Handbook of
Histopathological and Histochemical Techniques
(including museum techniques)

THIRD EDITION

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With a Foreword by

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Foreword

Despite the number of excellent texts on histological techniques and histochemistry now available, there is an unfortunate dearth of definitive works to guide the histopathologist or technologist in a practical fashion through the pitfalls of histological technique and at the same time provide in a clear, succinct manner the relevant supporting data and attendant bibliographical review. This new edition of *Handbook of Histopathological and Histochemical Techniques* does, I believe, fill this need.

During post-graduate study in England and the United States of America, I became aware of the meticulous and superb preparations that can be expected from first-class histological technologists. Charles Culling is an expert in his field and, utilizing his many years of experience in teaching and in applying these techniques, many of which he has in fact devised or modified, he has brought to a clear focus those methods which have proved most useful and practical.

He has added a wealth of new material to this edition, including an exhaustive survey of carbohydrate and protein histochemistry. The excellent chapters on fluorescent and immunofluorescent methods, the technical aspects of electron microscopy, and those dealing with fixation, lipids and enzymes, have all been revised and up-dated.

This work is, in fact, a complete book of methodology correlated with a discussion of the relevant theory for each method outlined.

When using this book one experiences the feeling that an expert is looking over your shoulder ready to offer advice. I believe it will be invaluable to all those with an interest in histology and histochemistry, both as a reference text and at the bench. It should prove of particular assistance to selected undergraduate students in the biological and health sciences, pathologists–both in the hospital setting and in experimental research–technologists and workers in various research fields.

W. L. DUNN

Preface to the Third Edition

Since the second edition, there has been a phenomenal expansion in the field of histochemistry and perhaps, even more particularly, in the literature pertaining to it. In our laboratory, over these nine years, we have endeavoured to test most of the new methods that have been described and to determine their specificity, usefulness and reliability of performance as routine methods. We have probably missed many of those published and perhaps been unfair to some we tried, but an effort has been made to incorporate in this new edition those methods that have been deemed useful and reasonably reliable. All books share the bias of their author and although I have endeavoured to reduce this to a minimum, the chapters on fixation and carbohydrates will, by their bibliographies, attest to mine. I have, for the first time, included a chapter on proteins which I hope embodies all the currently useful methods for their identification and/or demonstration. All the other chapters have been updated as far as possible; for example, the cell, enzymes, chromosome techniques, lipids, pigments, and so on. The chapters on museum technique, while unchanged, have been left in as one of the few textbook sources of information on the subject.

My continued association with students has helped tremendously with the preparation of the material and my association with post-graduate students in histochemistry, particularly those from the allied sciences, has broadened my concepts and will, I hope, make this volume more useful to them.

Due to the pressure of time and space, there are almost certainly errors of commission and omission and for these I apologize.

C. F. A. CULLING

Preface to the First Edition

When invited by the publishers to write this book, I was pleased to accept in view of the fact that having studied and practised this subject for over twenty years I felt there was a need for a textbook covering a wider field. Teaching and examining candidates for the Institute of Medical Laboratory Technology final examination in histopathological technique has emphasized this point, and since it embraces every aspect of the subject I have kept the Institute of Medical Laboratory Technology examination in mind while writing. I hope that this book will also be of use to those wishing to learn or practise histopathology or histology—such as students of biology, physiology or medicine. It should prove of value not only as a textbook from which to learn the subject, but also as an up-to-date reference book.

If the contents appear to be unbalanced in some respects, for example, the greater attention given to the anatomy of the central nervous system, and the composition and classification of the lipids and connective tissue, it is because my experience in teaching leads me to believe that a great deal of difficulty in learning and practising techniques is due to a lack of basic knowledge, particularly in these subjects. For similar reasons I make no apology for the amount of space given to microscopy.

Histochemical methods are playing an increasingly larger part in the histopathology laboratory and, although most of the traditional methods have been included, new methods are also given if they have proved reliable.

The term 'histochemistry' has come into prominence in recent years as the study of the chemistry of tissue components by histological methods, and it is probable that the impetus given in the post-war years

to this type of method, with its greater accuracy and control, is responsible for the impression that it is of recent origin. In fact Raspail, in 1830, wrote an essay (*Essai de Chimie Microscopique applique a la Physiologie*) which is generally accepted as the beginning of recorded histochemistry.

The point at which histopathology ends and histochemistry begins is impossible to determine and, although often regarded as an entirely separate subject, histochemistry is, in fact, the basis of many so-called histopathological methods.

. I have been fortunate in being able to call on many colleagues at the Westminster Hospital Medical School for advice and helpful criticism; to them I offer my sincere thanks.

I am especially grateful to Professor R. J. V. Pulvertaft for his constant encouragement and criticism of the script; Dr. E. Ball for his invaluable advice and correction of manuscript and proofs; Dr. J. D. Billimoria and Mr. J. F. Wilson for technical advice; Professor D. S. Russell, Director of the Institute of Pathology, the London Hospital, for the use of material; and Mr. H. J. Oliver and Mr. V. S. Trenwith, of the London Hospital, for advice and criticism of proofs; Mr. J. R. Stokes, who took many of the colour photographs; and Dr. P. Hansell and Mr. L. Hill, Department of Medical Photography and Illustration, for assistance in preparing photographic material.

I record my thanks to those commercial firms who have kindly supplied blocks or photographs for inclusion in the book; Miss A. M. R. Collard, for secretarial assistance; and to my publishers, who have been most co-operative and helpful at all times.

C. F. A. CULLING

Acknowledgements

If I have learned anything over the years, since writing the first edition of this work, it is that the quality and breadth of one's thinking is remarkably dependent upon one's colleagues, both past and present. I have been extremely fortunate in this regard and I thank them all most sincerely. Most particularly, I would thank Mr. J. F. Wilson at the Westminster School of Medicine, London, who first taught me how to cut and stain a section and who is perhaps more responsible than any other for any success I have achieved in this field—for me he will remain the doyen of histopathological technique.

I am especially grateful to Doctor W. L. Dunn, Professor and Head of the Department of Pathology at the University of British Columbia, and to Doctor H. E. Taylor (formerly Head of the Department who is now Director of Personnel Support for the Medical Research Council of Canada), who have both given unstintingly of their time to encourage, discuss and criticize material for me.

I would also particularly thank Doctor P. E. Reid, my research associate, whose knowledge and time I have drawn upon in large measure; he has shared my concerns, generally filled the role of critic, abstractor and enthusiast when the volume of literature seemed unending.

Doctor Philip S. Vassar has continued to give freely of his time, knowledge and up-to-date and encompassing files of published papers, all of which have been invaluable.

Doctor W. H. Chase, who wrote the chapter on the electron microscope in the last edition, has yet again generously assisted me with this task, for which I thank him most sincerely. My colleagues at the University of British Columbia, too numerous to mention, I thank no less sincerely.

To my technical assistants, particularly Mrs. Barbara Barkoczy, Mrs. Alison Russell, Mrs. Linda Trueman and Miss Maureen Day, I express my gratitude for their patience and perseverance with many of the more difficult techniques they worked on.

These acknowledgements would not be complete without mention of my wife, Lois, whose patience has been unending and support tireless.

Many of the new methods published or modified arose from research supported by grants to Doctor H. E. Taylor, Doctor W. L. Dunn or to myself from the Medical Research Council of Canada.

I would like to record my thanks to Mrs. Audrey Spencer for secretarial assistance, to the staff of the Woodward Library, University of British Columbia, who were of great assistance in literature surveys, and to my publishers who have been most understanding and co-operative at all times and in all ways.

Vancouver, B. C. C. F. A. CULLING

Part I - Introductory

Histopathological technique is that branch of biology concerned with the demonstration of minute tissue structures in disease. Since the differences between diseased and normal tissues are often slight, it follows that the majority of the methods involved may be used for both.

Before such structures can be demonstrated the tissue must be prepared in such a manner that it is sufficiently thin (one to two cells thick) to be examined microscopically, and that the many and complex structures which go to make up tissue may be differentiated. This differentiation is usually achieved by selective colouring, and, since it is impossible to demonstrate all these structures in one preparation, methods are employed which stain one or more in each section or slice of tissue.

The term 'histochemistry' has come into prominence in recent years as the study of the chemistry of tissue components by histological methods, and it is probable that the impetus given in the post-war years to this type of method, with its greater accuracy and control, is responsible for the impression that it is of recent origin. In fact, Raspail, in 1830, wrote an essay, *Essai de Chimie Microscopique Applique a la Physiologie,* which is generally accepted as the beginning of recorded histochemistry.

Mann (1902) said 'the object of all staining is to recognize microchemically the existence and distribution of substances which we have been made aware of macrochemically. It is not sufficient to content ourselves with using acid and basic dyes and speculating on the acid or basic nature of the tissues, or to apply colour radicles with oxidizing or reducing properties. We should find staining reactions which will indicate the presence of certain elements such as iron, phosphorus,

carbohydrates, nucleus or protamines, and so on.' Mann's words are as cogent today as they were 55 years ago when they were written.

There are special methods of preserving and preparing the tissue in mass, known as fixation, which precede the special staining methods employed. This process of fixation is used even when tissue or body fluid is smeared on glass slides.

When blocks of tissue are to be examined they must, after fixation, be cut into thin slices or sections. In order that such sections may be cut and manipulated they are normally impregnated and embedded in a firm medium, usually paraffin wax. The various methods of examination of tissue cells and structures are summarized in Chapter 2.

Chapter 1 describes the structure and contents of the cell, since it is considered that to attempt to practise histopathological technique without a knowledge of the cell is analogous to trying to drive a car without any knowledge of its controls.

Chapter 1

The Cell

The body is composed of tissues. Each tissue is composed of units of living matter (cells) and non-living fibres. The cells have certain common characteristics which are dealt with later in this chapter, but they are of various types, each of a specialized nature, differing from others in shape, size and function. The type of arrangement of cells and fibres enables the various tissues to be recognized.

As seen in the normal histological preparation, the fixed cell can do no more than bear a resemblance to the living, and the method of processing and staining will determine how near that resemblance will be. Long study of histological preparations leaves the observer unprepared for the fascinating picture of living cell cultures revealed by phase-contrast microscopy. By this technique the cells can be seen as living entities, sometimes actively moving in the preparation (for example, polymorphonuclears, lymphocytes, histiocytes), but always showing activity within. Many aspects of living cells can be seen by modern microscopy, which enables them to be studied in some detail, but even this detail is limited and by no means all the components can be seen and recognized.

A knowledge of normal histology is of immense help in practising histological technique, and those who hope to master the subject are advised to study it. If techniques are to be understood and controlled a knowledge of general cell structure is essential, and for that reason will be dealt with in some detail.

The cell *(Figure 1.1)* is to living tissue what the molecule is to chemistry; that is to say, it is the unit of which larger masses are built, and which cannot be further divided without losing its identity. The cell is composed of a *nucleus* surrounded by *cytoplasm*, each of these being enclosed by a membrane.

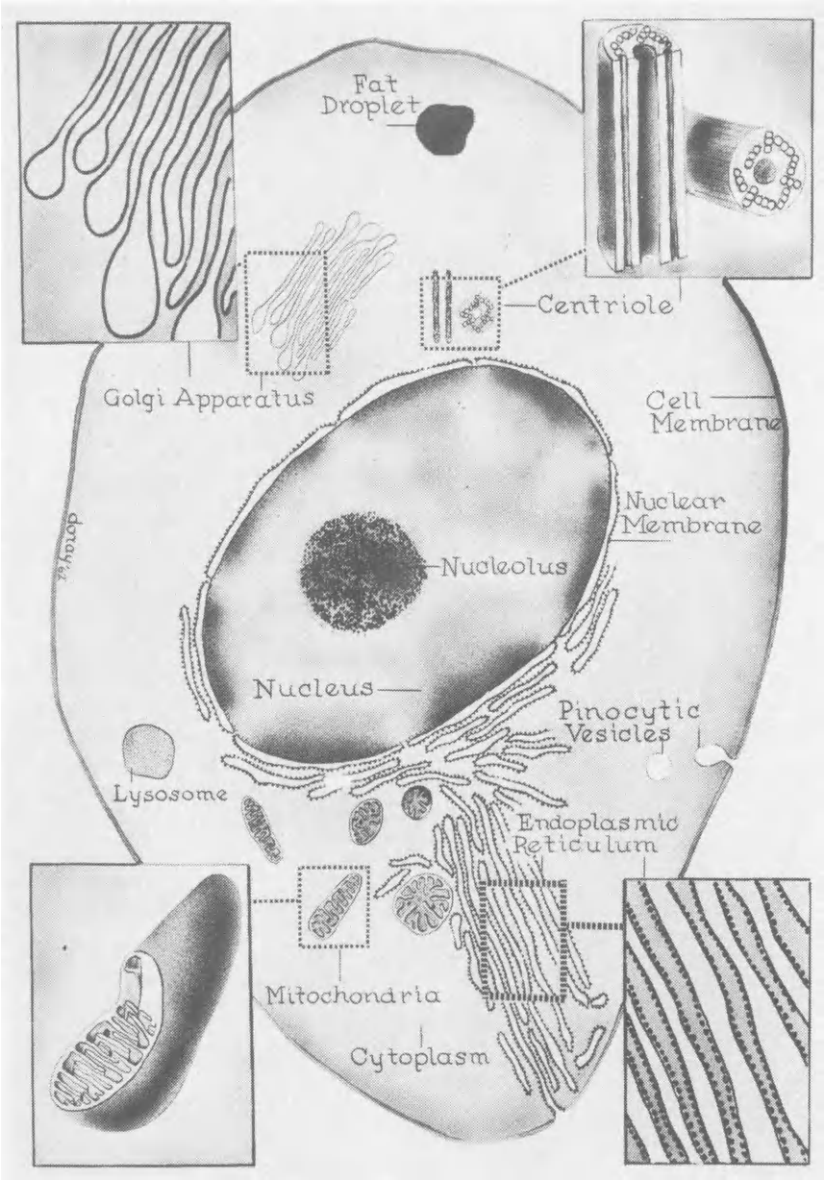


Figure 1.1 – Modern diagram of a normal cell

With modern methods of experimental cytology and cytochemistry allied to electron microscopy our knowledge of the cell in recent years has increased enormously. The modern diagram of a cell *(Figure 1.1)* based on electron micrographs and cytochemical research is vastly different from that of even a few years ago. It should however be remembered that the conventional diagram *(Figure 1.2)* is the image as seen by the light microscope and as such may still serve some purpose.

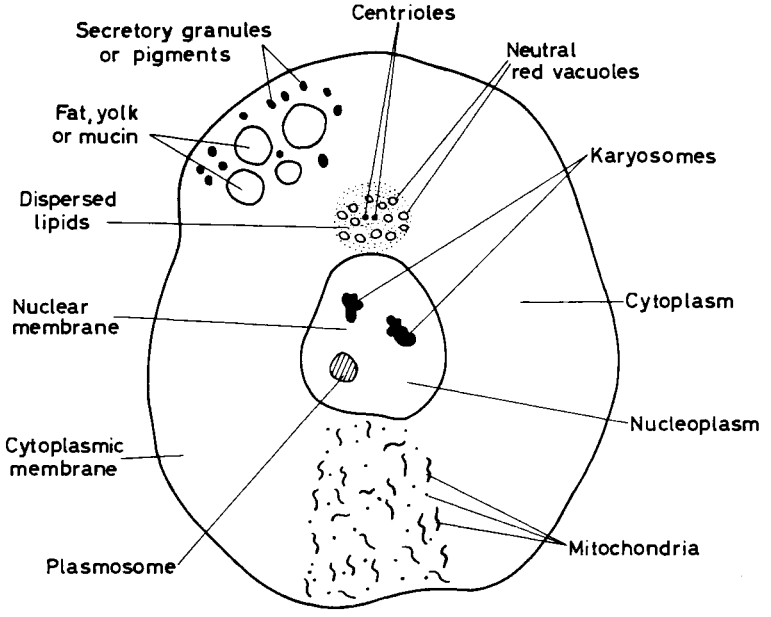


Figure 1.2 – Diagram of a living cell showing its component parts and possible inclusions. The Golgi apparatus would be present in place of the neutral red vacuoles, if the cell were fixed. The inclusion of fat, yolk, mucin, secretory granules, and pigments is purely diagrammatic; it is unlikely that more than one type would be present. Should such inclusions be present they are normally distributed throughout the cytoplasm

THE NUCLEUS

Nucleoplasm

The ground substance of the nucleus is a colloidal solution of proteins with various salts. The application of most fixatives causes the proteins

to be precipitated as a fine mesh with aggregates of protein at the intersections. Most of the protein is bound to nucleic acid (deoxyribonucleic acid–DNA) forming *nucleoprotein* which, because of its acid reaction, stains intensely with basic dyes. The darkly staining part of the nucleus is known as chromatin; the pale part is known as achromatin, and under certain circumstances as parachromatin.

The structure and function of the nucleic acids (DNA and RNA) is dealt with elsewhere (page 245), and will not be discussed here except to say they are the most important single cellular constituents. DNA, in addition to being self-replicating, has been shown to be the architect of the cell directing, through its intermediary the RNA, every function.

In human cells there are 46 chromosomes. These chromosomes carry in their DNA hereditary characteristics thought to be dependent on the code of nitrogenous bases, and on division split along their length, one half of each going to each new or daughter cell. In the resting cell, although they cannot at present be differentiated as separate structures, the chromosomes are believed to lie uncoiled in a delicate lace-like structure in which the DNA and other components are able to interact with the surrounding medium. It is in this form that the DNA is most active and when it is tightly coiled into the classical chromosome the DNA is inert.

Nucleoli

The nucleus generally contains one or more refractile particles which are known as nucleoli. These are easily recognizable in a resting nucleus by their regular spherical shape. Under the electron microscope they are seen to be composed of large numbers of small granules which are similar in appearance to the ribosomes of the cytoplasm. They are rich in RNA and are thought to be active in the synthesis of protein.

Nuclear Membrane

The nuclear membrane which surrounds the nucleus is not thought to be permeable in the normal sense of the word. Rupture of this membrane is a sign of imminent death of the cell.

It will be seen in *Figures 1.1* and *1.3e* that the nuclear membrane has now been shown to be a double membrane in which there appears to be annuli or holes (nuclear pores) in the outer layer which are open to the cytoplasm. Some workers believe that it is through these annuli that contact with the cytoplasmic or endoplasmic reticulum is made.

THE CYTOPLASM

Cytoplasmic Membrane

The cell is bounded by a semi-permeable membrane thought to be composed of protein and lipid particles; this permits the exchange of food and secretory products. This membrane has now been shown to be a double membrane (approximately 100 Å thick), the interruptions or holes in it connecting directly, by way of the endoplasmic reticulum, with the outer surface of the nucleus *(see Figure 1.1)*.

