent antiserum but sub-grouping may cause problems, as many group A infants appear A₂ at birth.

There are many antigen sites on a single red cell which express the presence or absence of the various blood groups and each of these has to be represented many times over. It has been calculated that the A antigen alone in a single adult red cell is represented approximately 1 000 000 times and the B antigen approximately 700 000 times. In the group AB adult the number of sites for each is reduced, there being approximately 500 000 A sites. The ABO antigens are also present in the white cells, platelets and tissue cells.

Group specific substances

Blood group factors A and B are not only present in red cells but are also widely distributed in tissue cells and body fluids in hapten form (carbohydrate) and are non-antigenic, hence the name given them. One of the richest and most readily available sources is saliva and therefore is used extensively.

Individuals whose saliva contains the appropriate ABO substances are called secretors, those whose saliva does not contain the substance are non-secretors. Absence of the substance in saliva indicates absence throughout all tissue and body fluids. Secretion is controlled by a pair of allelomorphic genes, Se and se giving rise to three genotypes:

$$\begin{array}{ll} \text{Se} & \text{Se} \\ \text{Se} & \text{se} \\ \text{se} & \text{se} \end{array} \left. \begin{array}{l} \text{Se} \\ \text{se} \\ \text{se} \end{array} \right\} 80 \text{ per cent}$$
$$\left. \begin{array}{l} \text{se} \\ \text{se} \end{array} \right\} 20 \text{ per cent}$$

There are two distinct forms of substance:

1. A water-soluble form present in body fluids and tissues.
2. Alcohol-soluble form present in red cells and some other tissues but absent from body fluids.

The presence of the water-soluble form is controlled by the secretor gene, the alcohol-soluble form is not. Thus red cells and tissue of all persons contains an alcohol-soluble form whether secretor or not, but the secretor in addition possesses the water-soluble form in the body fluids. Only the substances present in the red cells can also be present in the tissues and body fluids. Group O substance is not secreted and secretors' saliva contains H substance.

Gp A secretor	—A and H
Gp B	—B and H
Gp AB	—A, B and H
Gp O	—H

To detect substances in solution advantage is taken of the fact that soluble substance is capable of specifically neutralizing its corresponding agglutinin. The neutralization is reflected in the complete or partial inhibition of the agglutinin titre.

Bombay blood

These individuals apparently do not possess the H gene and are therefore unable to convert precursor substance to H substance; the red cells group as O. The serum of these individuals reacts with A, A₁, B and O cells. The cells can be demonstrated as being H negative by testing with lectin (*see* p. 634). Although rare in the United Kingdom, probably less than one per 1 000 000, it is thought to have a much higher incidence in the areas around Bombay.

The sub-groups of A

The A antigen can express itself with varying grades of antigenicity and, as previously mentioned, be subdivided into A₁ and A₂. More rarely, weaker forms of the A antigen can be demonstrated and have been differentiated into A₃, A₄, A_o and others. The differentiation of these weaker sub-groups is technically difficult and they are therefore often referred to collectively as A_x.

Incidence of the ABO groups

The incidence of the ABO groups varies strikingly in different parts of the world and certain races have a predominance of different groups to others, for example Negroes have a higher percentage of group B within their ethnic group. The approximate incidence of the ABO groups in Britain is given below, but even in this small part of the world there is a difference between the north and south of the country.

Group O	= 47 per cent
Group A	= 42 per cent
Group B	= 8 per cent
Group AB	= 3 per cent

ABO antibodies

These antibodies are frequently referred to as 'naturally occurring' but it seems unlikely that they should appear without any stimulation from an antigen. It is suggested that these antibodies, which are not present at birth, are stimulated by the inhalation or ingestion of bacteria, seeds and foodstuffs which have similar chemical structures to the ABO antigens. To support this theory experiments on animals, kept from birth in a sterile environment, do not produce any antibody. The production of ABO antibodies in infants does not begin until about four months of age, although antibody may be detected in the cord blood which has been transferred via the placenta from the mother. These 'natural' antibodies are also referred to as iso-* antibodies or iso-haemagglutinins, and as they react maximally at 4 °C are also called 'cold' antibodies. They have the following properties:

1. React maximally at 4 °C, but the thermal range of activity includes 37 °C.
2. Agglutinate cells suspended in saline.
3. Are absorbable.
4. The agglutinated cells adhere very strongly and agglutinates are difficult to break up.

ABO antibodies, like other antibodies, are found in the globulin fraction of plasma (for structure of immunoglobulins *see* p. 642). They can be demonstrated in other body fluids which contain plasma globulins, such as lymph, exudates and milk. They are not usually found in tears, saliva, urine, CSF or amniotic fluid.

Iso-haemolysins anti-A and anti-B

When fresh serum containing iso-antibody is combined with red cells containing the specific antigen the expected agglutination sometimes does not occur but the cells are disrupted, releasing their haemoglobin into the surrounding fluid. This phenomenon is called iso-haemolysis, and occurs in about 30 per cent of all fresh sera. This type of haemolysis requires the presence of complement (*see below*) and does not occur when this has been inactivated by ageing or by heating at 56 °C for 1 h.

Complement

Complement is a complex group of serum globulins mainly of β electrophoretic mobility which is present in fresh normal serum.

*iso—from the Greek word 'isos' meaning equal, alike or the same, and in this context—from the same species.

It can, in certain circumstances, be absorbed onto the surface of bacteria or red cells and bring about their lysis or accelerated removal from the circulation. Nine components of complement are now recognized and are called C1, C2, C3, etc. The components react in an ordered sequence (but not numerically) and their action may be likened to the 'cascade' theory of blood coagulation where the activation of one component leads to the activation of others and so on. A simplified sequence of the activation of complement may be schematically represented as follows:

E = erythrocyte A = antibody

$EA + C1 + C4 \rightarrow EAC14$
(in the presence of Ca^{++})

$EAC14 + C2 \rightarrow EAC142$
(in the presence of Mg^{++})

$EAC142 + C3 \rightarrow E \rightarrow$ ghost cell + free Hb

The binding of complement to a sensitized cell can be prevented by the addition of EDTA (sequestrene) which prevents the step $EA + C1 \rightarrow EAC1$, which requires calcium ions. Heating serum at 56 °C for 30 min completely inactivates C1 and C2. Complement is sensitive to changes in pH, the optimum being 6.8, and also to temperature; it shows optimum activity between the range of 30–37 °C. Haemolysis will not occur below a temperature of 15 °C.

ABO grouping serum

This is usually obtained from selected donors whose antibody content is suitable as a laboratory reagent. It is frequently stated that standard anti-A serum must have a titre of 1 in 512 and anti-B a titre of 1 in 256 when tested against A and B cells respectively. It is in fact unlikely that many standard antisera will react at all at these dilutions and it is of far greater importance that the weakest antigens can be detected. In the case of anti-A, provided it reacts with A_2B cells strongly at the same dilution and using the same technique as will be used routinely it must detect A_1 , A_2 and A_1B cells. The same is true of anti-B and its ability to react with A_2B cells. A suitable serum will therefore have good avidity (strength of reaction), specificity, and the ability to detect the weakest antigens. A serum showing a very high titre and low avidity is of no value as a standard grouping reagent.

The selection of O serum (anti-A + anti-B) should be performed in the same manner, using the weakest A antigen which will be Ax cells. Provided they are agglutinated satisfactorily and B cells

are strongly agglutinated the serum will react with A₁, A₂, A₁B and A₂B cells.

No other antibodies must be present other than those specifically required and thorough testing of the serum against as many red cell antigens as possible is necessary. The serum must also not cause cells to form rouleaux and be free from fat which gives the reagent a doubtful appearance suggestive of bacterial contamination and may accelerate loss of strength.

Lectins

Extracts from many plants and particularly seeds contain substances which will agglutinate red cells, in most cases regardless of the specific antigens present. These substances are not antibodies but happen to produce a similar effect. The extracts from the seeds of two plants are of immense value in blood grouping:

1. *Dolichos biflorus* (Indian cattle bean, commonly known as horse grain) can be diluted so as to react specifically with the A₁ antigen and therefore differentiate A₁ and A₁B cells from A₂ and A₂B cells.
2. *Ulex europaeus* (common gorse) has an anti-H specificity and agglutinates A₂, A₂B and O cells far more strongly than A₁B or B cells.

The extracts from these seeds may be produced in the laboratory but usually they are purchased commercially.

36

Inheritance of the ABO Blood Group

The presence of antigens in the red cells is determined by genes. The genes are carried on chromosomes which are present in the nucleus of all cells of the body. There are 46 chromosomes arranged in 23 pairs in each nucleus with the exception of the sex cells, which contain only 23 chromosomes, so that only one chromosome from each pair is present in the ovum and sperm. The fusion of the ovum and sperm brings the total number back again to 23 pairs. The two genes (one from each parent) which control the ABO group can be the same or different. If the two genes are the same the person is called *homozygous* for that character, and if different *heterozygous*. The genes which can occupy the same site or *locus* on a chromosome are called *allelomorphic* or *alleles*.

Ignoring the question of sub-groups, the inheritance of the ABO groups depends upon three genes A, B and O, which can be broadly described as co-dominant, although in practice the A and B genes express themselves dominantly to O. The O gene is called *an amorph* which is a recessive gene showing no observable change when present in the homozygous form. An individual inherits the A, B and O gene from one parent and the A, B or O gene from the other, thereby making a pair of genes called the *genotype*. Therefore six genotypes can occur: AA, AB, BB, AO, BO and OO. It is not possible to differentiate red cells of genotype AA or AO, BB or BO and the term *phenotype* is used to describe the observed reactions. AB cells and OO cells are both a phenotype and the genotype.

Laws of heredity

Two laws of inheritance have been proved in accordance with Bernstein's theory.

1. The offspring cannot possess the antigen A or B, alone or in combination, except that it be inherited from one or both parents.
2. The parent of group AB cannot produce an offspring of

group O, nor can a parent of group O give rise to a child of group AB. This is because the group AB is heterozygous so that the A gene must come from one parent and the B gene from the other.

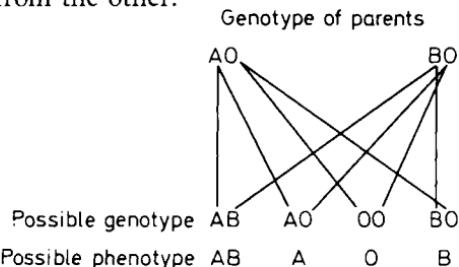


Figure 36.1. Diagrammatic representation of the possible genes being passed from parents to offspring

The only possible results from the matings of various blood groups are as follows:

<i>Phenotype of parents</i>	<i>Possible phenotypes of offspring</i>
O · O	O
O · A	O or A
O · B	O or B
O · AB	A or B
A · A	A or O
A · B	A, B, AB, or O
A · AB	A, B, or AB
B · B	B or O
B · AB	A, B, or AB
AB · AB	A, B, or AB

Inheritance of the sub-groups

The antigens A_1 and A_2 genetically are co-dominant, but in practice the genes A_1 and A_2 are expressed dominantly over the O gene; the A_1 gene is also expressed dominant to the A_2 gene. The following genotypes may therefore be present from each of the following phenotypes.

<i>Phenotype</i>	<i>Possible genotypes</i>
O	OO
A ₁	A ₁ A ₁ , A ₁ A ₂ or A ₁ O
A ₂	A ₂ A ₂ or A ₂ O
B	BB or BO
A ₁ B	A ₁ B
A ₂ B	A ₂ B

LAWS OF HEREDITY

The following laws of inheritance have been proved.

1. The antigen A_1 cannot occur in an offspring unless obtained from one or both parents, but since the phenotype A_1 can have the genotype A_1A_2 , two parents of this genotype can produce an A_2 offspring.
2. The matings $A_1B \times B$ and $A_1B \times A_1B$ cannot produce A_2B offspring because there is no genotype of A_1B that contains the A_2 gene.
3. In matings $A_1 \times O$, $A_1 \times A_1$, $A_1 \times B$ and $A_1 \times A_1B$ the sub-groups A_2 and A_2B are impossible in an offspring if it can be proved that a *sibling* (brother or sister) from the same parents is either B or O. The reason for this is that the genotypes of the B and O siblings must be BO or OO so that an O gene must have been obtained from an A_1 parent, thus revealing the genotype of this parent to be A_1O , which cannot give the offspring an A_2 gene.

The inclusion of the sub-groups of parents greatly increases the possible phenotypes of an offspring and the complete combinations are as follows:

<i>Phenotype of parents</i>	<i>Possible phenotype of offspring</i>
$A_1 \times O$	A_1, A_2 or O
$A_1 \times A_1$	A_1, A_2 or O
$A_1 \times A_2$	A_1, A_2 or O
$A_1 \times B$	A_1, A_2, B, A_1B, A_2B or O
$A_1 \times A_1B$	$A_1, A_2, B, A_1B,$ or A_2B
$A_1B \times O$	A_1 or B
$A_1B \times A_1$	$A_1, B, A_1B,$ or A_2B
$A_1B \times A_2$	$A_1, B,$ or A_2B
$A_1B \times A_1B$	$A_1, B, A_1B,$ or A_2B
$A_1B \times B$	$A_1, B,$ or A_1B
$A_1B \times A_1B$	$A_1, B,$ or A_1B
$A_2 \times O$	A_2 or O
$A_2 \times A_2$	A_2 or O
$A_2 \times B$	$A_2, B, A_2B,$ or O
$A_2B \times O$	A_2 or B
$A_2B \times A_2$	$A_2, B,$ or A_2B
$A_2B \times B$	$A_2, B,$ or A_2B
$A_2B \times A_2B$	$A_2, B,$ or A_2B

37

The Rhesus System

In 1940 Landsteiner and Wiener injected the red cells of the Rhesus monkey into rabbits and produced an antibody which not only agglutinated the monkey red cells but also the red cells of approximately 85 per cent of Caucasians (a term used by anthropologists for the white races). As these people apparently had an antigen similar to the Rhesus monkey these were called Rhesus positive and the remainder whose cells did not agglutinate were called Rhesus negative. The antigen was called D and the antibody anti-D. It is now known that the antigen in the monkey's cells and its corresponding antibody produced in rabbits is not identical with the human form but for all clinical purposes, time has sanctioned the continuance of the term Rhesus (Rh). Subsequent investigations proved the existence of further Rhesus antigens and antibodies and the basis of the Rhesus system was explained.

There are six common Rhesus genes which are called C, D and E and their allelomorphs \bar{c} , d and \bar{e} (i.e. a chromosome can carry C or \bar{c} but not both). Each chromosome can carry the genes in only eight possible combinations which are CD \bar{e} , \bar{c} D \bar{e} , \bar{c} D e , Cd \bar{e} , \bar{c} dE, CdE, CDE or \bar{c} d \bar{e} . As these combinations are difficult to say without causing some confusion, a shorthand system for easy identification is essential.

Rhesus antigens	Shorthand
CD \bar{e}	R ₁
\bar{c} D \bar{e}	R ₂
\bar{c} D e	R ₀
Cd \bar{e}	r'
\bar{c} dE	r''
CdE	r _y
CDE	R _z
\bar{c} d \bar{e}	r

The three genes, C or \bar{c} , D or d and E or \bar{e} , are carried on the same chromosome and are also close together, possibly adjacent. We know this because if a person received say \bar{c} d \bar{e} from one parent

and CDē from the other he passes on to his offspring either cde or CDē. If there were some distance between the genes and 'crossing-over' of the chromosomes occurred at all freely, the genetic frequencies would differ widely from those observed. We therefore say the linkage is close.

As far as this description has gone the gene triplets represent those present on a single Rh chromosome, as found in the sex cell. In the red cells there will be two such chromosomes and since eight combinations can be paired $\frac{1}{2}$ (8 + 1) or 36 different ways there are therefore 36 possible Rhesus genotypes. With the exception of the antigen d, the other five Rhesus antigens are capable of stimulating the formation of a specific antibody (see Rhesus antibodies). These antibodies may be used to determine the possible genotype of unknown red cells. The word *possible* is used because with the absence of anti-d, which has not as yet been discovered, the determination of a Rhesus genotype usually involves a guess, as it is not possible to say whether the D antigen is present in the homozygous form DD or the heterozygous form Dd. The Rhesus genotype is taken as that which has the highest percentage frequency from the phenotype expressed.

Table 37.1 shows the reactions of red cells with the five Rhesus antisera available, the Rhesus phenotype, commonest genotype, shorthand symbol and approximate percentage frequency in the United Kingdom.

The Rhesus phenotype of a person who gives a positive reaction with anti-D is only expressed as D because with the absence of anti-d it is not possible to state whether that person has a double dose of D (homozygous for D) or a single dose (heterozygous for D). If a negative reaction is given with anti-C the reaction with anti-*c* will be positive and therefore indicates that the person is homozygous for *c*, as these genes are allelomorphic. The Rhesus genotyping must be undertaken with extreme care, using only avid, specific antisera. The control of each specific antiserum must include both cells which are heterozygous and homozygous for the specific antigen (this will ensure that cells containing approximately half the antigenic sites in the heterozygote will be detected) as well as a negative control, which will be cells which do not contain the specific antigen.

The rhesus antigens are the strongest blood group antigens after the A and B antigens. The D antigen is by far the strongest followed by C, *c*, E and *e* in that order. Presumably d has little or no antigenic properties as, as previously stated, no example of anti-d has been discovered.

Table 37.1

<i>Anti-C</i>	<i>Anti-<i>c</i></i>	<i>Anti-D</i>	<i>Anti-E</i>	<i>Anti-<i>e</i></i>	<i>Rh phenotype</i>	<i>Commonest genotype</i>	<i>Symbol</i>	<i>Approximate % frequency in United Kingdom</i>
+	+	+	-	+	CcDee	CDe/cde	R ₁ r	31
+		+		+	CCDee	CDe/CDe	R ₁ R ₁	16
	+	-	-	+	ccdee	cde/cde	rr	15
+	+	+	+	+	CcDEe	CDe/cDE	R ₁ R ₂	13
+	+	+	+	+	ccDEe	cDE/cde	R ₂ r	13
+	+	-	-	-	ccDEE	cDE/cDE	R ₂ R ₂	3
+	+	-	-	+	ccDee	cDe/cde	R ₀ r	1

An individual is Rhesus positive if his red cells contain at least one of the large lettered antigens, i.e. C, D or E, and Rhesus negative if his cells contain only the small lettered antigens, i.e. c, d or e. In routine hospital laboratory practice it is usual to determine only the presence or absence of the D antigen, this being the most antigenic, and to report the Rhesus group as (D) positive or (D) negative. As the vast majority of patients in hospital are grouped as the potential recipients of a blood transfusion, the determination of the D antigen only is not hazardous. The reason for this is that if a person is grouped as (D) negative the transfusion blood will have been fully genotyped by the National Blood Transfusion Service and determined as a true Rhesus negative having the genotype cdē/cdē. A patient being grouped as (D) positive and for example lacking the E antigen, as in the genotype CDē/CDē, may well be transfused with blood containing the E antigen and the possibility of an anti-E developing cannot be discounted. Similarly any antigen transfused which is lacking in the recipient may well give rise to the production of the specific antibody, but in clinical practice the problem rarely exists with the exception, as previously stated, of the D antigen. It must be emphasized that determination of the presence or absence of the D antigen alone in a potential donor is not sufficient and the full Rhesus genotype must be determined.

The D^u antigen

The D^u antigen appears to be a weaker form of the D antigen although there is no specific antibody that will react with it. The D^u antigen does not usually react with complete anti-D but will react with varying numbers of different incomplete anti-D sera depending on whether it is a high-grade or a low-grade D^u antigen. When the D^u antigen is present, any anti-D serum which does not agglutinate the cells will have 'sensitized' the cells, that is to say the red cells will have been coated by antibody, and this may be demonstrated by using the antiglobulin test (*see p. 645*).

A D^u person if given D positive blood may, although rarely, form an anti-D, also D^u blood given to a D negative person may well stimulate the formation of an anti-D. It is therefore accepted that a D^u individual is considered as Rhesus positive as a donor and Rhesus negative as a recipient.

Although the D^u antigen has the highest incidence amongst the Negro races it also occurs in about 1 per cent of the population in the United Kingdom.

38

Blood Group Immunoglobulins

All antibodies are immunoglobulins because they are proteins which are synthesized in the lymphoreticular tissues. All the immunoglobulins have the same basic structure, four polypeptide chains composed of two long heavy chains which are distinct for each class of immunoglobulins, and two short light chains which exist in two forms, either Lambda or Kappa. Most antibodies are a mixture of Lambda and Kappa forms, although the individual molecules will be one or the other but not both. The heavy chains are joined to each other by disulphide bonds and the light chains

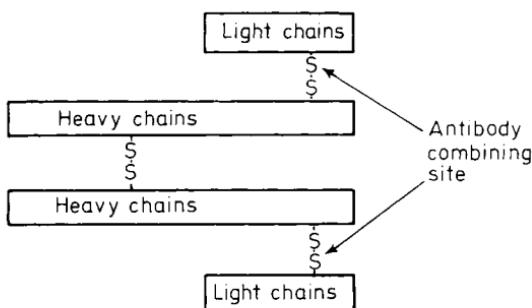


Figure 38.1. Diagrammatic representation of the immunoglobulin molecule—the general arrangement has been confirmed by electron microscopy

are joined to the heavy chains by similar bonding. The disulphide bonds give the molecule strength in its structure although allowing flexibility (Figure 38.1).

As there are marked differences in the structure of the heavy chains five classes of immunoglobulin have been defined by using immunoelectrophoresis. These are referred to as IgG, IgM, IgA, IgE and IgD (Ig = immunoglobulin). Only IgG and IgM antibodies have blood group specificity. Some IgA antibodies have been identified as having blood group specificity, but they have always been present with IgG antibodies with the same specificity and are probably of no importance. The differences in molecular

weight and size between IgG and IgM are marked and have *in vivo* as well as *in vitro* significance. The IgG antibody has a molecular weight of 155 000, is able to cross the placental barrier into the fetal circulation, but being small is unable to agglutinate red cells suspended in saline. The IgM antibody has a molecular weight of 900 000, is too large to cross the placental barrier, but is large enough to agglutinate red cells suspended in saline.

A physiological advantage of IgG antibody being able to cross into the fetal circulation is that during the first three months of extra-uterine life an infant is not able to synthesize immunoglobulin and therefore passive transfer of antibody from the mother acts as a protective mechanism. On the other hand a disadvantage is that under certain conditions, antibody which is harmful to fetal red cells is able to cross the placenta, causing a disease known as haemolytic disease of the newborn (HDN) (*see p. 647*).

There are a number of ways in which IgG and IgM antibodies may be distinguished, although the commonest method used in blood group serology is to treat the serum with a weak solution of 2-mercaptoethanol. This sulphhydryl compound is able to split the disulphide bonds, which in the case of IgM is sufficient to destroy the red cell agglutinating property of the molecule. After this treatment IgG is still able to agglutinate red cells using suitable techniques.

ABO antibodies

The 'naturally occurring' anti-A and anti-B are predominantly IgM immunoglobulins with some IgG. Only immune anti-A is predominantly IgG with some IgM. The anti-A₁, which is found in some A₂ persons is an IgM immunoglobulin.

Rhesus antibodies

The majority of Rhesus antibodies are immune and are developed from 'foreign' antigens which are given to patients either in the form of blood transfusions, or in particular cases a mother may form antibodies to fetal red cells during pregnancy. The only 'naturally occurring' examples of Rhesus antibodies are some cases of anti-E which only occur very rarely.

Rhesus antibodies occur in two distinct forms:

1. *Complete or saline agglutinating antibodies*—which are the first formed after stimulation by a foreign antigen and are composed of IgM immunoglobulins. They are able to agglutinate red cells suspended in saline and because they are immune react better at 37 °C.

2. *Incomplete or albumin agglutinating antibodies*—these are formed after the initial antibody response of complete antibody and become the predominant antibody component and therefore are the most common form found. They are composed of IgG immunoglobulins and will only agglutinate red cells suspended in a high protein medium such as bovine albumin. These also react maximally at 37°C. Incomplete antibodies are sometimes referred to as 'blocking' antibodies, as they effectively block the specific antigen receptor sites on the red cell, and will not agglutinate even after the addition of the same complete or saline operating antibody.

Red cells suspended in saline, although appearing to touch each other when viewed under the microscope, in fact do not, as they are surrounded by what is referred to as the *ionic cloud*. Red cells have a net negative electrostatic charge on their surface which attracts positive ions from the surrounding medium; this positive layer subsequently attracts negatively charged ions to its surface. This process continues in layers until there is insufficient force to attract more ions or anions and the outer edge is called the surface of shear. The electrostatic charge between the red cell surface and the surface of shear is referred to as the *zeta potential*.

The relative size of the immunoglobulins and the techniques

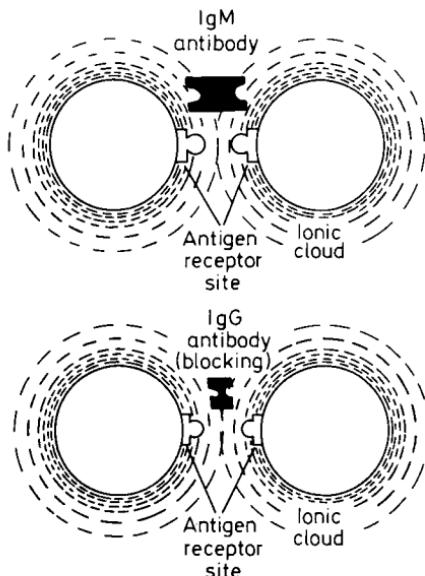


Figure 38.2. The effect of the ionic cloud on IgM and IgG antibody

for demonstrating specific red cell antigens and antibodies takes into account the surrounding ionic cloud. The IgM or complete antibodies which react in saline are large enough to straddle the ionic cloud and allow agglutination to take place. The IgG or incomplete antibodies are much smaller and unable to straddle this barrier, producing blocking of the receptor site, but the red cells will not agglutinate. To overcome this problem the red cells are suspended in 20 per cent bovine albumin which considerably reduces the size of the ionic cloud to such that the IgG antibody is able to straddle its effect (*Figure 38.2*).

The use of enzymes in the detection of IgG antibody

The reduction in the size of the ionic cloud allows red cells to come closer together and enables the small IgG antibody to agglutinate them. This may be achieved by modifying the red cells using enzymes, which digest about half of the sialic acid compounds from the cell surface and lessen the net charge. Albumin is therefore not necessary using enzyme techniques.

