General Pathology

J. B. WALTER
M. S. ISRAEL

5th Edition

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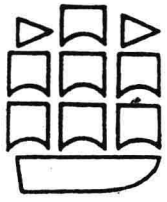
J. B. WALTER TD MD MRCP (Lond.) FRCPath
Department of Pathology, Banting Institute, University of Toronto, and Departments of Medicine and Pathology, Toronto General Hospital, Toronto, Canada

M. S. ISRAEL MB ChB MRCP (Lond.) FRCPath DCP
Department of Pathology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, and Lewisham Group Laboratory, London, England



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Preface

For over a hundred years pathology has been one of the keystones of medicine, and as an academic subject it has been taught as part of the medical curriculum to prepare the student for the more nebulous clinical programme that completes his training. Unfortunately the practice of pathology has come to be relegated to the full-time pathologist and clinical medicine to the specialised clinician. This separation of pathological practice from the direct care of the patient is quite artificial, and indeed is unnecessary and undesirable. Recently this cleavage has been bridged, and the process is being accelerated by the advances that have taken place during the last two decades in clinical pathology, namely cytology, biochemistry, haematology, and microbiology. Even the isolation of histopathology, surely the last bastion of the non-clinical medical pathologist, has eroded before the advancing techniques of needle biopsy and the various types of endoscopy. Diseases even of previously inaccessible organs like the kidneys, pancreas, and central nervous system can now be adequately investigated during life and their progress mapped with precision. The necropsy has ceased to be the final event of revelation, but has rather assumed the role of an epilogue to a drama already well documented. Nevertheless, the epilogue quite frequently provides engaging and disquieting twists to the story—but it is no longer the major part of the record of the tale itself.

Pathology is the scientific study of disease: it follows the morbid process from its inception to its termination and it investigates the lesions produced. Wherever possible it uses accurate measurement, for the scientific method demands reproducible data. Thus, the size of a cancer cell, its content of DNA, its characteristics in tissue culture, and the life span of the patient in whom it is found can all be measured; all these considerations fall within the scope of pathology. The subject is therefore the concern of clinicians no less than pathologists, a fact well attested by the many valuable contributions made to pathology by clinicians and to clinical medicine by pathologists. The medical graduate whose practice is restricted either to the bedside or the laboratory can be sure of having only an incomplete and unbalanced understanding of medicine.

The fifth edition of *General Pathology* is our continued effort to provide an account of the fundamental processes of pathology in relationship to medical practice. A full understanding of the subject involves the disciplines of morbid anatomy, biochemistry, haematology, microbiology, physiology, and clinical medicine; all these aspects of disease process are dealt with in this book, and in particular we have tried to give a careful description of the basic pathological processes, for both these and the terms that are commonly used to describe them are a constant source of difficulty to the beginner. We do not aim at giving a comprehensive account of recent advances; many fine books and reviews are available for such information.

Five years have now elapsed since the last edition, and although no outstanding breakthroughs in medicine have been evident, the many advances that have been recorded have necessitated a complete revision. Worn-out type and aged blocks have required that the whole text be reset, and we have taken advantage of this situation to recast the book. Three entirely new chapters have been added; in Chapter 33, Some Disorders of Metabolism, the section on glucose metabolism includes an account of diabetes mellitus and the glycogen-storage diseases; an account of gout and the porphyrias follows. Chapter 34, Disorders of Nutrition, encompasses the important topics of starvation, the role of vitamins, and the malabsorption syndrome. Chapter 35, Disturbances of Endocrine Function, includes a section on the APUD system of cells. The topic of calcium metabolism has been expanded and added to the existing section on heterotopic calcification: this now constitutes a new Chapter 36. The remaining new addition to the book is Appendix 4 which is devoted to the HLA histocompatibility antigens.

Over 150 illustrations have been added, either to replace unsatisfactory figures or to provide new material. Sections in which major revision has been necessary are those describing the chemical mediators of acute inflammation, the granuloma, the bactericidal mechanisms in polymorphs in relationship to immunity, immunology, viral diseases (especially the section on the oncogenic viruses and the viruses of hepatitis), rickettsial and chlamydial infections, and the pathogenesis of amyloidosis.

Among numerous topics which have either been expanded or introduced for the first time are the techniques and uses of freeze etching, horseradish-peroxidase staining, gel-filtration chromatography, banding of chromosomes, counter-current electrophoresis, the structure and function of the cell membrane, the Golgi complex, microtubules and microfilaments, Noonan's syndrome, myelin figures, unscheduled DNA synthesis, DNA repair, the metabolism of mucoproteins, the chemical composition of collagen, the collagenases, the functions of macrophages, axial regeneration in amphibians, regeneration of muscle, the tissue reactions in leprosy, silicosis, and asbestosis, the Jarisch-Herxheimer reaction, apoptosis, the anion gap, the platelet release reaction, the pathogenesis of atheroma, vasculitis, the Prinzmetal type of angina pectoris, mountain fever, and the clonal origin of tumours.

There have been two other departures from our previous custom. SI units have been introduced for the first time, since these are commonly used in many parts of the world. The traditional units have been retained for readers who are unfamiliar with the new nomenclature. The references at the end of each chapter have been rigorously scrutinised, and

most of those over 10 years old have been omitted. The old references can easily be obtained from the previous editions and standard texts of pathology, as well as from the current reviews. For the first time the full title of each reference has been included, and it is hoped that this will stimulate the reader to consult the material cited and adopt a critical approach.

J.B.W., Toronto, Canada
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Acknowledgements

We wish to thank W. B. Saunders Company of Philadelphia for generously allowing us to use over 45 illustrations from *An Introduction to the Principles of Disease* by one of us (J.B.W.). The great majority of the specimens illustrated are from the Wellcome Museum of Pathology, and we are grateful to the President and the Council of the Royal College of Surgeons of England for permission to reproduce them. In accordance with their wishes each is acknowledged at the end of the caption, and their catalogue number is indicated. A number of specimens illustrated are from the Boyd Museum, University of Toronto, and we thank Dr E. Farber of that institution for permission to use these.

We are indebted to a number of colleagues for providing valuable criticisms and for assisting in the realms of their particular expertise: Dr Y. Bedard (Toronto), for electron microscopy, Dr G. T. Simon (Toronto), for electron microscopy, Professor J. L. Turk (London) for his views on the granuloma, and Dr G. C. R. Morris (London) for electrolyte balance and renal failure. We thank Dr Leslie P. Spence for reviewing the section on virology; many of the new electronmicrographs of viruses have been provided by Micheline Fauvel of his department of virology at the Toronto General Hospital. We are grateful to Professor A. Fonnesu (Florence) for his helpful criticism, and also for allowing us to use material (especially Figures 33.4 and 33.5) that he added in the Italian translation of the Fourth Edition of *General Pathology*. We are particularly indebted to Mrs Mary Crookston for her continued help in writing Chapter 55 on Blood Grouping and Blood Transfusion. We owe special gratitude to those who have given us unpublished material or have allowed us to modify their original published work. Each figure is acknowledged separately under its caption.

Our thanks are due to Mrs Sonja Duda, librarian at the Banting Institute, Toronto, for valuable help in obtaining references, and to Mr A. A. Silcox and Mr J. T. Manders of the Royal College of Surgeons of England, London, for help with photomicrography.