When the movement of substances through the membrane is under the influence of osmotic pressure it is known as 'passive transport'; the name 'active transport' being given where chemical interaction and/or electrical charges are concerned in this interaction between the cell and its environment. Pinocytosis (cell drinking) and phagocytosis (cell eating) are terms used where the cell membrane invaginates material on its surface, the invagination then pinching off the material which floats free in the cytoplasm, usually appearing as a small vacuole. Using a micro-manipulation apparatus fine pipettes can be introduced through this membrane into the cytoplasm without, in themselves, causing death of the cell.

Cytoplasm (Ground Substance)

The cytoplasm is a homogeneous, watery solution, basic in reaction, of protein, various salts and sugars.

It has now been shown to have a 'cytoskeleton' known as the cytoplasmic or endoplasmic reticulum. This complicated system of internal membranes *(Figures 1.1* and *1.3d)* is seen by the electron microscope to be either smooth (agranular) or rough (granular) when it is lined by dots which are rich in RNA and known as ribosomes; they are the sites of protein synthesis.

These particles, which are remarkably uniform in size (120–150 Å), may also occur free in the cytoplasmic matrix. The free ribosomes are believed to be concerned with protein synthesis necessary for the cell itself, whereas those attached to membranes are thought to be concerned with the synthesis of protein to be secreted by the cell. Ribosomes are often found in clusters, consisting of 3–30 or more, held together by slender filaments 10–15 Å in diameter and of varying length. Such clusters are known as *polysomes* or *polyribosomes*.

In certain types of cell the cytoplasmic structure may be differentiated and such differentiation appears relatively fixed, like the striations seen in voluntary muscle fibres. The cytoplasm is a fluid of

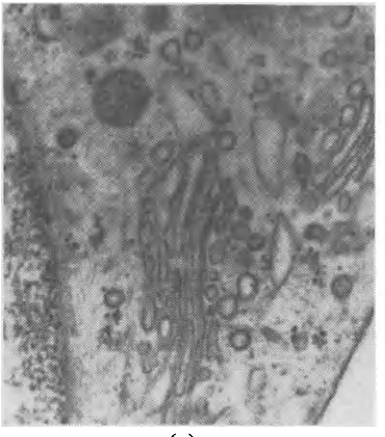
low viscosity, but towards the surface it is usually more of a gel in consistency. It should be remembered, however, that it is capable of rapidly reversible sol–gel transformation. It has an osmotic pressure equal to about 0.9 per cent sodium chloride.

Golgi Apparatus and Neutral Red Vacuoles

Neutral red vacuoles are minute spherical structures rendered visible in living cells by neutral red, such bodies being seen only in the living cell. The presence of a structure known as the Golgi apparatus has now been clearly established; it can be demonstrated by silver and osmium techniques (*see* frontispiece). By electron micrograph the structure is resolved as a series of flattened sacs with bulbous ends lying parallel to each other *(see Figures 1.1* and *1.3a).* The membrane surrounding these bodies is similar to that of the endoplasmic reticulum except that there are no granules lining it. The function of the Golgi apparatus is still unknown but in glandular cells it seems to be concerned in cellular excretion. There is some evidence that it is concerned in the synthesis of secretory products rich in polysaccharides. Since it may be well developed in cells which are not secretory in nature, its total function is still obscure.

Mitochondria

Mitochondria are minute bodies, of which there are usually several hundred in a cell. They are generally scattered throughout the cytoplasm, but occasionally congregated near the nucleus. In the living cell they are motile. In tissue sections they appear to be either filamentous or granular. Their structure and ultrastructure have now been proved and are shown diagrammatically (after Palade) in *Figures 1.1* and *1.3c.* This fluid-filled vessel is enclosed by two membranes, the inner one being involuted into folds or cristae. The membranes are believed to be constructed of outer single layers of protein molecules lined by double inner layers of phospholipid molecules. The thickness of the double membrane is 180 Å and this is consistent with the above theory since protein molecules have been shown to be 60 Å in length and phospholipid molecules 30 Å in length. The mitochondria are responsible for the process of respiration and phosphorylation in the cell, involving the interaction of at least seventy different enzymes and coenzymes. They are effectively the mobile 'power plants' of the cell supplying energy in the form of adenosine triphosphate (ATP). More recently, dense *granules* 300–500 Å in diameter have been described in the space between the cristae. These matrix granules have not been isolated or



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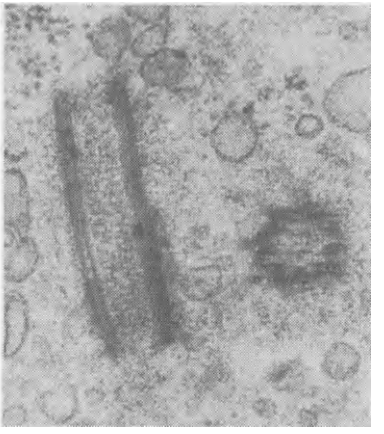

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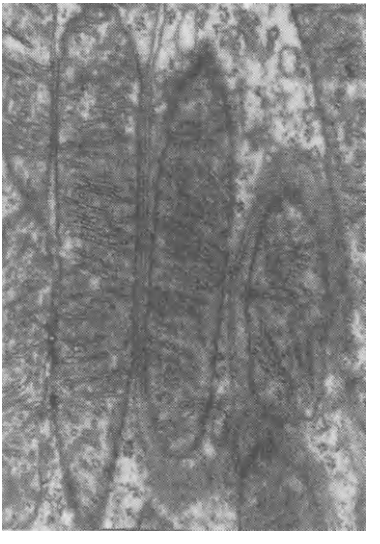
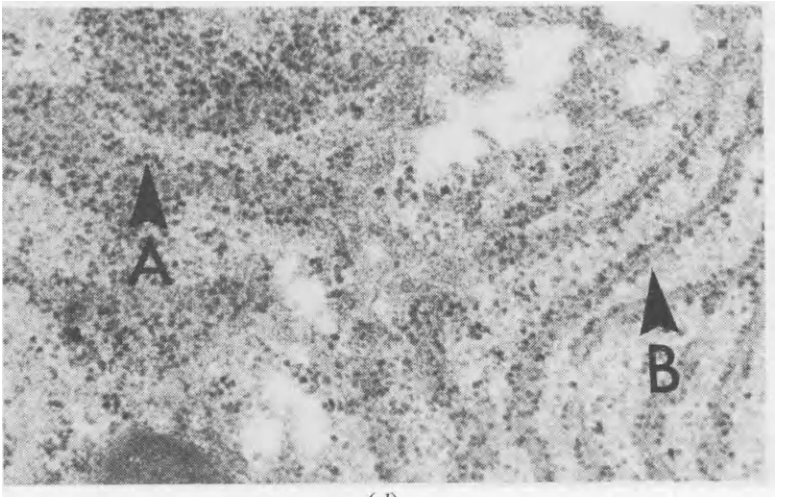


(*c*)

Figure 1.3 –Electron micrographs of various cell components: (a) Golgi apparatus; (b) centriole; (c) mitochondria (cont.)

analysed but they are thought to be concerned with the regulation of the internal ionic environment of the mitochondria. Filaments of DNA and small granules of RNA have been identified within the mitochondria matrix, their exact function is not yet known. They are quickly affected by temperature and autolysis and are among the first structures to disappear on death of the cell; for this reason early fixation is necessary for their demonstration.



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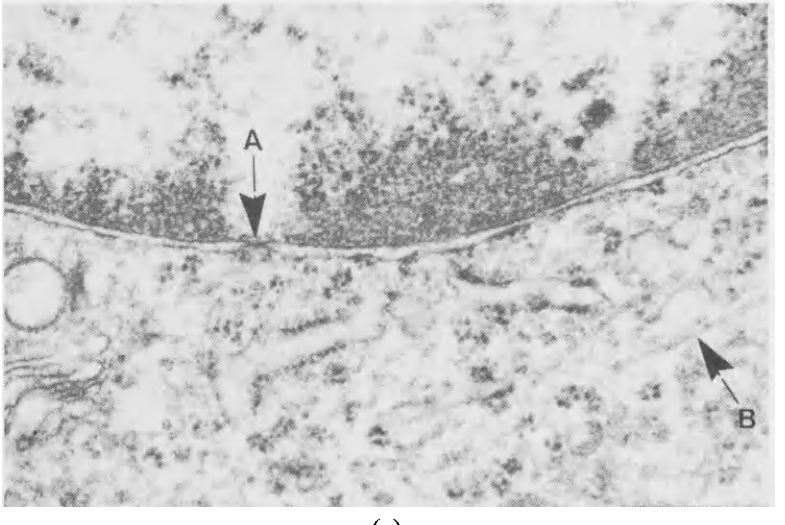


(*e*)

Figure 1.3 (cont.) – (d) ribosomes A and endoplasmic reticulum B; (e) nuclear pore A and microtubules B

Centriole (Centrosome)

Near the nucleus of most living cells can be seen a condensation of protoplasm which contains one or two small, refractile bodies known as centrioles. Electron micrographs have shown that a single centriole actually consists of two small cylinders at right angles to each other *(see Figures 1.1* and *1.3b).* These bodies are identical in every respect with the bodies found at the base of each cilium in ciliated cells (kinetosomes). In view of their diversity of function this relationship is difficult to explain, and no satisfactory theory has so far been advanced. During mitosis the two centrioles move to opposite poles of the cell and support a skein of fine protoplasmic rays, known as the achromatic spindle, which is crystallized protoplasm and may be demonstrated with a polarizing microscope. Along this spindle the chromosomes, after division, arrange themselves. The centriole is said to be absent from nerve cells, which, being highly differentiated, have lost the power of division.

Lysosome

The lysosome is similar in shape and size to a mitochondrion but, as seen by electron micrograph, it has a finely granular inner structure surrounded by a single membrane *(see Figure 1.1).* They are usually dense structures 0.25–0.5 μ in diameter and contain a number of hydrolytic enzymes known as *acid hydrolases.* Among the specific enzymes identified within the lysosome is *acid phosphatase,* and a histochemical acid phosphatase technique is usually used as a marker for their identification in light microscopy. The rupture or breakdown of the lysosomal membrane will allow the enzymes to escape and digest or lyse the cell. It is this activity that gave rise to the name 'lysosome'; however, it also plays an important part in the intracellular digestion of foreign matter within the cell. Phagocytic cells may be recognized, at least in part, by the number of lysosomal elements. When morphologically recognizable fragments of the cell's own structure are found within the lysosome it is known as an *autophagic lysosome* or *autosome;* if the material within the lysosome is an exogenous substance it is called a *heterolysosome.* Apparently not all substances can be broken down in the cell and with age the indigestible by-products of the cell associated with 'wear and tear' accumulate in the cell and are known as 'wear and tear pigment' or 'lipofuscin'.

Cytoplasmic Inclusions

These are inert, non-protoplasmic substances found within the cytoplasm, and may be products of the cell's own activity, or may be taken up from the surrounding medium.

The more important inclusions are:

(*a*) *Fat,* which occurs as globules within the cytoplasm, often forcing the nucleus and other intra-cellular materials to one side.

(*b*) *Yolk,* which is similar to fat but usually more yellow in colour. It is differentiated by dissolving out the fat, the yolk having a protein base which is subsequently demonstrable.

(*c*) *Glycogen,* which occurs in a watery solution of colloidal nature. After fixation it is seen as fine granules or an amorphous mass, dependent on the type of fixation technique employed.

(*d*) *Mucin,* which is first demonstrable in mucin-producing cells as minute granules known as mucigen. These become droplets of mucin which coalesce, producing the typical distension of the 'goblet cell'.

(*e*) *Secretory granules and pigments.* – Remnants of ingested material, such as cell membranes and bacteria, are seen occasionally in the cytoplasm of some cells; for example, histiocytes.

CELL DIVISION

Introduction

A typical cell division consists first of an equal division of the nuclear material (DNA) known as *karyokinesis,* followed by division of the cell body known as *cytokinesis* in which each of the two daughter cells receives one of the daughter nuclei. Occasionally, *karyokinesis* will be seen without *cytokinesis* giving rise to a multinucleated cell.

With each complete turn of the cell through its life cycle, all the structural elements and functional capacities of the nucleus and cytoplasm must undergo a doubling. All of these events, such as mitochondrion and ribosome reproduction, must be so inter-related and coordinated that cell growth and function are assured.

This area of cell reproduction is still not completely understood, although many of the control mechanisms involved have been demonstrated. Developmental biologists divide the cell reproduction cycle into four periods, G_1 S, G_2 and D (or M), with the latter D (division) or M (mitosis) period being the only one that can be easily studied by histological or light microscope techniques, although the S, DNA synthesis,

or chromosome replication cycle can be seen by autoradiography using radio-isotope labelled thymidine *(Figure 1.4).*

It has been shown that an initiator protein is produced that brings about the interaction between DNA and DNA polymerase necessary for

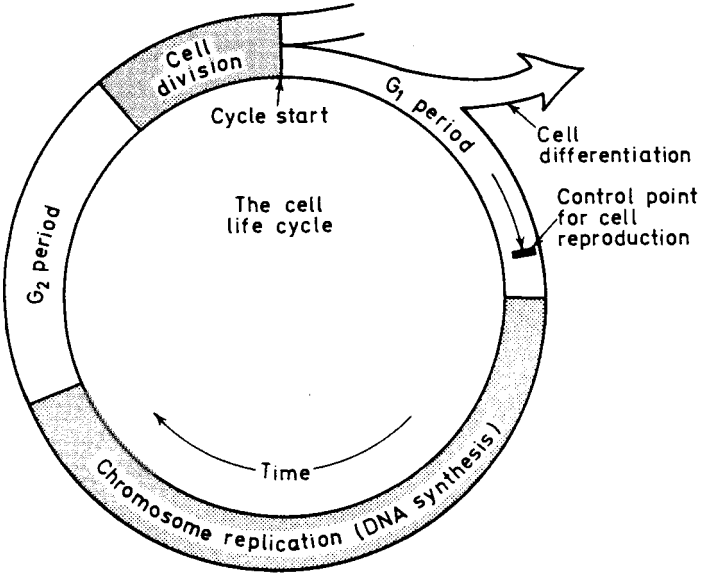


Figure 1.4 – The subsections of the cell life cycle are defined by the state of the nucleus. Regulation of cell reproduction is achieved by interruption and arrest of cycle progress in G_1 (Reproduced by courtesy of the Editor of Cancer Research *and Dr. D. D. M. Prescott.)*

DNA synthesis. A measurable G_1 phase may be absent during active cell proliferation, or prolonged when there is no apparent necessity or demand for cell growth, it is therefore an expendable time period. The S period is marked by DNA, RNA and protein synthesis. The G_2 period contains the events that link the end of chromosome replication with chromosome segregation. The mechanics of this segregation seem to involve a series of events, synthesis and assembly of the mitotic apparatus, alignment of the chromosomes on the apparatus, splitting of the centrioles, and so on. The G_2 period in animals is relatively short and of constant duration.

It will be evident that the subsections of this cycle, as described, are

defined by what the chromosomes are, or are not, doing rather than by any necessary state of the whole cell.

Mitosis

This section is concerned with mitosis as it has been seen and known for a number of years by examination with the light microscope.

With the exception of certain specialized cells (for example, nerve cells), cells multiply by division. The normal mechanism of division is a complicated one known as *mitosis,* but certain cells may possibly undergo simple division (*amitotic* division) like bacteria.

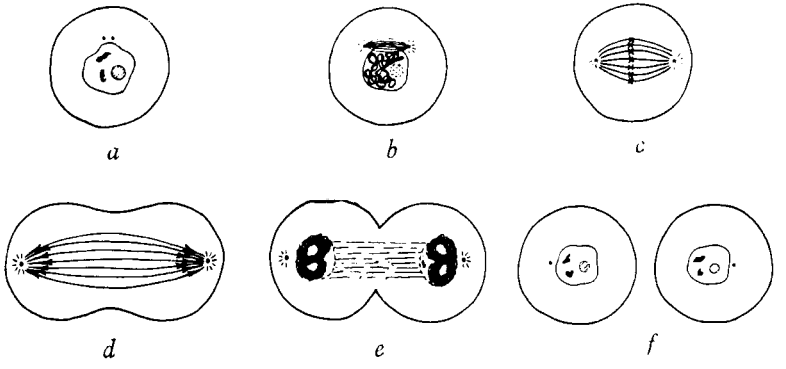


Figure 1.5 – Diagram showing the four phases of division of a cell. (a) Normal cell having a resting nucleus, with one plasmosome and two karyosomes. There are two centrioles. (b) Prophase: formation of the achromatic spindle has commenced, and the chromatin has condensed into a continuous skein. (c) Metaphase: the chromosomes have arranged themselves in the centre of the cell, and each has split into two. (d) Anaphase: the chromosomes have moved along the achromatic spindle to opposite poles of the cell, and the cytoplasmic membrane has become constricted. (e) Telophase: the chromosomes have begun to coalesce, and the cytoplasmic membrane has become more constricted. (f) Telophase: the cytoplasmic membrane has divided, and a nuclear membrane has formed in each of the two daughter cells

The process of mitosis is divided into four stages *(Figure 1.5)*: (1) Prophase; (2) Metaphase; (3) Anaphase; (4) Telophase.

Prophase

The chromosomes become visible by concentration of the chromatin; they at first form a long continuous skein which later

divides into separate chromosomes. At the same time the two centrioles move to opposite poles of the cell (if there is only one it first divides), and the nuclear membrane begins to disappear. Between the centrioles fine fibres appear, and it is on these fibres (the achromatic spindle) that the chromosomes arrange themselves, and appear to move after division *(Figure 1.5b).* The achromatic spindle, while not visible by normal staining methods, may be visualized with the polarizing microscope between crossed Nicol prisms or polaroids.

Metaphase

The centrioles are at opposite poles; the chromosomes are shorter and thicker and are arranged in the central region of the spindle. Each chromosome now splits into two *(Figures 1.5c* and *1.6a).*

Anaphase

At this stage the chromosomes move along the spindle to opposite poles *(Figures 1.5d* and *1.6b, c* and *d).*

Telophase

In this final stage, the sets of chromosomes having reached opposite poles, nuclear membranes are formed around each of the daughter nuclei, the chromosomes gradually expanding and dissolving. The cytoplasmic membrane itself divides, having become constricted during telophase and finally is only connected by a fine thread which ruptures as the cells move apart *(Figures 1.5e* and *f* and *1.6e* and *f).*

COLLOIDAL CONCEPTION OF TISSUE

In order to correlate the mobility of cells in certain conditions with the apparent immobility of tissues, it is necessary to have some knowledge of colloidal theory.

The cytoplasm of most cells allows free movement of granules within it, showing that it has a low viscosity, yet it maintains its shape by an outer area which is more of a gel. Both these areas are capable of reversal and the cell may, in certain circumstances, become almost liquid, or if injured or dead may revert wholly to a stiff gel. Damage to the cytoplasmic membrane will normally result in gel formation under the damaged area until repair has taken place.

This reversion from sol to gel and *vice versa* is possible because of the colloidal nature of protoplasm.