There are four enzymes available: (1) papain, extracted from paw-paw fruits, (2) bromelain, extracted from pineapples, (3) ficin, extracted from figs, and (4) trypsin, extracted from pancreas. It must be noted that ficin should be carefully handled, as the fine powder can cause serious damage to the mucous membranes.

The anti-human globulin (Coombs) test

This test was introduced by Dr Coombs in 1945 and is also known as the Coombs test or antiglobulin test. It is considered one of the most sensitive techniques in the detection of IgG antibodies or incomplete antibodies, which are antibodies which will not cause direct agglutination of red cells suspended in saline. Apart from its other important uses it is one of the essential tests in the crossmatching of blood to ascertain that the donor blood is compatible with the recipient's serum, and does not react with antigens in the donor's red cells.

The principle of the test is that as all antibodies are globulins an antibody against human globulin will attach itself to the specific blood group antibody which itself is attached to the red cell. In effect an antibody is prepared against another antibody and the red cells are used as an indicator of this reaction (*Figure 38.3*).

The anti-human globulin serum is prepared by injecting whole human serum into rabbits to produce what is called a 'broad-spectrum' serum which will contain IgG antibody and also anti-complement fractions. Specific antisera to IgG, IgM and IgA can

also be produced against the heavy chains of these immunoglobulins. It is essential that group O serum is used to prepare antiglobulin sera because anti-A and anti-B sera contain A and B antigens which may well stimulate the production of a large amount of unwanted anti-A or anti-B.

After an initial screening test to determine that the produced antiglobulin serum is potentially suitable for routine use, a full series of standardization tests is undertaken. The serum is inactivated to destroy complement and mixed with group A and group B cells to remove unwanted agglutinins. Doubling dilutions (1 in 2, 1 in 4, 1 in 8, etc.) of the antiglobulin serum are prepared and each dilution is tested against red cells which have been sensitized with varying amounts of IgG antibody; this is usually anti-D, as this antibody is freely available. The complexity of this standardization is to ensure that the antiglobulin serum will detect small

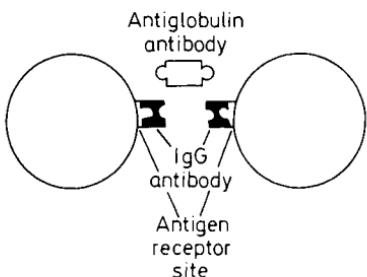


Figure 38.3. Diagrammatic representation of the principles of the antiglobulin test

amounts of IgG antibody and will not detect any red cell antigens causing a falsely positive reaction.

The detection of cells sensitized with IgG antibodies using anti-human globulin serum may be performed in two ways.

1. *The direct antiglobulin test*, also referred to as the direct Coombs test or DCT. This test is performed directly on the patient's washed red cells to establish if they have been coated with IgG antibody *in vivo*, i.e. antibody attaching itself to the patient's own red cells whilst still circulating in the vascular system. This test is often positive in haemolytic disease of the newborn, incompatible transfusion reactions and in some cases of auto-immune haemolytic anaemia.
2. *The indirect antiglobulin test* or indirect Coombs test. This test is used to detect antibody in a patient's serum by incubating fully grouped red cells with the patient's serum at 37°C to establish if any antibody present will sensitize the

red cells *in vitro*. After incubation the red cells are washed, antiglobulin serum is added and the presence or absence of agglutination is noted. By using a 'panel' of fully genotyped red cells and incubating the patient's serum with each, it is possible to determine the specificity of the antibody if an IgG antibody is present. Any red cells which do not agglutinate after the addition of antiglobulin serum will not contain the antigen to the specific antibody. The cells which do agglutinate will have been sensitized by the antibody and will contain the specific antigen to this antibody. By a series of eliminations the specificity of the antibody may be determined.

As all human serum contain globulins, it is essential that any globulin not attached to the red cells is removed by washing in copious volumes of saline before the addition of antiglobulin serum. If these are not removed the antiglobulin serum will be neutralized by the 'free' globulins and false negative reactions obtained. The cells are washed at least three times, and in laboratories which perform large numbers of antiglobulin tests this procedure is tedious and use is made of a cell-washing centrifuge which automatically washes the cells, and in some models automatically adds the antiglobulin serum.

All the common Rhesus antigens, with the apparent exception of d, are able to stimulate the production of their corresponding specific antibody in both the complete and incomplete forms. Mixtures of antibodies do occur with anti-C+D and anti-D+E being likely combinations.

A patient who has developed Rhesus antibodies from a previous blood transfusion, pregnancy or abortion and is given Rhesus incompatible blood will have a 'transfusion reaction'. This reaction, if detected at an early stage, may cause little damage, but if the patient is anaesthetized, the reaction may be masked and severe renal damage occur due to the large haemoglobin molecule being released into the blood stream, and being unable to pass through the kidney. The breakdown of red cells in this way is called 'intravascular haemolysis'.

Haemolytic disease of the newborn (HDN)

Haemolytic disease of the newborn is due to the destruction of fetal red cells by an IgG antibody which has crossed the placenta into the fetal circulation. The antibody is usually anti-D although, rarely, other Rhesus antibodies and other blood group system antibodies may be the cause. The mother, who will be D negative,

is usually sensitized at a previous delivery by her infant's D positive red cells entering the maternal circulation, and an IgG anti-D is produced. Occasionally sensitization is due to a Rhesus incompatible blood transfusion. During subsequent pregnancies the red cells of any D positive fetus will be haemolysed, and a jaundiced, anaemic infant, with a positive direct antiglobulin test, will result. The jaundice and anaemia may be severe enough to warrant the exchange of the infant's blood with fresh ABO compatible, Rhesus negative blood, this procedure being called an 'exchange transfusion'. Although the anaemia may be corrected by an exchange transfusion, it is important to estimate frequently the serum bilirubin level, as very high levels in an infant are predominantly in the form of unconjugated bilirubin, which can cause irreversible brain damage.

The infant's D positive gene will have come from its father and it is therefore of value to Rhesus genotype the father's red cells to establish whether the D antigen is present in the homozygous or heterozygous form. If he is homozygous for D it can be expected that all his offspring will be affected, whereas if he is heterozygous, some may be unaffected.

Haemolytic disease of the newborn can now be prevented by injecting soon after delivery any D negative mother, who produces a D positive infant, with a specific anti-D gamma globulin. The anti-D gamma globulin will destroy any D positive red cells which may have entered the maternal circulation during delivery, and thus prevent the formation of an IgG anti-D.

Occasionally HDN is caused by ABO incompatibility, although it is unusual for an exchange transfusion to be necessary. The infants affected will always be group A or group B and the corresponding immune anti-A or anti-B will have crossed the placenta into the fetal circulation. Contrary to expectation, the direct anti-globulin test in haemolytic disease of the newborn due to ABO incompatibility is invariably negative. The reason for this has not been established, although the offending antibody can be eluted from the infant's red cells.

Other blood group systems

Over one hundred blood group antigens may be demonstrated using the specific antisera, and these have been classified into blood group systems. Many of these antigens fortunately have no clinical significance, but as the blood group systems are inherited quite independently from each other, they are of immense value as genetic markers. Some of the other blood group systems apart

from the ABO and Rhesus are called as follows—MNSs, P, Kell, Lewis, Lutheran, Duffy, Kidd and I. For further information about these groups more specialized textbooks should be referred to.

Medico-legal aspects of blood groups

In paternity disputes the blood grouping of all the parties concerned can do no more than exclude one of the parents. Usually it is the father who is in dispute and he is excluded if antigens which he genetically must pass on are not present in the child, and also if the child possesses an antigen which both he and the mother lack the disputed father must be excluded. This type of work is not usually carried out in the hospital laboratory because of the legal implications.

Forensic aspects

The determination of the blood group antigens of an individual are nearly as exclusive as the fingerprints. This fact is used frequently by police departments throughout the world during the investigation of criminal cases. The techniques are often those used in the clinical laboratory, but highly sophisticated methods are available in detecting blood group antigens in dried stains and also the determination of blood group substances in saliva and other body fluids.

39

Blood Transfusion

Whole blood transfusions are used predominantly to replace blood loss from acute haemorrhage or surgical operations, whereas *packed red cells* are given to patients who need the additional haemoglobin but not the plasma fraction. Packed cells are prepared by removing approximately three-quarters of the plasma-anticoagulant solution from a pint of whole blood, using aseptic technique, immediately before the transfusion. If the enclosed system using a disposable plastic bag is used (see p. 661) the resulting packed cells, after removal of the plasma, may be used up to 21 days from the date of collection. The laboratory confirmation of anaemia does not in itself warrant a blood transfusion. The cause of the anaemia must be established, and any deficiency of iron, vitamin B₁₂ or folic acid should be corrected wherever possible before a transfusion is contemplated.

One of the necessary prerequisites to the storage of blood is the addition of a substance which prevents coagulation. The anticoagulant must not cause deterioration of the red cells nor must it be toxic to the recipients of the transfusion. Two factors are known to affect red cell preservation favourably: (a) slight acidification, and (b) the addition of dextrose. The solution used in blood bottles in Britain is *acid-citrate-dextrose (ACD)* and has the following composition:

Disodium hydrogen citrate	2 g
Dextrose	3 g
Distilled water (pyrogen-free)	to 120 ml

This is sufficient to anticoagulate 420 ml of blood in the standard MRC blood bottle having a total volume of 540 ml. The relatively large volume of anticoagulant solution is retained to limit the amount of blood taken from the donor.

There is nothing to be gained by raising the concentration of dextrose in the solution or by the use of other carbohydrates. The initial pH of the solution is about 5, and after addition of blood

the resulting pH of the plasma-anticoagulant solution is about 7. Blood stored at 4 °C is well preserved in ACD and may be used for up to 21 days from the date of collection. Although red cells may be satisfactorily stored, platelets survive storage poorly, and only 5 per cent are viable after 24 h in ACD and none viable after 48 h. Transfused white cells leave the recipients' circulation within a matter of minutes, and little research has been done to determine the viability of leucocytes in stored blood. As platelet and leucocyte antibodies can be demonstrated in recipients of multiple transfusions, it must be assumed that although neither of these cells survive storage, their antigenic structure remains intact. The important coagulation factor VIII or anti-haemophilic factor, diminishes rapidly on storage and disappears within 24 h of collection. It may be stored by freezing the plasma at -20 °C immediately after collection.

Heparin

Heparin is a very efficient anticoagulant and may be used both *in vitro* and *in vivo*, but has the disadvantage that it is gradually broken down, allowing clotting to begin. It can therefore only be used where the blood is to be used within 24 h of collection. Even so heparin has its uses, and blood collected for use in 'heart-lung' machines is anticoagulated in this manner.

National Blood Transfusion Service

Blood is predominantly collected by the National Blood Transfusion Service (NBTS) in Britain, and the country is divided into regions with each region having its own Transfusion Centre responsible for supplying blood, blood products and grouping sera to the hospital laboratories in its catchment area. Donors are bled twice a year and are notified when to attend a donor session which is operating in the locality where they live. The mobile collecting teams also visit factories and offices as well as local halls and establish a temporary but efficient blood collection service. The collected blood is then transported to the appropriate Transfusion Centre where it is fully tested for its suitability as donor blood, labelled and stored before its final journey to the hospital blood bank.

Some of the larger hospitals in the country supplement their supply of blood by maintaining an internal panel of donors. These consist of hospital staff which have been fully grouped, and are available at any time when fresh blood is required for a particular patient.

The British Red Cross also organize an efficient blood donor panel and acts as liaison between hospital and donor when fresh blood or that of a particular group is required.

The blood donor

Donors must be between the ages of 18 and 65, of either sex and conform to the National Standard of Fitness as laid down by Act of Parliament. Males must have a haemoglobin level of not less than 13.5 g per 100 ml and females not less than 12.5 g per 100 ml. The haemoglobin level of the donor is determined before each donation, and for convenience the copper sulphate specific gravity method is used. A drop of blood from a finger prick is carefully delivered into a copper sulphate solution of known specific gravity where it immediately forms copper proteinate. Male donors are accepted if their blood sinks in a copper sulphate solution of specific gravity 1.055, and female donors if it sinks in a solution having a specific gravity of 1.053. If the blood contains less than the required concentration of haemoglobin it will rise to the surface before finally sinking.

The amount of blood taken is limited by the size of the bottle, being 420 ml in the standard glass transfusion bottle, and approximately 430 ml in the disposable plastic bag which is gradually replacing the bottle. The plastic bag has a total capacity of 500 ml but only contains 70 ml of anticoagulant solution.

It is perhaps unnecessary to state that all donors must be fit and well before donating their blood, but certain diseases may remain hidden and must be excluded so as to prevent transmission to the recipient. Every donation is tested to exclude syphilis and any donor giving a positive result is discarded. Donors who have recently visited or who have lived in a country where malaria is present are also discarded, although their plasma may be used for the preparation of 'blood products' (*see p. 659*). The malarial parasites resist storage at 4 °C very well and are easily transmitted to the recipient of blood which contains them. Potential donors who give a history of jaundice, from whatever cause, have previously been automatically rejected from giving blood. This was to prevent the transmission of hepatitis to the recipient. This rejection may be unnecessary, although the possibility of transmitting hepatitis must be excluded and is dealt with in some depth below.

Hepatitis-associated antigen (HAA)

Hepatitis means inflammation of the liver, and in this context is thought to be due to a filterable virus which is sometimes referred

to as viral hepatitis. An antigen, first discovered in Australia and has been called Australia (Au) antigen, was found to be present in the serum of patients with viral hepatitis. The antigen is not thought to be a virus as it lacks nucleic acids, but it may be the sheath of a virus. This antigen is now generally known as hepatitis-associated antigen (HAA), and can be seen using electron microscopy in centrifuged deposits of serum. Investigations have now proved that blood containing the HAA when used for transfusion is followed by a high incidence of hepatitis in the recipients, whereas when transfusions of blood not containing the HAA are given, a very low incidence of hepatitis is recorded. As there are now a number of established techniques for the detection of HAA all blood from the NBTS is screened for the presence of this antigen. Although electron microscopy is the most sensitive technique for the detection of HAA, it is not practical to use this method for screening daily the huge number of donations passing through the Transfusion Centres. The main method employed is a gel diffusion technique (Ouchterlony plate) or a modification using immunoelectrophoresis. The antibody used to react with the HAA and cause a precipitation is often obtained from patients who have had numerous transfusions.

Note—Any blood samples entering the laboratory may contain the HAA, and if this enters the circulation of the laboratory worker, perhaps via a cut on the hand or finger, may result in hepatitis. The risk is particularly high in the patient with a diagnosis of hepatitis or possible hepatitis, and these so-called 'high risk' samples must be clearly identified, preferably with a specific yellow coloured label, and the request form must also state clearly that the patient is an 'HAA Suspect' or 'HAA Positive'. These samples must be treated with extreme care in the laboratory and the recommendations laid down in the Department of Health publication *Safety in Laboratories*, strictly adhered to.

STORAGE OF BLOOD

Blood deteriorates rapidly if not kept under ideal conditions, and haemolysed blood or infected blood may well be lethal to the recipient. Blood must be stored in specially constructed refrigerators which have high insulation properties and a very sensitive thermostat. The refrigerator must maintain a temperature of 4 °C with a maximum range of 2–6 °C. It is essential that the temperature does not exceed 6 °C or fall below 2 °C or damage to the red

cells may occur. To prevent this the blood bank must have a temperature recorder so that it is possible to tell the temperature at a glance, and also be able to keep a record of the stability of temperature. The blood bank must also be connected to an alarm system, preferably a loud bell or buzzer, which will give an audible signal if the temperature rises or falls outside the prescribed limits. The alarm must also sound in the casualty department or the porter's lodge, which are places where staff are always on duty, thereby ensuring that a responsible person will take the predetermined action even when the laboratory is closed. The alarm should also sound if the electricity supply fails; it therefore follows that the alarm system must be battery-operated.

On no account must blood be stored in a domestic refrigerator such as is found in most hospital wards. These refrigerators exhibit marked fluctuations in temperature and it is not unusual for this type of cabinet to fall below 0 °C.

If there is any doubt regarding the storage of blood once it has left the laboratory blood bank, it must be discarded and not returned to the stock to be re-crossmatched for another patient. It is the laboratories' responsibility to continually educate all persons who handle blood; this includes medical, nursing and portering staff as to the importance of the correct storage procedures in the individual hospital.

Transportation of blood

The National Blood Transfusion Service delivers the majority of its blood by road using specially designed refrigerated vans, or insulated vans with large ice containers, the bottles or bags of blood being held safely in metal crates. If the blood is to be sent by rail a purpose-built insulated box is used. The insulated box is very strong and is designed to take an ice insert in the centre. Insulated boxes are available in various sizes, the commonest size for inter-hospital use being for two bottles. Using this type of insulated box, blood will keep at a temperature of 4–6 °C for at least 6 h.

Storage of frozen blood

Blood for transfusion may be kept for a much longer period than 21 days if it is kept frozen solid at –20 °C, but to prevent damage to the red cells the addition of glycerol is essential. When the blood is required for transfusion, all traces of glycerol must be removed immediately the blood has thawed. This is achieved by washing the cells serially in lowering concentrations of isotonic glycerol

solutions. The recovery rate of red cells stored by this method is good and they may be kept at -20°C for up to two years.

Another low-temperature storage method is to freeze blood in liquid nitrogen which has a boiling point of -195.8°C . The frozen blood, which is in the form of corrugated slabs, is stored immersed in liquid nitrogen in specially constructed liquid nitrogen refrigerators. The thawing and recovery of cells ready for transfusing requires special techniques, but the advantage of this method is that red cells may be stored probably indefinitely.

Frozen blood, although very expensive to maintain, does have special advantages over blood stored in the usual way at 4°C . Red cells having rare antigen combinations or lacking in common antigens may be stored for extremely long periods, and will be available for patients who have developed antibodies to the majority of red cells. Alternatively, patients who have very rare blood groups may be bled at intervals so that they may receive their own blood during surgery; this is called *auto-transfusion*. Another advantage is that blood may be stored in large quantities as a reserve to offset any deficiency which may occur in normal supply of donor blood.

It is of value to store small quantities of red cells in glycerol and liquid nitrogen as reference cells in the laboratory. The recovery of small volumes of red cells frozen in glycerol is much easier to achieve as they may be dialysed against isotonic saline, and are ready for use in 2 h. Likewise with red cells stored in liquid nitrogen, these may be thawed quickly in a warmed sucrose solution. Red cells frozen in liquid nitrogen are in the form of small peas or pellets, and the appropriate number are removed as they are required.

PRINCIPLES OF REFRIGERATION

As the correct storage temperature of blood and its products is so vitally important, it is obvious that some form of refrigeration must be available to create these conditions. The principles and aims of refrigeration will therefore be discussed.

For small laboratories, a compact, suitably designed refrigerator, with a storage compartment for sera, will suffice, whilst a large blood depot will require a refrigerated room, and large deep-freeze cabinets for the storage of sera at temperatures of -20°C and lower. Whatever scale of refrigeration is used, however, the general principles are the same.

The aim of refrigeration is to draw heat from the material to be

cooled and transfer it to the air outside the refrigerator. This is achieved by the application of three basic facts:

1. Heat will pass spontaneously from a hot body to a colder one, but not vice versa.
2. The temperature at which a liquid boils depends on the pressure exerted on its surface; for example, water boils at below 100 °C where the pressure is below atmospheric pressure, and higher than 100 °C when the pressure exceeds that of the atmosphere.
3. A liquid, while it is boiling and being converted into a vapour, absorbs a considerable quantity of heat, called the 'latent heat of evaporation', for example, water boiling in a kettle is deriving latent heat of evaporation from the burning gas flame or electric hot plate.

METHOD OF OPERATION

A refrigerator operates as follows (*Figure 39.1*):

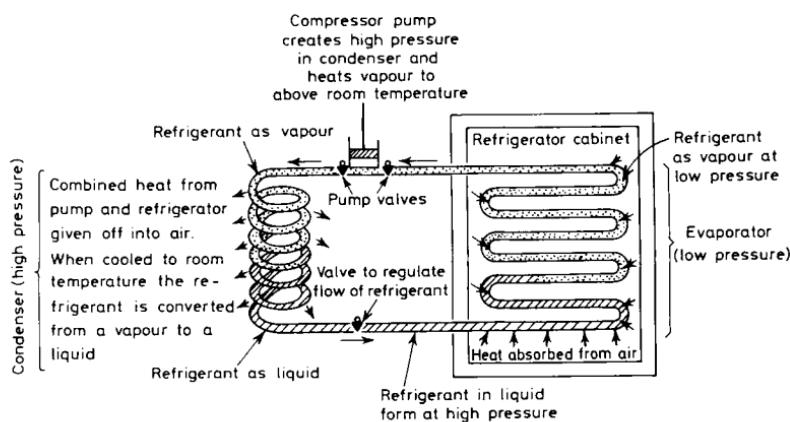


Figure 39.1. Diagrammatic illustration of principle of refrigeration

A liquid, called the *refrigerant*, is kept boiling in a coiled metal tube called the *evaporator*, which is usually built into the walls of the freezing compartment. By suitably adjusting the pressure, it boils at a temperature below that existing in the refrigerator; heat therefore passes to the refrigerant in the evaporator from the air inside the refrigerator cabinet, causing a fall in temperature. The rest of the cooling system serves to maintain a constant supply of refrigerant to the evaporator, and to remove the vapour as it is formed by the boiling refrigerant. The vapour is pumped into

another coiled metal tube, the *condenser*, the inside of which is maintained at a high pressure. This condenser is situated outside the refrigerator, so that it is kept at room temperature. The pumping action heats the vapour at above room temperature, so that when it reaches the condenser it cools down, and the heat which has been taken from the air inside the refrigerator is transferred by the vapour to the air outside the refrigerator. The pressure in the condenser is adjusted, so that when the temperature of the vapour falls, it condenses to a liquid, which is allowed to flow back into the evaporator, where it is boiled again under low pressure. In this way the same quantity of refrigerant is circulated over and over again. The flow of liquid from condenser to evaporator is adjusted so that there is a reservoir of boiling liquid maintained in the evaporator.

Refrigerants are liquids having a high latent heat, and which are converted into vapours under small changes of pressure. They must also be non-corrosive, and preferably non-toxic.

TYPES OF REFRIGERATOR

There are two main systems: (*a*) compression and (*b*) absorption. Both are based on the principles just described but differ essentially in the transfer of refrigerant from the evaporator to condenser.

Compression type

In this type the vapour is withdrawn from the evaporator by a mechanical pump, and passes through a valve into the condenser. Refrigerants used in the compression type of refrigerator are generally ammonia, carbon dioxide, sulphur dioxide, or the group of fluorocarbon compounds called the 'Arctons'.

Absorption type

In these refrigerators the vapour of the refrigerant, which in this case is liquid ammonia, is absorbed out of the evaporator by dissolving in water. This aqueous ammonia solution is then boiled by a flame, or other form of heater, and the ammonia gas so evolved passes to the condenser.

PRECAUTIONS IN THE USE OF REFRIGERATORS

Whetever type of refrigerator is used, there are certain elementary rules to be observed.

1. Never cover up the radiator or air vent. To do so means that no cool air can circulate, and the cycle of gaseous-liquid changes is hindered.

2. Never place hot liquids inside a refrigerator. To do so places an unfair burden on the refrigerant. The mechanism is only designed to cool from room temperature, not from higher temperatures.
3. Defrost the freezing compartment regularly, 7-10 days.
4. Keep the inside of the refrigerator dry, and always dry the interior after defrosting.
5. Never pack things inside the cabinet so tightly that air cannot circulate freely. Some refrigerators have a small fan connecting the freezing and general storage compartments. To block the air around such a fan leads to faulty working of the refrigerator.
6. Do not tamper with the mechanism if faults develop. A competent engineer should deal with the trouble.

Carbon dioxide refrigeration

When 'deep-freeze' refrigerators are not available special insulated containers can be used, which will maintain temperatures of about - 60 °C. This is achieved by placing blocks of solid carbon dioxide in the container. A block of frozen CO₂ is generally sufficient for 7 days deep-freezing.

PREPARATION OF SOLID CARBON DIOXIDE

Carbon dioxide gas is first liquefied by pressure and then subjected to a reduced pressure. This causes some of it to evaporate and extract heat, absorbed by the vapour, from the rest of the liquid. This heat extraction causes it to freeze to a hard, compact solid, with a temperature of - 78 °C at atmospheric pressure. It vaporizes without melting, and, as no liquid is produced, is much cleaner in use than ice, and gives a much greater cooling effect.

When using frozen carbon dioxide it is essential to wear heavy, insulated rubber gloves with long gauntlets, and to handle it with tongs. Never, under any circumstances allow the skin to come into contact with the carbon dioxide, as serious burns will result.

COMPATIBILITY TESTING OR CROSSMATCHING

Even though the blood donor's and the recipient's ABO and Rhesus groups are the same, it is essential that crossmatch tech-

niques are performed. This is to ensure that the donor blood will not give rise to any reaction from antibodies the recipient may have formed to any blood group systems. As it is probably true to say that no blood given will exactly match the antigenic structure of the recipient's red cells, it is vital that every precaution is taken to prevent harm to the patient. The crossmatch is performed by testing the donor red blood cells against the recipient's serum using a saline, albumin and anti-human globulin technique and incubating the tests for 2 h. Blood should never be given to a patient without crossmatching, and even in dire emergency situations a simple compatibility test should be performed before issuing the blood, always followed by the standard 2 h technique.

BLOOD SUBSTITUTES AND BLOOD PREPARATIONS

No perfect substitute for blood has been synthesized, and no synthetic plasma, which has all the properties of human plasma, is available at the present time. Nevertheless a number of solutions are available which enable either the blood volume to be replaced or specific substances which are depleted to be replaced. It must be emphasized that none of these substances will increase the oxygen-carrying capacity of the blood, only the transfusion of red cells will achieve this.

Dextran

Dextran is the collective name given to the polysaccharide formed when a solution of sucrose is broken down by the action of *Bacillus leuconostoc mesenteroides*. Dextrans of various molecular weights can be made, but the ideal molecular weight is approximately 70 000. If the molecules are too small they are rapidly excreted in the urine, and if too large may cause undesirable physiological effects. The solution is usually 6 per cent dextran in isotonic saline, and is used to replace the blood volume, particularly after acute haemorrhage, allowing time for whole blood to be crossmatched. All dextrans have the property of greatly increasing rouleaux formation, which can cause crossmatching errors unless the antiglobulin technique is used. It is advisable that blood for crossmatching is taken before dextran is given.