Contents

Chapter 1. *Introduction*

Pathology, the scientific study of disease processes, has its roots deeply implanted in medical history. The earliest observers, from Celsus (about 30 B.C.–A.D. 38) to Morgagni in the eighteenth century, based their work upon the naked-eye appearances of diseased individuals and organs. Only as the technique of microscopy improved was the Germanic School of Pathology, headed by Virchow (1821–1905), able to investigate changes at a cellular level.

In France, Pasteur, using the microscope, laid the foundation of the science of bacteriology, while later on the German dye industry enabled Koch, Ehrlich, and Domagk to extend this knowledge and open the era of chemotherapy. Advances in pathology thus have been closely related to advances in technology. This in no way belittles the efforts of inspired experimenters like Jenner, who in England pioneered the way to active immunisation.

Nowadays technological advances are occurring so rapidly that no doctor can hope to have a working knowledge of all the methods that are available. Nevertheless, it is by utilising all these complex techniques that problems are fully investigated. The student of medicine should therefore have some understanding of the techniques which are available, and the type of problem which they might be able to solve. In this way investigators conversant with many different disciplines are brought together to their mutual benefit.

TECHNIQUES AVAILABLE IN PATHOLOGY

MICRODISSECTION

Apparatus is available for dissecting cells. In this way the nucleus can be removed, and the effect of this on the deprived cell observed (p. 13). Micropipettes can be inserted into the capillaries, and microelectrodes placed in single nerve cells or fibres. More recently, microbeams of ultraviolet light, or Laser rays (*L*ight *A*mplification by *S*timulated *E*mission of *R*adiation), have been used to produce damage in a particular part of a cell.

The micromanipulation of embryos can produce some interesting results. Thus the developing eggs from two pregnant mice of different strains can be removed and made to adhere to each other *in vitro*. If reimplanted into a pseudopregnant female, the combined embryo can develop into one animal called an allophenic mouse. In this the two cell types form clones* of varying numbers in different organs. When hair colour is involved, roughly equal numbers of melanocytes of both genotypes are produced. If black and white strains of mice are used, the allophenic progeny have a series of transverse bands like a zebra![1] It has been suggested that even in the normal animal mutations could lead to the production of clones of different genetic constitution. Furthermore, even if all the cells of an individual have the same genotype, some could exhibit a separate phenotype due to suppression of certain genes. Perhaps some type of clonal segregation can account for the irregular and patchy distribution of the lesions seen in some human diseases.[2]

*A clone is a group of cells of like hereditary constitution which has been produced asexually from a single cell. The word is derived from the Greek *klon* meaning a cutting used for propagation.

MICROSCOPY

The Light Microscope

The light microscope has two disadvantages:

(*a*) Its *resolution* is limited by the wavelength of light. The ability to distinguish between two adjacent points is called the resolving power. In theory resolution should be possible up to a distance of half the wavelength of the light used, but in practice using green light the resolving power of the microscope is about 250 nm.† Thus, only the largest viruses (the poxviruses) can be seen with a good optical microscope. The shorter the wavelength of the light used the better is the resolution, and with ultraviolet light it can be improved two to three times. It is important to distinguish between *visibility* and *resolution*. Particles as small as 75 nm may be visible, but no detailed structure can be resolved. A simple analogy illustrates this difference. If an open book is placed at a distance from the eyes, the writing can easily be distinguished as such, i.e. it is visible, but it may be quite impossible to recognise individual letters because their details cannot be resolved.

(*b*) Living tissue is transparent, and the homogeneity in optical density of its components hides its detailed structure. Staining techniques must therefore be used to see cellular details, but these must almost invariably be performed on dead fixed tissue. It is possible to stain cells by supravital techniques, e.g. the mitochondria of living leucocytes can be stained by Janus green, but even this damages them rapidly so that the cells soon lose their motility and begin to die.

Three techniques have been developed to overcome these difficulties in examining living cells.

Dark-ground illumination relies upon the fact that objects placed in a beam of light may be seen by the rays which they reflect in much the same way that dust particles are rendered visible by a shaft of sunlight. The method finds particular application in the demonstration of organisms which cannot be readily stained, e.g. *Treponema pallidum* (Fig. 1.1).

Phase contrast microscopy takes advantage of the different refractive indices of various parts of the cell. These differences are converted into differences in optical density. In this way

†1 mm = 1000 μm, 1 μm = 1000 nm.

Figure 1.1
Dark-ground method for demonstrating *Treponema pallidum*. This came from a hanging-drop preparation of fluid from a chancre. It was illuminated by the dark-ground method. (*Photograph by courtesy of Dr. Marta Marschalko and the late Professor Kiraly, Institute of Dermatology and Venereology, Semmelweis Medical School, Budapest, Hungary.*)

living cells can be examined; the method is commonly used in virology (see Figs. 22.8 and 22.9).

Interference microscopy works on a different principle. The light that passes through the microscope is split into two beams. The light passing through the specimen is retarded, and interferes with the light of the other beam when the two are recombined in an image plane. The method finds little application in routine pathology.

Examination of Fixed Tissues[3]

Paraffin section technique. This is the most commonly used routine method of examination. Tissue is fixed, dehydrated in graded alcohols, cleared in xylol, chloroform, or other solvent which is miscible with both alcohol and wax, and finally embedded in paraffin wax.

Fixation. Fixation is a complex process which involves killing the cells rapidly and inhibiting the enzymatic processes of autolysis. Macromolecules are stablised and aggregated, proteins are denatured, and some active groups, e.g. $-NH_3^+$ of protein and $-PO_4^{--}$ of nucleic acid are exposed. The cell's membranes are altered in such a way that stains can penetrate into the cell and its organelles.

Formaldehyde, as a 10 per-cent aqueous solution called formalin, is the most useful, cheap, and commonly-used fixative. It penetrates tissue with ease and will therefore fix large specimens. It reacts by forming bridges between adjacent $-NH_2$ groups in the side-chains of proteins and in phospholipids. Only substances which are rendered insoluble, such as proteins, or which become attached to insoluble substances, such as nucleic acids, can be made visible by subsequent staining. Small soluble molecules, such as Na^+, glucose, and urea, cannot be localised with any degree of precision by present-day methods.

Frozen section technique. The paraffin-wax technique removes certain chemicals, e.g. fat, and alters others, e.g. enzymes and antigens. To meet this difficulty the cryostat has been developed: in essence this is a microtome with which sections are cut at −30°C. This is achieved by having the microtome enclosed in a refrigerator. The sections of frozen fresh material thus obtained are so little damaged that they are suitable for histochemical study. They may also be stained with haematoxylin and eosin, and the method can be used for quick examination of tissue. Some hospitals employ the method for the routine examination of surgical material, but the sections, although adequate for diagnosis, are nevertheless of poor quality.

Staining Techniques for Demonstrating Cell Structure[4,5]

Although many of the staining methods of histopathology have been developed empirically, some general principles can be discerned. There are three types of staining method:

Solution of lipid-soluble dyes in the liquid lipid of cells or tissue.

Staining of tissue by chemical union of coloured or chromogenic substances to reactive loci.

Coloration of added reagents by reactive loci (commonly enzymic) of tissue.