Colloids

When a powdered solid is put into a fluid, it may remain suspended in that fluid and be easily removable by filtration through paper; or it may disperse as single molecules which cannot be removed by such simple means, in which case the solid will have gone into true solution. The physical properties of solutions differ greatly, some diffusing readily through collodion membranes, while others do not. These differences are due to the degree of dispersion of the material within the solvent or in other words, to the size of the dispersed particles. If there is a state of true molecular dispersion, the particles are smaller than one-millionth of a millimetre, and the solution is a molecular solution. If the particles are so large that they can be seen microscopically, and can be removed by filtration through paper or porcelain, they are larger than one ten-thousandth of a millimetre (0.1 micron), and the solution is a coarse suspension. Between these limits there are the colloids, in which the dispersed material, while not in a state of true molecular dispersion, has been sufficiently reduced in size not to be classed, nor to act, as a coarse suspension.

Suspensoids and emulsoids. – Colloids may be sub-divided into two main types: (1) suspensoids and (2) emulsoids. The suspensoids with relatively large particles differ from coarse suspensions only in degree of suspension and size of particles, and do not combine to any degree with the solvent; they may be called anhydrocolloids.

In emulsoids, molecules of solvent (usually water) are firmly attached to the surface of the particles, rendering them miscible with water, or hydrophilic; they are therefore known as hydrocolloids. The two substances comprising a colloidal solution are known as phases, the solvent being the continuous phase, and the particles the dispersed phase.

Certain emulsoids, such as gelatin and protoplasm, are capable of reversal of their phases. In one phase they are liquid and are known as hydrosols, where semi-solid globules are dispersed throughout a fluid; in the other phase they are semi-solid and are known as hydrogels, where minute droplets of fluid are dispersed throughout the semi-solid material.

It must be remembered that protoplasm is capable of rapid reversal from sol to gel, unlike gelatin in which a slow reversal takes place on heating and cooling.

OSMOSIS

If red blood cells are placed in water they will swell and burst; conversely, if they are placed in a strong salt solution, they will shrink;

both of these reactions are due to osmotic pressure, a knowledge of which is essential to the understanding of the function and, especially, the preservation of cells and tissues.

Molecules of protein in colloidal solution are very much larger than those of simple salts or water, and it is possible to select membranes which will permit the passage of the small molecules but be impermeable to the larger ones. Such membranes are known as *semi-permeable*, and may be natural animal tissue (for example, pig's bladder), or artificial (for example, collodion). If two solutions are separated by such a semi-permeable membrane, small molecules will circulate freely between the two solutions, but large molecules, such as protein molecules, will be restricted to their own sides of the membrane;

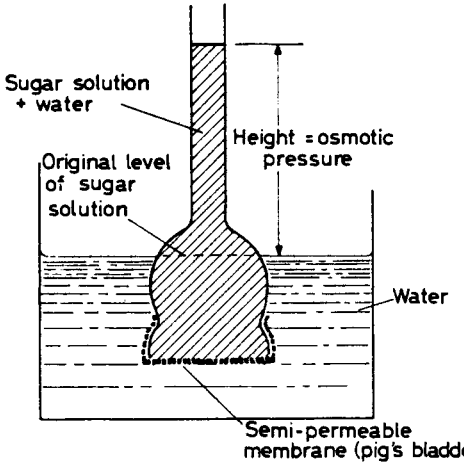


Figure 1.6 – Diagram to illustrate the movement of fluid due to osmosis

moreover, these large molecules will hinder the molecules of water attempting to pass through the membrane, the degree of hindrance being proportional to the concentration of protein in the solution. When the concentration of protein in the two solutions is different, water will pass more readily from the weaker to the stronger solution, and will continue to do so until the two solutions are of the same strength; if the volumes of the two solutions are fixed the transfer of water molecules will increase the pressure in the stronger solution. This process is known as *osmosis*, and the pressure created is called *osmotic pressure*. The movement of fluid may be demonstrated by covering the open end of a thistle funnel with a semi-permeable membrane, inverting

this in a beaker of water and pouring sugar or protein solution into the funnel. The level of fluid in the stem of the funnel will rise, showing that water has passed through the membrane into the funnel; after several hours this level will become constant, and the height of this surface above the surface of the water in the beaker will be an indication of the osmotic pressure of the solution in the funnel *(see Figure 1.7)*. The effect of osmotic pressure in an enclosed space may be demonstrated by filling a pig's bladder with sugar solution and immersing this in water. The bladder will be distended and will ultimately burst.

The walls of all living cells are semi-permeable membranes, and the cells contain colloidal solutions of protein. If such a cell is placed in a medium, the osmotic pressure of which is lower than that of the cell, water will pass into the cell and it will swell until it ruptures. This may be demonstrated with red blood cells. In the body these are surrounded by plasma which has osmotic pressure equal to that of the cells (about 7 atmospheres), and they will retain their normal shape if placed in 0.85 per cent sodium chloride solution, which has the same osmotic pressure as plasma (an *isotonic solution*). If placed in a weaker salt solution (a *hypotonic solution*) the red cells will be swollen by water passing through their cell walls, and will be ruptured (*lysed*), whereas if placed in a stronger salt solution (a *hypertonic solution*) water will leave the cells and they will become shrunken (*crenated*).

Chapter 2

Methods of Examination of Tissues and Cells

DISSOCIATION

The examination of teased specimens in normal saline or other indifferent medium is occasionally called for. The specimen is teased in a watchglass with needles, transferred to a microscope slide, a coverslip laid on, and the preparation examined under the microscope with a reduced cone of illumination.

A method likely to be of increasing importance in the future is the examination of dissociated tissues by the phase-contrast microscope. This type of examination has the advantage of showing the structure of the cell without staining, and while the cell is still alive. The arrangement and movement of mitochondria can, with experience, be seen quite clearly, and cells may be observed in mitosis, particularly in malignant tissue. Some cells show movement, and this may be so characteristic as to identify them.

These methods enable details of individual cells to be seen, but they inevitably suffer from the disadvantage that anatomical relationships are destroyed.

SMEAR TECHNIQUE

In the smear method the material to be examined is either pressed against a slide (*impression smear*), spread with a platinum loop, or crushed and spread with another slide. The preparation can then be fixed wet, stained to demonstrate specific structures and, after clearing, mounted in a medium having a high refractive index. This technique permits a much more detailed examination than a wet preparation but suffers from similar limitations.

THICK SECTION

In cases of emergency, very thin slices of fixed or unfixed material can be cut freehand with a sharp knife or razor, and by restricting staining to the surface of the tissue an urgently required diagnosis may sometimes be made. Control of the staining is effected by brushing a polychrome stain on the surface of the tissue and washing it off quickly. This method was devised by Terry (1929) and it has been used with a great deal of accuracy. A special microscope is available known as the Ultrapak microscope, but is by no mean essential. It should be remembered that only if the operator has a great deal of experience can this technique be relied upon.

VITAL STAINING

Some living cells will take up certain dyes (*vital stains*) which colour selectively elements in the cells, for example, mitochondria. Other cells (*phagocytic cells*) will engulf microscopic coloured particles which are then visible inside the cells; this may be used to demonstrate elements of the reticuloendothelial system.

Vital staining techniques are usually sub-divided into (*a*) *intravital* –within the body, and (*b*) *supravital*–with living cells outside the body. Unfortunately, these techniques are very limited and it would appear probable that they will be largely supplanted by phase-contrast microscopy.

SECTIONAL METHODS

Almost all the established techniques are embraced by sectional methods since the morbid anatomical relationships of cells are preserved, and extremely thin sections may be cut. The finished preparation is mounted in a medium having a high refractive index which enables it to be studied in detail.

The medium in which the tissue is to be embedded depends on the technique or techniques to be employed, and on the type of material to be sectioned. The most common medium for routine use is paraffin wax, but celloidin is used in certain instances, and gelatin occasionally.

Paraffin Sections

By this technique the tissue is finally embedded in paraffin wax, which permits easy sectioning. The first stage is the preservation of the tissue by a fixative, generally in aqueous solution, which, not being

miscible with paraffin wax, must first be removed and then replaced by a wax solvent. This is achieved by immersion in increasing concentrations of alcohol to remove the water, following which the alcohol is replaced by a wax solvent; this in turn is replaced by molten paraffin wax.

Water-soluble Waxes (Carbowax and Aquawax)

These materials are waxes which are miscible with water; they obviate the stages of dehydration and clearing with a consequent saving in time. A disadvantage of this technique is that it is difficult to remove creases in the tissue, since this is normally done in warm water: dilute alcohols and such materials have been used in place of water, but without consistent success. Only in rare instances are the results as good as those obtained by paraffin embedding, and this and the difficulties encountered in the method account for its relative unpopularity. It has, however, been used with success by some workers following fixation by the freeze-drying technique.

Celloidin Embedding

Celloidin, being a rubbery consistency, gives greater support to mixed tissues (for example, skin with subcutaneous fat) and to very hard tissues such as bone. It is also used when it is desired to avoid the use of heat, as with the central nervous system.

Celloidin is a nitro-cellulose soluble in equal parts of alcohol and ether. Therefore, with celloidin embedding one avoids the stage of clearing, tissues being fixed, dehydrated and transferred from absolute alcohol to thin celloidin (2 per cent), and finally embedded in thick celloidin (8 per cent). These blocks must be cut and stored in dilute alcohol, unless specially treated.

Giant Gelatin Sections

The technique of giant gelatin sections was devised by Gough and Wentworth (1949), especially for lungs. Here, after fixation, whole lungs are embedded in gelatin, which, having been allowed to set, is formolized, and sections 300–400 microns thick are cut on a special large microtome. These are then laid on sheets of Perspex, covered with filter paper and allowed to dry; the sections adhere to the filter paper and can then be filed in a book.

Frozen Sections

Unfixed or fixed tissue is frozen on a special microtome with carbon dioxide. It is especially valuable when a rapid diagnosis is required, or in order to demonstrate material which is soluble in alcohol or clearing agents. When material to be sectioned is friable, it can be embedded in gelatin prior to cutting.

Cryostat Cut Sections

The use of a deep freeze type cabinet to house the microtome, knife, tissue, etc. and to maintain them at a low temperature (-10° to -20°C) during the actual section cutting process has led to a series of new methods enabling the rapid sectioning of fresh unfixed tissues which have been quick frozen at a very low temperature (-160°C). The sections obtained in this manner are ideal for histochemical assessment as well as for the newer fluorescent antigen–antibody techniques. It has the great advantage over freeze drying of being rapid and presenting few technical problems. It is now being used increasingly as an alternative to the frozen section technique for rapid diagnosis.

FLUORESCENT ANTIGEN–ANTIBODY TECHNIQUES

This technique makes use of the fact that certain fluorescent dyes such as fluorescein isothiocyanate may be attached to proteins such as gamma globulins without interfering with their function as an antibody or antigen. Following the attachment of dye to protein (conjugation) the actual site of a specific antigen or antibody may be demonstrated by fluorescence microscopy. This technique has led to a greatly improved understanding of immunity at a cellular level and, in particular, of those diseases in which an auto-immune pathogenesis has been postulated.

POLARIZING MICROSCOPE

A polarizing microscope is particularly valuable for the detection and partial identification of birefringent or anisotropic objects such as certain foreign bodies, crystals, certain lipids, cross-striated muscle and myelinated nerve fibres. When these are placed between crossed Nicol prisms, or sheets of polaroid material, they rotate the plane of polarized light and appear bright against a dark background.

DARK GROUND ILLUMINATION

Dark ground illumination, with a one-seventh inch or a one-twelfth inch oil immersion objective fitted with a funnel stop, is obtained by fitting a special type of condenser giving a hollow cone of light. The numerical aperture of the objective must not exceed 1.0. Objects are seen by the light they scatter, no direct light being allowed to enter the objective. This method is used for the demonstration of *Treponema pallidum,* of blood flagellates and of other objects in wet preparations. Much experience is required to interpret the appearance of cells by this technique, internal structures tending to be hidden by the refracted light which forms a halo.

PHASE-CONTRAST MICROSCOPY

This type of microscope, which has been produced commercially only since 1946, was devised by Zernicke, and converts slight differences of refractive index into degrees of light and shade. This enables unstained objects to be examined in detail, and since such cell constituents as mitochondria, granules, nucleoli and so on may be seen clearly, it represents a great advance in microscopy. Living cells and organisms may be studied and photographed during division, and research workers are able to watch the effects on them of foreign substances. While still largely a research instrument, it is now being applied more and more in routine laboratory work. Great advantages of the present development are that a routine instrument may be adapted within minutes for phase microscopy, while the phase-contrast objectives may be used for routine work with no appreciable loss of definition.

INTERFERENCE MICROSCOPY

The interference microscope incorporates certain principles of both the phase-contrast and polarizing microscopes, and enables one to see unstained objects in great detail. It has an advantage over the phase-contrast microscope in that there is no halo whatsoever. Dependent upon the type of interference microscope used, objects are either differentially coloured, or viewed against a background of coloured wave-bands. By measuring the difference in colour between cell components, or the change in the wave front caused by particular objects, their weight may be calculated to 10^{-14} grammes. Being virtually a microscopical balance, the weight of objects, such as single mitochondria, may be estimated with great accuracy.

Although this microscope has been classed by some workers as the successor to the phase-contrast microscope, the adjustments are critical and complicated, and it is unlikely in its present form to become an instrument for routine use.

ULTRA-VIOLET LIGHT MICROSCOPY

Resolution of the normal compound microscope is limited by two factors: the numerical aperture of the objective used, and the wavelength of the light source employed. An increase in resolution is therefore attainable by using ultra-violet light which has a shorter wavelength than white light. As ultra-violet light is not in the visible spectrum the image must be recorded photographically, and the lenses employed must be made from quartz.

FLUORESCENT MICROSCOPY

Material which is fluorescent has the property of converting light rays from the invisible spectrum (ultra-violet) into the visible spectrum. Fluorescent material examined by this technique will appear bright on a dark background. Certain elements are naturally fluorescent (*innate fluorescence*), but the use of fluorescent dyes allows the specific demonstration of many tissue elements and bacteria.

ELECTRON MICROSCOPY

The use of electrons in place of light rays, and electrical lenses in place of glass lenses has greatly increased the degree of magnification and resolution that was previously attainable. Many ultra-microscopic particles, such as the smaller viruses, were seen for the first time by this technique.

A disadvantage of this method is that material has to be dried and examined *in vacuo,* introducing certain artefacts which detract from the value of the method.

MICRO-INCINERATION

Micro-incineration is used for determining the presence of mineral elements, and consequently fixation and processing of the tissue must not add to, or subtract from, its mineral content.

Duplicate sections are prepared, one being incinerated at a controlled high temperature, the other being stained by a routine method as a control.

Following incineration a coverslip is placed on the section at the earliest moment to prevent both the absorption of moisture from the air, and movement of ash. This section is then examined by normal microscopic methods, dark ground illumination, polarizing microscopy and, if possible, electron microscopy. Considerable experience may be required in the interpretation of results.

Chapter 3

Fixation

INTRODUCTION

With certain few exceptions (such as enzyme activity in tissue cultures, frozen or frozen-dried preparations) one does not conduct histochemical investigations upon living cells because it is difficult to maintain the integrity of the tissue and/or cells under such conditions. It is therefore necessary to 'fix' or preserve it in such a manner that subsequent examinations may be made to determine its micro-anatomy and allow localization of its various chemical constituents. Unfortunately, as will be seen below, it has not proven possible to perform all these functions with a single fixing reagent or group of reagents. One is therefore faced with the problem of selecting a fixative that will preserve (*a*) the gross and microscopical anatomy of the tissue to be examined, (*b*) those chemical compounds to be investigated and more particularly their histochemically reactive groups, and if possible, (*c*) other classes of compounds which may subsequently become important during the course of an investigation. The importance of (*b*) above was shown by Spicer (1963) in the demonstration of basic protein with Biebrich scarlet when he noted that metachromasia which was present after fixation in Carnoy's fluid was absent after formalin; this he attributed to the binding of the reactive amino groups by the formaldehyde rather than to their extraction by the fixative.

The foundation of all good histological preparations is adequate and complete fixation. Faults in fixation cannot be remedied at any later stage, and the finished section can only be as good as its primary fixation.

It is essential that tissues be fixed as soon as possible after death or removal from the body, and for this reason screw-capped specimen jars containing appropriate fixatives should be permanently kept wherever tissues for histological examination are taken regularly, for example in the operating theatre, the post-mortem room, or the animal house. The

amount of fluid in the jars should be 15–20 times the bulk of the tissue to be fixed. The easy availability of such jars will obviate drying of tissues. Care should be taken to ensure early despatch of the specimens to the histology laboratory in order to avoid over-fixation in intolerant fixatives.

Tissues selected for sectioning should be sufficiently thin to be adequately fixed throughout in a reasonable time. The overall bulk of the tissue determines the volume of fixative required; the thickness will determine the speed of fixation. The length and breadth of the tissue taken will often be determined by the type of microtome to be employed; the best thickness for routine use is 3–5 mm.

For complete fixation of whole organs the injection of an appropriate fixative, usually formol saline, in addition to immersion, is generally indicated. The brain in particular can be fixed adequately only by this combination of perfusion and immersion.

THE AIMS AND EFFECTS OF FIXATION

Fresh tissue which is left in a warm environment (at room temperature) will become liquefied with a foul odour, due mainly to putrefaction and autolysis. The examination of fresh tissue therefore requires the action of a preservative to prevent such deterioration. However, it is not sufficient to just preserve tissue, this may be done by quick freezing at low temperatures (*see* page 55 – **Quenching**), but such tissues must be kept frozen in air-tight containers, thawing will restart the process of deterioration.

Fixation must preserve the tissue in such a manner that it will retain, as nearly as possible, its original form and yet permit certain investigative procedures. Early workers in this field also required that the tissue be sufficiently hard to permit the cutting of thin slices by hand. Although modern techniques of embedding give adequate support to tissue and allow thin slices (or sections) to be cut, yet the hardening effect of fixatives is of value. We often employ tests that would smash a living cell yet fixed tissue sections easily withstand such treatment. It will be seen that many of the apparent by-products of fixation may, in fact, be of use in subsequent investigations.

The aim of fixation is to preserve tissue in as life-like a manner as possible. This preservative must also allow a variety of techniques to be performed upon it without destroying its integrity. The more common effects are summarized below.

Inhibition of Autolysis and Putrefaction

Among the common causes of poor cellular detail in histological

preparations are autolysis and putrefaction, defined as follows.

(1) *Autolysis.* – The lysis or dissolving of cells by enzymic action, probably as a result of rupture of the lysosome. The responsible group of enzymes are known as cathepsins; some of which are proteinases which shorten proteins to peptides and others, carboxypeptideases and aminopeptidases which cause the breakdown of peptides to individual amino acids.

(2) *Putrefaction.* – The breakdown of tissue by bacterial action, often with the formation of gas. This is sometimes called post-mortem decomposition and is most commonly encountered in the intestinal tract where there is normally a high bacterial content.

Both changes follow rapidly upon death of the cell, but this occurs a variable time after removal from the body. Cells of the central nervous system die quickly, but others will survive a considerable time. Pulvertaft (personal comminication) has obtained living cultures, showing mitosis, of bone marrow taken from a patient 30 minutes after death and stored for 3 hours in a refrigerator.