Other crystalloid solutions

These include 4 per cent glucose-saline, Hartmann's lactate solution and isotonic saline. These solutions are used predominantly to maintain the blood volume and prevent dehydration after major surgery.

Plasma protein fraction (PPF)

This is a solution of the proteins of human plasma and is a clear, amber fluid. It is prepared from pooled plasma which has been precipitated with suitable organic solvents, and re-dissolved in water. The final solution is made isotonic by adding sodium chloride, and further substances added to stabilize it to heat. It is sterilized by filtration and heated at 60 °C for 10 h to prevent transmission of viral hepatitis. PPF contains not less than 4.3 per cent *w/v* of total protein, contains no fibrinogen, little, if any immunoglobulin, and has a storage life of 3 years at 2–25 °C. It is used mainly to replace depleted plasma volume and may be used in place of blood in an emergency while awaiting issues of blood. It is likely that as more supplies of PPF become available it will completely replace dried human plasma.

Dried human plasma

This is prepared from pooled plasma and dried by freeze-drying; it is a light to deep cream coloured powder. To ensure cross-neutralization of the ABO antibodies by the soluble blood group substances, plasma from donors of A, O and either B or AB groups are mixed in the approximate ratio 9 : 9 : 2. No more than ten separate donations are pooled. The dried plasma must be stored at a temperature below 25 °C, preferably protected from the light. Providing the viscap remains intact, preventing the entry of moisture, the storage life of the dried plasma is six years. Dried plasma is reconstituted by adding 400 ml of sterile, pyrogen-free distilled water and should completely dissolve within 10 min at 15–20 °C. Any signs of lumpiness indicate the presence of denatured protein, and the bottle contents must be discarded.

Dried human fibrinogen

This is prepared from plasma by precipitating with organic solvents, the precipitate being re-dissolved in a solution of sodium chloride and sodium citrate and then freeze-dried. It is a white powder or friable solid and is reconstituted for use by the addition of sterile, pyrogen-free distilled water. Fibrinogen therapy is only

indicated for the specific replacement of fibrinogen when bleeding is due to lowered levels, or lack of this coagulation factor.

Cryoprecipitate

This is prepared from fresh plasma which is frozen solid in a mixture of solid CO₂ (dry ice) and ethanol, and allowed to thaw slowly at 4 °C for 24 h. Thawing leaves a cold-insoluble precipitate rich in anti-haemophilic factor (factor VIII), which is kept after the plasma has been centrifuged and the supernatant removed. The precipitate is stored at -20 °C, and for use thawed at 37 °C, re-suspended and usually injected intravenously using a syringe and needle. Cryoprecipitate contains about 56 per cent of the original factor VIII in less than 3 per cent of the original plasma volume, together with a small amount of fibrinogen, but no significant amounts of other coagulation factors are present. It is used predominantly in the treatment of classical haemophilia and sometimes in diseases where the factor VIII level is reduced to the extent that bleeding occurs.

BLOOD TRANSFUSION APPARATUS

With the obvious exception of the needle, all apparatus for the taking and giving of blood is manufactured from plastic, sterilized by irradiation, and is disposable. Many of the transfusion centres in Britain continue to use the standard MRC bottles to store the blood, together with the increasing use of disposable plastic bags. The plastic bag is a completely enclosed system and has the donor tube leading into the bag, which once the bag has been filled, is sealed off. Red cells may be obtained from this tube for subsequent crossmatching.

The plastic bags are resistant to breakage if accidentally dropped and require less space in the blood bank than the conventional bottle. It is possible by gently squeezing the bag, either by hand or using the special apparatus, to remove the supernatant plasma leaving 'packed cells', thereby eliminating the risk of bacterial contamination by inserting a needle and removing the plasma by suction.

The plastic 'giving' or 'recipient' sets are supplied individually in sterile, disposable packs. The set consists of a plastic pack or bottle attachment, a filter chamber leading directly into the drip

chamber, which is used to control the rate of flow, and a long tube ending with the needle attachment. This set is used for both the conventional glass bottle with cap and rubber liner, and the disposable plastic bag.

40

Blood Grouping Technique

GENERAL CONSIDERATIONS

The performance of any blood grouping technique demands a high degree of concentration and technical competence. Under ideal conditions the work should be carried out in a quiet atmosphere without disturbance by the telephone or by the talking of colleagues. In practice this is difficult to achieve, but every effort should be made to attain the quietest conditions possible.

Specimens

All blood for grouping and crossmatching must be correctly labelled, and as a minimum have the patient's name and hospital registration number clearly legible. If there is any doubt as to the identification of the sample it must be discarded and a fresh sample obtained. Blood group tests should be performed daily so that all samples are reasonably fresh and the risk of bacterial contamination and haemolysis minimized. After grouping, the specimens should be kept at 4 °C for one week, this will allow re-checking of the group should an anomaly subsequently come to light.

Apparatus

This is essentially very simple, but at all times must be kept scrupulously clean so as to ensure that no contamination will occur due to bacteria, chemicals or foreign proteins. The tubes used should preferably be clear polystyrene and disposable; glass tubes may be used but are difficult and time-consuming to keep clean. Basically only two sizes of tube are required; one approximately 75 × 12 mm (3 in × $\frac{1}{2}$ in) for cell suspensions, the antiglobulin test and various other procedures, sometimes called a 'postal-tube'; the other is the *precipitin tube*, 50 × 6 mm (2 in × $\frac{1}{4}$ in) and is used in all grouping procedures where the 'standard tube technique' is used. The precipitin tube being so small has the advantage that only small quantities of reagents are required; the column of serum/cell mixtures is relatively high in the narrow-bore tube and the cells will therefore take longer to fall allowing more contact

time for any antigen-antibody reaction, and the surface area is small and evaporation is minimal.

Pasteur pipettes should preferably be disposable, and one drop should approximately equal a volume of 0.03 mm^3 , which is the standard volume used in the tube technique.

Isotonic saline (0.9 per cent sodium chloride) should be prepared freshly each day. Large aspirators of isotonic saline should not be kept, as algae will grow and the pH will gradually fall due to absorption of CO_2 from the atmosphere. Wash-out pots must be cleaned at least once daily and preferably after each batch of tests. If distilled water is kept in the blood grouping laboratory it must be clearly labelled, and extreme care taken that it is in no way confused with the isotonic saline.

Racks or blocks for the tubes are usually the MRC Agriculture and Fisheries 50 hole wooden block or various racks prepared from sheet alloy. It is advisable that the blocks or racks are not identified by writing on them with wax pencils or felt-tip pens, but 'flagged' using a piece of paper cut into the shape of a flag and placed in one of the holes. It is strongly advised that the polystyrene tubes are identified with a felt-tip pen, and this is, of course, essential if the tubes are to be centrifuged.

Clerical errors

These are without doubt the commonest errors encountered in the blood group laboratory. These can only be avoided by careful checking and concentration; a quiet atmosphere is of considerable help in excluding clerical errors. All results must be recorded onto sheets which have a laboratory protocol printed on them. Results must be entered directly on the protocol and not transferred from a rough working sheet, thereby increasing the possibility of a clerical error.

Storage of grouping serum

The antibody content of untreated human serum deteriorates if kept at room temperature and therefore grouping antisera must be kept frozen preferably at -20°C , where its strength will be maintained almost indefinitely. Serum kept between 2°C and 4°C is preserved for a variable period. Serum should not be kept in large amounts so that continued thawing and freezing is a daily occurrence, as this will also accelerate deterioration and increase the risk of bacterial contamination, which is again another cause of deterioration.

Commercial antisera must be stored according to the manufac-

turer's instruction, and usually this is at 4 °C and not frozen. These sera do not resemble human serum in their composition and often contain bovine albumin. Although very expensive, commercial antisera from reputable sources have the advantage of being very potent and show consistency of reaction from batch to batch.

Bovine albumin

This is available in two concentrations, 20 per cent and 30 per cent. It is usually the 20 per cent concentration which is used in the clinical laboratory. It must be stored at 4 °C and on no account be frozen, as this will destroy its properties.

TECHNIQUES

There appear to be no 'standardized' techniques in the field of blood group serology, and the methods described are those which have given consistent results over many years of use. The inclusion of adequate controls to ensure that all reagents are reacting correctly must be set up with each batch of tests. It must be emphasized that proficiency in blood grouping and the detection of antibodies can only be achieved with considerable practice, and although the techniques described appear simple, experience is essential to obtain consistently reliable results.

Recording of results

As previously mentioned, all results must be recorded in a protocol, but the strength of reaction is important and therefore each laboratory must agree on a method of recording the various reactions which all staff must abide by. A suggested method of scoring agglutination is given below.

Macroscopic agglutination.

C = Complete—no free cells in surrounding fluid.

V = Visual—agglutinates easily visible with free cells.

Microscopic agglutination. + + + = Very large agglutinates with free cells.

+ + = Large agglutinates with many free cells.

+ = Agglutinates of 8–16 cells.

± or W = Agglutinates of 3–8 cells.

— = No agglutination.

R = Rouleaux formation.

H = Haemolysis.

ABO grouping—tile method

This method must only be used for emergency grouping and must always be confirmed using the tube technique. The use of commercial antisera is recommended for this technique, as being so potent will usually give clear-cut results extremely quickly, and easily detect the A₂ sub-group.

An opal glass tile previously etched or drawn with a wax pencil into 1-in squares, to prevent running together of the cell/reagent suspensions, is used for this method.

One volume of 20 per cent patients' red cells is added to 1 volume each of anti-A and anti-B and mixed with an applicator stick to a diameter of approximately 20 mm. Agglutination if present will easily be detectable within 2 min. The anti-A and anti-B must be controlled using both known A₂ cells and B cells.

		Patient's cells				A ₂ cells		B cells	
		A	B	AB	O	Controls			
Anti-A	+	+	-	-	+	-	+	-	-
	-	-	+	+	-	-	-	-	+
Anti-B	+	-	+	+	-	-	-	-	+
		A	B	AB	O	Controls			

Note—The opal glass tile must be scrubbed before use with soap and water, thoroughly rinsed, and dried using a clean cloth. The pasteur pipette used for the antisera and cell suspensions must be thoroughly rinsed with clean saline between each antiserum and cell suspension.

ABO grouping—standard tube technique

Using this method both the patient's cells and serum are tested and the results compared.

Into a 50 hole wooden block or metal grouping rack are placed the requisite number of precipitin tubes; these are used for the cell/serum mixtures. A postal tube for the patient's cell suspension is also required.

One volume, which is approximately 0.03 mm³ (usually one drop from a pasteur pipette is satisfactory) of both the unknown cells or serum, is added to each tube, and an equal volume of the known cells or serum added to the appropriate tubes.

<i>3 per cent patient's cells tested against:</i>	<i>Patient's serum tested against:</i>
Anti-A	A ₁ cells 3 per cent
Anti-B	B cells 3 per cent
Anti-A+B (O serum)	O cells 3 per cent
Patient's own serum	Patient's own cells 3 per cent
	(auto-control) ↔ (auto-control)

The tubes are gently mixed and allowed to stand undisturbed at room temperature for 2 h.

Controls are essential and are set up in the same way as the test, using the following combinations.

Anti-A, Anti-B and Anti-A+B against 3 per cent A₁ cells, A₂ cells, B cells and O cells.

The tests are read by gently tapping the tube and looking for the presence of agglutination and recording the strength on the protocol. All negative reactions must be examined microscopically; this is easily done by removing a little of the cell/serum mixture from the tube and gently spreading it out onto a glass slide. The slide is examined using the low-power objective.

The auto-agglutination control (patient's serum against patient's cells) must be negative; any agglutination of the A₁, B or O cells in the presence of a positive auto-agglutination control is discussed under causes of false positive results (*see below*).

Differentiation of group A₁ and group A₂

Using the tile method, one volume of 20 per cent patient's red cells are mixed with one volume of *Dolichos biflorus* lectin.

Agglutination is rapid and very strong if the cells are A₁. A negative reaction with *Dolichos biflorus* extract in conjunction with a positive reaction with standard anti-A indicates that the cells are group A₂.

CAUSES OF FALSE POSITIVE RESULTS IN ABO GROUPING

Rouleaux formation

Sometimes called 'pseudo-agglutination', this rarely gives trouble to an experienced worker. If the rouleaux formation is marked the auto-agglutination control will be positive and any cells added to

the patient's serum will give a similar reaction. If the serum is diluted 1 in 2 or 1 in 3 with physiological saline the rouleaux formation should disappear, whilst true agglutination will persist. Rouleaux formation is not true agglutination, as it is not due to an absorbable agglutinin. The commonest mistake due to formation of rouleaux is delay in reading the result when using a tile method, a 'graininess' appears which may be read as a weak positive but will usually disappear when a drop of saline or albumin is added to the mixture. Occasionally rouleaux may be present in the cell grouping tubes, in which case repeat the test using washed red cells.

Infected red cells (the Thomsen phenomenon)

Several types of bacteria are capable of causing red cells to agglutinate by any normal serum, animal or human, except young infants, and the cells are called polyagglutinable. The bacteria are able to

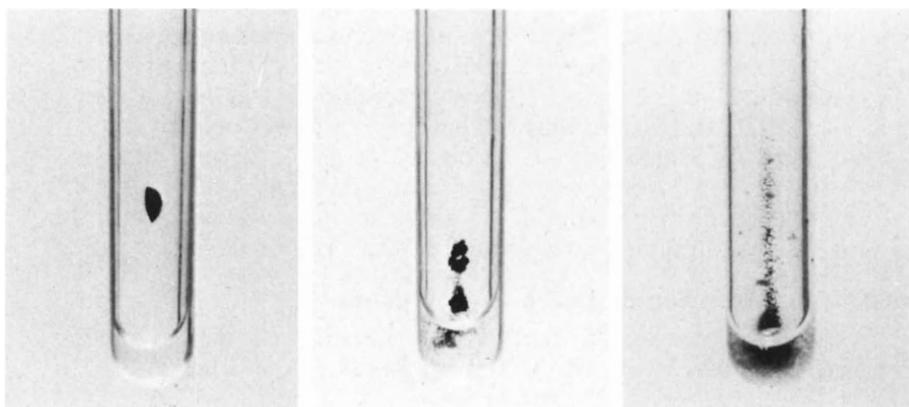


Figure 40.1. Macroscopic agglutination: (a) complete; (b) visual; (c) very large agglutinates and free cells

expose the T antigen and most sera contain the T antibody, thereby causing agglutination. The reaction may become apparent within 18 h at 4 °C or within shorter periods at room temperature. It is important to use only fresh cells or cells which have been stored for the shortest time in the refrigerator. In blood stored in the form of a clot, the phenomenon is rarely found.

Infected serum

Occasionally gives a false positive reaction but invariably gives a false negative reaction.

Table 40.1 RESULTS OF TYPICAL REACTIONS ENCOUNTERED ARE SHOWN IN THE FOLLOWING PROTOCOL

Cell group	1	2	3	4	5	6	7	8	9	10	A1	A2	B	O
Anti-A	C	—	C	—	+++	+++	++	++	—	—	C	+++	—	—
Anti-B	—	C	C	—	—	—	V	V	—	—	—	—	C	—
Anti-A+B	C	C	C	—	C	C	C	C	—	—	C	C	C	—
Patient's serum	—	—	—	—	—	—	—	—	—	—	<i>Controls</i>			
Serum group														
A ₁ cells	—	C	—	C	—	+	—	+	—	H				
B cells	C	—	—	C	C	C	—	—	—	H				
O cells	—	—	—	—	—	—	—	—	—	—				

Interpretation:

1. Group A
2. Group B
3. Group AB
4. Group O
5. Group A₁—confirm using lectin A₁ or specific anti-A₁.
6. Group A₁ with anti-A₁ in serum—confirm using lectin A₁ or specific anti-A₁. Test serum with A₂ cells—no reaction if anti-A₁.
7. Group A₁B—confirm using lectin A₁ or specific anti-A₁.
8. Group A₂B with anti-A₁ in serum—confirm using lectin A₁ or specific anti-A₁. Test serum with A₂ cells—no reaction if anti-A₁.
9. Group O. Cord blood. Note absence of 'naturally occurring' anti-A and anti-B.
10. Group O. Note presence of haemolysis rather than agglutination.

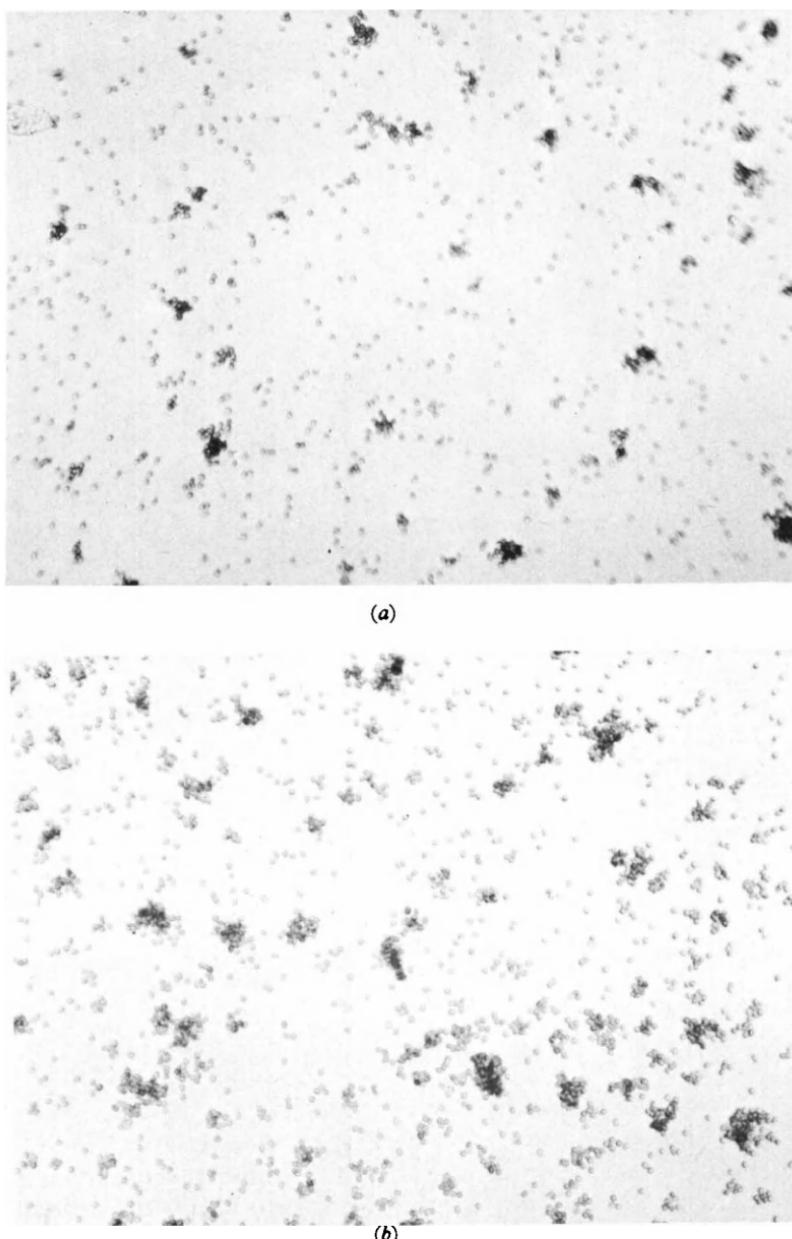


Figure 40.2. Microscopic agglutination: (a) + agglutination, (b) ++ agglutination

Cold agglutinins

The higher the titre of cold agglutinins the higher the temperature at which they will react (this will seldom be above 25 °C), and therefore if active at room temperature the auto-agglutination control will be positive as will the A₁, B and O cells, this type of reaction being termed pana-agglutination. High titre cold antibodies are sometimes formed during virus or atypical pneumonia (the causative organism often being *Mycoplasma pneumoniae*) lymphoma and rarely with infectious mononucleosis. They usually have blood group specificity within the I/i system (for further information of this system it is suggested that reference is made to a standard blood group serology textbook). When this type of reaction is encountered the patient's red cells should be washed in warm saline at 37 °C to remove the cold antibody, and the test repeated. The serum grouping must be repeated at 37 °C and the auto-agglutination control should be negative, although at this temperature any anti-A or anti-B present will still react.

CAUSES OF FALSE NEGATIVE REACTIONS IN ABO GROUPING

These are usually due to impotent sera which have deteriorated by being stored incorrectly or repeatedly frozen and thawed.

Failure to recognize the time factor in the tube method may cause false negative results to be reported.

Failure to recognize that haemolysis must be reported as such and not recorded as negative because no agglutination can be seen.

RHESUS GROUPING

A number of techniques are available for the detection of the Rhesus antigens but the commonest is probably the 'albumin addition' for the detection of the D antigen, which utilizes the incomplete or IgG antibody which is generally available. Complete or IgM sera are available, although not in such large quantities, but usually they are not as potent as the incomplete sera and interpretation of results tends to be more difficult. Ideally the patient's red cells should be tested with both an incomplete and a complete antibody, but usually two incomplete sera of different batches are used. As the Rhesus group cannot be 'back-checked' by using the

patient's serum, as in the ABO system, the use of two sera adds confidence to the results.

The detection of the Rhesus antigens C, D, E, c and e is technically the same for each, providing that care is taken to ensure that the relevant technique is used depending on whether the individual sera contain complete or incomplete antibodies.

Saline technique

To 1 volume of 3 per cent patient's red cells add 1 volume of saline operating antiserum, mix and incubate undisturbed at 37 °C for 2 h. Examine microscopically for agglutination. Positive and negative cells must also be included as controls.

Albumin addition technique

To 1 volume of 3 per cent patient's red cells add 1 volume albumin operating antiserum, mix and incubate undisturbed at 37 °C for 1½ h. Add, without disturbing the settled button of cells, 1 volume of 30 per cent bovine albumin, reincubate for a further 30 min at 37 °C. Examine for agglutination macroscopically by gently tapping the tube. Results are usually clear-cut but all apparent negative reactions must be examined microscopically.

Positive and negative cells must be included as controls using an identical technique to that of the test.

Emergency anti-D grouping

This is best performed with commercial anti-D sera, using a tile technique and following the manufacturer's instructions; these are usually similar to those described in the ABO tile method. D positive and negative cells must be included as controls. All emergency tile grouping must be confirmed using a tube technique.

Detection of Rhesus antibodies

As Rhesus antibodies exist in two different forms, namely IgM and IgG or saline and albumin operating, it is important that the method or combination of methods used will detect both forms. As a screening procedure the use of enzyme-treated red cells is ideal, and even small concentrations of Rhesus antibodies will be detected. The red cells usually used are of the genotype CD_e/cDE (R_1R_2) as these contain the Rhesus antigens C, D, E, c and e, and will be group O so that there will be no interference from any anti-A or anti-B which may be present in the serum. If the screening test is positive further tests will be necessary to determine the specificity of the antibody. A panel of red cells of various Rhesus

genotypes is used so that by a process of elimination the specificity of the antibody will be determined. The technique used will include a combination of the red cells being suspended in saline, 20 per cent bovine albumin, enzyme-treated red cells, and the use of the indirect antiglobulin test.

Enzyme-treated red cells

Treatment of red cells with enzymes will enable both IgM and IgG antibodies to be detected. The method of treating red cells with papain (Low's method) is described, which is the most commonly used enzyme.

Papain 2 g

*Sorensen's buffer pH 5.4 100 ml

Grind in a mortar and centrifuge for 10 min

Add 10 ml cysteine hydrochloride 0.5 mol

Make solution up to 200 ml with Sorensen's buffer and incubate at 37 °C for 1 h.

Add 1 volume of activated papain to 1 volume of patient's serum followed by 1 volume of the appropriate 3 per cent red cells. Incubate at 37 °C for 1½ h and read macroscopically.

The pre-treatment of red cells with papain gives greater sensitivity and this is usually the method of choice.

1 volume activated papain solution

9 volumes Sorensen's buffer pH 7.0.

Add equal volumes of papain and washed packed red cells and incubate in a water bath at 37 °C for 20 min. Wash the red cells twice in saline and dilute to 3 per cent. To 1 volume patient's serum add 1 volume 3 per cent pre-treated red cells and incubate at 37 °C for 1½ h. Read macroscopically.

THE ANTI-HUMAN GLOBULIN TESTS (COOMBS TESTS)

The direct anti-human globulin test (DAHGT)

This test is performed on the patient's red cells without any prior incubation, and demonstrates the sensitization of the red cells *in vivo* with antibody.

* Sorensen's buffer—stock solution: (a) M 15 potassium dihydrogen phosphate KH_2PO_4 (9.08 g/litre). (b) M 15 disodium hydrogen phosphate $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (11.88 g/litre).

For pH 5.4 add 9.6 parts of solution A to 0.4 parts solution B.

For pH 7.0 add 3.9 parts of solution A to 6.1 parts solution B.

The patient's red cells, preferably from a clot, are washed four times in isotonic saline using 75 mm × 12 mm disposable plastic tubes. A 20 per cent suspension of red cells in isotonic saline is prepared for the test.

Using a scrupulously clean opal glass tile place 1 volume of anti-human globulin serum (previously diluted to its optimum concentration) and 1 volume of isotonic saline into each of two previously marked or etched 1 in squares. Onto each add 1 volume of the previously washed 20 per cent suspension of patient's red cells. Mix using the end of a clean swab stick to a diameter of 20 mm and leave undisturbed for 1 min. After each minute view the cells for agglutination using transmitted light. The test is usually read for 5–7 min.

The agglutination of the red cells with anti-human globulin serum indicates that antibody has attached itself to the red cells in the patient's circulation. The red cells tested against isotonic saline should show no agglutination.

CONTROLS

To 1 volume of anti-human globulin serum is added 1 volume of 20 per cent red cells which have previously been sensitized with a weak IgG antibody, usually an incomplete anti-D, and washed as in the test. The use of this control indicates that the anti-human globulin serum being used is capable of detecting cells weakly sensitized with IgG, but in no way indicates that the anti-human globulin serum is operating on the site of the actual test. There are a number of reasons for this, but the commonest cause is that the patient's red cells have been inadequately washed leaving free globulin present which has neutralized the anti-human globulin serum. To ensure that the anti-human globulin serum is operating at the site of the test it is essential that to each negative test is added 1 volume of 20 per cent sensitized red cells and within 5–7 min agglutination is seen. If the test remains negative the patient's red cells must be re-washed in isotonic saline and the test repeated.