Each of these will be considered in more detail. It should be noted that those methods that are designed to demonstrate specific chemicals in cells or tissues fall within the domain of the sub-specialties of *cytochemistry* or of *histochemistry* respectively.

Lipid Soluble Dyes

An alcoholic solution of a lipid-soluble dye, such as Sudan III or Oil Red O, when applied to a frozen tissue section, will rapidly pass into cellular lipid droplets and thereby colour them. Similarly osmium tetroxide will dissolve in liquid lipid; it is then reduced by ethylenic double bonds to black lower oxides. Osmium tetroxide also acts as a fixative, and in this role finds particular application in electron microscopy (see p. 4).

Staining of Tissue by Chemical Union of Coloured or Chromogenic Substances to Reactive Loci of Cells

The attachment by chemical union depends on one of three mechanisms:

(*a*) electrostatic attraction of dye to oppositely charged tissue loci;

(*b*) precipitation of charged colloids on to oppositely charged tissue loci;

(*c*) covalent union to reactive sites.

(*a*) **Electrostatic attraction of dyes to oppositely-charged tissue loci.** *Acidophilic (positively-charged) tissue loci.* The principal cationic loci are the $-NH_3^+$ groups of basic amino acids. These can be demonstrated by anionic dyes such as eosin, picric acid, and acid fuchsin. If careful, controlled techniques are used, combinations of these dyes will stain cytoplasm and connective tissue selectively. Thus in Van Gieson's stain, picric acid stains cytoplasm and muscle yellow, whereas collagen takes up the red acid fuchsin.

Basophilic (negatively-charged) tissue loci. These include the $-PO_4^{--}$ groups of nucleic acids, $-SO_4^{--}$ of polysaccharides, and the $-COO^-$ of uronic and amino acids. The union of cationic dyes with the last group is tenuous and, in practice, the dyes stain nucleic acids, polysaccharides, and some acidic lipids. Important members of this group are methylene blue, Azur A (formed by the oxidation of methylene blue), pyronin, and methyl green. As with the anionic stains, combinations of dyes can produce differential staining. Thus in Leishman's stain DNA is stained violet by Azur A, RNA of cytoplasm and nucleoli is coloured blue by methylene blue, and cytoplasmic protein including haemoglobin is tinted

red by eosin (p. 600). The most useful cationic dye is derived from haematoxylin, a dye extracted from a tree. By oxidation haematein is produced, and this when mordanted with Al^{3+}, Cr^{3+}, or Fe^{3+} forms a useful deep purple, positively-charged dye, commonly referred to as 'haematoxylin' and used in combination with eosin.

(*b*) **Precipitation of charged colloids on oppositely charged tissue loci.** A good example of this method is Hale's colloidal iron stain. Positively-charged colloidal iron is precipitated and bound to sulphate esters and uronic acids of polysaccharides. The bound ferric ions are converted to Prussian blue by the addition of potassium ferrocyanide.

(*c*) **Covalent union of coloured or chromogenic substances to reactive tissue loci.** Reactive loci in the cell can be demonstrated by combining them covalently with coloured compounds. Two examples will be considered.

Periodic Acid-Schiff (PAS) reaction. When periodic acid is applied to a section, many carbohydrate components are oxidised to aldehydes. Aldehydes produce a red colour with Schiff's reagent (a solution of basic fuchsin decolorised by sulphurous acid). Therefore, if Schiff's reagent is applied to the treated section, the parts containing carbohydrate are stained red. The periodic acid-Schiff reaction is useful for the demonstration of glycogen, ground substance, and epithelial mucus. Glycogen can be differentiated from the others by pretreating the section with diastase, which removes it.

Feulgen stain. Tissue is treated with hydrochloric acid followed by Schiff's reagent. The reaction depends upon the hydrolysis by hydrochloric acid of the purine-deoxyribose linkages in the DNA molecule. The pentose-phosphoric acid complex which is released has a free aldehyde group, which can be detected by Schiff's reagent. Nuclei and chromosomes are therefore coloured magenta. The method is specific for DNA.

Coloration of added reagents by reactive loci of tissue. Some reactive loci, commonly enzymatic, can convert added reagents into coloured or opaque material. If this material is precipitated, the reaction product is located at, or very close to, the original locus. Several examples of this technique will be described:

(*a*) *Enzymatic localisation.* The demonstration of an enzyme is well illustrated by the method used for alkaline phosphatase. The section is placed in a solution of the substrate *4*-glycerophosphate in the presence of calcium. The enzyme splits off phosphate, and this forms calcium phosphate which is invisible. Addition of cobalt nitrate converts it into cobalt phosphate, and ammonium sulphide changes this to black cobalt sulphide.

Although the amount and situation of the black sulphide is indicative of the amount and site of the phosphatase originally present, it is evident that errors can creep in at each of the stages: glycerophosphate → calcium phosphate → cobalt phosphate → cobalt sulphide. The X-ray microscope demonstrates these stages very well.[6,7] This instrument takes radiographs of sections using X-rays generated at 5 to 10kV. The rays are absorbed by elements of high atomic number, and as in the conventional full-size radiographs show up deposits of calcium. Figure 1.2 shows how the original calcium phosphate is localised, whilst there has been considerable diffusion by the time the last step is reached. The X-ray microscope can, of course, be used on unstained tissues to show deposits of calcium as well as other structures.

Histochemical techniques are of value in many areas of pathology: nowhere are they of more value than in the study of disease of the muscles.[8] Two major types of muscle fibre can be identified, and the changes that take place in disease can be studied (Fig. 1.3). Histochemical techniques may also be applied to the study of cells by electron microscopy (see Fig. 2.6, p. 16).

(*b*) *Perls's Prussian-blue reaction for haemosiderin.* Acid is applied to the tissue to release Fe^{3+} from haemosiderin. Next, potassium ferrocyanide is applied; this reacts with the Fe^{3+} to form blue ferric ferrocyanide (Fig. 1.4).

(*c*) *Reduction of silver nitrate to metallic silver.* Some loci, such as those present on melanin granules and in the cells of Kultschitzky, which are called *argentaffin*, can reduce silver nitrate to metallic silver, and are thereby blackened.

Argyrophilic loci bind silver but do not reduce it. Subsequent treatment of the tissue with a reducing agent leads to the precipitation of metallic silver. This silver impregnation method is useful for demonstrating reticulin fibres and spirochaetes (Fig. 1.5).

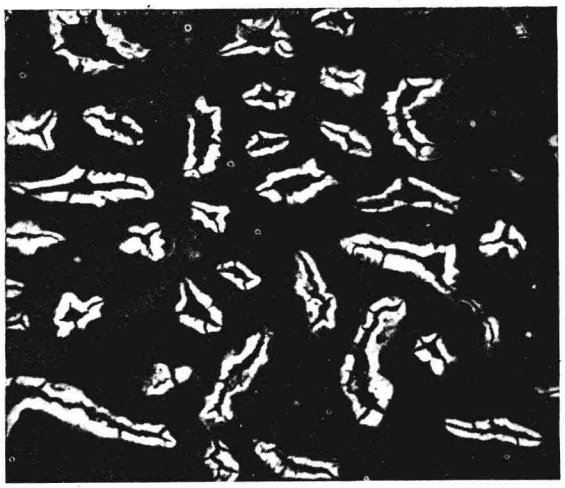
(*d*) *Metachromasia.* Some tissue components have the property of taking up certain dyes and so altering the dye's structure, usually by polymerisation, that the colour is changed. This is called metachromatic staining, and is a property exhibited by the granules of mast cells and by some acid mucopolysaccharides (p. 60). Dyes of the thiazine group are commonly used, and toluidine blue is the best known. It stains most tissues blue, but metachromatic material is coloured purple or even red.