Generally, however, the best results are obtained by putting tissues into fixative as soon after death as possible. If this is not possible, they should be placed in the refrigerator which slows down both autolysis and putrefaction.

Preservation

The preservation of cells and tissue constituents in as life-like a manner as possible is essential.

Hardening

The hardening effect of fixatives will allow easy manipulation of naturally soft tissues (for example, brain).

Solidification of Colloid Material

Fixation has the effect of converting the normal semi-fluid consistency of cells (sol) to an irreversible semi-solid consistency (gel).

Optical Differentiation

Fixation alters to varying degrees the refractive indices of the various cell and tissue components, which enables unstained components to be more easily seen than when unfixed.

Effect on Staining

Most fixatives affect histochemical reactions to some degree since, as has been shown, they generally bind to reactive groups in the tissue to achieve fixation. They may also affect routine staining, sometimes adversely (as with formalin and Biebrich S, *see* page 29), alternatively, they act as a mordant and improve staining (picric acid with trichrome stains).

Osmosis and Fixation

It would appear obvious that unless fixatives are isotonic solutions cells will become distorted before fixation. Baker (1933) has shown that this is not necessarily true; indeed, under certain circumstances cells reverse their expected behaviour. Tissue placed in 5 per cent acetic acid swells, yet acetic acid has a pressure of 20 atmospheres, which is about 3 times that of mammalian blood and would therefore be expected to shrink tissues. Saturated aqueous picric acid has a pressure of 2½ atmospheres, which is roughly one-third that of mammalian blood and should swell tissue, yet as it fixes it shrinks. It would seem, therefore, that the cause of the change in volume cannot be due to osmotic pressure. Those fixatives which are protein precipitants shrink tissues regardless of their osmotic pressure, and of non-protein precipitants the reverse is true.

Young (1925) has shown, however, that certain slowly penetrating fixatives (for example, formaldehyde) must have incorporated with them an indifferent salt to give an osmotic pressure roughly that of the cell. When distilled water is used instead of saline solution, vacuolation and swelling of cells takes place. Young's explanation is that the diluent penetrates more rapidly than the fixative, and if the diluent is distilled water, which is hypotonic, it will cause swelling and vacuolation of the cells before the fixative has penetrated. That this is not always true is probably due to varying speeds of penetration of fixing substances and the temperature and conditions under which they are employed.

In practice the osmotic pressures need only approximate that of the tissues. There is nothing gained by having the exact pressure, and there is no advantage in using a complex physiological saline solution when 0.85–0.9 per cent sodium chloride or 1 per cent calcium chloride work equally well.

Loss of Materials during Fixation

Recently, more emphasis has been placed upon measuring the loss of specific entities during fixation as an indicator of their efficiency. *It*

should be emphasized that such papers need to be read with great care, particularly with regard to the methods employed in the investigation. As will be seen below there is a great deal of disparity between the results obtained in different laboratories, although they may be of equal renown. Quite often this disparity is due to the fact that while the experiments are scientifically sound in themselves, they do not represent practical situations. Flitney (1965), using a model instead of tissue, found there was a loss of 76 per cent of protein after formalin fixation, while Jozsa and Szederkyi (1967), also using formalin, reported a 70 per cent loss of mucopolysaccharides from tissue; however, they washed their tissue in water immediately after fixation. In most routine and research laboratories tissues, after fixation in formol-calcium, are processed through graded alcohols, which has been shown by Culling and colleagues (1971) to act as a post-fixation process. They are then cleared and impregnated with paraffin (or Paraplast) at 60°C for 2–4 hours. The effect of the heat involved in this process should not be entirely discounted since 4 hours in wax at this temperature will coagulate egg albumen.

It will therefore be appreciated that correlation of the results of fixation experiments with those obtained in routine and research laboratories, after paraffin processing, is extremely difficult, if not impossible.

Protein

Osterowski (1961), investigating this aspect of fixation, showed the loss of protein to be nil with formaldehyde, 1.4 per cent with Carnoy's fluid, 8.3 per cent with ethanol, 13 per cent with acetone and 32–29 per cent with freezing techniques. Merriam (1958), following formaldehyde fixation, found a 6 per cent loss from liver and 4 per cent from muscle, whereas Flitney (1965) found that the loss of protein after 2 hours fixation in 4 per cent formaldehyde was 76 per cent compared to 11 per cent for 6 per cent glutaraldehyde; however, he was using an artificial albumin-gelatin model which presumably accounts for such high losses. Hopwood (1968) noted the efficiency of both glutaraldehyde and formaldehyde as protein fixatives with the former being more efficient. The loss of protein following osmium tetroxide is well documented (Dallam, 1957; Amsterdam and Schramm, 1966).

Lipids

Roozemond (1967) reported a 50 per cent lipid loss from hypothalamus by water which was markedly reduced by fixation in formol-calcium. The usefulness of formol calcium was further exemplified by

Deierkauf and Hesling (1962) who showed that such fixation did not interfere with subsequent lipid extraction with chloroform: methanol mixture and also by Urbanova and Adams (1970) who used an acetone extraction method; they noted the preservation of 80 per cent or more of phospholipids after formol calcium fixation. Dermer (1968) showed that even after fixation in osmium tetroxide there was a 16 per cent loss of lipid (which was mostly neutral or phospholipid).

Mucosubstances and Carbohydrates

Tock (1966) showed that freeze drying followed by formaldehyde vapour gave excellent preservation of mucin. Kugler and Wilkinson (1964), investigating the effect of fixation upon glycogen, found the best fixative to be ice-cold 80 per cent alcohol, this was confirmed by Hopwood (1967) who further showed glycogen preservation by formaldehyde to be 75 per cent and by glutaraldehyde 65 per cent. Jozsa and Szederkyi (1967) using 1 gramme pieces of aorta, skin and cartilage found the losses of acid mucopolysaccharides to be 60–70 per cent with formol-calcium; 2–3 per cent with formol/cetyl pyridinium bromide; 8 per cent with ethanol; 16–27 per cent with Rossman's fluid, while in Carnoy's fluid there was a loss of 40 per cent of hyaluronic acid and 10–20 per cent chondroitin sulphate A, B, and C. However, they washed with water immediately after fixation.

Nucleic Acids

Human cerebral tissue, following fixation in Carnoy's fluid or formaldehyde, was found by Schneider and Schneider (1967) to have lost DNA, RNA, protein and lipoprotein in both fixatives.

Low Molecular Weight Substances

The loss of low molecular weight substances may be quite high, for example, purines, phosphate esters, carbohydrates and indoles (Schneider and Schneider, 1967), catecholamines (Coupland and Hopwood, 1966; Hempel, 1965), ATP and proteins (Hopwood, 1968).

Tissue Shrinkage

Bahr, Bloom and Friberg (1957) studied changes in volume and weight in some 600 tissue specimens following fixation in formaldehyde or osmium tetroxide, and processing and embedding in methacrylate or paraffin wax. In both instances there was swelling in the

fixative; in formalin it amounted to 9 per cent in 30 minutes and 18 per cent in 12 hours, whereas in osmium tetroxide the tissue swelled 15 per cent in 15 minutes and 30 per cent in 24 hours. Subsequent *dehydration* in ethanol caused shrinkage (HCHO – 33 per cent, OsO_4 – 23 per cent), it is of interest to note that increasing the number of steps in dehydration caused less abrupt changes but with little difference in the end-result. Various dehydrants were used, with methanol being the only one superior to ethanol. *Infiltration* caused further shrinkage of the order of 20 per cent with methacrylate and 30 per cent with paraffin wax. From the data presented it appears that the final result of formalin fixation and paraffin processing is a total shrinkage of the order of 30–40 per cent. The authors note that the addition of dextran or other colloid osmotically active substance reduced these volume changes. Glutaraldehyde alone caused shrinkage of tissue (Hopwood, 1967).

The mechanism causing these volume changes is not completely understood but is thought to be associated with several factors, for example, changes in membrane permeability, inhibition of respiration and changes in sodium transport activity.

Rates of Penetration of Fixatives

Dempster (1960), using a variety of animal tissues (liver, heart, muscle, kidney, brain, and so on), studied the rates of penetration of nine standard fixing reagents when used alone or in mixtures prepared from standard formulae; the depth of penetration being measured at various time intervals. The behaviour of the standard fixing formulae was of particular interest since it was found that the ingredients of mixtures separated out during penetration with each proceeding at a rate comparable to that of the simple ingredient alone. Dempster showed that, after 8 hours in Zenker's fluid, rabbit liver had been penetrated to a depth of 0.85 mm by the bichromate, 2.6 mm by the mercuric chloride and 5.8 mm by the acetic acid. The implication being that the most rapid penetrant in a fixation mixture will have (?) fixed the tissue, with the other ingredients acting as post-fixatives (reacting only with those groups unaffected by the original or most rapid fixative), or alternatively, there is the possibility that reactions may take place between the secondary fixing agent and the fixed tissue constituents (tissue + fixative) which could be difficult to define at a chemical level.

A further complication was brought to light by Flitney (1966) who showed that the speed of fixation may be dependent upon the speed of reaction between fixative and tissue in addition to the rate of diffusion.

It will obviously be difficult therefore to predict the chemical reactions involved in complex mixtures of fixing reagents. Nine standard fixing reagents were tested on rabbit liver by Dempster with the results seen in *Table 3.1.* The figures for factor 'K' (constant) is simply the number of hours required for the penetration of the first millimetre of tissue; factor 'e' is an exponent relating to the rate of diffusion. Where factor 'e' (exponent) is not far from the square (e=2) the approximate time for complete penetration may be approximated by squaring the requisite depth in millimetres (that is, half the maximum tissue thickness and multiplying this factor by the value of the constant 'K' (this would apply to fixatives 1, 2, 4 and 7 in *Table 3.1*). The full equation

TABLE 3.1

		Depth of penetration (mm)			*Penetration factors*	
		4 hours	*8 hours*	*12 hours*	*K*	*e*
1	10% Acetic acid	3·8	5	5	0·25	2·11
2	5% Trichloracetic acid (c)*	2·7	4	5	0.67	1·78
3	10% Formalin	2·7	4·7	5	1·14	1·25
4	95% Ethanol	1·7	3·5	5	2·0	3
5	7·5% Mercuric chloride (c)	2·0	3·0	3.5	0·79	2·09
6	Sat. Aqueous picric acid (c)	1·0	1·5	1·75	3·9	2·03
7	2·5% Pot. bichromate	1·0	1·5	1·75	3·94	2·12
8	0·7% Chromic acid	0·6	1·0	1·2	8·4	1·99
9	4% Osmium tetroxide	0·3	0·5	0·7	23·9	1·94

*(c) = classified by Baker as coagulant fixative

was shown by Dempster to be $t = K.d^e$ where t = time and d = depth penetrated, factors K and e are as above. An example of the use of this equation, with a piece of tissue 20 X 10 X 6 mm to be fixed in formalin ($K = 1.14$, $e = 1.25$) would be $t = 1.14 \times 3^{1 \cdot 25} = 4.275$ hours.

REAGENTS EMPLOYED AS FIXATIVES

Formaldehyde (H.CHO)

Formaldehyde is a gas which is soluble in water to approximately 40 per cent by weight. This saturated solution is available commercially as

40 per cent formaldehyde, or formalin. In the past a great deal of confusion was caused by the careless use of these terms; a solution labelled '10 per cent formalin' might contain 10 per cent of formalin (4 per cent formaldehyde), or 10 per cent of formaldehyde (25 per cent formalin). It is now generally accepted that 40 per cent formaldehyde is formalin, and all references to it in this work will give actual percentages of this reagent. Thus, 10 per cent formalin consists of:

Formalin (40 per cent formaldehyde)	10 ml
Water	90 ml

Formalin consists mainly of the polymerized form of formaldehyde, whereas 10 per cent formalin consists principally of the monomeric form. However, it takes some time for depolymerization to take place unless the pH of the solution is neutral or alkaline since the rate of depolymerization is pH dependent. Since fixation is obviously much less efficient with the polymeric form, fresh unbuffered formalin fixatives should not be used.

It is well known that formaldehyde reacts with proteins (French and Edsall, 1945; Bowes and Cator, 1966; Hopwood, 1969) involving the formation of cross-links between the molecules giving rise eventually to an insoluble product. It is thought that two molecules may be involved in forming the cross-link since a single molecule would have steric difficulties in forming such a link; methylene bridges so formed are 2.4–2.5Å in length. Formaldehyde reacts readily with the amino groups and as the pH is raised to between 8 and 9 virtually all of these groups become involved. The main amino acids in proteins likely to react with aldehydes are: lysine, argenine, histidine, glutamine, asparagine, cysteine, tyrosine and tryptophan. The reaction of formaldehyde with lipids has been investigated and it has been shown to react rapidly with phosphatidyl ethanolamine causing degradation, there may also be very long-term effects associated with acid hydrolysis. Cholesterol, cerebrosides, sulphatides and sphingomyelin remain unaffected. Phospholipids, while not 'fixed' by formaldehyde, may be prevented from diffusing into the fixing fluid by the addition of calcium (Baker, 1944; Lillie, 1954) or cobalt (McManus, 1946) without affecting their solubility in lipid solvents. As a result of long storage, particularly in cold weather, formalin becomes turbid, owing to a white precipitate of paraformaldehyde. This can be filtered off and the filtrate used. Paraformaldehyde is a polymeric form of formaldehyde; polymerization is retarded in commercial formalin by the presence of methanol.

Adequate precautions should be taken when using formalin in large quantities for storing tissues. It may cause a dermatitis which can be a long-standing and painful complaint. Rubber gloves should, therefore,

always be worn when handling tissues from this fixative. The vapour is liable also to damage the nasal mucosa and cause sinusitis. Ventilation should always be adequate to reduce the concentration below the point at which it causes irritation.

Commercial formalin is usually acid, due to the formic acid content which is present either as an impurity or as a result of oxidation of the formaldehyde.

For the reasons given above, it should be made neutral or slightly alkaline before use as a fixative which can be achieved by the use of pH 7.2 phosphate buffer as a diluent, or by the addition of calcium acetate. The latter came into use following Baker's (1944) incorporation of calcium chloride in formalin solutions for the study of phospholipids. Subsequently, Lillie (1954) suggested the use of 2 per cent calcium acetate which also preserves the phospholipids and is almost as effective as a buffer, giving a pH of 7.1–7.3. In areas like Vancouver, Canada, where the tap water (being almost salt-free) has a pH of 7.0, prepared formal calcium has a pH of 6.6, this pH can be adjusted to 7.1–7.3 by the addition of 1–1.2 per cent sodium carbonate.

The use of neutral or slightly alkaline formalin fixatives results in a marked increase in the frequency with which ferric iron can be demonstrated and an almost complete absence of the formation of formalin pigment.

Glutaraldehyde (Glutaric Dialdehyde $(CH_2)_3$ CHO. CHO)

Glutaraldehyde was introduced as a fixative by Sabatini, Bensch and Barnett (1963); it has been used principally for electron microscopy, usually in combination with osmium tetroxide.

It is obtainable commercially as a 25 per cent solution in which form it contains various impurities; Hopwood (1967) noted that the glutaraldehyde could be fractionated from these impurities on Sephadex G–10. Chambers and his colleagues (1968) used activated coconut charcoal (10 g/100 ml 25 per cent glutaraldehyde) for this purpose, he found that, after exposure to the charcoal for several days at 4°C, the amber colour of the solution slowly diminished. It should be stored in the refrigerator without removal or change of the charcoal.

Studies by Bowes (1963) showed that, of several aldehyde fixatives tested, it was the most efficient cross-linking agent for collagen. Its cross-linking efficiency is said to give a better preservation of structure and a more rapid fixing action than formaldehyde, but poorer penetration. Hopwood (1969) stated that at pH levels greater than 8.0 it undergoes rapid polymerization.

Glutaraldehyde, which is a bi-functional aldehyde, reacts chiefly

with amino groups, its molecular size being particularly suited to bridge the gap between them on the polypeptide chain of collagen (Bowes and Cator, 1967). It also reacts with the amino acids tyrosine, tryptophane and phenylalanine (Hopwood, 1968), but to a lesser degree than formaldehyde. Chambers and his colleagues (1968) recommended the use of 4 per cent glutaraldehyde, in phosphate buffer at pH 7.4, as a routine fixative in histopathology. Non-specific P.A.S. staining is encountered after glutaraldehyde fixation due to unbound (free) aldehyde groups which result from some 10–15 per cent of the molecules being involved in unipointal fixation (only one end of the molecule bound or fixed). This false P.A.S. positivity can be eliminated by blocking with acetic-aniline (*see* page 289).

Acetaldehyde Acrolein

These aldehydes have been used as fixatives in electron microscopy but have not been very successful in histopathology.

Mercuric Chloride ($HgCl_2$)

Mercuric chloride is a protein precipitant which rapidly penetrates and hardens. In general, like other metallic ions, it combines with the acid groups of proteins and the phosphoric acid group of nucleoproteins, it also reacts specifically with thiol (SH) groups (Pearse, 1968). While most protein reactions can be utilized after Hg fixation, it is not recommended where nucleoproteins or sulphydryl groups are to be investigated. Unfortunately, the rate of penetration is decreased after the first few millimetres and pieces of tissue exceeding 5 mm in thickness will usually tend to be hard and overfixed at the periphery, and soft and underfixed in the centre. Because of this, and the great shrinkage caused, mercuric chloride is seldom used alone; it is a constituent of many good routine fixatives when combined with reagents which combat these defects, such as acetic acid, formalin, potassium dichromate, and so on.

Fixatives containing mercuric chloride should be listed among the *intolerant fixatives,* in that exposure of tissues to their action in excess of recommended times, will produce excessive hardness and make the cutting of thin sections difficult.

Although mercuric chloride neither attacks nor preserves lipids, its presence in tissue inhibits adequate freezing and makes frozen sections difficult to prepare.

Because it is radio-opaque the presence of mercuric chloride in calcified tissue precludes the use of x-rays to determine the end-point of decalcification.

Staining, particularly of the cytoplasm, tends to be more brilliant following mercuric chloride fixation.

Tissues fixed in any mercury-containing fixative will require treatment to remove the brownish mercury precipitate which will be found throughout. This is done by oxidation with iodine to mercuric iodide, which can be removed by treatment with sodium thiosulphate. This converts it to mercuric tetrathionate which is readily water-soluble.

As a routine the incorporation of 0.25 per cent iodine in the 80 per cent alcohol used in dehydrating tissues is recommended, and the following routine advised for individual sections before staining:

(1) Place section in 0.5 per cent iodine in 80 per cent alcohol for 3 minutes.

(2) Rinse in water.

(3) Place in 3 per cent aqueous sodium thiosulphate for 3 minutes.

(4) Wash in running water for 1–2 minutes.

Mercuric chloride corrodes metal and fixatives incorporating it must not be stored in containers with metal caps, unless such metal is 'Monel' metal, which is unaffected.