The indirect anti-human globulin test (IAHGT)

This test is usually used for the detection of antibody in a patient's serum using cells of known Rhesus genotype as the antigen and indicator. Occasionally this test is used in the reverse way to detect the presence of a red cell antigen, in which case known Rhesus antiserum is used against the unknown red cells.

Into a 75 mm · 12 mm disposable plastic tube place 2 volumes

of patient's serum and 1 volume of 50 per cent washed red cells*. Mix and incubate at 37 °C for 90 min. After incubation the test is performed exactly as for the direct anti-human globulin test described above.

*As a screening test the cells will be group O and usually have the genotype CDe/cDE (R_1R_2). If the screening test is positive a panel of various Rhesus genotyped cells must be used, and by a process of elimination the specificity of the antibody will be determined.

STORAGE OF RED CELLS IN GLYCEROL/CITRATE

The red cells required are collected in 3.8 per cent trisodium citrate and preparation for storage should commence immediately.

Centrifuge the sample and remove plasma. Re-suspend the packed red cells very slowly in an equal volume of the following solution:

5 per cent trisodium citrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) 6 parts
Glycerol 4 parts.

Distribute in 3 in $\times \frac{1}{2}$ in capped tubes in 1 ml amounts, or store in bulk and redistribute in smaller volumes as necessary. Store at $-20^{\circ}C$ or lower if possible. To recover the red cells, thaw at room temperature and re-suspend in solution 1, centrifuge and discard supernatant. Re-suspend in solution 2 and repeat washing followed by re-suspension in an equal volume of solution 2 and 0.85 per cent sodium chloride. The red cells are finally washed in 0.85 per cent sodium chloride and warmed to $37^{\circ}C$.

Solution 1. 12 per cent glycerol in 5 per cent trisodium citrate
Solution 2. 5 per cent glycerol in 5 per cent trisodium citrate.

It is easier to recover the red cells by dialysis using Visking tubing against physiological saline. The dialysis is continued overnight at $4^{\circ}C$, but reasonable red cell recovery is obtained after dialysis for 1 h.

AUTOMATED BLOOD GROUPING

With the increasing demand for blood grouping, both in the Blood Transfusion Centres and the larger hospital laboratories, a need

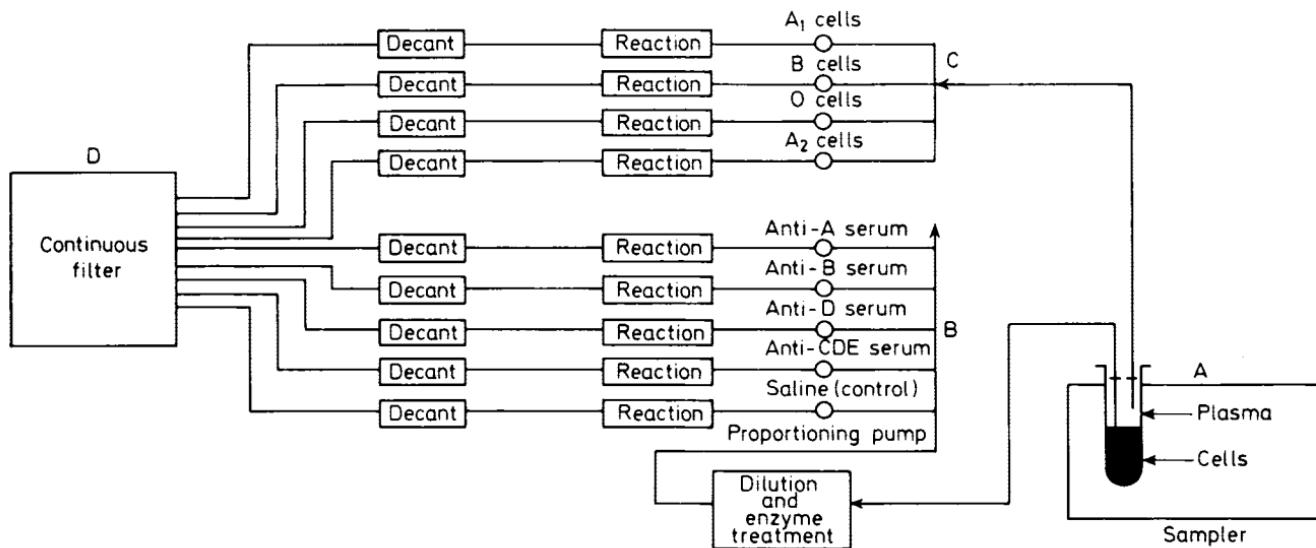


Figure 40.3. Diagram—simplified flow diagram of BG-9. (Reproduced by kind permission of Technicon Instruments Co. Ltd)

has been developed for the automation of blood groups. This need has been met by the Technicon BG-9* which allows both red cells and plasma to be typed, with the addition of an antibody screen, and operates at a speed of 120 samples per h (*Figure 40.3*).

ACD or EDTA samples in 5 ml amounts are centrifuged and placed in the Sampler module. At 30 s intervals a double sample probe dips into the centrifuged blood and simultaneously aspirates red cells and plasma into the system. Sampling occurs for 10 s followed by 20 s of physiological saline 'wash', segmented by air.

Each unknown plasma sample A is divided into four streams, which flow in identical paths through an all-glass manifold C. Sampler integrity is preserved by the conventional AutoAnalyser technique of segmenting sample and wash phases with air bubbles.

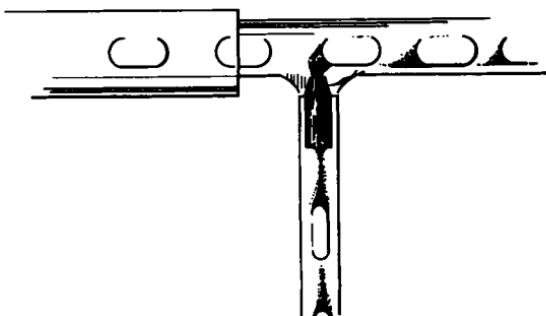


Figure 40.4. Decantation 'T'

Known red cells of group A₁, B, OR₁, R₂ and A₂ automatically join the plasma stream. Bromelin and polyvinylpyrrolidone are added to the reaction mix to enhance sensitivity. The polyvinylpyrrolidone, a macromolecule, induces rouleaux formation and further promotes the close physical contact of the cells. After dilution with physiological saline to disperse rouleaux, the mixture flows to a decant 'T'.

The agglutinated cells sediment more rapidly and are readily drawn into the limb of the tube, while unagglutinated cells pass to waste. The agglutinated cells are pumped onto the moving filter paper D, forming distinctive patterns for each blood group which are identified as agglutinated deposits.

The unknown cells which have been aspirated by the deep probe of the Sampler are diluted to 25 per cent with physiological saline, enzyme-treated with bromelin to enhance the reaction and then

* Technicon Instruments Company Limited.

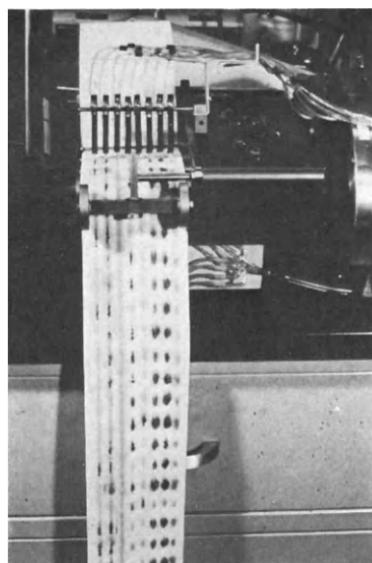


Figure 40.5. Continuous filter showing agglutinated cell patterns. (Reproduced by kind permission of Technicon Instruments Co. Ltd)

divided into streams B. The various antisera are added to each channel and the process continues in an identical manner to that of the plasma streams. The toughened 200 mm wide filter paper strip when dried may be kept as a permanent record.

Bibliography

Clinical Chemistry

- Foundations of Anatomy and Physiology* (1974). Ross, J. S. and Wilson, K. J. 4th Edn. Edinburgh; Livingstone
- Fundamentals of Clinical Chemistry* (1974). Tietz, N. W. 2nd Edn. Philadelphia; Saunders
- Microanalysis in Medical Biochemistry* (1964). Wootton, I. D. P. London; Churchill
- Review of Physiological Chemistry* (1969). Harper, H. A. Lange Medical Publications
- Textbook of Physiology and Biochemistry* (1972). Davidson, G. H. 8th Edn. Edinburgh; Livingstone

Haematology and Blood Transfusion Technique

- Bleeding Disorders, Investigation and Management* (1965). Hardisty, R. M. and Ingram, G. I. C. Oxford; Blackwell Scientific Publications
- Blood Groups in Man* (1975). Race, R. R. and Sanger, Ruth. Philadelphia; Lippincott
- Blood Transfusion in Clinical Medicine* (1967). Mollison, P. L. Oxford; Blackwell Scientific Publications
- Clinical Haematology* (1967). Wintrobe, M. M. 6th Edn. London; Kimpton
- Handbook of Haematological and Blood Transfusion Techniques* (1969). Delaney, J. W. and Garratty, G. 2nd Edn. London; Butterworths
- Practical Haematology* (1970). Dacie, J. V. and Lewis, S. M. 4th Edn. London; Churchill Livingstone

Histology

- Carleton's Histological Technique* (1967). Drury, R. A. B. and Wallington, E. A. 4th Edn. London; Oxford University Press
- H. J. Conn's Biological Stains* (1969). Lillie, R. D. 8th Edn. Baltimore; Williams and Wilkins

Handbook of Histopathological and Histochemical Techniques (1974). Culling, C. F. A. 3rd Edn. London; Butterworths
Histochemistry, Theoretical and Applied (1969). Pearse Everson, A. G. 3rd Edn. Baltimore; Williams and Wilkins

Microbiology

Clinical Bacteriology (1975). Stokes, E. J. 4th Edn. London; Arnold
Cowan and Steel's Manual for the Identification of Medical Bacteria (1974). Cowan, S. T. London; Cambridge University Press
Essential Immunology (1974). Roitt, I. Oxford; Blackwell Scientific Publications
Handbook of Bacteriological Technique (1967). Baker, F. J. London; Butterworths
Medical Microbiology (1974). Ed. by Cruickshank, R. 12th Edn. Edinburgh; Livingstone
Topley and Wilson's Principles of Bacteriology and Immunity (1975). Ed. by Wilson, G. S. and Miles, A. A. London; Arnold

Appendix

GENERAL NOTES

SI UNITS

SI units are basically divided into the following:
metre, kilogram, second, ampere, kelvin, candela, mole and litre.
All other units are derived from these.

<i>Physical Quantity</i>	<i>Name of SI unit</i>	<i>Symbol for SI</i>
length	metre	m
mass	kilogram	kg
time	second	s
electric current	ampere	A
thermodynamic temperature	kelvin	K
luminous intensity	candela	cd
amount of substance	mole	mol

Some derived SI units have special names and symbols; the ones we are concerned with are as follows:

<i>Quantity</i>	<i>Name of SI unit</i>	<i>Symbol</i>
work, energy, quantity, of heat	joule	J
power	watt	W
quantity of electricity	coulomb	C
electric potential, potential difference	volt	V
electric resistance	ohm	
pressure	pascal	Pa

PREFIXES FOR SI UNITS

<i>Fraction</i>	<i>Prefix</i>	<i>Symbol</i>	<i>Multiple</i>	<i>Prefix</i>	<i>Symbol</i>
10^{-1}	deci	d	10	deca	da
10^{-2}	centi	c	10^2	hecto	h

<i>Fraction</i>	<i>Prefix</i>	<i>Symbol</i>	<i>Multiple</i>	<i>Prefix</i>	<i>Symbol</i>
10^{-3}	milli	m	10^3	kilo	k
10^{-6}	micro	μ	10^6	mega	M
10^{-9}	nano	n	10^9	giga	G
10^{-12}	pico	p	10^{12}	tera	T
10^{-15}	femto	f			
10^{-18}	atto	a			

Although the SI unit of volume was given as the cubic metre (m^3), the litre (l) is still more generally recognized as the unit of volume and is exactly equal to one cubic decimetre (dm^3), i.e. $1000 \text{ litre} = 1 \text{ cubic metre}$. Because of its convenience the litre is used as the unit of volume in the laboratory. Multiples and submultiples of the litre should be used for all measurements of volume.

<i>SI Unit</i>	<i>Old Unit</i>
dl	100 ml
ml or cm^3	cc
μl	lambda
nl	
pl	$\mu\mu\text{l}$

The SI unit for mass is the kilogram (kg); the working unit is the gram (g). Multiples and submultiples of the gram should be used and not the kilogram.

<i>SI Unit</i>	<i>Old Unit</i>
kg	k, kg, kilogramme
g	gr, gm, gms, gramme
mg	mgm, mgms
μg	gamma
ng	mug
pg	$\mu\mu\text{g}$

Mass should not be confused with 'weight' which is measured in newtons. SI units for amount of substance is the mole (mol); this unit replaces the gram molecule, gram ion, gram equivalent and so on. It is recommended that the use of the equivalent and its submultiples commonly used for reporting the monovalent electrolyte measurements (sodium, potassium, chloride, bicarbonate) should be replaced by molar concentrations (mmol/l). For these four measurements the numerical value will not change.

<i>SI Unit</i>	<i>Old Unit</i>
mol	M, g-mol, eq
mmol	mM, mEq
μmol	μM
nmol	nM

The SI unit for length is the metre (m). The Ångstrom unit (\AA) should not be used and the measurements should be converted to nanometres (nm).

<i>SI Unit</i>	<i>Old Unit</i>
nm	$\text{m}\mu$
μm	μ (micron)

The SI symbol for 'day' (i.e. 24 hours) is 'd' but urine and faecal excretion of substances should be expressed as 'per 24 hours' (e.g. g/24 h).

The basic unit for thermodynamic temperature is the kelvin (K) not degree Kelvin ($^{\circ}\text{K}$). The customary working unit in medical laboratories is the degree Celsius (formally centigrade) ($^{\circ}\text{C}$).

BRITISH STANDARD COLOURS FOR MEDICAL GAS CYLINDERS

<i>Nature of gas</i>	<i>Colour of cylinder</i>
Oxygen	Black with white top
Nitrous oxide	Blue
Cyclopropane	Orange
Carbon dioxide	Grey
Ethylene	Mauve
Helium	Brown
Nitrogen	Grey with black top
Oxygen and carbon dioxide mixture	Black bottom with grey and white top
Oxygen and helium mixture	Black bottom with brown and white top
Air	Grey bottom with black and white top

CONVERSION FACTORS

FAHRENHEIT (F) AND CELSIUS (CENTIGRADE) (C)

TEMPERATURES

To convert F into C:

Subtract 32 and multiply by $\frac{5}{9}$

To convert C into F:

Multiply by $\frac{9}{5}$ and add 32

DILUTION OF SOLUTIONS

The following equation is useful when the dilution of solutions of known strengths is required.

Where R=Required concentration

V=Total volume of solution required

O=Original concentration

$$\frac{R \times V}{O} = \begin{matrix} \text{Volume of original solution to be diluted with} \\ \text{distilled water to the final volume required.} \end{matrix}$$

Example. The original solution is 70 per cent: 45 ml of 30 per cent solution is required.

Using the equation

$$\frac{30 \times 45}{70} = 19.3$$

Therefore, 19.3 ml of 70 per cent solution must be diluted with 25.7 ml of distilled water to obtain 45 ml of a 30 per cent solution.

MEASUREMENT CONVERSION

Kilometres-Miles			Temperature		
km	m or km	miles	centigrade		fahrenheit
1.609	1	0.621	- 30	-	22
3.218	2	1.242	- 20	-	4
4.827	3	1.864	- 10	+	14
6.437	4	2.485	- 5	+	23
8.046	5	3.107	0	+	32
9.655	6	3.728	+ 5	+	41
11.265	7	4.350	+ 10	+	50
12.874	8	4.971	+ 20	+	68

14.483	9	5.592	+ 30	+ 86
16.093	10	6.214	+ 36.9*	+ 98.4*
32.186	20	12.428	+ 40	+ 104
40.232	25	15.535	+ 50	+ 122
80.465	50	31.070	+ 60	+ 140
160.930	100	62.136	+ 70	+ 157
321.860	200	124.272	+ 80	+ 176
482.790	300	186.408	+ 90	+ 194
643.720	400	248.544	+ 100	+ 212
804.650	500	310.680	*normal body temp.	

Metres-Yards

<i>m</i>	<i>y or m</i>	<i>y</i>
0.914	1	1.094
1.829	2	2.187
2.743	3	3.281
3.658	4	4.374
4.572	5	5.468
5.486	6	6.562
6.401	7	7.655
7.315	8	8.749
8.230	9	9.843
9.144	10	10.936
18.288	20	21.872
22.860	25	27.340
45.720	50	54.681
91.439	100	109.361
457.195	500	546.805

Litres-Gallons

<i>l</i>	<i>g or l</i>	<i>g</i>
4.45	1	0.22
9.09	2	0.44
13.64	3	0.66
18.18	4	0.88
22.73	5	1.10
27.28	6	1.32
31.82	7	1.54
36.37	8	1.76
40.91	9	1.98
45.46	10	2.20
90.92	20	4.40
136.38	30	6.60
181.84	40	8.80
227.30	50	11.00

Metres-Feet

<i>m</i>	<i>f or m</i>	<i>f</i>
0.305	1	3.281
0.610	2	6.562
0.914	3	9.842
1.219	4	13.123
1.524	5	16.404
1.829	6	19.685
2.134	7	22.966
2.438	8	26.247
2.743	9	29.528
3.048	10	32.808
7.620	25	82.022

Kilograms-Pounds

<i>kg</i>	<i>lb or kg</i>	<i>lb</i>
0.453	1	2.205
0.907	2	4.409
1.360	3	6.614
1.814	4	8.818
2.268	5	11.023
2.721	6	13.228
3.175	7	15.432
3.628	8	17.637
4.082	9	19.841
4.535	10	22.046
11.339	25	55.116

BOILING POINTS

The following boiling points are correct to the nearest degree Celsius.

<i>Substance</i>	C
Acetic acid	118
Acetone	56
Amyl alcohol	130
Benzene	80
Butyl alcohol	118
Caprylic alcohol	180
Carbon disulphide	46
Chloroform	62
Ether	34
Ethyl alcohol	78
Methyl alcohol	65
Toluene	111
Water	100
Xylenes	138–144

SOLUTIONS OF ACIDS AND ALKALIS

Dilution of concentrated acids and alkalis to make approximately normal solutions.

<i>Acids</i>	<i>Ml diluted to 1000 ml with distilled water</i>
Acetic (glacial)	60
Hydrochloric	100
Nitric	63
Sulphuric	28

<i>Alkalis</i>	
Ammonium hydroxide	50
Potassium hydroxide (solid)	58 g
Sodium hydroxide (solid)	42 g

BUFFER SOLUTIONS*(After Clark and Lubs)*

To 50 ml 0.2 mol KH phthalate (40.844 g/l), add the following amounts of N/5 HCl and dilute to 200 ml.	To 50 ml 0.2 mol KH phthalate add the following amounts of N/5 NaOH (CO ₂ free), and dilute to 200 ml.		
pH	Amount of N/5 HCl in ml	pH	Amount of N/5 NaOH in ml
2.2	46.60	4.0	0.40
2.4	39.60	4.2	3.65
2.6	33.00	4.4	7.35
2.8	26.50	4.6	12.00
3.0	20.40	4.8	17.50
3.2	14.80	5.0	23.65
3.4	9.95	5.2	29.75
3.6	6.00	5.4	35.25
3.8	2.65	5.6	39.70
		5.8	43.10
		6.0	45.40
		6.2	47.00

BUFFER SOLUTIONS (cont)

To 50 ml 0.2 mol KH_2PO_4 (27.219 g/l) add the following amounts of N/5 NaOH and dilute to 200 ml.	To 50 ml 0.2 mol $\text{KCl} \cdot \text{H}_3\text{BO}_3$ (14.912 g KCl · 12.369 g H_3BO_3 l), add the following amounts of N/5 NaOH and dilute to 200 ml.
<i>pH</i>	<i>Amount of N/5 NaOH in ml</i>
6.0	5.70
6.2	8.60
6.4	12.60
6.6	17.80
6.8	23.65
7.0	29.65
7.2	35.00
7.4	39.50
7.6	42.80
7.8	45.20
8.0	46.80
<i>pH</i>	<i>Amount of N/5 NaOH in ml</i>
7.8	2.61
8.0	3.97
8.2	5.90
8.4	8.50
8.6	12.00
8.8	16.30
9.0	21.30
9.2	26.70
9.4	32.00
9.6	36.85
9.8	40.80
10.0	43.90

Notes

1. Providing the salts are in correct ratio to each other, the exact degree of the final dilution is not important.
2. Use the purest chemicals available when preparing buffer solutions.
3. Use freshly distilled or de-ionized water with pH 6.7–7.3.
4. Store the buffer solutions in polythene bottles or Pyrex glass with closely fitting stoppers.
5. Providing the reagents are in correct ratio to each other, the exact degree of the final dilution is not important.

SATURATED SOLUTIONS

(After Bayley)

Substance	Solubility in g per 100 ml of distilled water		
	0 °C	Various temperatures	100 °C
Ammonium chloride	29.7		75.8
Ammonium oxalate	2.54		34.8
Ammonium sulphate	70.6		103.8
Aniline		3.4 at 20 °C	
Barium chloride	31.0		59.0
Barium sulphate		0.00023 at 18 °C	
Barium sulphide		Decomposes in water	
Benzidine		Only slightly soluble	
Benzoic acid		0.27 at 18 °C	
Bromine		3.58 at 20 °C	
Calcium carbonate		0.0014 at 25 °C	
Calcium chloride (anhyd.)	59.5		
Calcium chloride (cryst.)	279.0		
Calcium hydroxide	0.185		0.077
Calcium oxalate		0.0014 at 95 °C	
Cholesterol		Only slightly soluble	
Citric acid	130.0	116 °C at 25 °C	
Copper hydroxide		Insoluble in water	
Copper oxide		Insoluble in water	
Cupric sulphate	31.6		203.3
Cuprous sulphate		Decomposes in water	
Ferric ammonium sulphate		124 at 25 °C	
Ferric chloride	74.4		535.7
Ferric oxide		Insoluble in water	
Lithium carbonate		1.33 at 20 °C	0.72
Magnesium carbonate		Only slightly soluble	
Magnesium sulphate		71.0 at 20 °C	
Mercuric chloride		6.9 at 20 °C	61.3
Naphthalene		Insoluble in water	
Osmium tetroxide		6.23 at 25 °C	
Oxalic acid		9.5 at 15 °C	
Phenol		6.7 at 16 °C	
Phloroglucinol		Only slightly soluble	
Phosphorus pentoxide		Decomposes in water	
Picric acid		1.4 at 20 °C	
Potassium acetate		253 at 20 °C	
Potassium carbonate		112 at 20 °C	156
Potassium chloride		34.7 at 20 °C	56.7
Potassium chromate		62.9 at 20 °C	79.2
Potassium dichromate	4.9		102
Potassium hydroxide		107 at 15 °C	178
Potassium iodide	127.5		208

SATURATED SOLUTIONS (cont)

Substance	Solubility in g per 100 ml of distilled water		
	0 C	Various temperatures	100 C
Potassium metabisulphite		Only slightly soluble	
Potassium nitrate		31.6 at 20 C	247
Potassium nitrite		31.3 at 25 C	413
Potassium oxalate		33 at 16 C	
Potassium permanganate		6.33 at 20 C	
		25.0 at 65 C	
Silver nitrate	122.0		
Sodium acetate	76.2		
Sodium carbonate	7.1		45.5
Sodium chloride	35.7		39.12
Sodium citrate		92.6 at 25 C	250.0
Sodium hydroxide	42.0		347.0
Sodium nitrate	73.0		180.0
Sodium nitrite		83.3 at 15 C	
Sodium oxalate		3.7 at 20 C	6.33
Sodium thiosulphate	79.4	291.1 at 45 C	
Zinc hydroxide		Insoluble in water	
Zinc sulphate		86.5 at 80 C	

SOME ELEMENTS AND THEIR SYMBOLS

Element	Symbol	Atomic No.	Atomic weight
Aluminium*	Al	13	26.9815
Arsenic	As	33	74.9216
Barium*	Ba	56	137.34
Bromine	Br	35	79.909
Calcium*	Ca	20	40.08
Carbon	C	6	12.01115
Chlorine	Cl	17	35.453
Chromium*	Cr	24	51.996
Copper (Cuprum)*	Cu	29	63.54
Gold (Aurum)*	Au	79	196.967
Hydrogen	H	1	1.00797
Iodine	I	53	126.9044
Iron (Ferrum)*	Fe	26	55.847
Lead (Plumbum)*	Pb	82	207.19
Lithium*	Li	3	6.939
Magnesium*	Mg	12	24.312
Manganese*	Mn	25	54.9380
Mercury (Hydrargyrum)*	Hg	80	200.59

Nitrogen	N	7	14.0067
Oxygen	O	8	15.9994
Phosphorus	P	15	30.9738
Potassium (Kallium)*	K	19	39.102
Silicon	Si	14	28.086
Silver (Argentum)*	Ag	47	107.870
Sodium (Natrium)*	Na	11	22.9898
Sulphur	S	16	32.064
Tin (Stannum)*	Sn	50	118.69
Tungsten (Wolfram)*	W	74	183.85
Uranium*	U	92	238.03
Zinc*	Zn	30	65.37

The atomic weights are taken from *International Atomic Weights*, 1961. The metallic elements are marked with an asterisk.

LOGARITHMS

	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	9	13	17	21	26	30	34	38
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	12	15	19	23	27	31	35
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	11	14	18	21	25	28	32
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	7	10	13	16	20	23	26	30
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	28
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	9	11	14	17	20	23	26
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	14	16	19	22	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	8	10	13	15	18	20	23
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8

Note.—These tables are so constructed that the fourth figure of a logarithm obtained by their use is never more than one unit above or below the best 4-figure approximation. E.g. if the logarithm found

LOGARITHMS

	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9083	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9963	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4

is 0.5014 the best 4-figure approximation may be 0.5013, or 0.5015. Greater accuracy than this cannot be obtained by the use of a uniform table of differences of this kind.