Immunological Techniques in Histochemistry

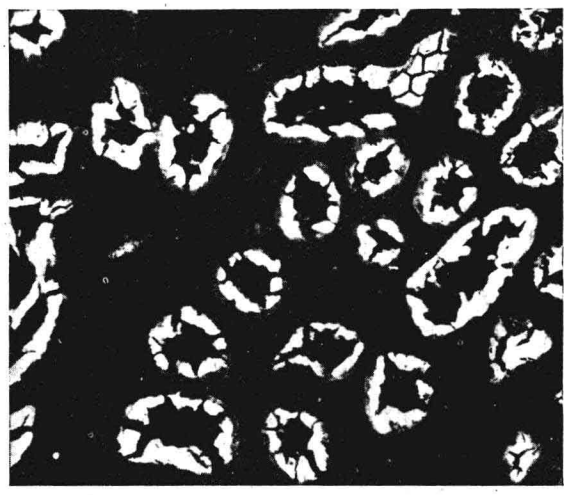
The highly specific binding of antibody to antigen can be utilised to great advantage in histochemistry. The antibody can be labelled with fluorescein; when it is applied to a section, it will become fixed to the appropriate antigen. This can be detected by examining the section under ultraviolet light. An alternative method is to apply unlabelled antibody to the section, thoroughly wash, and then detect the site of antibody attachment by using a second labelled anti-immunoglobulin (Coombs's reagent). This method is known as the *sandwich technique*, and has many advantages. Thus with each layer there is an amplification effect that renders the detection of specific fluorescence easier. Also commercial labelled Coombs's reagent can be purchased, whereas labelling of specific antibody must be done by the investigator himself. Sometimes complex multilayered techniques are used with each layer adhering to a specific receptor site of the previous reagent.

Labelling of antibodies can be done in other ways.

Radioactive isotopes can be used in conjunction with autoradiography both for light and electron microscopy (p. 9). A more recent histochemical method utilises *horseradish peroxidase.*[9,10] This enzyme splits hydrogen peroxide, and the released oxygen produces a brown oxidation deposit with 3-3′-diaminobenzidine. Since osmium tetroxide reacts with this end-product to form an electron-dense chelate, the method can be modified for use in electron microscopy. The peroxidase system can be used in several ways. The enzyme can



(a)



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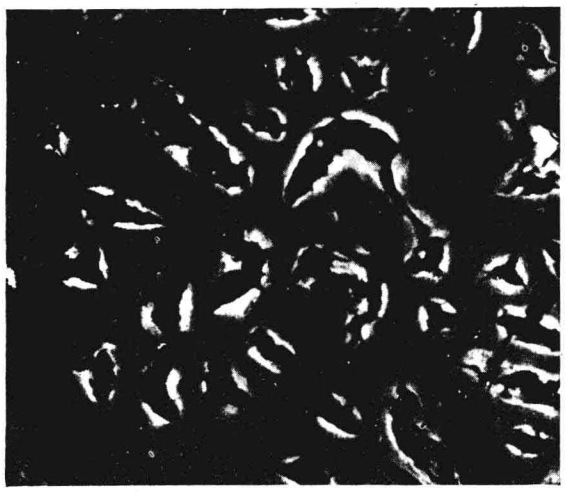

(c)

Figure 1.2
(*a*) Historadiograph of rat kidney after incubation of the section with β-glycerophosphate in the presence of calcium chloride. The deposits of calcium phosphate indicate the site of alkaline phosphatase activity. (*b*) Section treated as (*a*) above, but the calcium phosphate has been converted into cobalt phosphate by the action of cobalt nitrate. (*c*) Final treatment with ammonium sulphide converts the cobalt phosphate into cobalt sulphide. The historadiograph shows that there has been considerable diffusion, and that the distribution of the radio-opaque material no longer corresponds accurately to that of the alkaline phosphatase. Cobalt sulphide is black, and is visible under the light microscope. However, it is evident that a radiograph at stage (*a*) gives a clearer indication of the actual distribution of the enzyme. ×150. (*From Photograph by courtesy of Dr. A. J. Hale. Published previously in Hale, A. J.* (1961), J. biophys biochem. Cytol., **11**, 488)

be attached to the appropriate antibody, and the method then involves one step. Usually it is more convenient to use a multiple sandwich technique with the last reagent being an antibody to peroxidase. The addition of the horseradish peroxidase followed by 3-3′-diaminobenzidine and hydrogen peroxide produces the brown reaction product. This can be detected by routine light microscopy and the preparation is permanent, an advantage over the fluorescent techniques.

The immunoperoxidase technique can be used for many purposes, and has been employed to detect spirochaetal antigen, pituitary hormones, α-fetoprotein, and hepatitis antigen (HBsAg).

The Electron Microscope[11]

The electron microscope resembles the light microscope except that a stream of electrons is used instead of light, and electromagnetic fields instead of glass lenses. Its resolving power is 0·6 to 0·7 nm.* By its use viruses can be seen with ease, and our knowledge of detailed cell and tissue structure has increased enormously (see Ch. 2). The electron microscope of current design has, however, three disadvantages.

1. Only very small pieces of tissue can be examined; 1 to 2 mm blocks are usual. Orientation of the specimen under the electron microscope is aided by first preparing 'thick' sections; these are about 1μm thick as compared with the 60nm normally used in electron microscopy. The sections are stained with toluidine blue and examined under the light microscope.

2. The tissue must be examined in a vacuum—living cells cannot therefore be used.

3. Tissue must be fixed immediately, preferably *in vivo*, if artefacts are to be avoided. This must be borne in mind if it is proposed to examine human surgical material.

The most widely-used fixative is *osmium tetroxide*; this also acts as a stain by virtue of the increased electron scattering power of the tissue components after the deposition of osmium. Osmium tetroxide fixes unsaturated lipids and phospholipids well, but protein components are poorly preserved, and prolonged fixation causes considerable damage to the specimen. Penetration of the tissue is slow, particularly if it has been prefixed by aldehyde.

Glutaraldehyde is a commonly-used fixative that fixes protein well but lipids very poorly. Cytoplasmic organelles, e.g.

*For comparison the diameter of a globular protein of molecular weight 80 000 daltons is 6 nm, glucose is 0·5 nm, and water 0·28 nm.