Potassium Dichromate ($K_2Cr_2O_7$)

Potassium dichromate has a binding effect on protein similar to that of formalin, giving fixation of the cytoplasm without precipitation. It is thought that this is because chromium ions form complexes with water which combine with the reactive sites on adjacent protein chains. Their main affinity is for carboxyl and hydroxyl groups of protein which makes fixatives containing chromium ions unsuitable for histochemistry with the exception of the chromaffin reaction (page 464). It preserves phosphatides and is used for the fixation of mitochondria. It has a pH of 3.75 and, although it is said that when acidified both chromatin and cytoplasm are precipitated as meshworks, chromosomes are well fixed, and mitochondria destroyed. *Champy's fluid,* which contains potassium dichromate, chromic acid, and osmium tetroxide with a pH of 2.5, preserves mitochondria. This may, however, be due to the direct fixing action of osmium tetroxide on lipids.

Following fixation in potassium dichromate (or chromic acid), tissue must be well washed in running water before dehydration. The transfer of chromate-containing tissue direct to alcohol results in the formation of an insoluble lower oxide which cannot be removed.

Chromic Acid

Chromic acid is prepared by dissolving anhydrous chromium trioxide (CrO_3) in distilled water.

It precipitates all proteins, and preserves carbohydrates. It is a powerful oxidizing reagent, and should it be contained in a compound fixative such as Orth's fluid, or Zenker's fluid, then reducing agents such as formalin should only be added immediately before use.

Chromic acid hydrolyses DNA with the conversion of its pentose sugar to an aldehyde; it also converts carbohydrates to aldehydes. DNA and carbohydrates will therefore give positive staining with Schiff reagent without the normal pre-treatment associated with the P.A.S. or Feulgen reactions.

Tissues fixed in chromic-acid-containing fixatives will require thorough washing for the same reason as those fixed in potassium dichromate.

Osmium Tetroxide (OsO_4)

Osmium tetroxide is commonly, though incorrectly, known as osmic acid.

It demonstrates lipids (for example, myelin) but is very expensive. It gives excellent preservation of detail of single cells, or minute pieces of tissue, and for this reason it is used almost exclusively in electron microscopy (*see* page 661). With pieces of tissue over 2–3 mm thick, however, it penetrates poorly and unevenly, and several routine staining methods are difficult, if not impossible, following its use.

Osmium tetroxide is supplied in sealed glass tubes containing 0.5 or 1 g.

> To prepare the usual stock solution of 2 per cent, the label is removed, and the tube washed several times in pure glass-distilled water. A file mark having been made, the tube is then broken and dropped into a glass-stoppered, dark bottle containing the correct amount of glass-distilled water.

Care must be taken in handling since its vapour is very irritating and can cause a conjunctivitis. It is easily reduced by light and heat and should be stored in a dark, cool place. The addition of 1 drop of saturated aqueous solution of mercuric chloride to every 10 ml of solution will help to check reduction. All the osmium-tetroxide-containing fixatives give very uneven fixation; the resultant sections show an over-fixed and blackened zone at the periphery, and an under-fixed zone at the centre with little cellular detail.

While there is considerable disagreement as to the actual mechanism of fixation of osmium tetroxide, it is generally agreed that it oxidizes unsaturated bonds. Saturated lipids do not react but unsaturated lipids reduce OsO_4 with the formation of black compounds.

The chemistry of the reaction between lipids and osmium tetroxide in relation to electron microscopy has been reviewed by Korn (1966). Bahr (1954) has shown that the reaction of OsO_4 with proteins depends upon their histidine, cystein and tryptophan content; his view was supported by Hake (1965). Dallam (1957) who showed there was a 37 per cent loss of protein during OsO_4 fixation and dehydration, and Leonard and Singer (1968) who showed that glutaraldehyde-osmium tetroxide fixation produced marked changes in the helical structure of proteins, have again indicated the need for caution in the interpretation of electron micrographs. It fixes and blackens lipids (for example, mitochondria and Golgi apparatus). The vapour alone can be used to fix very small pieces of tissue, or unfixed sections such as those prepared by the freeze-drying technique.

This blackening of certain elements is due to the conversion of the colourless OsO_4 to the black hydrated form, $OsO_2\,5H_2O$.

Picric Acid ($C_6H_2(NO_2)_3OH$)

Picric acid, owing to its explosive nature when dry, must be kept damp, preferably stored under a layer of water. The trichrome stains generally give a more brilliant contrast after fixation in a picric-acid-containing fixative.

It precipitates proteins and combines with them to form picrates, some of which are soluble in water. These water-soluble picrates must be rendered insoluble by treatment with alcohol before tissue so treated comes into contact with water.

It generally lakes red blood cells, quite often removes ferric iron, particularly if it is only present in small amounts and may render RNA resistant to ribonuclease digestion.

Ethyl Alcohol (C_2H_5OH)

Alcohol alone is of little use as a fixative except occasionally for blood films and smears. It penetrates rather slowly and tends to harden tissue after long exposure. When combined with other reagents, as in Carnoy's fluid, fixation is very rapid.

It denatures protein by precipitation, and precipitates glycogen. Denaturation of a protein may theoretically change the reactivity of its groups (Pearse, 1968) and while I have not seen an example of a change in staining pattern due to alcohol fixation, such a possibility should be

kept in mind when alcohol fixation is employed. It is used in histochemical methods for enzymes because, to some extent, it leaves them in their original state; being a fat solvent it dissolves fats and lipids.

Acetone (CH_3COCH_3)

Used cold, acetone is sometimes used as a fixative for the histochemical demonstration of tissue enzymes, notably the phosphatases and lipases. Its action as a fixative is almost identical to that of alcohol, except that glycogen is not well preserved.

Acetic Acid (CH_3COOOH)

Commonly called glacial acetic acid because it is solid at temperatures below 17°C, this is never used alone because of its swelling effect on collagen fibres; but in compound fixatives it is used to counteract the shrinkage effects of other reagents.

Nucleoproteins are precipitated by acetic acid, mitochondria and Golgi apparatus are destroyed or distorted.

Trichloracetic Acid (CCl_3COOH)

Trichloracetic acid, like acetic acid, is never used alone, but because of its swelling effect on many tissues it may be usefully employed in a compound fixative. It is a general protein precipitant, and has some slight decalcifying properties.

COMPOUND FIXATIVES

Choice of a Fixative

The choice of a fixative will be governed by the type of investigation required, both immediately and in the future. There would be little point in using Carnoy's fixative because of a primary interest in chromosomes, if a need to demonstrate lipids was likely to arise later. Large pieces of tissue should be fixed in a tolerant fixative, such as formol saline, which will allow subsequent treatment; smaller pieces can be taken from the mass either before or following formol fixation and given specialized treatment if required. It should also be remembered that museum specimens to which colour is to be restored can only be prepared from formalin-fixed tissues.

Rarely will one fixative be suitable for a variety of methods; for this reason it is convenient to divide them into three main groups: *micro-anatomical, cytological,* and *histochemical.*

(1) *Micro-anatomical fixatives*

These are used when it is desired to preserve the anatomy of the tissue, with the correct relationship of tissue layers and large aggregates of cells. It is obvious that fixatives for routine use should be drawn from this group.

(2) *Cytological fixatives*

Cytological fixatives are used when the preservation of intracellular structures or inclusions is of first importance. Often these elements are preserved at the expense of even penetration, ease of cutting, and the loss of other cell structures.

(3) *Histochemical fixatives*

When histochemical tests are to be applied, it is essential that the fixative employed produces minimal changes in the element that is to be demonstrated. Whilst the freeze-drying technique is probably almost ideal for this purpose it is far too troublesome and time-consuming for routine work.

While the more common fixatives employed in histochemistry are given under this heading, the fixative used should be that recommended for the specific technique to be employed.

The fixatives given in this chapter are the more common and reliable. Fixatives for special techniques are given under their appropriate headings.

MICRO-ANATOMICAL FIXATIVES

ROUTINE FORMALIN FIXATIVES

Although formol saline (10 per cent formalin in 0.9 per cent sodium chloride) has been the routine fixative of choice for many years it has now been largely supplanted by either buffered formalin (Lillie, 1954) or formol calcium acetate (Lillie, 1965). They have the same advantages and are used in the same manner as formol saline. Since they have a near neutral pH, formalin pigment (acid formaldehyde haematin) is not formed since its occurrence is due to the interaction of formalin solutions, at an acid pH, with haemoglobin or its products; it is seen most commonly in sections of the spleen, liver, bone marrow, and so on. Should this pigment be encountered it is easily removed from sections, before staining, by treatment with picric alcohol or a 1 per cent alcohol solution of sodium hydroxide.

The incorporation of calcium chloride in 10 per cent formalin was

designed by Baker (1944) to preserve phospholipids, but the use of calcium acetate has the added advantage of buffering the solution, and since it is easily and rapidly prepared we prefer it as a routine fixative.

Buffered formalin, formol calcium and formol saline, because of their tolerance are probably the most useful and most widely used fixatives. Tissue can be left in them for long periods without excessive hardening or damage, and may be sectioned easily after as long a period as one year. Other than a slight decrease in basophilia, and in the reactivity of myelin to Weigerts haematoxylin technique, those tissue elements not preserved are not destroyed which enables most of them to be demonstrated after further treatment.

Fixation of tissue blocks, not exceeding 5 mm in thickness, is usually complete in 6–12 hours at room temperature. Fixation by formalin, and other chemical fixatives, is influenced by heat and pieces of tissue up to 3 mm in thickness can be fixed in 1½–2 hours at 55°C with, however, some loss of detail.

Formol Calcium (Lillie, 1965)

Formula

Formalin	10 ml
Calcium acetate	2 g
Water	to 100 ml

Formol Calcium (Baker, 1944)

Formula

Formalin	10 ml
Calcium chloride	2 g
Water	to 100 ml

This solution should be made from formalin that has been neutralized with buffer or marble chips.

Buffered Formalin

Formula

Formalin	10 ml
Acid sodium phosphate monohydrate	0.4 g
Anhydrous disodium phosphate	0.65 g
Water	to 100 ml

Buffered Formol Sucrose (Holt and Hicks, 1961)

Formula

Formalin	10 ml
Sucrose	7.5 g
M/15 phosphate buffer (pH 7.4)	to 100 ml

This fixative gives excellent preservation of fine structure, phospholipids and some enzymes. It is recommended for combined cytochemistry and electron microscopical studies. To get the best results it should be used refrigerator cold (4°C) on fresh tissues.

Electron microscope pictures show well preserved mitochondria, endoplasmic reticulum, etc., after fixation by this method.

Alcoholic-Formalin

Formula

Formalin	10 ml
70–95 per cent Alcohol	90 ml

If desired, 0.5 g of calcium acetate may be added to ensure neutrality.

Acetic-Alcoholic-Formalin (Tellyesniczky's)

Formula

Formalin	5 ml
Glacial acetic acid	5 ml
70 per cent alcohol	90 ml

Either alcoholic-formalin or acetic-alcoholic-formalin are excellent for glycogen although not ideal as routine fixative. It is said to prevent the solution of carbohydrates before the fixation of the protein component is complete. The addition of acetic acid ensures fixation of the nuclear protein with an improved histological picture. Since both alcohol and formalin are rapidly penetrating agents, this is a reasonably rapid fixative. Tissues up to 5 ml in thickness are fixed in 4 hours.

FORMALIN FIXATIVES FOR CARBOHYDRATES

The addition of various salts to formalin solution as precipitating agents for mucopolysaccharides and mucoproteins has been recommended by

many workers in this field. Williams and Jackson in 1956 recommended the addition of quarternary ammonium salts to 10 per cent formalin such as 1 per cent cetylpyridinium chloride (CPC), 1 per cent cetyltrimethylammonium bromide (CTAB). There may be changes in staining reactions due to their binding of reactive sites and the same criticism might also be applied to the use of 1 per cent 5-aminoacridine hydrochloride in 10 per cent formalin. These methods and others for carbohydrates are discussed in the appropriate chapter (page 266).

Buffered Glutaraldehyde

Formula

Glutaraldehyde stock 25 per cent solution (charcoal treated)	16 ml
Phosphate buffer pH 7.4	84 ml

This fixative is discussed on page 38. It will, it should be remembered, give a false P.A.S. positive reaction. The purification of the stock 25 per cent glutaraldehyde is also discussed on page 38.

Heidenhain's Susa

Formula

Mercuric chloride	4.5 g
Sodium chloride	0.5 g
Trichloracetic acid	2 g
Acetic acid	4 ml
Formalin	20 ml
Distilled water	to 100 ml

This is an excellent fixative available for routine biopsy work, allowing brilliant staining with good cytological detail. It is well balanced and gives rapid and even penetration with a minimum of shrinkage. It is, however, an intolerant fixative, and tissues left in it over 24 hours are bleached and excessively hardened. The incorporation of trichloracetic acid is said to give it slight decalcifying powers, but this can only be relied upon for minute calcium deposits.

Tissue must be transferred direct to 96 per cent absolute alcohol to avoid swelling of connective tissues. Since this solution contains mercuric chloride it must not be kept in a container with a metal cap. Although it has been claimed that tissues fixed in 'Susa' do not contain mercury pigment, this is not universal experience, and the tissues should be treated with iodine to remove it (page 40).

Tissues not exceeding 7–8 mm in thickness are fixed in 12–24 hours. Small pieces, not thicker than 3 mm are fixed in 2–3 hours.

Zenker's Fluid

Formula

Mercuric chloride	5 g
Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water	to 100 ml
Add glacial acetic acid immediately before use	5 ml

This solution does not keep after the acetic acid has been added, but without acetic acid (Zenker's stock fluid) it keeps well and has the advantage that either acetic acid or formalin may be added immediately before use (*see* below).

Zenker's fluid is a good routine fixative giving fairly rapid and even penetration. Following its use, tissues must be washed in running water overnight to remove the excess dichromate (page 40), and mercuric chloride pigment must be removed with iodine (page 40).

Fixation is usually complete in 12 hours. Small pieces, not thicker than 3 mm are fixed in 2–3 hours.

Zenker Formol (Helly's Fluid)

Formula

Mercuric chloride	5
Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water	to 100 ml
Add formalin immediately before use	5 ml

This fixative is stock Zenker mixture to which formalin is added instead of acetic acid. It is irrational in that it contains potassium dichromate which is an oxidizing agent, and formaldehyde which is a reducing agent, but it is an excellent micro-anatomical fixative.

It is variously known as Helly's, Spuler's, or Maximow's fluid, although the formalin content and the amount of mercuric chloride and potassium dichromate varies. Carleton and Leach (1947) recommend 3.5 g and 1.5 g respectively, and Lillie (1954) 7 g and 2.5 g. In the author's experience, results do not justify alteration of the stock Zenker's fluid given above.

As with Zenker-acetic the excess dichromate must be washed out, and mercuric pigment removed with iodine.

It is an excellent fixative for bone-marrow and spleen, and is recommended for blood-containing organs in general.

Zenker-formol is slower than Zenker-acetic; fixation is usually complete in 6–24 hours.

Bouin's Fluid

Formula

Picric acid, saturated aqueous solution	75 ml
Formalin (40 per cent formaldehyde)	25 ml
Glacial acetic acid	5 ml

This fixative, which keeps well, penetrates rapidly and evenly and causes little shrinkage. Tissue fixed in it gives brilliant staining by the trichrome methods. The excess picric acid, to which the yellow colour of the tissue is due, should be removed from the section by treatment with alcohol or prolonged washing.

Bouin's fluid can be used to demonstrate glycogen, but Gendre's fluid is a better picric-acid fixative for this purpose. Owing to the formation of some water-soluble picrates, tissues must be transferred from the fixative direct to alcohol (page 42).

Fixation is usually complete in 24 hours, but small pieces not exceeding 2–3 mm in thickness are fixed in 2–3 hours.

Gendre's Fluid

Formula

Picric acid, saturated solution in 95 per cent alcohol	80 ml
Formalin (40 per cent formaldehyde)	15 ml
Glacial acetic acid	5 ml

This fluid is said to give good fixation of glycogen, after 3–4 hours at room temperature; however, see the discussion on page 298.

Rossman's Fluid (1940)

Formula

Formalin (neutralized)	10 ml
Absolute ethyl alcohol saturated with picric acid (approximately 8.5–9 per cent)	90 ml

It will be seen that this is similar to Gendre's fluid but without the acetic acid. This fixative has also been recommended for carbohydrate fixation.

CYTOLOGICAL FIXATIVES

For convenience, this group of fixatives is sub-divided into (1) nuclear, and (2) cytoplasmic.

NUCLEAR FIXATIVES

Carnoy's Fluid

Formula

Absolute alcohol	60 ml
Chloroform	30 ml
Glacial acetic acid	10 ml

Carnoy's fluid penetrates very rapidly, and gives excellent nuclear fixation with preservation of Nissl substance and glycogen. This has been recommended by many workers for the fixation of carbohydrates. It causes considerable shrinkage, and destroys or dissolves most cytoplasmic elements. The degree of shrinkage can be reduced by fixation at 0°C for 18 hours (Lillie, 1954).

Being a rapid fixative it is sometimes used when a diagnosis is urgently required.

Fixation is usually complete in 1–2 hours; small pieces 2–3 mm in thickness are fixed in 15 minutes.

Clarke's Fluid

Formula

Absolute alcohol	75 ml
Glacial acetic acid	25 ml

This fixative penetrates rapidly and gives good nuclear fixation and reasonably good preservation of cytoplasmic elements. It is excellent for smears or coverslip preparations of cell cultures for general fixation or chromosome analyses.

Newcomer's Fluid

Formula

Isopropanol	60 ml
Propionic acid	30 ml
Petroleum ether	10 ml
Acetone	10 ml
Dioxane	10 ml

This fixative, which penetrates rapidly, was devised for the fixation of chromosomes, and preserves the chromatin better than Carnoy's fluid, giving an improved Feulgen reaction. It was recommended by Saunders (1964) for the fixation and preservation of mucopolysaccharides.

Fixation is complete in 12–18 hours; small pieces of tissue not exceeding 3 mm in thickness are fixed in 2–3 hours.

Flemming's Fluid

Formula

1 per cent aqueous chromic acid	15 ml
2 per cent aqueous osmium tetroxide	4 ml
Glacial acetic acid	1 ml

Flemming's fluid, originally a nuclear fixative, is rarely used as such because of its poor and uneven penetration. For chromosomes, the full 1 ml of acetic acid is used, but when this is omitted, the fluid is used as a cytoplasmic fixative. Unlike other fixatives the bulk need only be 5–10 times that of the tissue. Following fixation tissue should be washed overnight.

In common with all the osmium tetroxide fixatives, pieces of tissue must be small (not more than 2 mm in thickness), when fixation will be complete in 12 hours.

CYTOPLASMIC FIXATIVES

Champy's Fluid

Formula

3 per cent potassium dichromate	7 ml
1 per cent chromic acid	7 ml
2 per cent osmium tetroxide	4 ml

This fixative does not keep and should be prepared freshly from stock solutions. It penetrates poorly and unevenly (page 41 on osmium tetroxide); consequently, only thin pieces of tissue should be treated.