ANTILOGARITHMS

	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9
.00	1000	1002	1005	1007	1009	1012	1014	1016	1019	1021	0	0	1	1	1	1	2	2	2
.01	1023	1026	1028	1030	1033	1035	1038	1040	1042	1045	0	0	1	1	1	1	2	2	2
.02	1047	1050	1052	1054	1057	1059	1062	1064	1067	1069	0	0	1	1	1	1	2	2	2
.03	1072	1074	1076	1079	1081	1084	1086	1089	1091	1094	0	0	1	1	1	1	2	2	2
.04	1096	1099	1102	1104	1107	1109	1112	1114	1117	1119	0	1	1	1	1	1	2	2	2
.05	1122	1125	1127	1130	1132	1135	1138	1140	1143	1146	0	1	1	1	1	1	2	2	2
.06	1148	1151	1153	1156	1159	1161	1164	1167	1169	1172	0	1	1	1	1	1	2	2	2
.07	1175	1178	1180	1183	1186	1189	1191	1194	1197	1199	0	1	1	1	1	1	2	2	2
.08	1202	1205	1208	1211	1213	1216	1219	1222	1225	1227	0	1	1	1	1	1	2	2	3
.09	1230	1233	1236	1239	1242	1245	1247	1250	1253	1256	0	1	1	1	1	1	2	2	3
.10	1259	1262	1265	1268	1271	1274	1276	1279	1282	1285	0	1	1	1	1	1	2	2	3
.11	1288	1291	1294	1297	1300	1303	1306	1309	1312	1315	0	1	1	1	1	2	2	2	3
.12	1318	1321	1324	1327	1330	1334	1337	1340	1343	1346	0	1	1	1	1	2	2	2	3
.13	1349	1352	1355	1358	1361	1365	1368	1371	1374	1377	0	1	1	1	1	2	2	3	3
.14	1380	1384	1387	1390	1393	1396	1400	1403	1406	1409	0	1	1	1	1	2	2	3	3
.15	1413	1416	1419	1422	1426	1429	1432	1435	1439	1442	0	1	1	1	1	2	2	2	3
.16	1445	1449	1452	1455	1459	1462	1466	1469	1472	1476	0	1	1	1	1	2	2	2	3
.17	1479	1483	1486	1489	1493	1496	1500	1503	1507	1510	0	1	1	1	1	2	2	2	3
.18	1514	1517	1521	1524	1528	1531	1535	1538	1542	1545	0	1	1	1	1	2	2	2	3
.19	1549	1552	1556	1560	1563	1567	1570	1574	1578	1581	0	1	1	1	1	2	2	3	3
.20	1585	1589	1592	1596	1600	1603	1607	1611	1614	1618	0	1	1	1	1	2	2	3	3
.21	1622	1626	1629	1633	1637	1641	1644	1648	1652	1656	0	1	1	2	2	2	3	3	3
.22	1660	1663	1667	1671	1675	1679	1683	1687	1690	1694	0	1	1	2	2	2	3	3	3
.23	1698	1702	1706	1710	1714	1718	1722	1726	1730	1734	0	1	1	2	2	2	3	3	4
.24	1738	1742	1746	1750	1754	1758	1762	1766	1770	1774	0	1	1	2	2	2	3	3	4
.25	1778	1782	1786	1791	1795	1799	1803	1807	1811	1816	0	1	1	2	2	2	3	3	4
.26	1820	1824	1828	1832	1837	1841	1845	1849	1854	1858	0	1	1	2	2	2	3	3	4
.27	1862	1866	1871	1875	1879	1884	1888	1892	1897	1901	0	1	1	2	2	2	3	3	4
.28	1905	1910	1914	1919	1923	1928	1932	1936	1941	1945	0	1	1	2	2	2	3	3	4
.29	1950	1954	1959	1963	1968	1972	1977	1982	1986	1991	0	1	1	2	2	2	3	3	4
.30	1995	2000	2004	2009	2014	2018	2023	2028	2032	2037	0	1	1	2	2	2	3	3	4
.31	2042	2046	2051	2056	2061	2065	2070	2075	2080	2084	0	1	1	2	2	2	3	3	4
.32	2089	2094	2099	2104	2109	2113	2118	2123	2128	2133	0	1	1	2	2	2	3	3	4
.33	2138	2143	2148	2153	2158	2163	2168	2173	2178	2183	0	1	1	2	2	2	3	3	4
.34	2188	2193	2198	2203	2208	2213	2218	2223	2228	2234	1	1	2	2	2	3	3	4	4
.35	2239	2244	2249	2254	2259	2265	2270	2275	2280	2286	1	1	2	2	2	3	3	4	4
.36	2291	2296	2301	2307	2312	2317	2333	2328	2333	2339	1	1	2	2	2	3	3	4	4
.37	2344	2350	2355	2360	2366	2371	2377	2382	2388	2393	1	1	2	2	2	3	3	4	4
.38	2399	2404	2410	2415	2421	2427	2432	2438	2443	2449	1	1	2	2	2	3	3	4	4
.39	2455	2460	2466	2472	2477	2483	2489	2495	2500	2506	1	1	2	2	2	3	3	4	5
.40	2512	2518	2523	2529	2535	2541	2547	2553	2559	2564	1	1	2	2	2	3	3	4	4
.41	2570	2576	2582	2588	2594	2600	2606	2612	2618	2624	1	1	2	2	2	3	4	4	5
.42	2630	2636	2642	2649	2655	2661	2667	2673	2679	2685	1	1	2	2	2	3	4	4	5
.43	2692	2698	2704	2710	2716	2723	2729	2735	2742	2748	1	1	2	2	2	3	4	4	5
.44	2754	2761	2767	2773	2780	2786	2793	2799	2805	2812	1	1	2	2	2	3	4	4	5
.45	2818	2825	2831	2838	2844	2851	2858	2864	2871	2877	1	1	2	3	3	4	4	5	6
.46	2884	2891	2897	2904	2911	2917	2924	2931	2938	2944	1	1	2	3	3	4	4	5	6
.47	2951	2958	2965	2972	2979	2985	2992	2999	3006	3013	1	1	2	3	3	4	4	5	6
.48	3020	3027	3034	3041	3048	3055	3062	3069	3076	3083	1	1	2	3	4	4	4	5	6
.49	3090	3097	3105	3112	3119	3126	3133	3141	3148	3155	1	1	2	3	4	4	4	5	6

These logarithm and antilogarithm tables originally appeared in *Four Figure Tables and Constants for the Use of Students*, published by Her Majesty's Stationery Office. They are reproduced here by courtesy of the Controller of Her Majesty's Stationery Office.

ANTILOGARITHMS

	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9	
-50	3162	3170	3177	3184	3192	3199	3206	3214	3221	3228	1	1	2	3	4	4	4	5	6	7
-51	3236	3243	3251	3258	3266	3273	3281	3289	3296	3304	1	2	2	3	4	5	5	6	7	
-52	3311	3319	3327	3334	3342	3350	3357	3365	3373	3381	1	2	2	3	4	5	5	6	7	
-53	3388	3396	3404	3412	3420	3428	3436	3443	3451	3459	1	2	2	3	4	5	6	6	7	
-54	3467	3475	3483	3491	3499	3508	3516	3524	3532	3540	1	2	2	3	4	5	6	6	7	
-55	3548	3556	3565	3573	3581	3589	3597	3606	3614	3622	1	2	2	3	4	5	6	7	7	
-56	3631	3639	3648	3656	3664	3673	3681	3690	3698	3707	1	2	3	3	4	5	6	7	8	
-57	3715	3724	3733	3741	3750	3758	3767	3776	3784	3793	1	2	3	3	4	5	6	7	8	
-58	3802	3811	3819	3828	3837	3846	3855	3864	3873	3882	1	2	3	4	4	5	6	7	8	
-59	3890	3899	3908	3917	3926	3936	3945	3954	3963	3972	1	2	3	4	5	5	6	7	8	
-60	3981	3990	3999	4009	4018	4027	4036	4046	4055	4064	1	2	3	4	5	6	6	7	8	
-61	4074	4083	4093	4102	4111	4121	4130	4140	4150	4159	1	2	3	4	5	6	7	8	9	
-62	4169	4178	4188	4198	4207	4217	4227	4236	4246	4256	1	2	3	4	5	6	7	8	9	
-63	4266	4276	4285	4295	4305	4315	4325	4335	4345	4355	1	2	3	4	5	6	7	8	9	
-64	4365	4375	4385	4395	4406	4416	4426	4436	4446	4457	1	2	3	4	5	6	7	8	9	
-65	4467	4477	4487	4498	4508	4519	4529	4539	4550	4560	1	2	3	4	5	6	7	8	9	
-66	4571	4581	4592	4603	4613	4624	4634	4645	4656	4667	1	2	3	4	5	6	7	9	10	
-67	4677	4688	4699	4710	4721	4732	4742	4753	4764	4775	1	2	3	4	5	7	8	9	10	
-68	4786	4797	4808	4819	4831	4842	4853	4864	4875	4887	1	2	3	4	6	7	8	9	10	
-69	4898	4909	4920	4932	4943	4955	4966	4977	4989	5000	1	2	3	5	6	7	8	9	10	
-70	5012	5023	5035	5047	5058	5070	5082	5093	5105	5117	1	2	3	4	6	7	8	9	11	
-71	5129	5140	5152	5164	5176	5188	5200	5212	5224	5236	1	2	4	5	6	7	8	10	11	
-72	5248	5260	5272	5284	5297	5309	5321	5333	5346	5358	1	2	4	5	6	7	9	10	11	
-73	5370	5383	5395	5408	5420	5433	5445	5458	5470	5483	1	3	4	5	6	8	9	10	11	
-74	5495	5508	5521	5534	5546	5559	5572	5585	5598	5610	1	3	4	5	6	8	9	10	12	
-75	5623	5636	5649	5662	5675	5689	5702	5715	5728	5741	1	3	4	5	7	8	9	10	12	
-76	5754	5768	5781	5794	5808	5821	5834	5848	5861	5875	1	3	4	5	7	8	9	11	12	
-77	5888	5902	5916	5929	5943	5957	5970	5984	5998	6012	1	3	4	5	7	8	10	11	12	
-78	6026	6039	6053	6067	6081	6095	6109	6124	6138	6152	1	3	4	6	7	8	10	11	13	
-79	6166	6180	6194	6209	6223	6237	6252	6266	6281	6295	1	3	4	6	7	9	10	11	13	
-80	6310	6324	6339	6353	6368	6383	6397	6412	6427	6442	1	3	4	6	7	9	10	12	13	
-81	6457	6471	6486	6501	6516	6531	6546	6561	6577	6592	2	3	5	6	8	9	11	12	14	
-82	6607	6622	6637	6653	6668	6683	6699	6714	6730	6745	2	3	5	6	8	9	11	12	14	
-83	6761	6776	6792	6808	6823	6839	6855	6871	6887	6902	2	3	5	6	8	9	11	13	14	
-84	6918	6934	6950	6966	6982	6998	7015	7031	7047	7063	2	3	5	6	8	10	11	13	15	
-85	7079	7096	7112	7129	7145	7161	7178	7194	7211	7228	2	3	5	7	8	10	12	13	15	
-86	7244	7261	7278	7295	7311	7328	7345	7362	7379	7396	2	3	5	7	8	10	12	13	15	
-87	7413	7430	7447	7464	7482	7499	7516	7534	7551	7568	2	3	5	7	9	10	12	14	16	
-88	7586	7603	7621	7638	7656	7674	7691	7709	7727	7745	2	4	5	7	9	11	12	14	16	
-89	7762	7780	7798	7816	7834	7852	7870	7889	7907	7925	2	4	5	7	9	11	13	14	16	
-90	7943	7962	7980	7998	8017	8035	8054	8072	8091	8110	2	4	6	7	9	11	13	15	17	
-91	8128	8147	8166	8185	8204	8222	8241	8260	8279	8299	2	4	6	8	9	11	13	15	17	
-92	8318	8337	8356	8375	8395	8414	8433	8453	8472	8492	2	4	6	8	10	12	14	15	17	
-93	8511	8531	8551	8570	8590	8610	8630	8650	8670	8690	2	4	6	8	10	12	14	16	18	
-94	8710	8730	8750	8770	8790	8810	8831	8851	8872	8892	2	4	6	8	10	12	14	16	18	
-95	8913	8933	8954	8974	8995	9016	9036	9057	9078	9099	2	4	6	8	10	12	15	17	19	
-96	9120	9141	9162	9183	9204	9226	9247	9268	9290	9311	2	4	6	8	11	13	15	17	19	
-97	9333	9354	9376	9397	9419	9441	9462	9484	9506	9528	2	4	7	9	11	13	15	17	20	
-98	9550	9572	9594	9616	9638	9661	9683	9705	9727	9750	2	4	7	9	11	13	16	18	20	
-99	9772	9795	9817	9840	9863	9886	9908	9931	9954	9977	2	5	7	9	11	14	16	18	20	

The copyright of the part of the tables giving the logarithms of numbers from 1,000 to 8,000 is the property of Messrs. Macmillan & Co. Ltd., who have authorized their reprint for educational purposes.

SQUARE ROOTS OF 0·1 TO 2·09

(After G. A. Harrison)

	0·00	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09
0·1	0·32	0·33	0·35	0·36	0·37	0·39	0·40	0·41	0·42	0·44
0·2	0·45	0·46	0·47	0·48	0·49	0·50	0·51	0·52	0·53	0·54
0·3	0·55	0·56	0·57	0·57	0·58	0·59	0·60	0·61	0·62	0·62
0·4	0·63	0·64	0·65	0·66	0·66	0·67	0·68	0·69	0·69	0·70
0·5	0·71	0·71	0·72	0·73	0·73	0·74	0·75	0·76	0·76	0·77
0·6	0·77	0·78	0·79	0·79	0·80	0·81	0·81	0·82	0·82	0·83
0·7	0·84	0·84	0·85	0·85	0·86	0·87	0·87	0·88	0·88	0·89
0·8	0·89	0·90	0·91	0·91	0·92	0·92	0·93	0·93	0·94	0·94
0·9	0·95	0·95	0·96	0·96	0·97	0·97	0·98	0·98	0·99	0·995
1·0	1·00	1·01	1·01	1·02	1·02	1·03	1·03	1·03	1·04	1·04
1·1	1·05	1·05	1·06	1·06	1·07	1·07	1·08	1·08	1·09	1·09
1·2	1·10	1·10	1·11	1·11	1·11	1·12	1·12	1·13	1·13	1·14
1·3	1·14	1·15	1·15	1·15	1·16	1·16	1·17	1·17	1·18	1·18
1·4	1·18	1·19	1·19	1·20	1·20	1·20	1·21	1·21	1·22	1·22
1·5	1·23	1·23	1·23	1·24	1·24	1·25	1·25	1·25	1·26	1·26
1·6	1·27	1·27	1·27	1·28	1·28	1·29	1·29	1·29	1·30	1·30
1·7	1·30	1·31	1·31	1·32	1·32	1·32	1·33	1·33	1·33	1·34
1·8	1·34	1·35	1·35	1·35	1·36	1·36	1·36	1·37	1·37	1·38
1·9	1·38	1·38	1·39	1·39	1·39	1·40	1·40	1·40	1·41	1·41
2·0	1·41	1·42	1·42	1·43	1·43	1·43	1·44	1·44	1·44	1·45

Glossary

Abscess: a localized formation of pus in a cavity produced by the breakdown of tissues.

Absorption: in bacteriology and haematology, the removal of specific antibodies from a serum by mixing with their specific antigen.

Absorption band: that point in the spectrum, seen in a spectroscope, which is blocked out by the absorption of the solution under test. It is expressed in terms of the wavelength of the obliterated light, either in nanometres or Angström units.

Acidophilic: staining readily with acid dyes.

Acute: short and severe; not chronic.

Adipose: of a fatty nature, fatty, fat.

Adsorption: condensation of gases on surfaces of solids (distinct from absorption).

Aerobe: an organism requiring oxygen for multiplication.

Aerosol: 1. A colloid in which the dispersion medium is a gas.

2. A solution finely atomized to sterilize air.

3. A medicinal solution atomized to a fine mist for inhalation.

Afferent: conducting inwards, or towards, for example, afferent nerves.

Agglutination: clumping together of red cells or bacteria, usually the result of antigen-antibody reaction.

Agglutinin: antibody capable of causing the agglutination of its specific antigen.

Agglutinogen: an antigen which will react with a specific anti-serum.

Albumin: a water-soluble heat-coagulable protein found in animal tissues.

Alicyclic compounds: ring compounds resembling aliphatic compounds.

Aliphatic compounds: open chain compounds. One of the major groups of organic compounds.

Allergy: a specific hyper-sensitiveness to a substance which is harmless for the majority of members of the same species.

Allotropy: the existence of a chemical element or compound in two or more forms, each with different physical properties, for example, carbon as diamond, charcoal or blacklead.

Ambulant: able to walk, not confined to bed.

Amoeba: a minute unicellular protozoan.

Amorphous: substances which are not crystalline, for example, amorphous urates and phosphates in urine.

Ampere: the current produced by the electromotive force of one volt in a wire having the resistance of one ohm.

Ampoule: small glass container capable of being sealed to preserve the contents in a sterile condition.

Amyloid disease: a disease in which liver, spleen and kidney tissue is replaced by an amorphous wax-like material.

Amylopsin: enzyme of pancreas which hydrolyses complex carbohydrates into soluble sugars.

Anabolism: the elaboration of complex substances from simpler compounds, with absorption and storage of energy—a metabolic process.

Anaerobe: an organism not requiring oxygen for multiplication.

Analytical reagents: pure chemicals of known composition.

Anaphylosis: a reaction produced in a sensitized animal by injection of a substance to which the animal has been sensitized.

Anatomy: the study of the structure of the animal body.

Anatoxin or toxoid: a toxin which has been inactivated by formalin and heat.

Aneurysm: a sac produced by the dilatation of the walls of a blood vessel.

Angström unit: one ten-millionth of a millimetre. Unit of length used for measurement of wavelengths of light.

Anhydride: the anhydride of a substance is that substance after chemical removal of water, which gives the substance itself; for example, acetic acid minus water forms acetic anhydride.

Anhydrous: without water. Often refers to a salt which crystallizes without water, for example, NaCl.

Anion: negatively charged ion attracted to the anode during electrolysis.

Anisocytosis: variation in the sizes of red cells, greater than is normally present, usually in association with an anaemic state.

Anode: positive electrode.

Ante-: prefix meaning before, in time or place, for example, antecubital, situated in front of the cubitus, or forearm.

Anterior: situated in the forward or frontal part.

Anti-: prefix denoting opposite.

Antibiosis: an association between two organisms which is detrimental to one of them.

Antibiotic: an antibacterial substance of biological origin.

Anticoagulant: a substance which when added to blood prevents clotting.

Antiseptic: a substance that will inhibit the growth of organisms without necessarily destroying them.

Antitoxin: a substance that will neutralize toxins.

Aorta: main artery of the body, leaving the left ventricle of the heart.

Aqueous: watery, especially with reference to solutions having water as solvent.

Arachnoid: a membrane surrounding the brain and spinal cord and interposed between the dura mater and pia mater.

Aromatic compounds: ring compounds containing at least one benzene ring.

Artefact (microscopical): an artificial optical effect.

Artery: a vessel through which blood is conveyed from the heart.

Ascitic fluid: an accumulation of fluid in the peritoneal cavity.

Ascorbic acid: vitamin C.

Asepsis: freedom from infection.

Aspiration: 1. Drawing in of breath.

2. Withdrawal of fluid from a cavity by means of suction.

Assimilation: the absorption and utilization of digested food-stuffs by the tissue cells.

Atmospheric pressure: the pressure of the air upon the earth. At sea level approximately 15 lb per in².

Atomizer: apparatus for producing a fine spray.

Atypical: differing from type.

Auto-: a prefix signifying self, for example, auto-agglutination—agglutination of blood corpuscles of an animal by its own serum.

Auto-agglutinin: an antibody present in a donor's serum which will react with the donor's red blood cells.

Autogenous: self-produced; originating within the organism.

Autologous: a normal occurrence in a tissue structure.

Autolysis: the destruction of tissues by autogenous enzymes.

Autolytic: pertaining to autolysis.

Autopsy: post mortem examination of a body.

Bacillus: a rod-shaped organism.

Bacteria: minute simple-celled organisms.

Bacterial: pertaining to bacteria.

Bacteriology: the study of bacteria.

Bacteriostatic: preventing the multiplication of bacteria.

Bence-Jones protein: a protein which coagulates at 60 °C but redissolves at higher temperatures.

Benign: not malignant.

Benzene: benzol (C_6H_6), hydrocarbon found in coal tar.

Bi-: prefix denoting two, or twice.

Biconcave: having two concave surfaces.

Biconvex: having two convex surfaces.

Bifurcation: a division to form two branches.

Bile: a fluid produced by the liver which assists in emulsification and absorption of fats.

Bilirubin: the pigment of bile, derived from haemoglobin breakdown.

Binocular: pertaining to both eyes, for example, binocular microscope.

Biochemistry: the chemistry of living matter.

Biology: the study of life in general.

Biopsy: diagnostic examination of tissue removed during life.

Botulism: food poisoning due to a toxin produced by *C. botulinum*.

Bovine: pertaining to ox, cow or heifer.

British Thermal Unit: amount of heat required to raise the temperature of 1 lb of water through 1 °F.

Bronchus: either of the two main branches of the trachea.

Budding: asexual reproduction by division into two parts, the larger parent body, and smaller bud, for example, yeast.

Buffer solution: a solution which resists change of pH.

Buffy coat: the layer of white cells and platelets between the red cells and the supernatant plasma of centrifuged blood.

Bouillon: a meat broth used as a culture medium.

Calculus: an abnormal concretion of mineral salts formed within the body, for example, renal calculus.

Calorie: amount of heat required to raise the temperature of 1 g of water through 1 °C.

Cane sugar: sucrose, saccharose ($C_{12}H_{22}O_{11}$). Identical with beet sugar.

Capillary: hair-like, for example, capillary tube.

Caramelization: the process whereby a brown complex is produced when sugar is heated.

Carbohydrate: organic compounds of carbon, hydrogen and oxygen, for example, sugars, gums, starches and cellulose.

Cardiac: relating to the heart.

Carrier: in bacteriology, a person who harbours specific organisms without necessarily having symptoms of disease, but is capable of spreading the infection to other persons.

Casein: principal protein of milk.

Catabolism: that part of metabolic process dealing with the breakdown of complex substances into simpler compounds with a release of energy.

Catalyst: a substance which alters the rate of a reaction.

Catheter: a tube for withdrawing fluids, for example, urine.

Cathode: negative electrode.

Cation: positively charged ion which is attracted to the cathode during electrolysis.

Cell (Biol.): a minute protoplasmic mass containing a nucleus.

Cellular: composed of cells.

Cellulose: a carbohydrate forming the outer walls of plant cells.

Centi-: prefix denoting 1/100th part, for example, a centimetre is 1/100th of a metre.

Centrifugation: the process of separating a lighter portion of a solution, mixture or suspension from the heavier portion, by centrifugal force.

Cerebral: pertaining to the cerebrum.

Cerebrum: the main portion of the brain—consists of two hemispheres.

Chemotherapy: the treatment of disease by the administration of chemicals.

Chlorophyll: the green colouring substance of plants.

Chromatic: pertaining to colour, for example, monochromatic.

Chromatin: deeply staining portion of the cell nucleus.

Chromosomes: small dark staining rod-shaped bodies present in the cell nucleus during division.

Chronic: long-standing; not acute.

Chyle: milky looking fluid of lymph and emulsified fat absorbed into the intestine after digestion of food.

Clarify: to clear of turbidity.

Cocci: spherical bacteria.

Colloidal state: the condition in which molecules in solution are aggregated together to form solid particles held in suspension.

Colony (Bact.): a collection or group of bacteria on a solid culture medium.

Combustion: a chemical combination with oxygen with the production of heat, light and flame.

Complement: a thermolabile substance formed in normal blood serum. Used in complement fixation tests.

Congenital: existing at the time of birth; born with.

Crenated: with a notched or toothed edge, for example, crenated red blood cell.

Crystal: the definite geometric form of solidified substances. Most pure substances have characteristic crystalline forms.

Culture: the propagation of micro-organisms.

Current: a flow of electricity.

Cutaneous: relating to the skin.

Cyst: a hollow sac containing a liquid or semi-solid fluid.

Cytology: the study of cells.

Cytolysis: the destruction of cells.

Cytoplasm: the protoplasmic structure of a cell, other than the nucleus.

Dark ground illumination: the system used in microscopy in which the central rays of light are blocked out and the peripheral ones are directed obliquely against the object, showing it up brightly on a dark background.

Defibrination: the removal of fibrin.

Dehydration: loss or removal of water.

Deliquesce: the absorption of water from the air by certain chemicals so that they eventually dissolve, for example, NaOH and P₂O₅.

Density: the weight of a substance divided by its volume.

Dermatology: the study of skin.

Dermis: the true skin.

Desiccation: the removal of water: drying.

Dextrin: mixture of carbohydrates obtained by partial hydrolysis of starch.

Dextrose: see **Glucose**.

Diastase: enzyme which converts starches into sugars.

Diphasic: occurring in two phases.

Disinfectant: a substance capable of destroying micro-organisms.

Distillation: the process of converting a liquid into a vapour, condensing the vapour and collecting the condensed liquid or distillate.

Dorsal: relating to the back.

Dura mater: the outermost of the three membranes (meninges) surrounding the brain and spinal cord.

Dysgonic: poorly growing.

Efferent: conducting outwards or away.

Efflorescence: the loss of water of crystallization on exposure to air, for example, crystals of washing soda crumble into powder.

Electrolysis: Chemical decomposition of certain substances (electrolytes) by an electric current.

Embryology: the study of the development of the embryo.

Emulsion: a milky fluid containing fatty droplets in a state of suspension.

Endo-: a prefix denoting within, for example, endogenous, occurring within the body.

Endothermic reaction: a chemical process accompanied by absorption of heat.