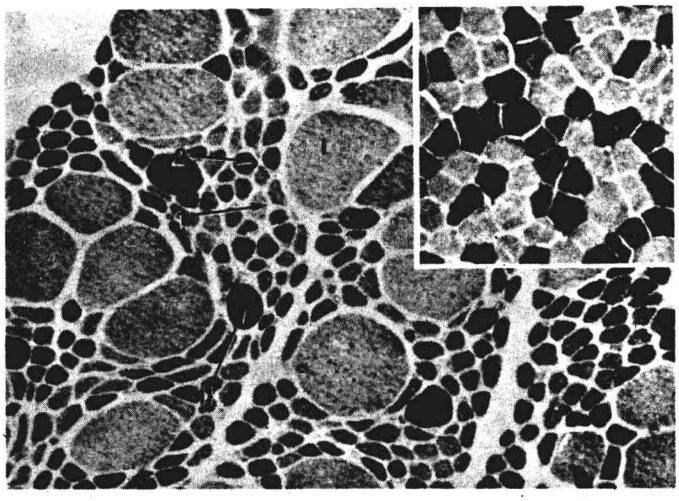

Figure 1.3
Myosin ATPase reaction in skeletal muscle. The routine reaction for ATPase clearly separates muscle fibres into two groups. Type I fibres are lightly stained, and type II fibres are heavily stained. The insert shows normal muscle with the characteristic checkerboard appearance. The fibres are of equal size. Compare this with the muscle from a case of type-I spinal muscular atrophy (Werdnig-Hoffmann disease), which is also shown. Many of the fibres are atrophic, and both types of fibres are affected (1) and (2). A few type I fibres are hypertrophied (1), whereas type II fibres are of normal size. (*Photographs by courtesy of Dr. Dawna L. Armstrong.*)

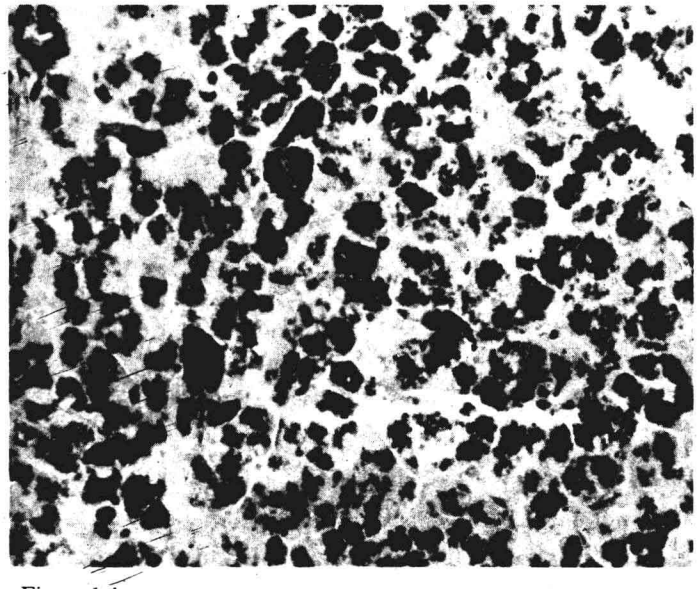

Figure 1.4
Section stained for haemosiderin by the Prussian-blue method. The section shows a massive deposition of haemosiderin in the liver cells. The pigment is stained brilliant blue by this method, but appears black in the photograph. The detailed structure of the liver cells is not well shown. The tissue was from a patient with haemochromatosis. (× 380).

microtubules and microfilaments, are well preserved. Prolonged fixation causes less damage than with osmium tetroxide, an asset when dealing with human biopsy material that has to be transported to the laboratory. *Formaldehyde*

Figure 1.5
Smear from syphilitic lesion stained by a silver-impregnation method. The field is crowded with *Treponema pallidum*. (*Photograph by courtesy of Dr. Marta Marschalko and the late Professor Kiraly, Institute of Dermatology and Venereology, Semmelweis Medical School, Budapest, Hungary.*)

penetrates tissue very rapidly but fixes it slowly. Proteins, e.g. enzymes, are less damaged than by glutaraldehyde, and it is therefore used as a fixative in histochemical studies. Glutaraldehyde can also be used, but osmium is quite unsuitable. Potassium permanganate was used as a fixative, but has largely been replaced by glutaraldehyde.

Double fixation. It is now a common practice to combine several fixatives in the hope of bringing together their various virtues. A popular method is to use glutaraldehyde as a primary fixative and follow this by 'post-fixation' in osmium tetroxide. *Universal fixative* contains formaldehyde and glutaraldehyde; an advantage is that tissue can remain in it with little damage. Regardless of the fixative used, sections can usefully be stained by a salt of a heavy metal; alkaline lead hydroxide and uranyl acetate are the ones most often used.

Although fixation is essential if cellular details are to be examined, remarkably good results can be obtained with poorly fixed material under some circumstances. Thus even formalin-fixed tissue embedded in paraffin wax can be examined usefully by electron microscopy. In this way melanosomes or neurosecretory granules can be identified in tumour cells if it is desired to re-investigate the nature of a tumour in old surgical material (see p. 310).

Histochemistry. Whereas in light microscopy the final reaction product of a histochemical stain is coloured, in electron microscopy it contains a heavy metal and is therefore electron-dense (see Fig. 2.6). Another approach is to conjugate ferritin with protein; in this way intracellular antigens and antibodies may be localised[1,2] (see p. 152).

Methacrylate was the first embedding medium to gain popularity, but this plastic tends to disintegrate in the electron beam and artefacts are produced. It has therefore been replaced by more stable plastics, e.g. araldite and epon, and pictures of much higher quality can now be obtained. Two other developments of electron microscopy are of note:

Replication. A surface can be examined by making a replica of it, e.g. with a carbon film, and then examining the replica with the electron microscope. The technique is commonly combined with freeze fracturing.[13] Tissue is frozen to the temperature of liquid nitrogen, placed in a vacuum chamber, and fractured with a special knife. The surface so exposed is sprayed immediately with evaporated platinum and carbon from an angle of 45°, and the replica so formed is strengthened by further deposition of carbon applied vertically. The tissue itself is dissolved away, and the replica is studied by transmission electron microscopy. The process is known as *fracture replication*. If the fractured tissue surface is partially freeze-dried, ice is sublimated, and a deeper surface is revealed. This process is referred to as 'etching'. A replica is subsequently made in this process of 'freeze-etching' When tissue is fractured, the lines of cleavage tend to follow membranes, and indeed may actually split the unit membranes (p. 14). Hence these techniques have been of greatest value in the study of cell membranes.

Negative staining. The method consists of embedding the particles to be examined in a layer of electron-dense material, e.g. containing sodium phosphotungstate or a uranium salt, so that the objects stand out against a dense background. Viruses and amyloid fibres have been usefully studied by this technique (Figs. 22.1 and 2 and 44.6 and 7).

Instruments with a high accelerator voltage are now available, and by their use it has been possible to photograph living bacteria. Although useful in metallurgy, their contribution to biological knowledge has so far been small.

Scanning electron microscope. This instrument works on a different principle from the conventional transmission electron microscope: a very fine beam of electrons is focused to a point and made to scan the surface of the specimen. The secondary electrons scattered from the surface are collected and amplified. The current so generated is used to modulate the brightness of a television tube which is scanned in synchronicity with the electron beam scanning the object. The method is useful for examining the surfaces of objects, and the pictures so obtained have a remarkable three-dimensional effect (Fig. 1.6). Micro-organisms, red cells, and intestinal epithelium have been studied with advantage.