Champy's fluid preserves mitochondria, fat, yolk and lipids, and gives results similar to those of Flemming's fluid without acetic acid, although Champy's fluid is preferred for mitochondria.

Tissue must be washed overnight after fixation which, for pieces of tissue not thicker than 2 mm will be complete in 12 hours.

Règaud's Fluid

Formula

3 per cent potassium dichromate	80 ml
Formalin (40 per cent formaldehyde)	20 ml

Régaud's fluid does not keep, and the solutions should only be mixed immediately before use. It penetrates evenly and fairly rapidly, but has a tendency to overharden tissue. It may be used as a routine fixative, but is particularly good for mitochondria if followed by 4–8 days chromation in 3 per cent potassium dichromate. Chromaffin tissue is well demonstrated by the same method, but fluids may be improved for this purpose by the addition of 5 per cent acetic acid.

Fixation is usually complete in 24 hours, small pieces not more than 3–4 mm in thickness are fixed in 4–6 hours.

Müller's Fluid

Formula

Potassium dichromate	2.5 g
Sodium sulphate	1 g
Distilled water to	100 ml

This is rarely used as a fixative except for bone specimens (page 64). It may be substituted for 3 per cent potassium dichromate for post-chroming.

Formol Saline and Formol Calcium

Fixation in formol saline, followed by post-chromatization (page 55) gives good cytoplasmic fixation in most instances, with improved preservation of the micro-anatomical features of the tissue.

Zenker Formol (Helly's Fluid)

Like formol saline it can be used with good results, both as a cytoplasmic fixative and a micro-anatomical fixative, particularly for bone-marrow and the blood-forming organs. When used for mitochondria, it is advisable to transfer tissues to 3 per cent potassium dichromate for 6–8 days, or treat sections before staining in the dichromate solution for 24 hours.

Schaudinn's Fluid

Formula

Mercuric chloride, saturated aqueous solution	2 parts
Absolute alcohol	1 part

This fixative has been popular for many years as a cytoplasmic fixative for wet smears. It is not recommended for tissue, being harsh in action and causing excessive shrinkage; probably due to the time of exposure needed for penetration.

Tissue or smears need treatment with iodine-alcohol and sodium thiosulphate to remove mercury deposit (page 40).

Wet smears are well fixed in 10–20 minutes and unless too thick they rarely become subsequently detached from the slide.

HISTOCHEMICAL FIXATIVES

A good histochemical fixative should: (*a*) preserve the constituent to be demonstrated, preferably preserving its morphological relationships; (*b*) bind or otherwise preserve the specific tissue constituent, without affecting the reactive groups to be used in its visualization (*see* the example given on page 29 regarding basic protein); (*c*) not affect the reagent to be used in the process of visualization; for example, glutaraldehyde fixation leaves the tissue proteins so fixed with a coating of free reactive aldehyde groups which give a positive Schiff or P.A.S. reaction.

For the majority of histochemical methods it is best to use cryostat cut sections of rapidly frozen tissue (*see* page 142), or sections of frozen dried tissue. Such sections may be used unfixed or they may be fixed by a vapour fixative.

Formol Saline

As formalin is the most common fixative, it is likely that much of the material on which histochemical methods are to be applied will have been so fixed. Provided it is buffered to prevent the formation of formalin pigment, and the tissue is well washed to remove the excess fixative, the majority of histochemical techniques are applicable.

Cold Acetone

Immersion in acetone at 0–4°C is widely used for the fixation of tissues in which it is intended to study enzymes, particularly the phosphatases.

Absolute Alcohol

Fixation of sections cut from freeze-dried material may be effected by immersion in absolute alcohol for 24 hours.

It is occasionally recommended as a basic fixative, but in most histochemical techniques formalin can be used as an alternative with a consequent improvement in micro-anatomical and cytological preservation.

VAPOUR FIXATIVES

Vapour fixatives may be used to fix cryostat cut sections of fresh tissue and sections or blocks of frozen dried tissue.

Pearse has designed for this purpose a useful vapour fixation chamber with controlled heat and humidity. We have successfully fixed cryostat sections using a glass slide holder (the type used for staining) inside an air-tight glass container, in a 60°C oven. Rost and Ewen (1971) used vapour fixatives on frozen dried tissue to demonstrate amines, the amines being converted to a fluorescent isoquinoline, quinonoid or beta carboline, they heated all their fixatives to 80°C.

Formaldehyde

Vapour is obtained by heating paraformaldehyde at temperatures between 50°C and 80°C. Blocks of tissue require 3–5 hours at 50–60°C, sections require from ½ to 1 hour.

Acetaldehyde

Heat met-aldehyde ('meta') at a temperature of 80°C for 1–4 hours.

Rost and Ewen (1971) obtained poor general fixation but good fluorescence due to amines with acetaldehyde.

Glutaraldehyde

Rose and Ewen used a 50 per cent aqueous solution at 80°C for 2 minutes to 4 hours; Pearse recommends 60°C for 7 hours.

Acrolein or Chromyl Chloride

The liquid reagent may be used at 37°C for 1–2 hours.

POST-CHROMATIZATION

Post-chromatization is the treatment of tissues with 3 per cent potassium dichromate following normal fixation. Post-chromatization may be carried out either before processing, when the tissue is left for 6–8 days in dichromate solution, or after processing when sections, before staining, are immersed in dichromate solution for 12–24 hours, followed in each case by washing well in running water.

This technique is employed to mordant tissues, particularly mitochondria, and gives improved preservation and staining of these elements.

FREEZE DRYING

The preservation of tissues by freeze drying is often discussed as a method of fixation, but it is, correctly, an alternative to fixation. It is a method of initially preserving tissue with little alteration in cell structure or chemical composition which permits embedding in wax without the normal intermediate stages of dehydration by alcohols and clearing. The resultant sections may be examined unfixed in an inert medium, by micro-incineration or after extraction with buffered solutions; or they may be fixed and normal histochemical or histological techniques applied.

The technique of freeze drying consists of the following three stages:

(1) Initial rapid freezing, known as 'quenching.'
(2) Drying of the frozen tissue.
(3) Embedding, sectioning and mounting.

Quenching

In our laboratory, small pieces of tissue, not more than 1 mm in thickness, are placed on a thin strip of folded aluminium foil or copper

foil and plunged into isopentane cooled to a temperature of – 160° to – 180°C with liquid nitrogen; the exact temperature of the isopentane is not important; cooling should be continued until the isopentane becomes more viscous. The frozen tissue is then removed from the foil, and the isopentane poured off. The use of Freon 12 (dichlorodifluoromethane) in place of isopentane was recommended by Bell (1952), however, Freon 22 (monochlorodifluoromethane) was investigated and recommended by Rebhun (1965), and Pearse (1968) employs it routinely in his laboratory.

. It is essential that tissues be absolutely fresh so that the initial rapid freezing not only inhibits autolysis and putrefaction, but also prevents any diffusion of substances within the cells. The low temperature used for the initial freezing is important, for unless the whole of the tissue is frozen rapidly large ice crystals are formed which will disrupt cell structure. Pieces of tissue more than 1 mm in thickness will probably show this disruption artefact at the centre, and for this reason are to be avoided.

Drying

The frozen tissue is transferred quickly to the drying apparatus, where a high vacuum is established and the ice in the tissue transferred by sublimation to a vapour trap. The rate of drying depends on the following three factors:

(1) Temperature.
(2) Vacuum pressure.
(3) Distance of tissue from vapour trap.

Temperature. – The higher the temperature at which the tissue is maintained the more rapid is the drying: for example, raising the temperature from – 60° to – 40°C increases the rate of evaporation tenfold: to prevent the formation of ice crystals the temperature should theoretically be maintained below the eutectic point of the tissue (about −55°C); and noticeable deterioration of the tissue occurs if it is allowed to rise to −20°C. In practice, satisfactory results are combined with reasonable speed of drying if the temperature is maintained between −30° and −40°C.

Vacuum pressure and the distance of tissue from vapour trap. – In the process of drying, water molecules sublime from the frozen tissue to form water vapour and this condenses on a vapour trap maintained at a temperature below that of the tissue; for example, liquid nitrogen

(−185°C) or an acetone-CO_2 mixture (−70°C). This transfer of water molecules is hindered by the presence of air, or by bends or constrictions obstructing the flow of vapour: if the pressure is kept below 10^{-3} mm Hg, and the vapour trap is within 5 cm of the tissue, molecules of water leaving the tissue will cross directly to the vapour trap and condense upon it, and the maximum rate of drying will be achieved. If the pressure is higher the distance must be reduced (and *vice versa*) to achieve the same rate of drying for example with a vacuum of 5×10^{-5} mm the vapour trap may be up to 30 cm distant.

The vapour trap may be a 'cold finger' type, where a small tube containing liquid nitrogen is inserted into the drying tube so that it is within 5 cm of the tissue; on the surface of this 'cold finger' the sublimed water condenses as ice, and when drying is completed the cold finger is removed. A secondary vapour trap is incorporated between the drying tube and the vacuum pump which removes water from the system and protects the pump.

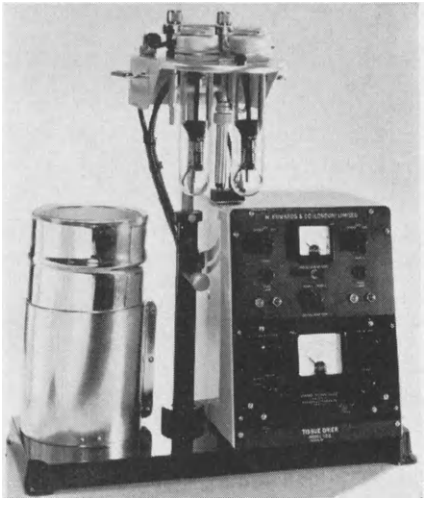
Another type is that used by Edwards High Vacuum Ltd., in their tissue dryer model TD.2 *(Figure 3.1a)*. In this the vapour trap is the wall of the drying tube, which is immersed in a Dewar flask containing the cooling mixture, the tissue being held in the middle of the tube on a special carrier *(Figure 3.1b)*. Phosphorus pentoxide is used as a secondary vapour trap.

Drying is usually complete within 24–48 hours for small pieces of tissue.

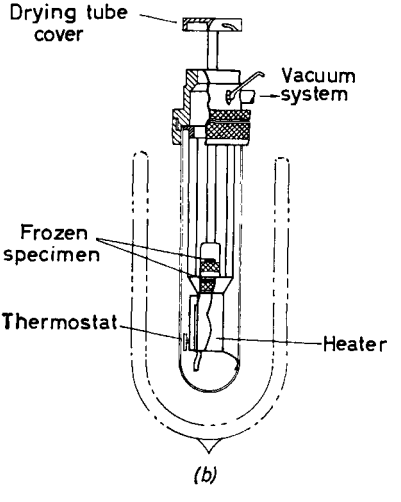
Method of use. – Dehydration of tissue is carried out in an evacuated drying tube, inside which the specimen is suspended in a carrier. The drying tube is placed in a cooling mixture consisting of solid carbon dioxide (CO_2) and methylated spirits at −78°C contained in a Dewar flask. Water vapour sublimed from the specimen is condensed on the drying tube walls.

The drying tube is evacuated by a two-stage rotary pump, and drying is accelerated by applying a controlled amount of heat to the specimen. For this purpose the specimen carrier is surrounded by an electrical heater which can be thermostatically controlled at any temperature between −50° and −10°C.

A phosphorus pentoxide (P_2O_5) moisture trap must be placed in the pipeline close to the pump. This trap in addition to preventing backstreaming of water vapour from the pump is also used at the end of the drying operation to absorb re-vaporized ice from the walls of the drying tube while the tube is brought up to room temperature prior to the removal of the specimen. By this means the condensate is prevented from re-hydrating the specimen.



(a)



(b)

Figure 3.1 – (a) Tissue dryer, model TD.2; (b) Diagram of drying tube unit. (By courtesy of Edwards High Vacuum Ltd.)

Thermo-electric Tissue Dryers

These tissue dryers are based upon the cooling of the tissue and its maintenance at a temperature approaching its eutectic point (−55°C)

by thermo-electric cooling elements. The Edwards-Pearse (Model EPD2)*, costing approximately $2,000 (in Canada) is of such a type. One point of interest is that only a phosphorus pentoxide vapour trap is used, in addition to vapour traps in the vacuum line. The thermo-electric assembly will cool to −60°C (from room temperature) in 20 minutes which makes the unit convenient and rapid in use. The average drying time for 2 mm thick pieces of tissue is 3½–4 hours at −40°C on this apparatus.

The provision of boats for use with degassed paraffin wax (or a resin embedding accessory which is not recommended) together with a simple pushbutton type of operation may encourage more workers to use this technique.

Embedding, Sectioning, Mounting, Fixation and Storage

Embedding. – The embedding of the tissue in wax may be carried out in one of two ways.

The use of Paraplast is preferred to other embedding waxes, since it contains a high concentration of plastic polymers.

(*a*) The dried tissue may be transferred quickly to a vacuum embedding oven containing molten wax, with a vacuum of routine pressure. The tissue on sinking to the bottom of the wax bath will be impregnated with wax, a period of only 5–10 minutes being required.

(*b*) The embedding media, thoroughly degassed, may be placed at the bottom of the drying tube prior to the drying operation. When drying is complete the tube is allowed to reach room temperature and then heated slowly until the wax just melts: the dried tissue sinks and is impregnated.

While the first method will give satisfactory results, the second is obviously the better technique, since at no time is there a possibility of absorption of moisture from the air.

The embedding medium may be paraffin wax, or one of the water-soluble waxes, the latter being indicated for the demonstration or investigation of fats or fatty compounds.

Complete avoidance of heat may be obtained by embedding in celloidin but, generally, workers in this field have preferred to use the standard method of double embedding.

Sectioning. – This is generally carried out on a standard microtome, sections being cut slightly thicker than usual to facilitate handling.

*Although there have been a number of papers (and techniques) published on this subject since the 2nd Edition of this book, in my opinion it remains inferior to existing methods; for example, vapour fixed, or unfixed, cryostat sections.

The floating out of sections presents difficulties, since the use of warm water would result in disintegration. Sections may be directly affixed to warm albuminized slides (page 131) by finger pressure or light blotting, or they may be floated on warm mercury to remove creases and picked up by contact with the albuminized side of a slide.

If fixation will not interfere with the technique to be employed, sections may be floated on warm formol saline or formol calcium; this method may be used with success for the demonstration of fats and fatty substances in tissues embedded in water-soluble wax.

Mounting. – Unfixed, unstained sections can be mounted for microscopic examination in an inert medium such as liquid paraffin or nonane (Bell, 1952); either of these media dissolves the paraffin wax and the preparations are suitable for examination by phase-contrast, fluorescent, polarized light, or ultra-violet light microscopy. For the examination of lipids by polarized light, thick Carbowax-embedded sections are taken quickly through water and mounted in Karo corn syrup.

Fixation. – Fixation of sections is easily effected, either by floating on formalin, as already described, or by immersion of the mounted sections in 80 per cent alcohol or acetone for 12–24 hours after removal of the wax.

Storage. – Embedded blocks of tissue and sections of freeze-dried material must *not* be stored in the conventional manner. They may be kept in a desiccator over calcium chloride in a cold room to avoid the absorption of moisture from the air, (with resultant dissolution of the tissue). Sections are best kept by fixation in alcohol, or formalin, but if such solutions must be avoided, then fixation may be effected by the vapour of osmium tetroxide, or formaldehyde, with consequent denaturing of proteins.

For a more complete discussion on the theory and practice of freeze drying, the appropriate chapter in *Histochemistry, Theoretical and Applied,* by Everson Pearse (1968) is recommended.

FREEZE SUBSTITUTION

Simpson (1941) described a freeze substitution technique as an inexpensive alternative to freeze drying. While in general the results are not always as good as with the latter it does offer a method which can be employed in a routine laboratory without the purchase of expensive equipment.

FREEZE SUBSTITUTION

This method is based on the quenching of the tissue to (1) inhibit autolysis and putrefaction; (2) prevent diffusion and dissolution of the substances within the tissues; (3) prevent the formation of large ice crystals in the quenched tissues. This is done at low temperatures in liquid dehydrating agents which are also fixatives. It has been shown that many substances which are soluble in these agents at room temperature are insoluble, and thus preserved, at the low temperatures used.

Tissues are quenched, as for freeze drying, in either isopentane in liquid nitrogen (−160°C) or in acetone containing, and surrounded by, dry ice. They are then transferred to Rossman's fluid (which is Gendre's fluid without acetic acid (Peyrot 1956)) or into 1 per cent osmium tetroxide in acetone (Feder and Simpson, 1958). These are kept at −60° to −70°C in an insulated box with dry ice in a freezing cabinet or in a low temperature (−70°C) freezer for 1–6 days. The tissues are then allowed to reach room temperature slowly when they are processed by the normal paraffin embedding technique. Balfour (1961) used freeze substitution as a method of preparing tissue sections for fluorescent antibody staining. After quenching, pieces of tissue not more than 1–2 mm thick are transferred to test tubes containing absolute alcohol and a small amount of sodium sulphate as a drying agent at a temperature of −70°C for three days when substitution should be complete. They are then rapidly embedded in polyester wax or in ester wax if the tissue is hard. Sections are cut at 3 μ thick and flattened on 18 per cent sodium sulphate to prevent solution of gamma globulins. After removal of the wax, staining is carried out as described on page 187.

Chapter 4

Decalcification

The presence of calcium salts in tissue prevents the preparation of good sections by routine methods. The incomplete removal of these salts results in torn and ragged sections, and in damage to the cutting edge of the microtome knife.

An exception may be made to the above in the case of moderately calcified material, such as human rib, reasonable sections of which may be prepared by cutting on a freezing microtome.

Technique

The technique of decalcification may be divided into the following stages:

(1) Selection of tissue.
(2) Fixation.
(3) Decalcification.
(4) Neutralization of acid.
(5) Thorough washing.

On occasions, fixation of a gross specimen will precede selection of a piece for decalcification, and there are certain fluids which have a fixing and decalcifying action. The above scheme sets out a general plan of work, but it should not be taken as a rigid schedule.

Selection of Tissue

Bone. – Thin slices of bone are obtained using a fine-toothed bone saw, or hacksaw. To ensure adequate fixation and complete removal of the calcium, slices should not exceed 4–5 mm in thickness. Following decalcification and washing, the cut surfaces should be re-trimmed to remove the areas damaged by the saw.

Calcified tissue. – Thin slices of calcified tissue can usually be cut with a sharp knife, but when difficulty is encountered a saw should be used to avoid damage to the tissue surrounding the calcified area. The type and duration of treatment of such tissue (for example, chronic tuberculosis foci, calcified scar tissue) will depend on the degree of calcification. Tissues containing only small areas should be tested after 2–3 hours in a decalcifying fluid.