Enzymes: organic substances produced by living cells, which act as catalysts.

Equilibrium: a state of balance.

Erythroblast: a nucleated red cell of any development stage, whether involved in normal or abnormal maturation.

Eugonic: growing luxuriantly.

Exo-: a prefix denoting outside, for example, exospores, spores occurring outside a hair fibre.

Exothermic reaction: a chemical process accompanied by release of energy in the form of heat.

Expectoration: clearing of secretions from the respiratory tract by coughing.

Faeces (stool): matter excreted by the bowels.

Filtration: passing of a fluid through a filter to separate fluids from solids.

Flaccid: soft, flabby, not firm.

Flash point: the temperature at which the vapour of a combustible fluid will ignite if exposed to a naked flame.

Flocculation: a flaky precipitation.

Fractional crystallization: a process for the separation of a mixture of dissolved substances by making use of their differing solubilities.

Freund's adjuvant: dead mycobacteria in an oil base, to which may be introduced antigens of various biological types.

Fructose: fruit sugar, $C_6H_{12}O_6$.

Fungi: a class of vegetable organisms of a low order of development and lacking chlorophyll.

Gland: an organ capable of secretion.

Globulin: a characteristic type of protein insoluble in water.

Glucose (dextrose): grape sugar, $C_6H_{12}O_6$.

Glycogen: a carbohydrate stored in various tissues (mainly the liver). It can be converted into glucose and released into the blood stream.

Habitat: the natural abode or home of an organism.

Haem-: a prefix meaning pertaining to blood.

Haematology: the study of blood and blood-forming tissues.

Hepatic: pertaining to the liver.

Heterocyclic compounds: organic ring compounds which include atoms of elements other than carbon, for example, pyridine C_5H_5N .

Histology: the study of the structure of tissues.

Homo-: prefix denoting the same.

Homogeneous: of uniform composition throughout.

Hormones: substances, produced by endocrine glands, which specifically regulate organic functions; for example, insulin, produced in pancreas, regulates blood sugar levels.

Hyaline: glass-like; transparent.

Hydrates: compounds forming crystals which contain water of crystallization, for example, copper sulphate crystals.

Hydro-: prefix denoting water.

Hydrolysis: the chemical decomposition of a substance by water, the water being decomposed during the process.

Hydrometer: instrument for measuring the specific gravity of liquids.

Hygrometer: instrument for measuring humidity of the atmosphere.

Hygroscopic: solids which absorb water from the air without liquefaction, for example, $NaCO_3$, and liquids which absorb moisture, for example, H_2SO_4 .

Hyper-: prefix denoting over or above, for example, hypertonic meaning of greater osmotic pressure.

Hypo-: prefix denoting under or below, for example, hypotonic.

Hypo: abbreviation for sodium thiosulphate.

- Immune:** protected against any particular disease.
- Immunize:** to render immune.
- Inactivation:** destruction of the activity of serum by action of heat, that is at 56° C for 30 min.
- Indicator:** substance which indicates the pH of a liquid or which denotes the end of a chemical reaction by a colour change.
- Inert:** chemically inactive.
- Infection:** invasion and injury of the tissues of the body by pathogenic organisms.
- Inferior:** situated below.
- Inflammatory:** relating to inflammation.
- Inhibition:** arrest or restraint of a process.
- Inoculation:** the introduction of any organism or substances into the body tissues.
- Inoculum:** the substance used in inoculation.
- Insipillation:** the process of thickening or condensing often by heat or evaporation, for example Loeffler's serum slopes.
- Inter-:** prefix denoting between, among. Intercellular means between cells.
- Intra-:** prefix meaning within, for example, intracellular.
- Ion:** a charged atom.
- Iso-:** prefix denoting equal, for example, isotonic solutions, meaning of equal osmotic pressure.
- Iso-agglutinin:** an antibody which will agglutinate all red blood cells of the same species.
- Isomerism:** phenomenon occurring where compounds have the same molecular formulas but different physical and chemical properties for example, lactose, maltose and sucrose.
- Isotonic saline:** 0.85% sodium chloride in distilled water, which has the same osmotic pressure as red blood cells.
- Jaundice:** a yellow discolouration of the skin due to an excess of bilirubin in the blood.
- Lactose:** milk sugar, $C_{12}H_{22}O_{11}$.
- Laevulose:** fruit sugar; see **Fructose**.
- Lake:** 1. the colour formed between a dye and a mordant.
2. to cause blood to lyse.
- Latent heat:** heat required to convert a solid into a liquid, or a liquid into a vapour without change of temperature.
- Lesion:** an alteration in the structure of living tissues by injury or disease.
- Leucopenia:** a total white cell count lower than normal.

Lipids: fats.

Litre: metric unit of volume. The volume of 1 kilogram of pure air-free water at 4°C and 760 mm pressure. A litre is equivalent to 1000 ml or 1000.027 cc.

Lumen: the interior of a tubular structure.

Lymph: a transparent, alkaline fluid filling lymphatic vessels.

Lysis: a breaking up or dissolution, for example, haemolysis, disintegration of red blood cells.

Macro-: large, the opposite of **Micro-**.

Macrophage: a large mononuclear phagocytic cell.

Macroscopic: visible without the aid of a microscope.

Malignant: virulent; tending to deteriorate.

Maltose: malt sugar, $C_{12}H_{22}O_{11}$.

Manometer: an instrument for measuring the pressures exerted by liquids and gases.

Medial: relating to the middle.

Metabolism: the sum of all the physical and chemical processes of a living organism.

Micro-: 1. very small;

2. one millionth part (metric system), for example, microgram.

Microscopy: the use of the microscope.

Microtomy: the preparation of thin sections.

Miscible: capable of being mixed to form a homogeneous substance, for example, ethyl alcohol and water.

Mitochondria: rod-shaped structures found in the cytoplasm of cells.

Mitosis: indirect cell division. The nucleus resolves into chromosomes, which then divide longitudinally. The halves separate and form a complete nucleus in each daughter cell.

Mono-: prefix meaning one, for example, mononuclear.

Monomorphism: uniformity of structure.

Mordant: substance used to bring about a staining reaction which otherwise would not occur.

Morphology: the study of the structure and forms of organized beings.

Motility (Bact.): the ability to move spontaneously through the surrounding medium.

Multi-: prefix meaning many.

Necrosis: death of a circumscribed piece of tissue.

Neo-: prefix meaning new growth, for example, neoplasm.

Nephro-: prefix denoting kidney.

Nerve: structure which conveys impulses and sensations in the body.

Neurology: the study of the nervous system.

Non-volatile: liquids which can remain exposed to air for an indefinite period without evaporating, for example, oil.

Normochromia: stained red cell appearance when each cell has the normal content of haemoglobin.

Normocytosis: blood film term denoting red cells of normal size.

Nucleus: in cytology, a spheroid body within a cell, with a dense structure, and containing chromatin; essential and vital part of a cell.

Oedema: excessive fluid in the tissues.

Oesophagus: the gullet.

Ohm: the resistance of a column of mercury at 0 °C, 14.452 g in weight with a cross-sectional area of 1 mm² and 106.30 cm long.

Optimum: most favourable conditions.

Organic chemistry: the chemistry of carbon compounds.

Osmosis: a process whereby liquids of different concentrations, separated by a semi-permeable membrane, percolate and mix until their concentrations are equal.

Osteology: the study of the skeleton.

Oxidation: combination with oxygen (or removal of hydrogen).

Para-: prefix meaning beside, against, apart from.

Parallax: a shift in the apparent position of an object due to a change in position of the observer.

Parasite: a plant or animal living upon or within another living organism at whose expense it gains advantage, without any compensations.

Parasitology: the study of parasites.

Parentral: not through the alimentary canal.

Pathogen: any disease-producing organism.

Pathology: science dealing with the nature of disease and the structure and functional changes it brings about.

Pellicle: a thin skin or film: the scum on the surface of a liquid.

Pentose: a sugar with the formula C₅H₁₀O₅.

Pepsin: a gastric enzyme which converts proteins into peptone in an acid medium.

- Pepsin:** a gastric enzyme which converts protein into peptone by the action of pepsin.
- Peri-:** prefix denoting around, about, for example, periphery.
- Peristalsis:** muscular contractions of the alimentary tract which propel food material along.
- Peritoneum:** the membrane lining the abdominal cavity and the organs contained within it.
- pH:** the symbol used to denote acidity and alkalinity. It is the logarithm of the reciprocal of the hydrogen ion concentration, to base 10.
- Phagocyte:** a cell capable of ingesting micro-organisms and other substances.
- Photosynthesis:** process by which plants containing chlorophyll can manufacture carbohydrates in the presence of light and air.
- Physiology:** science dealing with structure and function of living organisms.
- Pia mater:** the innermost of the three membranes surrounding the brain and spinal cord.
- Plasma:** fluid portion of the blood.
- Plasmolysis:** contraction of cells due to loss of water by osmosis.
- Pleomorphic:** occurring in various distinct forms.
- Pleura:** membranes which line the thoracic cavity and surround the lungs.
- Poly-:** prefix denoting many.
- Post-:** prefix denoting behind or after, for example, posterior, post-mortem.
- Pre-:** prefix meaning before.
- Proteins:** characteristic constituents of animal tissues. They are combinations of α amino acids mostly condensed together by the peptide linkage.
- Proteolytic:** having the power to decompose proteins.
- Protoplasm:** the main constituent of cells, of a colloidal nature, with water, proteins, carbohydrates, lipids and inorganic salts.
- Protozoa:** unicellular animal organisms.
- Pseudo-:** prefix meaning false.
- Pyrogens:** substances, generally of bacterial origin, which are thermostable and able to pass bacterial filters. May cause a reaction in the recipient if present in intravenous solutions.
- Qualitative analysis:** the determination of the chemical nature of substances present.

Quantitative analysis: the determination of the amount of substances.

Reduction (Chem.): removal of oxygen (or the addition of hydrogen).

Renal: pertaining to the kidney.

Respiration: the act of breathing, that is, inspiration and expiration.

Reticular: resembling a network.

Retro-: prefix denoting behind or backwards.

Rigor: 1. a chill accompanied by a fever;
2. stiffening, for example, rigor mortis.

Ringer's solution: solution of same osmotic pressure as body fluids.

Rouleaux formation: a property of serum which causes red blood cells to stack like a pile of coins. Must not be confused with agglutination.

Saponin: a compound capable of laking blood.

Saprophytes: organisms which live on dead organic matter.

Saturated (solution): one that can exist unchanged in contact with the solid (solute) which is partly in solution.

Scarification: the making of many small cuts in the skin.

Serology: the study of serum.

Serous: pertaining to serum.

Serum: fluid portion of blood which separates after blood has clotted.

Slough: a mass or sheet of dead tissue cast off from living tissue.

Solubility: the extent to which a solute will dissolve in a solvent. Usually measured in g per 100ml at a given temperature. The substance in solution is called the solute. The liquid in which the solute is dissolved is called the solvent.

Spatula: a flat blunt instrument used for depressing tongues and transferring samples of chemicals.

Specific gravity: the ratio of the mass of a given substance to the mass of an equal volume of water, at the same temperature.

Spectroscope: an instrument for viewing a spectrum.

Spectrum: the coloured bands into which light is split when passed through a prism.

Sphygmomanometer: an instrument for measuring blood pressure.

Starch: carbohydrate stored by certain plants, $(C_6H_{10}O_5)_n$.

Sterile: free from contamination with living organisms.

Stool: faecal discharge from the bowels.

Striated: having lines or furrows.

Sub-: a prefix denoting beneath or under.

Sublimation (Chem.): the conversion of a solid direct into a vapour and its subsequent condensation. A method of purification.

Sucrose: cane sugar, $C_{12}H_{22}O_{11}$.

Superior: higher, above.

Supernatant: the liquid above a solid deposit.

Supersaturated solution: a solution containing more solute than the saturation value.

Symbiosis (Bact.): an association between two different organisms which is of mutual benefit.

Tachometer: an instrument for measuring velocity, for example, speed of a centrifuge in revolutions per minute.

Tertiary: third.

Therapy: treatment of disease.

Thermo-: prefix denoting relation to heat, for example, thermometer.

Thermolabile: easily altered or decomposed by heat. As applied to serum, losing its activity at a temperature of 55–56 °C.

Thermostable: not easily affected by heat.

Thorax: the chest and its enclosed organs.

Tincture: a solution of a medicinal substance in alcohol.

Tissue: an aggregation of cells similar in structure and function.

Titration: volumetric determination using standard solutions.

Titre: the highest dilution of a serum that will still react with its specific antigen.

Tonicity: condition of tension, for example, tonicity of muscle.

Toxic: poisonous.

Trachea: wind pipe.

Turgid: firmly distended with blood or fluid.

Ultra-: prefix denoting beyond, in excess of, for example, ultracentrifuge.

Uraemia: condition due to the accumulation in the blood of toxic substances normally excreted by the kidneys.

Urobilin: Brownish pigment found in faeces; an oxidized form of urobilinogen.

Urobilinogen: colourless compound formed in intestines by reduction of bile and excreted in faeces where it is oxidized to urobilin. Occasionally found in urine.

Vacuole: a clear space filled with fluid, such as found in the protoplasm of amoebae.

Vaporization: the conversion of a liquid into a vapour.

Vein: a vessel through which blood is conveyed towards the heart.

Velocity: rate of motion in a given direction.

Venepuncture: introduction of a needle into a vein for the withdrawal of blood or the injection of a fluid.

Virulence(Bact.): the relative infectiousness of a micro-organism.

Virus: a micro-organism so small that it will pass through Chamberland sterilizing filter, cannot generally be seen by ordinary microscopy and will only grow in the presence of living cells.

Viscera: the large interior organs, for example, abdominal viscera.

Viscosity: quality of being viscous.

Viscous: sticky, gummy, viscid.

Vitamins: accessory food factors necessary for a well-balanced diet.

Volatile: liquid capable of rapid evaporation, for example, acetone.

Volt: the unit of electromotive force which would cause the flow of 1 ampere through a resistance of 1 ohm.

Xanthochromic: having a yellow colour.

Xanthous: (pronounced zanthus) yellow.

Yeast: a unicellular fungus lacking in mycelium.

Zymase: enzyme found in yeasts, acts on glucose to form ethyl alcohol and CO₂.

Zyme: a ferment.

Index

- Aberration in microscopes, 29
Absorptiometers, 117
calibration for haemoglobin, 602
cells and cuvettes, 119
galvanometers, 121
light sources, 117
photoelectric cells, 120
wavelength selection, 118
- Absorption, 172
intestinal, 185
- Absorption bands, 128
- Accidents, treatment of, 64
- Acetest, 265
- Acetic acid,
fixative, as, 316
mode of action of, 84
- Acetoacetic acid in urine, 262
- Acetone, dehydration of tissue with, 339
- Achromatic spindle, 305
- Acid balsam, 399
- Acid dyes for staining, 388
- Acids,
poisoning with, 64
storage of, 62
strong, 80, 93
weak, 93, 94
- Acids and bases, 80
- Acid-base balance, 158
- Acid-base chart, 81
- Acid-base titrations, 92
- Acid-citrate-dextrose, 650
- Acid haematin method of haemoglobin estimation, 599
- Acridine orange technique, 435
- Addison's disease, 166
- Adrenal glands,
function of, 165
hormones from, 164
- Adrenaline, 162
- Adrenocorticotrophic hormone, 161
function of, 163
- Adsorption indicator method for chloride, 107
- Agar, 482
blood slopes, 491
chocolate slopes, 491
deoxycholate-citrate, 497
Fildes' slopes, 491
filtration of, 490
glucose, 492
MacConkey's, 496
mannitol salt, 504
nutrient, 489
media made from, 491
serum slopes, 491
thiosulphate citrate bile salt, 504
- Agar diffusion method of antibiotic assay, 541
- Agglutination tests, 517
- Albert's stain, 459
- Albuminized slides, 380
- Albumin, bovine, 665
- Albumin addition technique, 672
- Albuminuria, 243
pseudo-, 248
- Albustix, 247
- Alcian blue-chlorantine fast red, 419
- Alcian blue-periodic acid Schiff staining, 418
- Alcohol,
dehydration of tissue with, 338
sterilization with, 470
storage of, 62
- Alcohol-ether fixative, 324
- Aldosterone, 163, 165
- Alimentary tract, 181
- Alkaline haematin method of haemoglobin estimation, 600
- Alkaline methylene blue stain (Loeffler's), 463
- Aloxite sharpening stone, 369
- Alpha rays, 135
- Alum haematoxylin (Harris), 402, 432
- Ames reagents, precautions in use, 268
- Amino acids, 174
- p-Aminosalicylic acid (PAS), 263

- Ammonia, storage of, 62
 Ammonium, identification, 88
 Ammonium thiocyanate solution,
 0.1 mol, 105
 standard, 274
 Anabolism, 154
 Anaemia, 549
 haemolytic, 245
 hypochromic, 609
 iron deficiency, 627
 Mediterranean, 558
 Anaerobic jar, 511
 carbon dioxide cultivation, for, 514
 testing of, 512
 Anaerobic organisms, culture media for,
 506
 Analysis,
 automated, 138
 discrete systems, 141
 sequential multiple analysis sys-
 tems, 141
 blood gases, 156, 159
 continuous flow systems, 138
 coulometric, 137
 potentiometric, 132
 qualitative, 87
 quantitative, 89
 sequential multiple systems, 141
 volumetric, 89
 golden rules, 95
 preparation of solutions, 94
 use of factors in, 95
 Andrade's indicator, 494
 Androgens, 165
 Aneurine, 177
 Anions, identification, 88
 Anisochromasia, 594
 Anthrax, demonstration of bacilli,
 464
 Antibiotics,
 assay of, 540
 sensitivity tests, 537
 Antibodies,
 ABO, 632, 643
 Rhesus, 643
 Anticoagulants, 562
 blood transfusion, in, 650
 Antidiuretic hormone, 170
 Antigens,
 ABO, 629
 D^e, 640, 671
 H and O, 516
 hepatitis associated (Australia), 652
 Antigens—contd.
 particulate, 515
 Rhesus, 647
 Antigen-antibody reactions, 515-519
 Anti-human globulin test (*See Coombs*
 test)
 Antiseptics, 469
 Apparatus, 20-24 (*See also under specific*
 items)
 Arkansas sharpening stone, 368
 Aschoff's gelatin embedding method,
 352
 Ascitic fluids, smears from, 430
 Ascorbic acid, 179
 Asparagin-mineral salt solution, 502
 Augmented histamine test, 190
 normal values of, 194
 results of, 193
 Auramine stain, 457
 Australia antigen, 652
 Autoanalyser, 138, 141
 Autoclaves, 441, 468
 Automated analysis, 138
 Automatic tissue processors, 344
 Autopsy specimens, collection of, 74
 Avogadro's law, 79
 Ayre spatula, 429
 BTL anaerobic jar, 511
 Baar's fluid, 614
 Bacilli,
 acid fast, stains for, 457
 morphology, 445
Bacillus anthracis, 545
Bacillus cereus, 545
Bacillus stearothermophilus, 469
Bacillus subtilis, 525, 528, 545
 Bacteria, 443
 acid-fast, staining of, 421
 aerobic, 447
 anaerobic, 447
 cultivation of, 510
 associations, 449
 biology of, 447
 carbon sources, 448
 classification, 445
 cultures (*See under Cultures*)
 endotoxins, 450
 energy sources of, 448
 enzymes, 447
 examination of specimens, 522
 genetic changes in, 449

- Bacteria—*contd.*
Gram-positive and Gram-negative, 454
identification tests, 535
intestinal, media for culture, 496
metabolism, 447
microscopic examination of, 451–464
hanging drop preparations, 451, 453
smear making, 452
stains for acid-fast bacteria, 457
wet preparations, 454
morphology, 445
sensitivity tests for antibiotics, 537
specimen examination, 520
sporing, 446
staining, 454
acid-fast, 457
capsule, 462
- Bactericides, 469
Bacteriostats, 469
Balsam, neutral, 399
Barr bodies, demonstration of, 436
Barrett's alcoholic picric acid, 402
Basal metabolic rate, 155
Bases, 80
strong, 81, 93
weak, 93, 94
Basic dyes for staining, 388
Basins, evaporating, 11
Basophilia, 594
Basophils, polymorphonuclear, 551
Battlement method of differential leucocyte count, 584
Beakers, 8
Beer's law, 114, 117, 122, 125
Beer Lambert law, 115
Bence-Jones protein, detection, 249
electrophoresis, 250
Harrison's method, 249
urine, in, 248
Benedict's qualitative test, 251
Benedict's solution, standardization of, 271
Benzene, clearing with, 340
- Bertalanffy acridine orange technique, 435
Beta rays, 135
Bial's test for pentose, 257
Bicarbonate, measurement, 171
Bile,
composition of, 220
function of, 220
secretion of, 219
Bile duct, 185
Bile pigments, 222
detection of, 224
jaundice, in, 223, 224
metabolism of, 221
urine, in,
Fouchet's test, 224
ictotest, 225
Bile salts, 220, 222
urine, in, 230
Bilirubin, 222, 223, 556
detection of, 224
faecal, 226
Biliverdin, 222
meconium, in, 226
Biopsy specimens, collection of, 74
Biotin, 178
Biuret reaction, 272
Bleeding time, 613, 614
Blood,
antibiotic assay in, 540
buffering system, 158
cerebrospinal fluid, in, 288
collection of specimens, 67, 68, 564
frozen, 654
pH, renal maintenance of, 234
storage of, 653
transportation of, 654, 658
urea in, 275, 276
diacetyl monoxime estimation, 280
Nessler's reagent for, 277
urine, in, 244
Blood banks, 654
Blood cells,
enumeration of, 564
sources of error, 578
maturation of, 553
staining methods, 586
structure and function of, 549
Blood clotting time, 613, 615
Blood coagulation, 559–563
factors, 560, 616, 651
intrinsic-extrinsic mechanism, 560
Morawitz theory of, 559

- Blood coagulation—*contd.*
 tests for, 613–618
- Blood counts, 566
 bulk dilution method, 572
 chambers and coverglass, 566
 diluting pipettes, 571
 electronic machines, 579
 pipettes for, 571
- Blood cultures, 523
- Blood dilution
 fluids, 572
 for red cell counting, 568
- Blood donors, 652
- Blood gas analysis, 156, 159
- Blood glucose, 207
 estimation of, 208, 212
 following meals, 215
- Blood groups and grouping,
 ABO, 627
 antibodies, 632, 643
 antigens, 629
 cold agglutinins, 671
 false negatives, 671
 false positives in, 667
 incidence of, 631
 incompatibility, 648
 infected red cells in, 668
 infected serum in, 668
 inheritance of, 635
 rouleaux formation, 667
 serum, 633
 technique of, 666
 apparatus for, 663
 automated, 675
 Coombs test, 645, 673
 errors in, 664
 I, 649
 immunoglobulins, 642
 iso-haemolysins anti-A and anti-B,
 632
 Kell, 649
 Kidd, 649
 lectins, 634
 Lewis and Lutheran, 649
 MNSs system, 649
 medico-legal aspects, 649
 P, 649
 recording of results, 665
 Rhesus system, 638
 detection of, 671
 detection of antibodies, 672
 emergence detection, 672
 specific substances, 630
- Blood groups and grouping—*contd.*
 storage of serum, 664
 sub-groups of A, 628, 631
 differentiation of, 667
 inheritance of, 636
 technique, 663–678
- Blood platelets, 549, 553
 counts, 582, 613
 development of, 555
- Blood preparations, 659
- Blood substitutes, 659
- Blood sugar, 207
 estimation of, 208
- Blood transfusion, 627, 650–662
 anticoagulants, 650
 apparatus, 661
 compatibility testing or cross matching,
 659
 donors, 652
 National Blood Transfusion Service,
 651
 pH and, 650
 packed red cells, 650
 storage, 653
 frozen blood, of, 654
 substitutes and preparations, 659
 transportation, 654
 refrigeration and, 658
 whole, 650
- Body fluids, 167
- Body temperature, 156
- Boiling water, sterilization with, 467
- Bombay blood, 631
- Bone, for sectioning, 328
- Bordetella*, identification, 542
- Bordetella pertussis*, 546
 culture media for, 504
 transport media, 507
- Bordet–Gengou medium, 506
- Bottles, 9
 heparin, 72
 polythene, 9
 reagents, for, 9
 sequestrene (EDTA), 72
 specific gravity, 239
 sterile universal, 72
 ‘trap’, 18
- Bouin’s solution, 321
- Boyle’s law, 78
- Brain,
 fixation, 325
 structure of, 283
- Brain thromboplastin, 616

- Breast smears, preparation of, 428
Brewer's broth, 506
British Drug Houses capillitor outfit, 486
Bromide, identification, 88
Broths, 481
 blood, 489
 chocolate, 489
 digest, 482, 488
 Fildes', 489, 492
 glucose, 489, 511
 infusion, 481, 487
 meat extract, 481, 488
 preparation of, 487
 serum, 489
 thioglycollate (Brewers), 506
 types of, 481
Browne's sterilizer control tubes, 468
Brownian movement, 453
Brucella, identification, 542
Brucella abortus, 546
Brucella melitensis, 546
Buccal smears, Barr bodies in, 436
Buffering system of blood, 158
Buffer solutions, 84
Burettes, 7, 9
Burker counting chamber, 568
Burns and scalds, treatment of, 64
- Calcified tissue, sectioning, 329
Calcitonin, 160, 167
Calcium, 179
 identification, 88
 in tissue, 328, 423
 section cutting and, 375
Calibration curves, colorimetry, for, 124
Calories, 155, 181
Cambridge rocking microtome, 358
Canada balsam, 396, 399
Cancer, diagnosis by smears, 426, 435
Candida albicans, 531
Capillary blood, collection of, 564
Capsule staining, 462
Carbohydrates, 172
 blood, in, 207
 culture media, 493
 function of, 173
 metabolism, 220
 staining, 462
Carbol-fuchsin, dilute, 456
Carbol-fuchsin-tergitol staining, 421
¹⁴Carbon, 136
- Carbon dioxide,
 cultivation, anaerobic jars for, 514
 dissolved in blood, 157
 refrigeration, 658
 solid, 658
Carbon disulphide, clearing with, 341
Carbon monoxide poisoning, 157
Carbon tetrachloride, clearing with, 341
Carbonate, identification, 89
Carbonic anhydrase, 157
Carboxyhaemoglobin, 556
Carlson-Ford filter sheets, 474
Carmine, 384
Carnoy's fluid, 323
Castaneda, 524
Catabolism, 154
Catalase activity, 535
Cations, identification of, 87
Cedar wood oil, clearing with, 341
Celestin blue stain, 406
Celloidin sections, 311, 353, 366
 cutting, 382
Celloidinization, 380
Cells,
 blood (See Blood cells)
 centrosomes, 303
 chromatin, 304
 cytoplasm, 299
 inclusions, 303
 organelles in, 302
 staining, 388, 391, 404
 division (mitosis), 305
 endoplasmic reticulum, 302
 Golgi apparatus, 302
 lysosomes, 303
 membrane, 300
 mitochondria, 302
 nucleus, 304
 membrane, 304
 staining, 388, 402, 404, 405, 406, 407
 nucleolus, 304
 pigments in, 304
 secretion granules, 304
 structure of, 299, 300, 301
 substances binding, 307
Central nervous system, fixation of, 325
Centrifuges, 20
 tubes, 10, 21
Centromeres, 305
Centrosomes, 303
Cerebrospinal fluid, 283-296
 appearance of, 288
 bacteriological examination of, 524