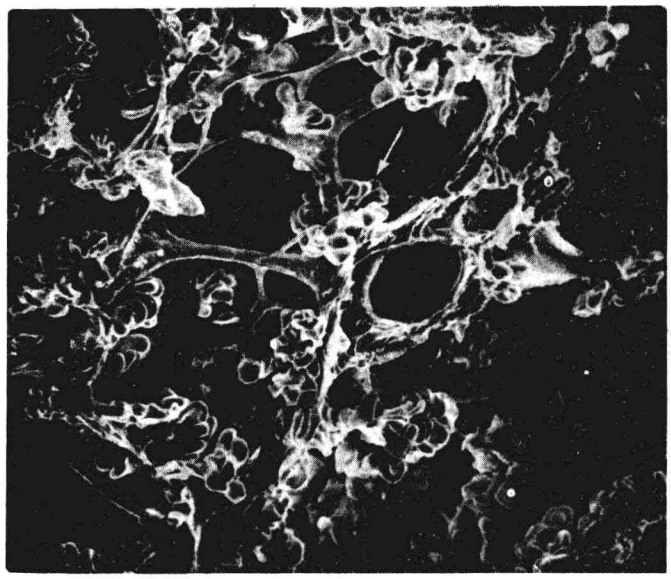

Figure 1.6
Scanning electron micrograph showing the sinuses of a haemolymph node of a rat. Trabeculae are seen crossing the sinuses, and in this mesh-work there are macrophages surrounded by red cells which are being phagocytosed. The sinus-lining cells cannot be discerned, but the groups of red cells forming a rosette around each macrophage are clearly seen and one of them is indicated by an arrow. (*Photograph by courtesy of Drs. C. Nopajaroonsri, S. C. Luk and G. T. Simon*)

THE ULTRACENTRIFUGE

The tremendous centrifugal force that can be applied by this machine is utilised to separate mixtures of large molecular chemicals. The present-day instrument can develop a centrifugal field sufficient to spin down partcles as small as 1 nm. The velocity with which sedimentation occurs can be measured, and the ratio:

$$\frac{\text{Sedimentation velocity}}{\text{Centrifugal field}}$$

is called the Sedimentation Constant. It is measured in Svedberg units (S).

The ultracentrifuge has been used to separate mixtures of proteins; it was in this way that the macroglobulins were identified (see Ch. 49).

The various components of cells can also be separated and examined by physical and chemical means. If cells are disrupted either physically or by ultrasonic vibration, the cell membranes, nuclei, mitochondria, and ribosomes can be isolated in a fairly pure state after centrifugation. By analysing these fractions the results of histochemistry may be correlated with those of the well-established procedures of chemistry. The many enzyme systems of mitochondria have been investigated in this way. The granules in cloudy swelling have been isolated and identified as altered mitochondria. (See p. 52.)

CHEMICAL AND PHYSICAL ANALYSIS OF SUBSTANCES OF BIOLOGICAL INTEREST

It is beyond the scope of this book to deal with this subject. It is sufficient to note that methods are available which have led to the complete analysis of substances as complex as the insulin molecule (51 amino-acid residues), the enzyme ribonuclease (124 amino-acid residues), and more recently immunoglobulin. X-ray diffraction techniques have revealed the complex folded structure of a large molecule like myoglobin. Modern methods of chemical analysis combined with automation have made it possible to estimate the concentration of a large number of chemical substances in a small quantity of body fluid, e.g. blood. With the SMA-12 AutoAnalyzer, which is routinely used in large centres, it is currently possible to estimate the concentration of 12 substances from a single

sample of blood. It is often cheaper to obtain this *biochemical profile* than to make individual chemical estimations.

The techniques of immunology have been of great value in the identification and assay of proteins. The radioimmunoassay method has been adapted to measure the blood levels of certain peptide hormones, e.g. insulin, glucagon, TSH, and ACTH, which are present in picograms or nanograms per ml.* The method depends upon the ability of unlabelled hormone to inhibit, by simple competition, the binding of labelled hormone (e.g. with ^{131}I or ^{125}I) by specific antibody. The hormone content of an unknown sample is determined by comparing the degree to which it inhibits the binding of labelled antigen with the inhibition produced by a series of standard solutions containing known amounts of hormone. The reagents required are therefore pure hormone, labelled hormone, and specific antibody. The amount of labelled hormone bound to antibody is determined after separating it from the free labelled hormone. This separation is done by electrophoresis or by chemical means.

Two other methods have found particular application in pathology:

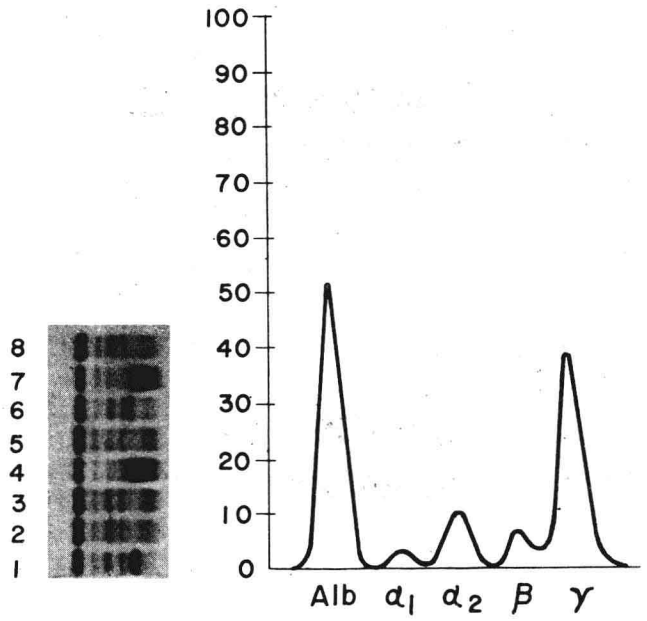

Figure 1.7
Cellulose acetate electrophoresis of serum. On the left electrophoretic strips from 8 separate sera are shown. The anode is on the left, and the dense band that has moved furthest to the left is due to albumin. No. 8 is normal serum, whereas Nos. 4 and 7 show a diffuse increase in the γ-globulins; each shows the picture of a polyclonal gammopathy. No. 1 shows a dense band in the γ-globulin area; the density of the bands of strip No. 1 is depicted in graphic form on the right. The various serum proteins are in the same relative positions on both the strip and the graph. The sharp spike in the γ region is characteristic of a monoclonal gammopathy. (*Reproduced, with permission, from Hall, C. A.:* Gammopathies. In Halsted, J. A. (Ed.) (1976) The Laboratory in Clinical Medicine, p. 493. Philadelphia: Saunders.)

Electrophoresis

If a mixture of proteins is placed in an electric field at a known pH, individual proteins move at particular rates dependent to a great extent upon their size and charge. The test is conveniently performed on filter paper, starch or other gel, or cellulose acetate strip, the choice depending upon the nature of the substances being investigated. After passing an electric current for a suitable time the strip is dried, and the separated proteins are stained with a simple dye, e.g. light green. Figure 1.7 shows a typical electrophoretic separation of the plasma proteins. Combined with the agar-diffusion technique, electrophoresis has proved invaluable in separating protein mixtures (see Fig. 1.8 and caption).

Chromatography[14]

Chromatography is an important technique for separating pure substances from mixtures. The chromatographic system consists of two immiscible phases, a *stationary phase* which is fixed and granular, and a *moving phase* which flows through the interstices of the stationary phase. The moving phase is fluid (liquid or gas), and its movement is effected by gravity, applied pressure, or capillarity. The stationary phase is usually a finely divided insoluble solid.