Fixation

The general rules for fixation are applicable, and a fixative is chosen which will best preserve the tissue elements to be demonstrated. As a routine fixative, formalin is preferred, but bone marrow is best fixed in Zenker formol. Some fine preparations of bone have been produced following immersion in Müller's fluid for up to 3 months, followed by decalcification in 3 per cent formic-acid–formalin.

It has been shown by Cook and Ezra Cohn (1962) that *tissue damage during acid decalcification is approximately four times greater when the tissue is unfixed.*

Decalcification

Decalcification may be effected by the following:

(*a*) *Simple solution* of the calcium by an acid reagent, usually a dilute mineral acid.

(*b*) *Simple solution* aided by ion exchange resins which keep the decalcifying fluid free calcium and thus give more rapid results.

(*c*) Use of chelating agents.

(*d*) *Electrophoretic* removal of the calcium ions from tissues by the use of an electric current.

All these methods are greatly accelerated by the application of heat. Decalcification carried out at a temperature of 56°–60°C is very rapid, but it causes undue swelling of tissues, and complete digestion can occur quite rapidly. Even a temperature of 37°C causes swelling of tissue and impairs subsequent staining. The use of heat to accelerate decalcification cannot, therefore, be recommended.

The criteria of a good decalcifying agent are:

(i) Complete removal of calcium..
(ii) Absence of damage to tissue cells or fibres.
(iii) Non-impairment of subsequent staining techniques.
(iv) Reasonable speed of decalcification.

Determination of end-point. – Tissues should not be exposed to decalcifying fluids for longer than necessary to ensure the complete removal of calcium salts, and some method of determining the absence of calcium is therefore needed. The most satisfactory method is undoubtedly radiography of the tissue, but it must be remembered that tissues fixed in mercuric chloride-containing fixatives cannot be tested by this method since they will be radio-opaque.

A satisfactory alternative to x-rays is a chemical test for calcium which may be performed in the following manner.

> Five ml of decalcifying fluid are nearly neutralized with N_2.NaOH, then 1 ml of 5 per cent sodium or ammonium oxalate added. Turbidity of the fluid indicates the presence of calcium. Absence of turbidity after a delay of 5 minutes indicates that the decalcifying fluid is free of calcium.

While calcium is still present in the tissue, some calcium ions will be dissolved in the decalcifying fluid; therefore, when this is free of calcium it may be inferred that decalcification is complete. It will be obvious that the fluid must be completely changed each time it is tested, and that an interval of 2–3 hours must elapse between tests to allow solution of the calcium.

Testing for decalcification by the flexibility of the tissue is unreliable, and the out-moded practice of inserting a needle to feel for calcium deposits causes damage to the tissue.

Neutralization of acid

It has been said that, following immersion in mineral acids, tissues should be de-acidified or neutralized, before washing, by treatment with an alkali; this may be effected by treatment overnight in 5 per cent lithium or sodium sulphate. Although failure to do this is said to cause swelling, if the tissues are transferred direct to dilute alcohol (70 per cent) and given two changes over 12–18 hours, there is not only little evidence of swelling but staining reactions are improved in most cases. Following this step, dehydration is continued in the usual way.

Washing

Thorough washing of the tissue is essential before processing to remove the acid (or alkali, if neutralization has been carried out) which would otherwise interfere with staining reactions.

Washing should be carried out for 3–4 hours in alcohol, or overnight in water in a tissue washer – an automatic syphon type which ensures a complete change of water every few minutes is convenient and efficient. These can be purchased, or made quite simply in the laboratory.

DECALCIFYING FLUIDS

Gooding and Stewart's Fluid

Formula

Formic acid	5–25 ml
Formalin	5 ml
Distilled water	to 100 ml

The use of 5 ml of formic acid gives a good routine decalcifying fluid, with reasonable speed and a minimum of damage to tissue. The increase of the formic acid content up to 25 per cent results in increased speed of decalcification, the formaldehyde content giving the tissue some protection against damage by the acid, but it should be remembered that this increase in speed can only be attained at the sacrifice of cellular detail.

Decalcification in the 5 per cent solution will usually be complete in 2–4 days, dependent on the thickness of the tissue and the degree of calcification. A cross-section of human rib will require 36–48 hours.

Decalcifying paraffin-embedded calcified tissues (Culling and Barkoczy, 1972)

It has been known for many years that paraffin is pervious to aqueous solutions, for this reason paraffin sections may be stained before removal of the wax (Culling, 1949) and paraffin-embedded frozen dried tissues must be stored in a desicator over calcium chloride (*see* page 60). We have made use of this characteristic in a rapid decalcification technique by routinely processing calcified tissue (rib, and so on) and then decalcifying the Paraplast-blocked tissue immediately before cutting.

The surface of the block to be decalcified is trimmed with a scalpel or knife to expose the tissue. The block is then placed, face downward, in 5 per cent hydrochloric acid (Hcl). If only one or two sections are required the block need only be left in the Hcl for a short period. We were able to get 30 sections of human rib after 4 hours treatment.

It is suggested that a routine hospital laboratory could process bone specimens together with soft tissues, and that the embedded blocks of

calcified tissues are treated with Hcl while the soft tissues are being cut. After a sufficient time interval, the bone sections may then be cut in the usual manner. This avoids the necessity for special treatment and allows a more rapid diagnosis to be made. The cut sections showed less artefact by this method than by the usual decalcification procedures and did not damage the cutting edge of the knives employed. The blocks may be treated in this manner as required, and as often as required without the fear of excessive exposure to acid with a consequent loss of staining. It follows, of course, that blocks containing unsuspected calcium deposits can be treated in the same manner; thus avoiding having to deblock, rehydrate and decalcify tissue.

Citrate–Citric Acid Buffer (pH 4.5)

7 per cent citric acid (monohydrate)	5 ml
7.54 per cent ammonium citrate (anhydrous)	95 ml
1 per cent zinc sulphate	0.2 ml
Chloroform	a few drops

As calcium ions are soluble at pH 4.5, buffer solutions have been used recently to decalcify tissues. They are generally much slower than the more established fluids, but since they cause no perceptible damage to cells or tissue fibres they are of use when time is not an important factor.

Cross-section of human rib (5 mm) is decalcified in 4–6 days.

Jenkins' Fluid

Formula

Absolute alcohol	73 ml
Distilled water	10 ml
Chloroform	10 ml
Glacial acetic acid	3 ml
Hydrochloric acid	4 ml

Jenkins' fluid not only decalcifies but also dehydrates. The swelling action of the hydrochloric acid is counteracted by the shrinking effect of the alcohol.

Large amounts of this fluid should be used, between 40 and 50 times the bulk of the tissue. After decalcification the tissue is transferred directly to absolute alcohol in which it is given several changes to remove the acid.

Cross-section of human rib is decalcified in 4–6 days.

von Ebner's Fluid

Formula

Concentrated hydrochloric acid	15 ml
Sodium chloride	175 g
Distilled water	to 1,000 ml

Hydrochloric acid (0.5 per cent) should be added daily until decalcification is complete.

This fluid is popular in certain parts of Great Britain as a routine decalcifying agent. It is moderately rapid in action, but the results are, in the opinion of the writer, not quite as good as those obtained with Gooding and Stewart's, or Perenyi's fluids.

Cross-section of human rib (5 mm) is decalcified in 36–72 hours.

FLUIDS CONTAINING NITRIC ACID

A disadvantage of the use of nitric acid as a decalcifying fluid is the yellow colour which develops owing to the formulation of nitrous acid. This causes alteration in the speed of decalcification which gets more rapid as the colour develops: it also causes yellow discolouration of the tissue which subsequently interferes with staining reactions. The yellow colour can be obviated by the addition of 0.1 per cent urea to pure nitric acid, which should be colourless: as the urea has only a temporary effect further additions should be made when the acid becomes tinged with yellow (Clayden, 1952).

Formol Nitric Acid

Formula

Formalin	5 ml
Nitric acid (specific gravity 1.41)	7.5–15 ml
Distilled water	to 100 ml

In this formula the formaldehyde partially inhibits the tendency to maceration by the nitric acid. In practice, aqueous nitric acid (*see below*) gives better cell preservation and staining.

Discoloration may be prevented by stabilization with urea.

Cross-section of human rib (5 mm) is decalcified in 1–2 days using 7.5 per cent nitric acid.

Phloroglucin–Nitric Acid

(1) Place 10 ml of concentrated nitric acid (specific gravity 1.41) in an evaporating dish.
(2) Add 1 g of phloroglucin.
(3) When bubbling ceases add 100 ml of 10 per cent nitric acid.

The use of phloroglucin is said to protect the tissue from maceration, and allows good subsequent staining. In the author's experience, although decalcification is extremely rapid, subsequent staining has proved to be very poor, and the method cannot be recommended.

Cross-section of human rib (5 mm) is decalcified in 12–20 hours.

Aqueous Nitric Acid

Formula

Nitric acid (stabilized with 0.1 per cent urea)	5–10 ml
Distilled water	to 100 ml

This fluid, recommended by Clayden (1952), is in use in many establishments as a routine decalcifying agent. It is rapid, causes little damage to tissue if the time of decalcification is carefully controlled, and allows most staining techniques to be applied.

Cross-section of human rib (5 mm) is decalcified in 12–24 hours.

Perenyi's Fluid

Formula

10 per cent nitric acid	40 ml
Absolute alcohol	30 ml
0.5 per cent chromic acid	30 ml

These solution are kept in stock, and mixed freshly when required. The solution acquires a violet tinge after a short while.

Perenyi's fluid is rather slow for decalcifying dense bone, but is an excellent reagent for small deposits of calcium. It has little hardening effect on tissue, and excellent cytological preparations are possible after its use. In the laboratories at Westminster Hospital Medical School it has been the decalcifying fluid of choice for some years. The chemical test for decalcification cannot be carried out with this fluid: x-rays should be used.

A cross-section of rib will be decalcified in 2–4 days, but a 5 mm cross-section of femur may require up to 10–14 days.

USE OF ION-EXCHANGE RESINS

Ion-exchange resins in decalcifying fluids are used to remove the calcium ions from the fluid, thus ensuring a more rapid rate of solubility of the calcium from the tissue, and a reduction in the time of decalcification.

The resin, an ammonium form of a sulphonated polystyrene resin, is layered on the bottom of the container to a depth of approximately ½ inch (it should not be less than 10 per cent of the bulk of the decalcifying agent), and the specimen is allowed to rest on it. The volume of the fluid by this technique need be only 20–30 times the bulk of the specimen. X-rays must be used to determine the end-point of decalcification as, for obvious reasons, the chemical test cannot be applied.

The use of these resins is limited to those decalcifying fluids not containing mineral acids: formic-acid-containing fluids give good results.

After use the resin is regenerated by washing twice with dilute (N/10) hydrochloric acid, followed by three washes in distilled water. This procedure allows the resin to be used over a very long period without renewal.

CHELATING AGENTS

Chelating agents are organic compounds which have the power of binding certain metals. Ethylene diamine tetracetic acid, disodium salt (Sequestrene or Versene) has the power of binding calcium, and its use as a decalcifying agent has been described by Hilleman and Lee (1953) and Cook and Ezra Hohn (1962).

Tissues decalcified by this method show a minimum of artefact, and may be subsequently stained by most techniques with first class results.

Technique

Following fixation in 10 per cent neutral formol saline, tissue is transferred to 50 times its bulk of 5.5 per cent Sequestrene buffered to pH 7.4 with phosphate buffer.

For pieces of tissue not more than 5 mm in thickness the fluid is changed every 4–5 days. After three such changes, the fluid may be changed daily in order to determine the end-point of decalcification more accurately. This end-point may be determined either by x-rays, or by the chemical test using oxalate.

Following complete decalcification, tissues are transferred directly to 70 per cent alcohol, dehydrated in the usual manner, and impregnated and embedded in paraffin wax, or celloidin.

ELECTROPHORETIC DECALCIFICATION

Electrophoretic decalcification, first described in 1947, is based theoretically on the attraction of the calcium ions to a negative electrode in addition to the solution of the calcium in the electrolyte. While a considerable decrease in the length of time required for decalcification may be achieved by this method, Clayden (1952) demonstrated that a similar decrease in time was possible by an increase in the temperature of the electrolyte to 40°–45°C without a current flowing. Since during electrolysis the temperature of the fluid rises to 40°–45°C, it would appear that the increase in the speed of decalcification is due primarily to the increase in temperature with possibly a small increase due to the action of electrolysis in keeping the electrolyte free of calcium. In the above experiment two comparable pieces of bone were used, the first being decalcified by the normal electrolytic method, described below, and the second by immersion in a bath of equal parts of 8 per cent hydrochloric acid and 10 per cent formic acid at 40°C. Decalcification of both specimens took alnost the same time.

By cooling the electrolyte during electrolytic decalcification, improved staining reactions are obtained, but with a consequent increase in the time of decalcification.

Although this technique is used in some establishments, it has been abandoned at most institutes in favour of other methods.

Technique

A 6 volt DC supply is required and, although in the original paper a 6 volt battery was used, a rectifier can easily be obtained which will give 6 volts, with also 8 volt and 10 volt tappings. The latter has been used by some workers to give greater speed of decalcification, or for decalcifying dense bone.

The electrolyte used is equal parts of 8 per cent hydrochloric acid and 10 per cent formic acid. This is placed in a glass museum jar or dish and, if freely movable electrodes are used, a perforated Perspex screen should be employed to prevent them touching and causing a short circuit *(Figure 4.1)*.

Flower (1952) recommends the use of a brass plate as the negative electrode, and platinum wire as the positive. In this method the positive electrode is wound around the specimen with the coils not more than 4 millimetres apart. When the wire is removed following complete decalcification if will be found to have burned the tissue with which it was in contact: this may be overcome by using close fitting electrodes of brass plate on each side of a square or rectangular flat dish, and laying the tissue to be decalcified between them without being in

contact with either. Speed of decalcification may be increased by moving the elctrodes closer together, or decreased by moving them farther apart *(Figure 4.1)*.

Decalcification should be cheched by x-rays every 2–3 hours. A 5 mm cross-section of human rib will be decalcified in 6–8 hours.

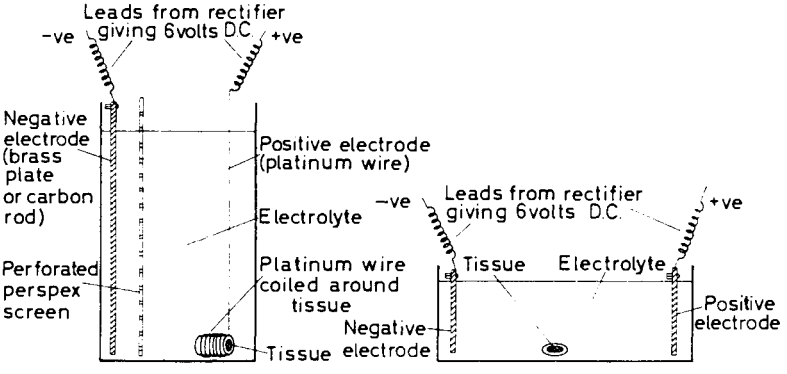


Figure 4.1 – Diagram illustrating two methods of decalcification by electrophoresis

TREATMENT OF HARD TISSUES

Perenyi's Fluid

Perenyi's fluid is recognized as a decalcifying fluid and is today quite widely used, but it was originally devised for softening chitinous material. Immersion of hard tissues in this fluid for 12–24 hours will make sectioning approciably easier, and excellent preparations of calcified arteries, thyroid, calcified glands, and so on, are possible.

Lendrum's Technique

Lendrum's technique is a very useful method treating tissues known to be hard at the time of fixation, for example, small fragments of skin. Following the washing out of the fixative, tissue is immersed in a 4 per cent aqueous solution of phenol for 1–3 days.

The treatment of wax-embedded blocks of hard tissue is dealt with on page 133.

Chapter 5

Processing

Introduction

The most commonly used method of examining tissues microscopically is by sectioning and, with the exception of frozen sections of tissue of normal consistency, this requires the embedding of the tissues in a medium which will permit thin sections to be cut easily. These embedding techniques may be classified as follows.

Paraffin Wax Embedding

The majority of routine techniques are performed on sections from paraffin wax embedded material. Paraffin wax, being of a fairly hard consistency, allows sections of only 3–6 microns in thickness to be cut routinely, at the same time giving moderate ease of handling and storage.

Water-soluble Waxes

Tissues embedded in water-soluble waxes have many of the advantages of those in paraffin wax, with the added advantage that tissue can be embedded directly from water. The use of this method, however, is restricted, owing to the fact that the floating of sections on water is made difficult by the solubility of the wax, and that the somewhat violent diffusion currents which are set up can lead to complete fragmentation of the section.

Celloidin

Tissues of particularly hard consistency, such as bone, are more easily cut if the embedding medium has a certain amount of resilience. Celloidin has a rubbery consistency and is ideal for this purpose; it has the additional advantage that heat is not needed during processing, therefore there is minimal shrinkage and distortion.

Double Embedding

Double embedding is a technique by which tissues are embedded in celloidin within a block of paraffin wax. Although tedious, this enables certain advantages of both methods to be combined: serial sections are easily prepared, and an extra degree of resilience is given when cutting hard tissues.

Gelatin Embedding

As a routine, frozen sections of fixed or unfixed tissue are cut without further processing. If sections are to be prepared of fragments of friable tissue it may be necessary to embed them in gelatin in order that they may be floated on to slides and the creases removed.

Whilst each of the above methods requires a separate technique, there are certain stages which are common to all, and these will be dealt with in detail only in the first technique in which they occur.

Labelling of Specimens

Correct labelling and identification of specimens are the first essentials of any technique of processing, since a mistake in labelling may well lead to a major operation on the wrong patient, or an incorrect diagnosis which could eventually cause the death of the patient. It is therefore impossible to overstress the amount of care that should be given to (*a*) a foolproof system of labelling, and (*b*) ensuring that the correct label remains with the appropriate specimen throughout. Ordinary ink should not be used as this may be dissolved in the reagents used during processing. Printed, graphite pencilled, typewritten, stencilled or Indian ink written labels are satisfactory. Tissue to be fixed in a solution containing osmium tetroxide should not be labelled by the foregoing methods as the label will be blackened. In such cases it is

advisable to fix a label to the outside of the jar during fixation, the orthodox method being used during processing and embedding.

Some institutions number every specimen as it is received at the laboratory by using a perforated roll of printed numbers; one of these accompanies the specimen throughout. If the name of the patient is used for identification it should always be accompanied by a number to obviate mistakes due to similarity in names. It is recommended that all histological specimens are entered into either a clinical report book, or a post-mortem specimen book which should contain the patient's Christian name and surname, age, ward, diagnosis, tissue and description; the latter is useful in the event of loss or confusion of labels. Details of clinical biopsies are entered in the appropriate book, including a record of the number of pieces taken, and each specimen is numbered. The numbers run concurrently for each year, and are followed by an oblique sign (/) and the last two figures of the year; for example, the first specimen of the year 1974 would be 1/74. This number is written with Indian ink on a small oblong of white paper, 2 × 1 cm, and is dropped into the bottle containing the specimen in fixative, and subsequently transferred along with the specimen at every stage. Post-mortem specimens may be treated in a similar manner except that the last two figures of the year are written in roman numerals, for example, the first specimen in 1974 would be labelled 1/LXXIV. This slight change in the method of labelling enables one to see at a glance to which category the specimen belongs.