- Cerebrospinal fluid—*contd.*
 blood in, 288
 cell count, 288
 chlorides in,
 EEL chloride meter method, 294
 Mohr's method, 293
 coagulum, 288
 composition of, 286
 formation of, 284
 function of, 284
 globulin in, 291
 glucose in, 295
 importance of examination, 286
 obtaining of, 286
 pathological changes in, 287, 288
 protein in, 288
 sulphosalicylic acid-sodium sulphate method, 291
 trichloracetic acid method, 289
 turbidimetric estimation, 289
 turbidity of, 288
- Cervical smears, preparation of, 428, 429
- Charles' law, 78
- Chelating agents, decalcification with, 336
- Chemicals, storage of, 62
- Chemistry,
 clinical, 154–171
 fundamentals of, 77–111
 laws of, 77 (*See also under specific laws*)
- Chemolithotrophs, 448
- Chloride meter method of estimation, 275
- Chlorides,
 CSF, in,
 EEL chloride meter method for, 294
 Mohr's method, 293
 estimation of, 137
 identification, 88
 sweat, in, 206
 titration of,
 adsorption indicator method, 107
 Volhard's method, 106
 urine, in, 273
 chloride meter method, 275
 Volhard's test, 273
- Chlorine, sterilization with, 470
- Chloroform,
 clearing with, 340
 sterilization with, 470
- Cholesterol, 176
- Chromatids, 305
- Chromatin, 304
- Chromatography, 143–153
 absorbents, 149
 adsorption, 143
 application of sample, 149
 column, 143
 ion exchange, 145
 one-dimensional, 144, 147
 paper, 143
 paper partition, 143, 146
 preparation of plates, 149
 terminology, 143
 thin layer, 143, 144, 148
 two-dimensional, 144
 visualization, 150
- Chromic acid as fixative, 316
- Chromophores, 386
- Chromosomes, 305
- Chylomicrons, 175
- Citrate–citric acid buffer in decalcification, 335
- Clayden test, 330
- Clinical chemistry, 154–171
- Clinistix reagent, 253
- Clostridium* spp., 545
 identification, 542
- Clostridium perfringens*, 515, 525, 528, 531
- Clotting time, 615
- Coagulase activity, 535
- Cochineal, 385
- Cold agglutinins, 671
- Cole and Onslow's pancreatic extract, 489
- Cole's haematoxylin, 406
- Cloiform bacteria, identification, 542
- Collagen, 307
- Colorimetry, 112
 absorptiometers, 117
 cells and cuvettes, 119
 galvanometers, 121
 light sources, 117
 photoelectric cells, 120
 wavelength selection, 118
- calibration curves, 124
 preparation of, 125
- Dubosq instrument, 113
- flow-through instruments, 122
- Lovibond comparator, 112
- photoelectric absorptiometers, 115
- requirements of, 127
- spectrophotometers, 121

- Calorimetry—*contd.*
 spectroscopy, 128
 visual instrument, 112
- Commensal organisms, 449
- Complement, 515, 632
- Computers, 70
- Condensers for microscopes, 41
 bright-field, 42
 dark-field, 42
 fluorescence, use in, 56
- Connective tissue, staining of, 419
- Conservation of mass, law of, 77
- Constant composition, law of, 77
- Cooke-Arneth count, 594
- Cooked meat medium, 507
- Coombs test, 645
 direct, 646, 673
 indirect, 646, 674
- Corynebacterium*,
 culture media, 494, 500
 identification, 542
 stains for, 459
- Corynebacterium diphtheriae*, 545
 culture media for, 500, 501
 staining, 459, 460
- Corynebacterium hofmannii*, 545
- Cortisol, 165
- Coulometric analysis, 137
- Coulter counters, 580
- Cryoprecipitate, 661
- Cryostats, 310
- Crystal violet capsule stain, 462
- Culture(s),
 anaerobic bacteria, 510
 blood, 523
 colonies forming, 478
 disposal of, 60
 media, 476–514
 adjustment of pH, 484
 agar, 489
 anaerobic organisms, for, 506
 B. pertussis, for, 504
 broths, 481, 487, 506, 511
 carbohydrate, 493
 differential, 483
 egg, 501
 enriched, 482, 483, 489, 508
 essential requirements, 476
 fermentation, 508
 glucose agar deeps, 510
 liquid, 477
 Loeffler's serum slopes, 501
 nutrient gelatin, 492
- Culture(s)—*contd.*
 media—*contd.*
 pancreatic extract, 489
 peptone, 493
 preparation of, 481, 487
 quality control of, 508
 selective, 483, 508
 selenite F, 500
 solid, 478, 482
 solid sugar, 495
 special purposes, for, 496
 staphylococci, for, 503
 sterility testing, 508
 storage of, 483
 transport, for, 507
 V. cholerae, for, 504
 Wilson and Blair's, 499
- phases of growth, 477
- plate methods, 478
 inoculation, 478
- slide agglutination test, 517
- temperature and, 477
- tube methods, 479
 deep, 480
 roll tubes, 480
 slope, 479
- Curie, 135
- Cushing's syndrome, 166
- Cyanide, storage of, 62
- Cyanmethaemoglobin method of haemoglobin estimation, 601, 603
- Cyanocobalamin, 179
- Cylinders, measuring, 7, 12
- Cysteine-magnesium salt mixture, 505
- Cystic fibrosis, 205
- Cystine-lactose-electrolyte deficient medium (CLED), 498
- Cytological techniques, 426–437
- Cytology, exfoliative, 426
- Cytoplasm, 299
 staining, 388, 391, 404
- Cytoplasmic inclusions, 303
- DPK mountant, 400
- D^u antigen, 640, 671
- Decalcification, 328–336
 assessment of, 330
 solutions for, 331
 technique of, 329
- Dehydration, 337
- De-ionization of water, 23, 81
- Deoxycholate-citrate agar, 497

- Desiccators, 10
 Desoxyribonucleic acid (DNA), bacterial, 444
 Detergents, cleaning glassware with, 6
 Dextran, 659
 Diabetes mellitus, 188, 214
 cerebrospinal fluid in, 296
 glucose tolerance test, in, 217
 urinary ketones in, 261
 urine analysis in, 238, 271
 Diacetyl monoxime estimation of blood urea, 280
 Diatomaceous earth filters, 471
 Differential leucocyte counts, 583
 normal value, 593
 Digest broth, 488
 Digestion, 172, 182-188
 accessory organs in, 185
 Dilute carbol-fuchsin, 456
 Dilutions, 518
 Dioxane, dehydration with, 339
 Diplococci, morphology, 445
 Disaccharides, 173
 Disinfectants, 469
 Distillation, 81
 chemically pure water, of, 22
Dolichos biflorus, 634, 667
 Dorset egg medium, 502
 Drabkin's solution, 601
 Drugs, renal excretion of, 235
 Dubosq colorimeter, 113
 Duke's method for bleeding time, 614
 Duodenum, 184
 Dyes for staining, 385
 basic, acid and neutral, 387
 natural, 385
 properties of, 389
 synthetic, 386 (*See also under specific dyes*)
- EEL chloride meter method, 294
 Effusions, specimens, 69
 Egg media, 501
 Ehrlich's haematoxylin, 404
 Elastic fibres, 307
 staining of, 409, 410, 416
 Electric shock, 65
 Electrolytes, 170
 balance, kidney, in, 234
 Electrophoresis, 150-153
 cellulose acetate versus paper, 153
 Elliptocytosis, 594
 Embedding and impregnation, 337-357
 automatic tissue processors, 343
 celloidin, 353, 366
 gelatin, 352
 inadequate, 373
 low viscosity nitrocellulose, 355
 moulds for, 349
 paraffin wax, 341, 366
 preparing sections, 371
 staining, 394
 vacuum technique, 347
 Embedding rings, 350
 Endocrine function tests, 165
 Endocrine system, 160
 Endoplasmic reticulum, 302
 Endotoxins, 450
 Enzymes, 447
 pancreatic, 198
 faeces, in, 204
 Enzymes, serum, determination of, 198
 Eosinophils,
 polymorphonuclear, 551
 total count, 578
 Equivalents, 77
 Erythrocytes, 549
 counting,
 bulk dilution method, 572
 methods, 573
 pipettes, 571
 development of, 553
 diluting fluids, 572
 enzyme-treated, 673
 fragility test, 609
 infected, in blood grouping, 668
 mean corpuscular diameter, 621, 622
 mean volume, 620
 osmotic fragility, 611, 612
 sedimentation rate, 605
 Westergren method, 606
 Wintrobe method, 605
 storage in glycerol, 655, 675
 structure and function, 549
Escherichia coli, 487, 500, 545
 growth characteristics, 498
 Ether, storage of, 63
 Ethyl alcohol as fixative, 316
 Ethylene diamine tetra-acetic acid, 563
 decalcification with, 336
 Ethylhydrocuprein hydrochloride, inhibition, 536
 Evaporating basins, 11
 Extracellular fluids, 167

- Eye swabs, 73
 bacterial examination of, 531
- Faeces,
 bacteriological examination of, 525
 bilirubin in, 226
 collection of specimen, 69
 fat globules in, 203
 microscopical examination, 200
 muscle fibre in, 202
 occult blood in, 195
 pancreatic enzymes in, 204
 pancreatic function tests, in, 200
 soaps in, 203
 starch in, 201
- Farrant's medium, 398
- Fats, 175
 absorption of, 175
 cells, in, 303
 classification of, 175
 demonstration of, 423, 424
 faeces, in, 203
 metabolism, 221
 simple, 176
- Fearon's methylamine test for lactose, 257
- Fermentation, 441
 media, 508
- Ferric salts, demonstration of, 422
- Ferritin, 222
- Fibrin formula, demonstration of, 411
- Fibrinogen, dried, 661
- Fibrous tissue, softening of, 336
- Fildes' broth, 489, 492
- Filters,
 diatomaceous earth, 471
 membrane, 431, 474
 porcelain, 472
 sintered glass, 474
- Filter funnels, 11
- Filtration, sterilization with, 471
- Fire, precautions against, 61
- Fixation and fixatives, 312-328 (*See also under specific substances*)
 compound, 313, 317
 cytological, 314, 322
 cytoplasmic, 314, 322, 324
 gross specimens, of, 325
 micro-anatomical, 314, 317
 nuclear, 314, 322, 323
 post-chromatization, 327
 requirements, 312
- Fixation and fixatives—*contd.*
 secondary, 326
 simple and compound, 313
 actions and properties, 314
 smears, of, 324
 temperature affecting, 313
 washing out, 327
- Flame photometry, 130, 171
- Flasks, 11
 volumetric, 7, 12
- Fleming fuchsin-nigrosin spore stain, 461
- Flemming's fluid, 323
 without acetic acid, 324
- Fluids, bacteriological examination of, 526
- Fluorochromes, 57
- Folic acid, 178
- Follicle stimulating hormone, 161
 function of, 163
- Food, absorption of, 172
- Formaldehyde, 314
 as fixative, 318
- Formaldehyde post-mortem precipitate, 395
 removal of, 401
- Formic acid, decalcification with, 331
- Formol citrate for blood dilution, 573
- Formol-saline, 317, 324
- Formol-saline-sublimate, 320
- Foucher's test, 224
- Fraunhofer lines, 129
- Frozen sections, 309
 cutting methods, 377
 floating out, 379
 handling, 379
 microtomes, 363
 preparation of, 377
 staining, 394
 urine, in, 260
 Seliwanoff's test, 256
- Fructosuria, 260
- Fuchsin-methylene blue spore stain, 461
- Fuchsin-nigrosin spore stain, 461
- Fuchs-Rosenthal counting chambers, 568
- Fuelgen reaction, 416
- Fungi, 443
 demonstration of, 414
 identification, 543
- Funnels, 11

- Galactose in urine, 261
 Galactosuria, 261
 Gall bladder, 185
 function of, 186
 Galvanometers, 121
 Gamma rays, 135
 sterilization with, 465
 Gas constant, numerical value of, 79
 Gas Pak system of bacteria cultivation, 512
 Gases,
 blood, 156, 159
 general equation, 79
 laws, 78
 Gastric content, collection of, 69
 Gastric function tests, 189
 Gastric juice,
 composition of, 189
 secretion, stimulants of, 189
 Gastric lavage, bacteriological examination of, 532
 Gastric mucosa, 182
 Gastric washings, smears from, 430
 Gastrin, 183
 Geiger-Muller tubes, 136
 Gelatin,
 culture media, as, 482
 nutrient, 492
 Gelatin embedding, 352
 Gelatinized slides, 380
 Gendre's fluid, 322
 Genito-urinary swabs, bacteriological examination of, 531
 Gerhardt's test for acetoacetic acid, 262
 Germicides, 469
 Gibson and Harrison haemoglobin estimation, 600
 Giemsa stain, 589
 Glass, composition of, 3
 Glass electrodes in pH meter, 134
 Glassware, 3-19 (*See also under specific items*)
 biochemical, 5
 calibrated, cleaning, 6
 care of, 4
 colorimetry, for, 128
 cleaning of, 4
 biochemical use, for, 5
 detergents for, 6
 infection from, 60
 safety precautions in use, 59
 standardized, 7
 sterilization of, 4
 Globulin in cerebrospinal fluid, 291
 Glomerular filtration, 235
 Glucagon, 188
 Glucocorticoids, 163
 Gluconeogenesis, 221
 Glucose,
 blood, in, 207
 estimation of, 208, 212
 following meals, 215
 cerebrospinal fluid, in, 295
 determination of, 211
 renal threshold for, 217, 236
 'true' estimation of, 208
 urine, in, 260
 Benedict's test for, 269
 quantitative estimation, 269
 Glucose agar, 492
 Glucose agar deeps, 510
 Glucose broth, 489, 511
 Glucose oxidase, 211, 254, 271
 Glucose-saline, 660
 Glucose tolerance test, oral, 215
 Glycerine jelly, 399
 Glycerol, sterilization with, 470
 Glycerol citrate, red cell storage in, 675
 Glycerol egg medium, 502
 Glycogen,
 cells, in, 303
 demonstration of, 414
 Glycogenolysis, 221
 Glycolipids, 176
 Glycolysis, 208
 Glycosuria, 217, 260
 Golgi apparatus, 302
 Gomori's aldehyde fuchsin, 416
 Gonococcus, identification, 542
 Gooding and Stewart's fluid, 332
 Gordon and Sweets' reticulin stain, 412
 Gram's stain, 420, 454
 counterstains for, 455, 456
 Graupner and Weissberger's dehydration method, 339
 Group specific blood group substances, 630
 Growth hormone, 161
 function of, 163
 Gutter plate method of sensitivity testing, 539
 Haematology, 549
 indices, 619
 Haematoxylin, 384, 385

- Haematoxylin staining solutions for nuclei, 402
Haematuria, 244
Haematopoiesis, 221
Haemoglobin, 179, 555
A, 557
A2, 558
deficiency of, 594
estimation of, 598–604
absorptiometer calibration, 602
acid haematin method (Sahli), 599
alkaline haematin method, 600
cyanmethaemoglobin method, 601,
 603
oxyhaemoglobin method, 601
preparation of curve, 603
F, 557
fate of, 556
iron in, 598
mean cell, 620
mean cell concentration, 608, 619
oxygen in, 598
pigments, 556
S, 558
Haemoglobinopathies, 557
Haemoglobin pipettes, 572
Haemoglobinuria, 245
Haemolysis, 609
Haemolytic disease of newborn, 647
Haemophilus spp., identification, 542
Haemophilus influenzae, 525, 529, 546
Haemosiderin, 222, 556, 304–396
Hair, specimens of, 69
Half life, biological, 136
 radioactive, 135
Halides, identification, 88
Hanging drop preparations, 451, 453
Harleco carbon dioxide apparatus, 171
Harris alum-haematoxylin, 405, 432
Harrison's three-tube test, 249
Hartmann's lactate solution, 660
Hayem's fluid, 572
Hay's test for bile salts, 230
Health and Safety at Work Act 1975, 59
Heart, fixation of, 326
Heat, sterilization with, 466, 467
Heidenhain's iron-haematoxylin stain,
 402, 408
Heidenhain's susa, 319
Heiffor knife, 366
Heller and Paul oxalate mixture, 72
Helly's fixative, 321
Helly's fluid, 324
Hemalog, 582
Hemming's filter, 473
Heparin, 563, 651
Heparin bottles, 72
Hepatitis-associated antigen, 652
Hereditary spherocytosis, 609
Heredity, laws of, 635, 637
Hiss' serum water sugars, 494
Histamine, stimulating gastric juice, 190
Histamine test, 190
Histology, 299
Hormones, 160
 assessment of, 433
 function of, 163
Hoyle's medium, 500
Human chorionic gonadotrophin, 164
Human placental lactogen, 164
Hydrochloric acid
 digestion, in, 183
 0.1 mol solution, 97
 preparation of solutions, 97
 standardization of solution, 97
Hydrogen fluoride, 63
Hydrogen ion concentration, 82
 measurement of, 131
Hydrogen peroxide, storage of, 63
β-Hydroxybutyric acid in urine, 267
Hyperglycaemia, 188
 fasting, 214
Hypochromia, 594
Hypochromic anaemia, 609
Hypoglycaemia, 188
 fasting, 214
Hypothalamus, 160

Ictotest, 225
Ilford spectrum filters, 118
Immunoglobulins, 515, 642–649
 molecular structure, 642
Immunoglobulin A, 642
Immunoglobulin D, 642
Immunoglobulin E, 642
Immunoglobulin G, 642
 antibodies, 646, 647, 671, 673, 674
 ionic cloud, 644
 use of enzymes in antibody detection,
 645
Immunoglobulin M, 642, 671
 antibodies, 673
 ionic cloud, 644
Impregnation, (*See under Embedding and impregnation*)

- India ink preparation, 463
 Indicators, theory of, 91
 Indole production, tests for, 536
 Infection, precautions against, 60, 65
 Inoculating cabinet, 521
 Inoculating cabins, 61
 Instrumentation, 112-153 (*See also under specific techniques*)
 Insulin, 188
 Intercellular substances, 307
 Interstitial cell stimulating hormone, 161
 Intestinal bacteria, culture media, 496
 Intestinal juice, 185
 Intestines, 184
 absorption in, 185
 fixation of, 326
 Intracellular fluid, 167
 Iodide, identification, 89
 Iodine, 180
 radioactive, 136
 storage of, 63
 Ion exchange chromatography, 145
 Ion exchange resins, 81, 145
 chemically pure water, for, 23
 decalcification, in, 335
 Iron, 179, 180
 demonstration in tissue, 422
 haemoglobin, in, 598
 identification, 88
 Iron deficiency anaemia, 627
 Iron-haematoxylin solutions, 402
 Iron strips, 511
 Islets of Langerhans, demonstration of, 416
 Isotonic saline, 660, 664
 Ivy's method for bleeding time, 615
- Jansky blood groups, 628
 Jaundice, 223
 Jenner's stain, 590
 Jenner-Giemsa stain, 591
 Joules, 155
- Karo corn syrup, 396
 Karyopyknotic index, 433
 Ketone bodies, 261
 Ketones,
 urine, in, 261
 acetest for, 265
 ketostix for, 266
- Ketones—*contd.*
 urine—*contd.*
 Rothera's test, 264
 Ketostix, 266
 Kidney (*See also headings beginning Renal*)
 anatomy of, 233
 drug excretion by, 235
 electrolyte balance and, 234
 filtration by, 235
 fixation of, 326
 functions of, 234
 maintenance of blood pH by, 234
 reabsorption by, 236
 urine formation by, 235
 water balance, in, 234
 Kinyoun's carbol fuchsin, 421
Klebsiella spp., growth characteristics, 498
Klebsiella pneumoniae, 529, 546
 identification, 542
 Kohler illumination for microscopes, 51, 52
 Kohn and Kelly orthotolidine test, 197
 Kovacs reagents, 536
 Kristenson's fluid, 614
- Labstix, 267
 Lacey's medium, 504
 Lactose,
 Fearon's methylamine test, 257
 urine, in, 260
 Lactosuria, 260
 Laevulose (fructose) syrup, 398
 Lambert's law, 115
 Lange colloidal gold curve, 286
 Langerhans, islets of, 188
 Laryngeal swabs, 73
 bacteriological examination of, 532
 Lectins, 634
 Lee and White's clotting time method, 615
 Lempert's modification of Kristenson's fluid, 614
 Lens,
 converging, foci, 30
 diverging, foci, 31
 focal length, 31, 38
 image formation, 32
 optical centres, 31
 real images, 32
 resolving power, 38

- Lens—*contd.*
 virtual images, 33
- Leuckhard embedding boxes, 350
- Leucocytes, 549
 counting,
 bulk dilution method, 572
 differential, 583, 593
 diluting fluids, 573
 method, 576
 pipettes, 571
 development of, 555
 polymorphonuclear, 551
 staining, 586, 589, 590
 structure and function, 550
 urine, in, 243
 viability in stored blood, 651
- Leucocytosis, 550
- Leucopenia, 550
- Leukaemia, chronic lymphatic, 552
- Lipids (*See* Fats)
- Litmus, 386
- Litmus milk, 495
- Litmus solution, 496
- Liver,
 bile secretion in, 219
 carbohydrate metabolism in, 220
 detoxication in, 221
 excretion by, 220
 fixation of, 326
 function of, 220
 haematopoiesis in, 221
 lipid metabolism in, 221
 protein synthesis in, 221
 structure of, 218
- Loeffler's alkaline methylene blue stain, 463
- Loeffler's serum slopes, 501
- Longitudinal method of differential leucocyte count, 584
- Loops, making, 451
- Lovibond comparator, 112, 485
- Lowenstein-Jensen medium, 502
- Low viscosity nitrocellulose, embedding with, 355
- Ludlam's medium, 503
- Lugol's iodine, 401, 455
- Lungs, fixation of, 326
- Luteinizing hormone, function of, 164
- Lymphoblasts, 555
- Lymphocytes, 552
- Lymphocytosis, 552, 594
- Lyphogel pellets, 250
- Lymphopenia, 594
- Lysosomes, 303
- MacConkey's agar, 496
- McFadyean's reaction, 464
- Magnesium, 179
- Malachite green stain, 459
- Malaria, 652
- Mannitol salt agar medium, 504
- Marrow cells, staining, 591
- Martius scarlet blue (MSB), 411
- Mayer's acid-alum-haematoxylin and eosin, 403
- Mayer's glycerol-albumin mixture, 377
- Mean cell diameter, 622
- Mean cell haemoglobin, 580, 620
- Mean cell haemoglobin concentration, 580, 608, 619
- Mean cell volume, 580, 620
- Mean corpuscular average thickness, 622
- Mean corpuscular diameter, 621
- Mean corpuscular volume, 622
- Measuring cylinders, 7, 12
- Meat extract broth, 488
- Meconium, 226
- Median corpuscular fragility, 612
- Megakaryocytes, 555
- Melanocyte stimulating hormone, 164
- Membrane filters, 431, 474
- Meningitis, 287, 525
- Meningococcus, identification, 542
- Mercuric chloride, 315
- Mercuric chloride deposit, 395
- Mercuric chloride precipitate, removal of, 400
- Mercury vapour lamps, 46
- Metabolic rate, 155
- Metabolism, 154
- Metamyelocytes, 555
- Methaemoglobin, 557
- Methyl benzoate, clearing with, 341
- Methylene blue stain, 434, 458
- Microbiology, 441
- Micrometry, 47
- Micro-organisms (*See also* Bacteria)
 anaerobic, culture of, 480, 506
 classification of, 442
 motile, 453
 tests for identification, 535
- Microscopes and microscopy, 25–58
 aperture diaphragm, 52
 barrier filters, 56

Microscopes and microscopy—*contd.*
 binocular, 26, 27
 chromatic aberration, 29
 component parts, 34
 mechanical, 46
 optical, 34
 condensers, 41
 fluorescence work, for, 56
 critical illumination, 50
 do's and don'ts of, 58
 draw-tubes, 41
 empty magnification, 50
 exciter filters, 55
 eyepieces, 40
 fluorescent work, for, 57
 micrometry, for, 47
 field diaphragm, 52
 filters, 44, 55, 56
 fluorescence, 54
 condensers, 56
 eyepieces, 57
 filters, 55, 56
 fluorochromes for, 57
 light source, 54
 objectives, 56
 heat-absorbing filters, 55
 image formation in, 32
 lens, 30
 light sources, 43, 44, 50
 fluorescence, for, 54
 Kohler, 51, 52
 magnification, 49
 mechanical tube length, 40
 micrometer eyepieces, 47
 monocular, 26
 numerical aperture, 39
 objectives, 37
 fluorescent work, for, 56
 optical tube length, 41
 refraction, 28
 resolving power, 38
 setting-up, 25, 50
 dark field illumination, for, 53
 spherical aberration, 29
 stage micrometers, 48
 stages, 46
 types and design of, 25
 Microtomes, 358–366
 base sledge, 360
 Cambridge rocking, 358
 freezing, 363
 knives, 366
 imperfect edge of, 373