Chromatographic separation depends on the fact that different substances follow the moving solvent at different rates. All these substances must be soluble in the moving phase, but some become distributed on the surface (adsorption) or throughout the interior (partition) of the particles of the stationary phase (Fig. 1.9). Those substances whose distribution favours the moving phase pass more rapidly through the chromatogram than those which favour the stationary phase. If the column is sufficiently long, a complete separation of substances between successive bands of pure solvent can be achieved, as is seen in Figure 1.8.

There are three main types of chromatography: (*a*) *column chromatography*, in which liquid passes down through particles of solid packed in a glass tube; (*b*) *paper chromatography*, in which an organic solvent moves by capillary action through the pores of filter paper, the fibres of which contain a little water; (*c*) *thin-layer chromatography*, which resembles (*b*) except that the stationary phase in the form of a gel or plastic sheet, is made to adhere to a glass up which the solvent moves by capillarity.

Paper or thin-layer chromatography has found great use in the separation of amino acids and sugars in a solution such as urine. Each component moves at a specific rate along the paper depending on the solvent, so that the ratio

$$\frac{\text{Distance travelled by component}}{\text{Distance travelled by solvent}},$$

or the R_f value, is a constant for each component under the conditions of the test. After allowing the solvent to move a certain distance the paper is dried, and the area occupied by the component is 'developed' by spraying the paper with a chemical that reacts with that component, e.g. ninhydrin turns purple with amino acids. If a series of known substances are run in parallel with the unknown components, their R values

*1 milligram = 1000 micrograms (g). 1 microgram = 1000 nanograms (ng). 1 nanogram = 1000 picograms (pg).

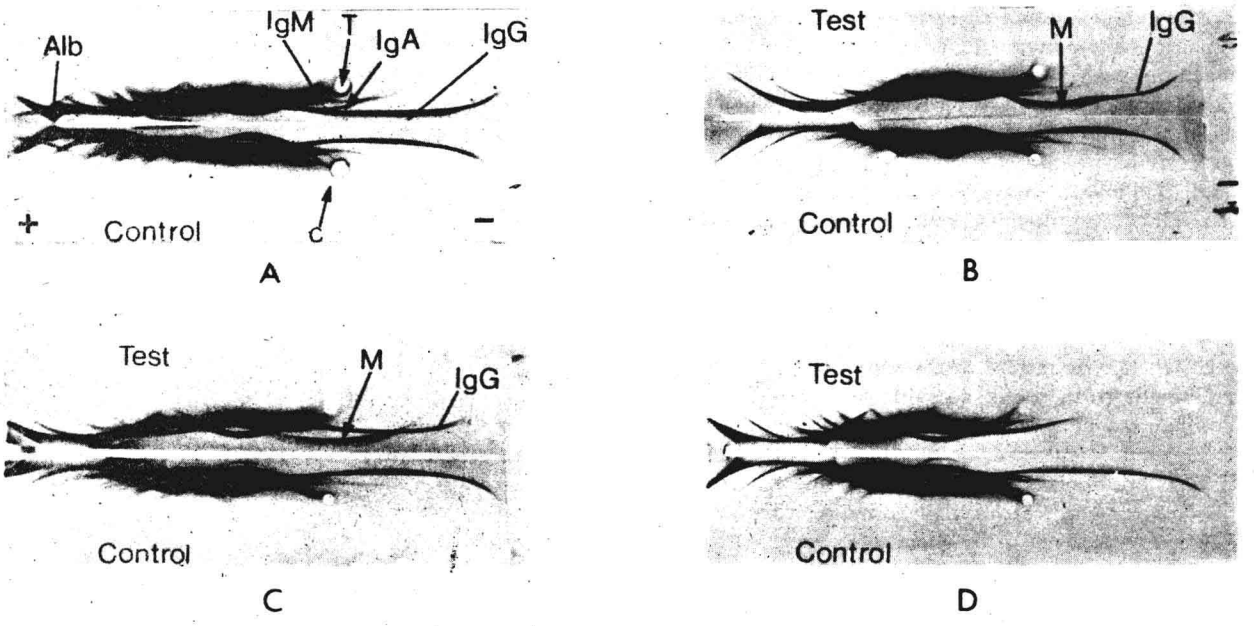

Figure 1.8
Immunoelectrophoresis of serum. This method entails the separation of the proteins by electrophoresis in a gel, and then demonstrating each fraction by means of a precipitin reaction using an antibody.

Sera (test and control) are placed in the two cups in a sheet of agarose gel. Albumin has a strong negative charge, and γ-globulin has a weak negative charge. The application of an electric field results in a tendency for the proteins to move towards the anode. Their actual movement, however, is determined not only by their molecular size and charge but also by a flow of water towards the cathode due to the phenomenon of electro-osmosis. This is due to a negative charge on the gel itself, and as the gel is fixed, so its tendency to move towards the anode actually results in a movement of water to the cathode. The weakly charged γ-globulins are thus carried towards the cathode.

The separated plasma components are demonstrated by placing antiserum to whole plasma down the central strip. From there the antibodies diffuse to form precipitin lines with each separated serum protein. A stain to accentuate these lines has been used.

A. The IgG band is heavier than that of the control and is closer to the central strip. Serum tested here was from a case of systemic lupus erythematosus with polyclonal hypergammaglobulinaemia. The IgA and IgM bands are well shown. *B*. An M protein is present and distorts the normal IgG band (from a case of multiple myeloma with an IgG M protein). *C*. An M protein is present and is in the position of the normal IgA band; note how the normal IgG band crosses the M protein (from a case of multiple myeloma with an IgA M protein). *D*. Note the deficiency of the IgG band (from a case of congenital hypogammaglobulinaemia). (*Photograph by courtesy of Dr. K. C. Carstairs.*)

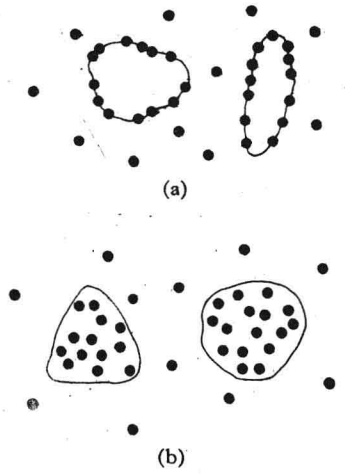

Figure 1.9
Distribution of a substance between moving and stationary phases (*a*) adsorption and (*b*) partition mechanisms. (*Eastoe, J. E.* (1964), Ann. roy. Coll. Surg. Engl., **35**, 234)

can be compared. In this way an unknown component can be identified.

Column chromatography is used to separate and purify the individual components of a solution containing a mixture. In *ion-exchange chromatography*[15] a cellulose resin is packed into the column, and proteins are bound in varying degree to the particles by electrostatic forces. The diffusion of small molecules into the pores of a gel from which large molecules are excluded because of their size forms the basis of *gel-filtration (exclusion) chromatography*.[15] The original starch gel has now been replaced by standardised cross-linked dextran known as *Sephadex*. Many grades are available and vary in the degree of cross-linkage—this determines the upper limit of size of molecule that can enter the pores. Gel-forming beads are allowed to swell in water, and are then packed in the column. Large molecules cannot enter the beads, and so pass rapidly through the column. Small particles enter the beads and pass slowly through the column. The method has found great use in the separation and purification of proteins; in general they appear in the column eluent in order of decreasing molecular size (Fig. 1.10).

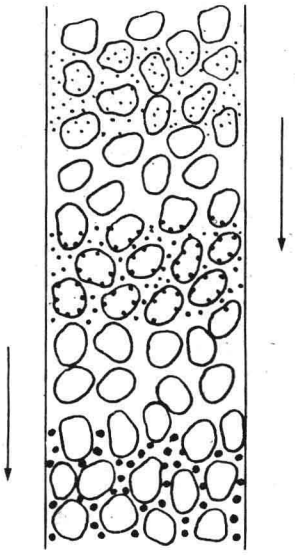

Figure 1.10
Gel filtration or exclusion chromatography.
Molecules of three sizes are seen being separated by passage down a column of gel beads. Large molecules are excluded from the beads, and pass rapidly through the column. Small molecules enter the beads, and pass slowly down the column.

FLUORESCENT TRACING TECHNIQUES[16]

The ability of certain compounds to convert the energy of ultraviolet light into visible light can be utilised in various ways. Fluorescent dyes, e.g. auramine, have been used to stain tubercle bacilli, which are then easily visible under the ultraviolet microscope. The use of the fluorescent dye quinacrine for Q-banding of chromosomes and for demonstrating the Y body is described in Chapter 3.

Fluorescein and lissamine rhodamine B can easily be attached to protein molecules. Labelled antibodies are widely used in immunopathology (see p. 152).

RADIOACTIVE ISOTOPES

The radioactive isotopes are treated by living cells in the same way as the normal elements. Their radiation can be detected by suitable counters, and they may usefully be employed as labels in a wide range of fields. Clinically, thyroid function can be investigated by estimating the distribution of an administered dose of radioactive iodine in the plasma and over the thyroid gland. Radioactive chromium can be attached to the red cells, and their length of survival estimated (see Ch. 52). The clearance of a small quantity of injected radioactive sodium from the skin has been used to measure blood flow. In the experimental animal many compounds can be tagged and their metabolism followed, e.g. albumin.

The introduction of the man-made element *technetium* has revolutionised the use of radioactive isotopes in humans. This element emits gamma radiation only, and is therefore safer to use than isotopes that emit other more damaging radiations. It has a half-life of six hours. When injected intravenously with pyrophosphate, technetium is taken up selectively by the bones, probably by the osteoid tissue. Using this technique the whole skeleton can be surveyed, and the method now augments radiography in the detection of bony lesions, particularly metastatic carcinoma. If the technetium is attached to aggregated albumin, it becomes concentrated in the lung; an estimate of the blood flow through this organ is thereby obtained. By various techniques it is now possible to scan many other organs; it is particularly useful in the investigation of lesions in the liver and the thyroid gland.

Autoradiography

The isotopes can also be used at a microscopic level. By placing a section of tissue on a photographic film, subsequent photographic development will reveal the site of isotope localisation as a series of black grains. As an example of the value of this it is found that tritiated thymidine given to a cell is incorporated into its nucleus. This may therefore be used as an indicator of DNA synthesis (see Fig. 1.11).

PHOTOGRAPHY

Apart from the value for record purposes photography plays an important role in many other fields. It is essential in ultraviolet, electron, and X-ray microscopy. The ultrafast films now available have made high-speed cine photomicrography possible, and blood flow can be examined from films shot at 7600 frames per second (the 'normal' is 24 per sec.). Time-lapse cine photography, on the other hand, has made it possible to 'speed up' the movement of living cells grown in tissue culture. This technique, as yet but little used, has provided interesting information on the behaviour of phagocytes, lymphocytes, and tumour cells.

TISSUE CULTURE[17]

Tissues of various types can be cultured outside the body with comparative ease. The usual surface employed is glass or plastic, and the tissues can be grown in test-tubes, medicine-flat bottles, flasks, or simply on the surface of a cover-slip inverted over a slide with a hollow depression.

There are two types of tissue culture: (*a*) *cell culture*, in which the cells of an organ or tumour are allowed to grow out. The organ is first cut up into small fragments which are then digested with trypsin at 37°C until there is complete disintegration. The cells derived from the tissue are centrifuged, suspended in a liquid growth medium that usually contains serum and a balanced salt solution, and allowed to sediment in a container; they rapidly become attached to the surface, and within a few days multiplication is sufficient to cause it to be covered with a monolayer of confluent cells. Another method consists of attaching a tissue fragment, or explant, to the glass surface, adding a growth medium, and then waiting for an outgrowth of cells from it. Cell culture is an extremely important technique for cultivating viruses, and is described in Chapter 22. Its use in cancer research has proved disappointing except in the study of tumours of nervous tissue, where the cells growing out sometimes differentiate into specific elements, e.g. astrocytes or ganglion cells, thus allowing the tumour to be more easily classified. (*b*) *Organ culture*, in which a fragment of tissue is grown on a grid so that there is no cellular outgrowth, but the organ is kept as normal

(a) (b)

Figure 1.11
DNA localisation. Autoradiograph of a Feulgen-stained anaphase figure in a squash preparation of the root tip of *Allium cepa* fed with tritiated thymidine. (*a*) Photographed at the level of the section to show the chromosomes. (*b*) The same cell photographed at the level of the photographic emulsion. The labelled thymidine has been taken up by the cell and incorporated into the DNA. Note how the silver dots correspond to the chromosomes. × 3200. (*Photographs by courtesy of Dr. P. B. Gahan*)

histologically as is possible. Limb buds, eye rudiments, and bones have been studied while growing in an artificial medium, and their differentiation observed. The effect of vitamin A on the skin of chick embryos has been investigated in this way (see Ch. 34).

The culture of human cells has yielded much information.[18] Some normal human cells, e.g. fibroblasts, can be sustained in tissue culture for about 50 generations. Serially propagated mammalian cells do not necessarily carry out the same specific functions as when situated in the organ from which they were isolated. However, enzyme studies on human cell cultures have shed some light on the genetic enzyme defects seen in some diseases, and have proved to be useful in the antenatal diagnosis of some inherited diseases (p. 38).

Another interesting aspect of cell culture is the technique of making two somatic cells fuse to form a common cell. Sometimes the stimulus of a virus infection has been used, and the mononuclear hybrid cell may be capable of proliferation. For example, hybrid cells between a human cell line and a mouse cell line have been obtained.[19] Another method of combining DNA from two cells is based on the finding that certain DNA viruses, such as polyoma and S.V. 40, can add new genes to cells in culture when these are infected.[20] The viral DNA can become incorporated in the host's DNA and reproduce with the host-cell chromosomes. Sometimes the descendants of these infected cells can be stimulated to synthesise infectious virus particles again, and, in the case of the polyoma virus infecting mouse cells, the infected virus particles contain mouse genes.[21]

Tissue culture has played a useful part in adding to our knowledge of cellular metabolism, but its use is greatly limited by the fact that the cells are surviving in a completely abnormal environment; the vascular arrangement of the living body is replaced by a synthetic fluid medium which bathes the tissue.

It is certain from this bewilderingly large number of specialised techniques at our disposal that many of the intimate secrets of cellular physiology are now being revealed. Nevertheless, it is still as true as ever that the most important investigation into the nature of disease is based on a careful study of the patient.

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