Microtomes—*contd.*
 knives—*contd.*
 incorrect setting of, 374
 sharpening machines, 370
 sharpening of, 367
 sharpening stones, 368
 stropping, 369
 orientation of block to, 372
 rotary, 359
 sliding, 362
 Mineral salts, 179
 Mitochondria, 302
 Mitosis, 305
 anaphase, 307
 metaphase, 306
 prophase, 305
 telophase, 307
 Mohr's method of chloride estimation, 293
 Mohr's method of silver nitrate solution titration, 103
 Molar solutions, 87, 95
 Monoblasts, 555
 Monocytes, 552
 Monocytosis, 594
 Monosaccharides, 173
 Morawitz theory of blood coagulation, 559
 Mordants, 385, 389, 402
 Mosmols, 240
 Moss blood groups, 628
 Mounting media, 397
 resinous, 399
 synthetic resins, 400
 Mucin, 182
 demonstration of, 414, 419
 Mucoviscidosis, 205
 Mucus in urine, 244
 Multiple myeloma, 248
 Multiple proportions, law of, 77
 Muscle fibres in faeces, 202
 Mutorotation, 211
Mycobacterium tuberculosis, 457, 526, 527, 528, 545
 identification, 542
 routine examination for, 529
 urine, in, 533
 Myeloblasts, 555
 Myelocytes, 555
 Nagler reaction, 515
 Nasal swabs, bacteriological examination of, 530

- National Blood Transfusion Service, 651
Nebulizer-burner system of spectroscopy, 130
Necol, 353
Neisseria catarrhalis, 545
Neisseria gonorrhoea, 531, 544
demonstration of, 456
transport media, 507
Neisseria meningitidis, 544
Neisseria pharyngis, 545
Neisseria spp., 525, 537
culture media, 495
Nephritis, specific gravity of urine in, 238
Nessler's reagent for blood urea, 277
Neubauer counting chambers, 568
Neutral dyes for staining, 388
Neutralization indicators, 91
Neutral red stain, 456
Neutropenia, 594
Neutrophilia, 594
Neutrophils,
polymorphonuclear, 551
classification of, 594
Newborn, haemolytic disease of, 647
Nicotinamide, 177
Microme wire for loops, 451
Nigrosin-methylene blue capsule stain, 462
Nitrate, identification, 89
Nitric acid, decalcification with, 332
Nitric acid-formaldehyde,
decalcification with, 332
Nitrogenous foods, 174
Nonne-Apelt's method for CSF globulin, 292
Normal solutions, 87, 94
Normoblasts, 553
Nucleolus, 304
Nucleoprotein, 304
demonstration of, 416
Nucleus of cell, 304
staining, 388, 402, 404, 405, 406, 407

Occult blood in faeces, 195
Oestrogens, 165
assessment of activity, 433
Oil red O in isopropanol, 423
Okokit, 196
Optochin sensitivity, 536
Orcein, 385
Osazone test, 255
Osmium tetroxide, 315
Osmolality, measurement of, 137
Osmometers, 137
Osmometry, 240
Ovalocytosis, 594
Ovens, hot-air for sterilization, 466
Oxalate mixture, 72
Oxalates, blood coagulation and, 562
Oxford staphylococcus, 540, 541
Oxidase test, 537
Oxygen in haemoglobin, 598
Oxyhaemoglobin method of haemoglobin estimation, 601

PAS, 263
pH, 82
blood, role of kidney in maintenance, 234
culture media, of, 484
measurement of, 131
culture media, in, 484
glass electrodes, 134
standardization of meter, 134
temperature variation, 133
Packed cell volumes, 580, 607
Pancreas, 186
endocrine function, 188
exocrine function of, 186
fibrocystic disease of, 205
Pancreatic function tests, 198
endocrine, 207
exocrine, 198
faecal studies, 200
serum amylase, 198
Pancreatic juice, 187
Pandy's method for CSF globulin, 291, 292
Pantothenic acid, 178
Papanicolaou fixing fluid, 427
Papanicolaou staining methods, 324, 426, 431
Paper boats for embedding, 350
Paraffin wax embedding, 337, 341, 366
attachment of section to slide, 376
cutting of sections, 310, 371
faults encountered, 375
dispensers, 343
moulds for, 349
ovens for, 342

- Paraffin wax embedding—*contd.*
 preparation of section, 371
 size of block, 343
 staining, 394
 times, 342
 tissue density and, 343
- Parasites, 449
- Parathormone, 167
- Parathyroid glands, 167
- Paratrophs, 448
- Parietal cells, direct stimulus of, 190
- Pasteurella*, identification, 543
- Pasteurella multocida*, 546
- Pasteur pipettes, 664
- Paternity disputes, 649
- Pathogens, 449
- Peltier effect, 364
- Pentagastrin, stimulating gastric juice, 190
- Pentagastrin test, 194
- Pentose,
 Bial's test for, 257
 urine, in, 261
- Pentosuria, 261
- Pepsinogen, 183
- Peptone, 481
- Peptone water, 493
- Peptone water sugars, 494
- Perenyi's fluid, 332
- Periodic acid Schiff reaction, 414
- Peritoneal fluids,
 bacteriological examination of, 526
 specimens, 69
- Perls' method for ferric iron, 422
- Pernasal swabs, 74
- Pestle and mortars, 13
- Peterfi's double-impregnation method, 356
- Petri dishes, 13, 442, 478
- Petroff's method, 529
- Pharyngeal swabs, 74
 bacteriological examination of, 530
- Phenistix, 263
- Phenol, sterilization with, 470
- Phenylalanine in urine, 262
- Phenylketonuria, 264
- Phenylpyruvic acid, 263
- Phosphate, 179
- Phospholipids, 176
- Photoelectric absorptiometers, 115
- Photoelectric cells,
 barrier layer, 120
 photoemissive, 120
- Phototrophs, 448
- Picric acid, 316
- Pigments, 395
 artificial, 395
 bile (*See* Bile pigments)
 cells, in, 304
 natural, 396
 removal of, 400
- Pilocarpine iontophoresis, 206
- Pineal gland, aldosterone secretion and, 164
- Pipettes, 7, 13
 automatic and dispensing, 13
 bulb type, 18
 cleaning of, 7
 delivery, 17
 tolerances for, 8
- diluting, 571
- graduated, 18
- haemoglobin, 572
- pasteur, 664
- safety precautions, 59
- Thoma, 571
- 'to contain', 18
- white cell, 571
- Pituitary gland, 160
 anterior, hormones secreted by, 161
 mid-lobe, hormones from, 164
 posterior, hormones from, 164
 staining cells in, 417
- Placenta, hormones from, 164
- Plasma, 549, 565
 acid-base balance, 158
 bilirubin in, 224
 dried, 660
- Plasma bicarbonate, 171
- Plasma protein fraction, 660
- Plasma proteins in liver, 221
- Pleural fluids,
 bacteriological examination of, 526
 smears from, 430
 specimens, 69
- Pneumococcus*, identification, 543
- Poikilocytosis, 594
- Poisoning, treatment of, 64
- Poisons, safety precautions, 62
- Poliomyelitis, CSF glucose in, 296
- Polyanethyl sulphonate, 524
- Polychromasia, 594
- Polychrome methylene blue stain, 464
- Polymorphonuclear leucocytes, 551
- Polymorphonuclear neutrophils, classification of, 594

- Polysaccharides, 173
Porcelain filters, 472
Porphobilinogen in urine, 231
Post-chromatization, 327
Post-mortem changes, 312
Post-mortem tissue,
 formaldehyde precipitate, 395
 removal of, 401
Post-nasal swabs, 74
Potassium, 170, 180
 depletion, 169, 170
 estimation, 171
 identification, 130
Potassium dichromate as fixative, 317
Potassium hydrogen phthalate, standardization of sodium hydroxide with, 102
Potassium hydroxide, storage of, 63
Potassium permanganate,
 solution of, 107
 standardization against sodium oxalate, 107, 110
Potato extract, 506
Potentiometric analysis, 132
Precipitin tubes, 663
Pregnancy, proteinuria in, 251
Proerythroblasts, 553
Prolactin, 161
 function of, 163
Propylene glycol sudan method, 424
Proteins, 174
 Bence-Jones, 248
 test for, 249
 cerebrospinal fluid, in, 288
 sulphosalicylic acid-sodium sulphate method, 291
 trichloracetic acid method, 289
 turbidimetric estimation, 289
 classification, 174
 colorimetric analysis and, 124
 plasma, synthesis of, 221
 serum (*See* Serum proteins)
 synthesis, 221
 urine, in, 245
 albustix, 247
 boiling test, 246
 colorimetric (Biuret) test, 272
 differentiation from radio-opaque substances, 248
 qualitative tests, 246
 quantitative tests, 271
 sulphosalicylic acid test, 247
 turbidimetric tests, 271
Proteinuria, 245, 251
Proteus spp., 546
 culture media, 498
 growth characteristics, 498
 identification, 543
Prothrombin index, 617
Prothrombin test, 616
Prothrombin time, 613
Protoplasm, 299
Protozoa, 442
Pseudomonas aeruginosa, 537, 546
Pseudomonas pyocyanne, growth, 499
Pseudomonas spp., identification, 543
Pteroylglutamic acid (*See* Folic acid)
Pugh's stain, 460
Pumps, water-vacuum, 19
Pus,
 bacteriological examination of, 527
 specimen collection, 69
Putrefaction, 312
Pyridoxine, 178
Qualitative analysis, 87
Quantitative analysis, 89
Quartz-iodine vapour lamps, 45
Quaternary ammonium compounds, sterilization with, 471
Quick's one-stage prothrombin test, 613, 616
Radiation, 135
 sterilization by, 465
Radioactive isotopes, 134-137
 detection of, 136
 half-life, 135
 measurement of, 136
 stable, 135
 unstable, 135
Radioactivity, units of, 135
Reagent bottles, 9
Reagents, storage of, 62
Reciprocal proportions, law of, 77
Red cells (*See* Erythrocytes)
Redox potential, 510
Reduced phenolphthalein test, 196
Reducing substances (*See also under specific substances*)
 Fructose, Pentose etc.)
urine, in, 251
 Benedict's test, 251
 chromatography for, 258

- Reducing substances—*contd.*
 urine—*contd.*
 clinstix, 253
 identification of, 253
 osazone test, 255
 quantitative tests, 269
 yeast fermentation test, 254
- Refraction, 28
- Refractive index, 29
- Refrigeration, principles of, 655
- Renal disease, proteinuria in, 251
- Renal failure, urea in urine in, 282
- Renal function tests, 232–282
- Renal glycosuria, 217
- Renal threshold, 236
- Resins, synthetic, for mountants, 400
- Resorcin fuchsin stain, 410
- Respiration exchange, 156
- Reticular fibres, 308
 staining of, 412
- Reticulocyte count, 596
- Rhesus system, 638
 antibodies, 643
 antigens, 647
 detection, 671
 D^u antigen, 640, 671
 detection of antibodies, 672
 emergency grouping, 672
- Riboflavine, 177
- Ribonucleic acid, 304
 bacterial, 449
- Rickettsiae, 443
- Ringer's solution, 529
- Romanowsky stains, 585
- Rothera's nitroprusside test, 264
- Roughage, 181
- Rouleaux formation, 667
- Rye's halometer, 622
- Safety hoods, 521
- Safety precautions, 59–65
 Australia antigen and, 653
 bacteria, for, 521
 fire, against, 61
 infection, against, 60, 65
 poisons, 62, 64
 radioactivity, 137
 refrigerators, with, 657
 safety hoods, 521
 treatment of accidents, 64
- Saffron, 386
- Safranin, 455
- Sahli method of haemoglobin estimation, 599
- Salicylates in urine, 262
- Saliva, 182
 specimen of, 69
- Salmonella* spp., 546
 culture media for, 483, 497, 498
 growth, 499
 identification, 543
- Salmonella typhi*, 499, 516, 524
- Salt depletion, 169, 170
- Saprophytes, 449
- Saturated solutions, 85
- Scalds, treatment of, 64
- Schaudinn's fluid, 325
- Schlesinger's test for urobilin, 229
- Schriddé's method for post-mortem formalin pigment removal, 401
- Secretion granules, 304
- Sections,
 automatic tissue processors, 344
 clearing, 340
 dehydration, 337
 embedding and impregnation (*See under Embedding*)
 size of block, 343
- Section cutting, 358–383
 attachment to slides, 376
 calcium in tissue, 375
 celloidin sections, 382
 cryostats, 364
 preparation of, 381
 frozen, 377
 floating out, 379
 handling sections, 379
 preparation of, 377
 staining, 394
- microtomes, 358
 base sledge, 360
 Cambridge rocking, 358
 freezing, 363
 incorrect setting of, 374
 orientation of block to, 372
 rotary, 359
 sliding, 362
- microtome knives, 366
 imperfect edge on, 373
 sharpening of, 367
 sharpening machines, 370
 sharpening stones, 368
- Peltier effect, 364
- technique, 371

- Section cutting—*contd.*
use of adhesives, 376
- Seitz filters, 473
- Selenite F, 483, 500
- Seliwanoff's test for fructose, 256
- Sensitivity tests, 537
- Separating funnels, 11
- Septicaemia, 524
- Sequestrene, 563
- Sequestrene bottles, 72
- Serology, 528
- Serratia marcescens*, 474
- Serum, bilirubin in, 224
- Serum amylase, 198
- Serum enzymes, determination of, 198
- Serum proteins, electrophoresis of, 151
- Sex chromatin, 436
- Sex hormones, 160
- Shandon-Elliott bench type processor, 344
- Shigella* spp., 546
culture media, 483
identification, 543
transport media, 507
- Shock, electric, 65
- Shorr staining method, 433
- Sickle cells, 558
- Silver nitrate, storage of, 63
- Silver nitrate solution,
0.1 mol, 103
titration of, 103
Mohr's method, 103
Volhard's method, 105
- Sintered glass filters, 474
- Slide agglutination test, 517
- Slides,
albumenized or starched, 380
attaching sections to, 376
attachment of frozen sections to, 380
cleaning of, 5
gelatinized, 380
- Smears, 309
bacterial, 452
staining of, 454
cancer diagnosis from 426, 435
fixation of, 324, 427
making,
liquid media, from, 452
solid media, from, 453
postal specimens, 428
preparation of, 428
- Smith-Noguchi method of bacterial cultivation, 511
- Soaps in faeces, 203
- Sodium, 170, 180
depletion, 169, 170
estimation, 171
identification, 130
storage of, 63
sweat, in, 206
- Sodium acetate, mode of action, 84
- Sodium citrate,
as anticoagulant, 562
preparation of, 73
- Sodium deoxycholate, 497
- Sodium fluoride-potassium oxalate,
preparation of, 73
- Sodium hydroxide,
0.1 mol solution of, 100
preparation of, 111
standardization of solution, 101
- Sodium nitroprusside, storage of, 63
- Sodium oxalate, standardization of
potassium permanganate against, 107, 110
- Solubility, 86
temperature effects on, 86
- Solutes, 85
concentration of, 137
- Solutions, 85
colorimetry, for, 127
coloured measurement of (*See under Colorimetry*)
concentration of solutes in, 137
concentrations of, 86
molar, 87, 95
normal, 87, 94
saturated, 85
- Solvents, 85
- Somatotrophic hormone, 161
- Somogyi amylase unit, 199
- Sorensen's buffer, 673
- Southgate's mucicarmine, 414
- Specific gravity of urine, 269
measurement of, 238
- Specimens, 66–74 (*See also under specific specimens*)
autopsy, 74
bacteriological examination of, 520–546
cerebrospinal fluid, 524
faecal, 525
general, 520, 534
macroscopic, 522

- Specimens—*contd.*
 bacteriological examination of—*contd.*
 microscopic, 522
 biopsy, 74
 blood grouping, for, 663
 collection of, ward etiquette, 71
 containers for, preparation of, 72
 postage of, 71, 522
 receipt of, 66
 reporting on, 70
 Spectrophotometers, 121
 Spectroscopy, 128
 detector system, 130
 direct vision, 129
 flame emission, 130
 nebulizer-burner system, 130
 Spermatozoa in urine, 244
 Spherocytosis, hereditary, 609
 Spinal cord, fixation, 325
 Spirillum, morphology, 446
 Spirochaetes, 446
 Spleen, fixation of, 326
 Spore stains, 460
 Sputum,
 bacteriological examination of, 528
 smears, 430
 specimen, 66, 69
 Squash preparations, 309
 Stains and staining, 384–392 (*See also under specific stains and methods etc.*)
 accelerators, 390
 bacteria, of,
 acid-fast, 457
 counterstains, 455, 456
 Gram's stain, 454
 simple, 458
 basic, acid and neutral dyes, 387
 cell nuclei, 402
 Ci number and solubility table, 388
 colour index, 384
 control and test slides, 395
Corynebacteria, for, 459
 cytological, 389, 431
 direct, 389
 dishes for, 393
 dyes, 385
 equipment, 393
 formaldehyde post-mortem precipitate, 395
 removal of, 401
 hormone assessment by, 433
 impregnation, 390
 indirect, 389
 Stains and staining—*contd.*
 leucocytes, 586, 589, 590
 machines, 393, 592
 marrow cells, of, 591
 mercuric chloride deposit, 395
 removal of, 400
 metachromatic, 390
 micro-anatomical, 389
 mordants, 385, 389, 402
 mounting media, 397
 natural dyes, 385
 negative, 390
 pigments, 395
 artificial, 395
 natural, 396
 removal of, 400
 procedures, 393
 progressive, 390
 racks for, 393
 regressive, 390
 Romanowsky, 585
 section mounting, 396
 specific, 390
 spore, 460
 synthetic dyes, 386
 techniques, 403 (*See also under specific techniques*)
 theory of, 391
 vital, 390
Staphylococci
 culture media for, 503
 identification, 543
 morphology, 445
Staphylococcus aureus, 524, 525, 527, 528, 531, 533, 544
 culture media, 503
 growth, 499
 identification of, 535
Staphylococcus epidermidis, 503, 527, 544
Staphylococcus pneumoniae, 524, 525, 536
Staphylococcus pyogenes, 524, 525
 Starch granules in faeces, 201
 Starched slides, 380
 Steam sterilization, 467
 Stercobilinogen, 222
 Sterility testing of culture media, 508
 Sterilization, 465–475
 alcohol, with, 470
 boiling water, with, 467
 chemical agents, with, 469
 chlorine, with, 470

- Sterilization—*contd.*
chloroform, with, 470
cresols and phenols, with, 470
diatomaceous earth filters, with, 473
dry heat, by, 466
filtration, with, 471
glycerol, with, 470
low-temperature, 469
membrane filters, with, 474
moist heat, with, 467
physical methods, 465
porcelain filters, with, 472
quaternary ammonium compounds,
 with, 471
radiation, with, 465
Seitz filters, with, 473
sintered glass filters, with, 474
steam, 467
- Steroid hormones, 160, 161
Stokes method of sensitivity testing,
 538
- Stomach, 182, 189 (*See also headings
beginning Gastric*)
 lining, 182
- Stomach content, collection, 69
- Streptococci*, 445
 identification, 543
 morphology, 445
- Streptococcus faecalis*, 525, 533, 536,
 544
 growth, 499
- Streptococcus pneumoniae*, 544
- Streptococcus pyogenes*, 544
- Stuart's transport medium, 507
- Subarachnoid haemorrhage, CSF in,
 287
- Succus entericus (*See Intestinal juice*)
- Sugar,
 blood (*See Blood sugar*)
 thin-layer chromatography, 258
 urine, in, 251
- Sugar medium, 495
- Sulphaemoglobin, 557
- Sulphate, identification, 89
- Sulphosalicylic acid-sodium sulphate
 method for CSF proteins, 291
- Sulphosalicylic acid test for protein,
 247
- Susa, 319
- Swabs,
 bacteriological examination of, 530
 collection of, 73
- Sweat, collection of, 206
- Sweat test in cystic fibrosis, 206
- Symbiosis, 450
- Tanzer-Unna orcein stain, 385
- Technicon BG-9, 677
- Teeth for sectioning, 329
- Temperature,
 body, of, 156
 cultures and, 477
 fixation and, 313
- Thalassaemia, 558
- Thermophiles, 448
- Thiamine, 177
- Thioglycollate broth, 506
- Thiosulphate citrate bile salt agar,
 (TCBS), 504
- Thoma pipettes, 571
- Thomsen phenomenon, 668
- Throat swabs, 73
 bacterial examination of, 530
- Thrombocytes (*See Blood platelets*)
- Thrombocythaemia, 553
- Thrombocytopenia, 553
- Thyroid function tests, 166
- Thyroid gland, hormones from, 166
- Thyrotrophic stimulating hormone,
 161, 163
- Thyroxine, 162, 166
- Thyroxine binding globulin, 166
- Tissue, automatic processors, 344
 examination of, 308
 celloidin sections, 311
 fixed, 310
 fresh specimens, 308
 frozen sections, 309, 310
 paraffin sections, 310
 smears, 309, 324
 squash preparations, 309
 teased preparations, 308
 selection of for sectioning, 337
- Tissue Tek, 350
- Titrations, acid-base, 92
- Tocopherol, 177
- Toisson's fluid, 573
- Toluene, clearing with, 340
- Total eosinophil count, 578
- Toxins, 450
- Trap-bottles, 18
- Trichloracetic acid
 decalcification with, 334
 fixative, as, 317
- Trichrome-PAS method (Pearse) of
 staining, 417

- Triiodothyronine, 166
 Tritium, 136
 Trypsin, 187
 faeces, in, 204
 in culture media, 482
 Tube dilution, 537
 Turk's solution, 573
 Tyndallization, 467
- Ulex europaeus*, 634
 Ultraviolet light, sterilization by, 465
 Urea,
 blood, in, 275, 276
 diacetyl monoxime estimation, 280
 Nessler's reagent, 277
 detection, 275
 renal threshold of, 236
 urine, in, 282
 Urinary swabs, bacteriological examination of, 531
 Urine,
 acetoacetic acid in, 262
 acid-base balance and, 158
 analysis of, 236
 quantitative, 269
 antibiotic assay in, 540
 appearance of, 237
 bacteriological examination of, 532
 culture media, 498
 bile pigments in, 224
 Fouchet's test, 224
 ictotest, 225
 bile salts in, 230
 blood in, 244
 casts, 243
 cells in, 242
 chlorides in, 273
 chloride meter method, 275
 Volhard's method of detection, 273
 collection of, 241
 composition of, 236
 deposits in, 242
 examination of, 268
 formation of, 235
 fructose in, 260
 Seliwanoff's test, 256
 galactose in, 261
 glucose in, 217, 260
 Benedict's test, 269
 quantitative estimation of, 269
 β -hydroxybutyric acid in, 267
 ketones in, 261
- Urine—*contd.*
 ketones—*contd.*
 acetest reagent, 265
 ketostix for, 266
 Rothera's test, 264
 lactose in, 260
 Fearon's test, 257
 leucocytes in, 243
 microscopic examination of, 242
 mucus in, 244
 odour of, 238
 organisms in, 243
 osmolarity of, 240
 pentose in, 261
 Bial's test, 257
 phenylalanine in, 262
 porphobilinogen in, 231
 preservatives, 241
 protein in, 245
 albustix; 247
 boiling test, 246
 colorimetric (Biuret) test, 272
 electrophoresis of, 151
 qualitative tests, 246
 quantitative tests, 271
 sulphosalicylic acid test, 247
 turbidimetric tests, 271
 pus cells in, 243
 reaction, 238
 reducing substances in, 251
 Benedict's test, 251
 chromatography of, 258
 clinstix reagent, 253
 identification of, 253
 osazone test, 255
 quantitative tests for, 269
 yeast fermentation test, 254
 salicylates in, 262
 smears from, 430
 specific gravity of, 238, 269
 measurement of, 239
 specimens, 66, 69
 spermatozoa in, 244
 sugars in, 251
 urea in, 282
 urobilin in, 229
 urobilinogen in, 227
 urobilistix, 228
 Wallace and Diamond test, 227
 volume of, 237
 Urinometers, 239
 Urobilin in urine, 229

- Urobilinogen, urinary, 227
uroblistix, 228
Wallace and Diamond test, 227
Uroblistix, 228
- Vaccination, 60
Vaccines, sterilization of, 469
Vacuum-impregnation technique, 347
Vaginal smears, 433
preparation of, 428, 429
Vaginal swabs, 73
Van Gieson's stain, 408
Venous blood, collection of, 565
Venturi pumps, 19
Verhoeff's elastic fibre stain, 409
Verocay's method of post-mortem pig-
ment removal, 401
Vibrio, morphology, 446
Vibrio cholerae, 526
culture media for, 504
Viridans streptococci, 544
Viruses, 443
Vitamins, 175, 176
absorption of, 176
deficiency, 176
fat-soluble, 176
water-soluble, 177
Vitamin A, 176
Vitamin B₁, 177
Vitamin B₂, 177
Vitamin B₆, 178
Vitamin B₁₂, 179
Vitamin C, 179
Vitamin E, 177
Vitamin K, 177
Volhard's method for urinary chlorides, 273
Volhard's method of chloride titration, 106
Volhard's method of silver nitrate solu-
tion titration, 105
Volumetric analysis, 89
golden rules of, 95
primary standards, 90
secondary standards, 91
use of factors in, 95
Volumetric solutions,
preparation of, 94
Von-Ebner's fluid, 334
Von Kossa's method for calcium
demonstration, 423
- Wallace and Diamond reaction for uro-
bilinogen, 227
Ward etiquette in specimen collection, 71
Washing out, 327
Watch glasses for embedding, 350
Water, 167, 180
absorption, 185
balance, 168
role of kidney in, 234
chemically pure, 81
production of, 22
conductivity, 81
dissociation of, 81
hormonal control of output, 170
loss and balance, 168
Water bath method of attaching section
to slide, 376
Water sugars, 494
Waterfield halometer, 622
Water-vacuum pumps, 19
Weigert's iron-haematoxylin and Van
Gieson stain, 407
Weigert's primary mordant, 385
Weigert's solution, 402
Westergren method of erythrocyte sedi-
mentation rate, 605
White cells (*See* Leucocytes)
White fibres, 307
Wilson and Blair's medium, 499
Wintrobe's method of erythrocyte sedi-
mentation rate, 605
Wright's stain, 588
- Xanthochromia, CSF in, 287
Xylene, clearing with, 340
Xylene-balsam, 394
Xylene damar, 400
- Yeast fermentation test, 254
Yeasts, identification, 543
Yellow fibres, 307
- Zenker-formol, 314, 321
Zenker-formol (Helly's), 321
Zenker's solution, 320
Ziehl-Neelsen's stain, 421, 457, 458