Transference of Labelled Tissues

The ideal method of ensuring that labels remain with their correct specimens is probably by the use of tissue processing baskets. These are small metal containers inside which pieces of tissue, together with an appropriate reference number, may be clipped. The containers are perforated and may be transferred from reagent to reagent. They have been primarily designed for use with automatic tissue processers, but may be used in a manual system. Small porcelain pots which are perforated have also been used for this purpose (Hatcher and Hoskins, 1948).

In the absence of such tissue baskets great care must be taken to instruct junior laboratory assistants that only one pot containing tissue should be dealt with at a time, that when empty the container should be shaken to ensure the label has not been left behind, and that no

small pieces of tissue remain which, by adhering to subsequent specimens, can cause great confusion.

PARAFFIN WAX EMBEDDING

Since most of the tissue fixatives employed are in aqueous solution, the water has to be removed in order to embed the tissue in paraffin wax. This is generally achieved by immersion in increasing strengths of ethyl alcohol (ethanol), and is known as dehydration. Since alcohol and wax are not miscible, the alcohol must be replaced by a wax solvent, and since the majority of wax solvents have the effect of raising the refractive index of tissue, which makes them appear clear, this stage has become known as clearing. Finally, there is the impregnation of the tissue with wax, and its casting into a solid block.

Routing paraffin wax embedding can, therefore, be conveniently discussed under the following five headings.

(1) Fixation.
(2) Dehydration.
(3) Clearing.
(4) Impregnation.
(5) Casting or 'blocking'.

Fixation has been dealt with in the previous chapter.

Dehydration

The concentration of alcohol in the first bath depends on the fixation, size, and type of tissue to be dehydrated. After fixation in aqueous solutions delicate tissue needs to be dehydrated slowly, starting in 50 per cent ethyl alcohol, whereas most tissue specimens may be put into 70 per cent alcohol. Tissue immersed in too great a concentration of alcohol after an aqueous fixative will usually show a high degree of shrinkage, due to the too rapid removal of the water; an exception is made in the case of Heidenhain's Susa, which must be followed directly by 96 per cent alcohol. Tissue from alcoholic fixatives such as Carnoy's fluid, may be placed in higher grade alcohols or even in absolute alcohol, but it should be remembered in these instances that several changes are needed to remove acids.

The minimum duration of treatment in graded alcohols will depend on the size and type of tissue, but it can be accepted that long periods in dilute alcohol will not harm tissue; indeed, tissues may be stored in

70 per cent alcohol after fixation in intolerant fixatives such as those containing mercuric chloride. Giant sections of whole organs are best dehydrated successively in 50, 70, 96 and 100 per cent alcohol for 24–48 hours in each, dependent on their thickness, using three changes of each strength. The same series of dilutions may be used for delicate tissue, or for cytological research, with a reduction in time to 2–4 hours in each, according to their size. For routine biopsy specimens and post-mortem tissue of not more than 7 mm in thickness, 70 per cent (usually with added iodine to remove mercury pigment), 90 per cent, and absolute alcohol (2 or 3 changes, for 2–4 hours each) are sufficient to give reasonable results compatible with urgency (*see* page 106).

Agitation of the Tissue

To speed the process of dehydration the tissue may be agitated either mechanically in an automatic tissue processer (page 91) or by shaking the container periodically.

Heat has also been used to speed dehydration: the specimen is placed in a tightly-corked bottle in the embedding oven, thus producing a positive pressure and an increase in convection currents. This method is not to be recommended except in cases of urgency because of excessive hardening caused by the hot alcohol, and the danger of explosion.

The Use of Copper Sulphate in Final Alcohols

This is now a generally accepted practice in most parts of the world. A layer of anhydrous copper sulphate, ¼–½ inch deep, is placed at the bottom of a dehydrating bottle or beaker and is covered with two or three filter papers of an appropriate size to prevent staining of the tissue.

Anhydrous copper sulphate, which is white, removes water from the alcohol as it, in turn, removes it from the tissue. This action not only speeds the process of dehydration of the tissue, but also prolongs the life of the alcohol. The change of colour of the copper sulphate from white to blue indicates that the alcohol and copper sulphate should be changed. In the interests of economy this alcohol can be used with fresh anhydrous copper sulphate for the first and second absolute alcohol baths, but the alcohol of the final bath should be renewed as soon as a trace of blue shows in the copper sulphate.

Substitutes for Absolute Alcohol

Cheaper substitutes for absolute alcohol are available, such as 74 O.P.* spirit (which is about 99 per cent alcohol) and isopropyl alcohol can be used in conjunction with copper sulphate. Such a substitute for alcohol should not be used to dissolve stains or reagents until they have been tested for each use by control with pure absolute alcohol.

Dioxane (diethyl dioxide). – Its advantage is said to be that it mixes freely with water, alcohol, hydrocarbons and paraffins and should therefore eliminate some of the shrinkage and hardening normally encountered, but this is not our experience. It is toxic and areas of use should be well ventilated. It is more expensive than alcohol. Miller (1937) recommends that, following fixation, tissue is transferred to 75 per cent Dioxane for 1 hour, 2 changes of 100 per cent Dioxane for 1 and 2 hours respectively, and then into equal parts of Dioxane and wax for 2–4 hours followed by wax overnight.

Tetrahydrofuran (THF). – Daria Haust (1959) recommends THF in place of Dioxane, she used 50 per cent THF for 2 hours, 100 per cent THF for 3 changes of 2 hours each, THF and wax (equal parts) for 2 hours followed by 2 hours in wax.

A complete schedule of times for processing various types if tissue is given in page 106.

*The purchase and use of absolute ethyl alcohol is subject to restrictions for customs and excise purposes: 74° O.P. spirit (Absolute Industrial Methylated Spirit) is not subject to these restrictions.

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 S.G. 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 (O.P.) or under-proof (U.P.). Proof spirit is the standard and is referred to as 100°. A spirit stated as 70° would therefore be 30° U.P. (100°–70°). A spirit stated simply as 160° would be 60° O.P. (100° + 60°).

Ninety-five per cent alcohol is equivalent to 66° O.P., 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 O.P. (174°) would contain

$$\frac{57 \times 174}{100} \text{ per cent ethyl alcohol} = \text{approx. 99 per cent.}$$

From *An Introduction to Medical Laboratory Technology, 2nd Ed. London Butterworths.*

Technique of Dehydration

Following fixation, and having completed any after-treatment that may be necessary, the tissue is transferred to the first alcohol bath. In a manual system it is best to use glass-stoppered or screw-capped glass jars to prevent evaporation and to allow the specimens to be seen. The volume of reagent should be 50–100 times the bulk of the specimens, but if this is not possible (for example, with giant sections) several more changes should be made.

At the appropriate time the tissue, with the identifying label, is removed from the first bath with fine forceps, taking care not to damage the tissue. It is then laid on a piece of filter paper and lightly blotted to remove excess fluid before being transferred to the next reagent. Should tissue baskets be used in a manual system they are shaken lightly, and are transferred to the next reagent. This process is continued until the tissue is completely dehydrated.

Clearing

The most common clearing agents in use are chloroform, benzene, xylene, toluene, carbon tetrachloride and cedar-wood oil.

Chloroform is probably the commonest reagent in routine use by manual methods, because of its tolerance: tissues may be left in it overnight, withour rendering them unduly brittle – an advantage over benzene, xylene and toluene. It has the disadvantage of not affecting the refractive index of the tissue, and the end-point of clearing cannot easily be determined; the tissue must therefore be immersed for a rather longer period than is actually necessary to ensure complete penetration and replacement of the alcohol. With the exception of cedar-wood oil, chloroform is the most expensive of the common clearing agents.

Carbon tetrachloride, which is a toxic substance, has a similar action to chloroform and is much cheaper. Both these reagents are non-inflammable in contrast with the other members of this group, except possibly cedar-wood oil.

Xylene, benzene and toluene are fairly rapid in action, small pieces of tissue being cleared in ½–1 hour, and biopsy specimens of 5 mm in thickness in 2–4 hours. They possess the advantage that the tissue becomes clearer as the alcohol is replaced, owing to the difference in the refractive index. It is then possible to determine the end-point with some accuracy and to avoid over-exposure of the tissue to the hardening effects of these reagents. Benzene has a slightly slower action than xylene and toluene, but has less tendency to render the tissue brittle.

Cedar-wood oil is the best reagent for research and treatment of delicate tissue, since it has the least hardening effect. Tissue may be left in this clearing agent for long periods, even for months, without damage. In some institutions it is used as a clearing agent for hard tissue such as skin, and dense fibrous tissue, since sections are easier to cut after such treatment. When ordering it should be specified that the cedar-wood oil is for use as a clearing agent.

Even very small pieces of tissue need to be left overnight in cedar-wood oil; specimens from 5 to 7 mm in thickness will need 2–5 days.

Cedar-wood oil may be poured into a small specimen jar, and a similar quantity of absolute alcohol superimposed on it, avoiding any mixing at the junction of the two fluids: the specimen is then placed gently into the alcohol when it will float at the interface of the two fluids. As clearing takes place the specimen slowly sinks into the cedar-wood oil; the alcohol is then removed by pipette or syphon, the specimen transferred to fresh cedar-wood oil for a few hours, and finally transferred to paraffin wax.

Following clearing in this reagent, several extra changes of paraffin wax will be required to remove the oil. This technique is rarely, if ever, used today.

Technique of Clearing

The technique of clearing is the same as that used in dehydration, tissues being lightly blotted during transfer from one reagent to the next. The volume of clearing agent should be 50–100 times that of the tissue.

Tissues being cleared in chloroform or carbon tetrachloride are best left overnight, and those in xylene, benzene or toluene should be given one change after 30–60 minutes, and transferred to wax when they are seen to be clear (translucent). The special technique for cedar-wood oil has been dealt with above.

Impregnation with Wax

Ovens or Incubators

Impregnation with paraffin wax takes place in an oven heated to 54–60°C, the temperature depending on the melting point of the wax in use.

The oven may be electrically heated or gas heated, either water-jacketed or not. Care must be exercised when using the gas heated variety: a major explosion in a laboratory many years ago led to a standing rule that not more than one gas heated piece of apparatus

should be employed in any one department. The explosion occurred when the by-pass of one incubator was blown out by an unusual draught. As the temperature in the oven fell the flow of gas increased until the room, which was moderately air-tight for tissue culture, was full of gas. The gas was then ignited by the by-pass of another incubator with a consequent explosion which completely wrecked the laboratory. While the above rule will prevent an exact repetition of this type of accident, the author's personal preference is now for electrically heated apparatus.

The ovens used for wax embedding should have a temperature range of 50–60°C. They should be sufficiently large to accommodate an enamel jug (with a funnel inserted for filtering) for the storage of paraffin wax, one or two Coplin jars (for staining techniques in which heat is required), and a sufficient number of containers for wax impregnation of tissues *(Figure 5.1)*. In a routine laboratory, ovens will be normally controlled at a temperature of 58°C. An additional oven, regulated at 37°C, is useful for drying sections on slides, and for certain other staining techniques, but in a small laboratory one oven can be adapted to most uses.

Paraffin Wax

Paraffin wax, bought from a reliable source, should be free from dust, grit, and other foreign matter. It should not contain water, which causes it to crystallize and turn white. All wax should be filtered routinely before use by keeping it in an enamel jug in the embedding oven, into which is put a funnel with an ordinary filter paper. New blocks of wax are broken up and put into the funnel, being filtered as they melt. This ensures a readily available stock of filtered wax. Should new wax crystallize because of its water content, it may be heated and stirred to remove the water, but this method is not always satisfactory and it is generally advisable to return it to the suppliers.

The melting point of wax to be used will depend on personal preference, the type of tissue, and the general temperature of the laboratory; the most popular melting point for routine work is 54°C which gives support for hard tissues and yet allows easy ribboning of sections.

While the use of hard wax (melting point 60°C) for hard fibrous tissue, and soft wax (melting point 45°C) for soft tissue such as fetal and areolar tissue is recommended, this will be possible only for research workers. A routine laboratory system which segregates tissue by its type and consistency, keeping a range of wax ovens and waxes for the various specimens, would be impracticable and uneconomical.

Figure 5.1 – Embedding bath and oven. The top surface is chromium plated and recessed to contain 4½ inch diameter chromium plated pot with spout for stock paraffin, 2½ inch diameter chromium plated embedding pots, and 3 X 1 inch glass tubes. A drying slot with sliding tray is provided for microscopic slides (by courtesy of Messrs. Baird and Tatlock Ltd.)

In view of the general use of ice when cutting sections, it is possible to use the same wax in winter or summer, regardless of the atmospheric temperature. For three years in India the author consistently used wax of 54–55°C melting point with good results, by using ice when cutting.

To harden the lower melting point waxes 10–20 per cent of beeswax may be added. This permits the easier cutting of sections

associated with higher melting point waxes without the tissue being exposed to the higher temperature during impregnation.

*Paraplast**

This material is a mixture of highly purified paraffin and several plastic polymers. It possesses a greater degree of elasticity than normal paraffin wax giving results superior even to double embedding, allowing sections of bone to be cut with ease. It ribbons well allowing almost 'wrinkle' free serial sections to be cut with ease at 4μ thick. It does not require cooling before cutting, therefore ice cubes are eliminated. No changes in routine processing technique are required since the usual clearing agents can be used and it has a melting point of 56°C. This material represents a major technical advancement and is recommended for routine and research work on both human and animal tissues. Paraplast and Paraplast Plus (*see below*) were recently tested together with various other available embedding materials as part of an investigation into methods and times of processing (*see* page 96).

Paraplast Plus

This embeddant contains dimethyl sulfoxide (DMSO) which, since it gives more rapid penetration (approximately two-thirds of that with normal Paraplast) reduces the time of tissue processing.

Technique of Impregnation

After blotting lightly with filter paper, the tissue is transferred from the clearing agent to molten paraffin wax.

If the vacuum embedding oven is not being used, the wax – in open glass containers, or copper pots – is kept in the embedding oven which has been regulated to maintain a temperature of 56°C (using wax with a 54°C melting point). The volume of wax should be about 25–30 times the volume of the tissue, and must be changed at least once during impregnation. This change is effected by simply lifting the tissue from one pot of wax to the next with warmed forceps. A useful routine is to give three changes. The first wax should be discarded by tipping it in the used wax bowl. Refill the container with fresh wax and use it for the last change, the previous first change becoming the first wax and so on.

*Obtainable from laboratory suppliers, or Sherwood Medical Industries Inc., 1831 Olive Street, St Louis, Missouri, U.S.A. 63103.

Time of Impregnation

The length of time and the nimber of changes required for thorough impregnation of the tissue with wax will largely depend on the following three factors.

(1) The size and type of the tissue.
(2) The clearing agent employed.
(3) The use of a vacuum embedding oven.

The size and type of the tissue. – The size of the specimen will, as throughout the whole process, influence the time required for complete impregnation. The thicker the tissue the longer will be the time required for wax to penetrate to the centre; moreover, thick tissue will carry over more of the clearing agent, and more changes of wax will be necessary to remove it. Even small amounts of clearing agent contaminating the wax will cause crystallization, and crumbling of the sections during cutting.

The type of tissue should also be taken into account since dense tissue, such as bone, skin, and central nervous system, will require nearly twice as long as soft tissue such as liver and kidney. Tissue containing a high proportion of blood, muscle, and fibrous strands has a tendency to over-harden and become brittle in the wax bath, and consequently the time of impregnation in such cases must be kept to a minimum. This reduction in time of impregnation can be achieved quite satisfactorily by using a vacuum embedding oven, except in the case of tissue from the central nervous system. The cutting of such sections is made easier if the tissue has been left in paraffin wax overnight in the ordinary oven, with two or three changes; sections from tissue so impregnated do not curl and crack in the drying oven.

The clearing agent employed. – Some clearing agents are more easily replaced and eradicated than others. Xylene, toluene, and benzene are the easiest to remove, and one change of wax will normally be sufficient. Chloroform and carbon tetrachloride are almost as easy but two changes of wax will be found a safe routine. Cedar-wood oil, as has been mentioned, will require several changes dependent on the size of the tissue: small pieces should be given at least three changes, and larger pieces correspondingly more.

When impregnating delicate tissue, such as embryonic tissue, it is recommended that it be transferred from the clearing agent to a mixture of clearing agent and paraffin wax. This mixture should be a nearly saturated solution of paraffin wax in the clearing agent at room temperature. This method is used as a routine in some institutions but,

with the possible exception given above, it would seem to be an unnecessary and time-wasting procedure.

The use of a vacuum embedding oven. – Using the normal paraffin oven, tissues are as a routine given two changes of paraffin wax (melting point 52–54°C) over a period of 4 hours; by the use of a vacuum embedding oven this time may be halved.

Vacuum embedding ovens

The apparatus consists of an air-tight embedding oven attached to an exhaust pump, the degree of vacuum achieved being controlled by an attached mercury manometer or vacuum gauge. By reducing the pressure during wax impregnation air bubbles and clearing agent are more speedily removed from the tissue, resulting in more rapid impregnation.

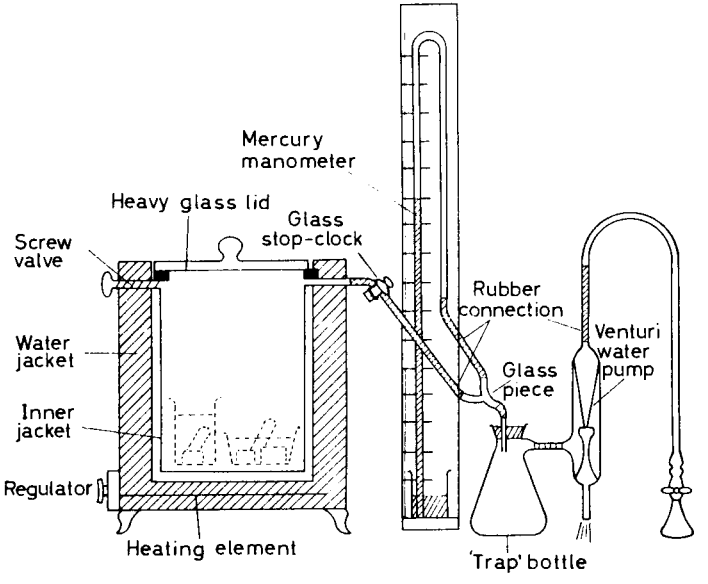


Figure 5.2 – Diagram illustrating the vacuum embedding apparatus (from An Introduction to Medical Laboratory Technology, 2nd Ed. London; Butterworths.)

One type of apparatus in common use is shown in *(Figure 5.2)* It consists of an outer bath, normally controlled at 56°C (or 2°C above the melting point of the wax being used), into which the vacuum

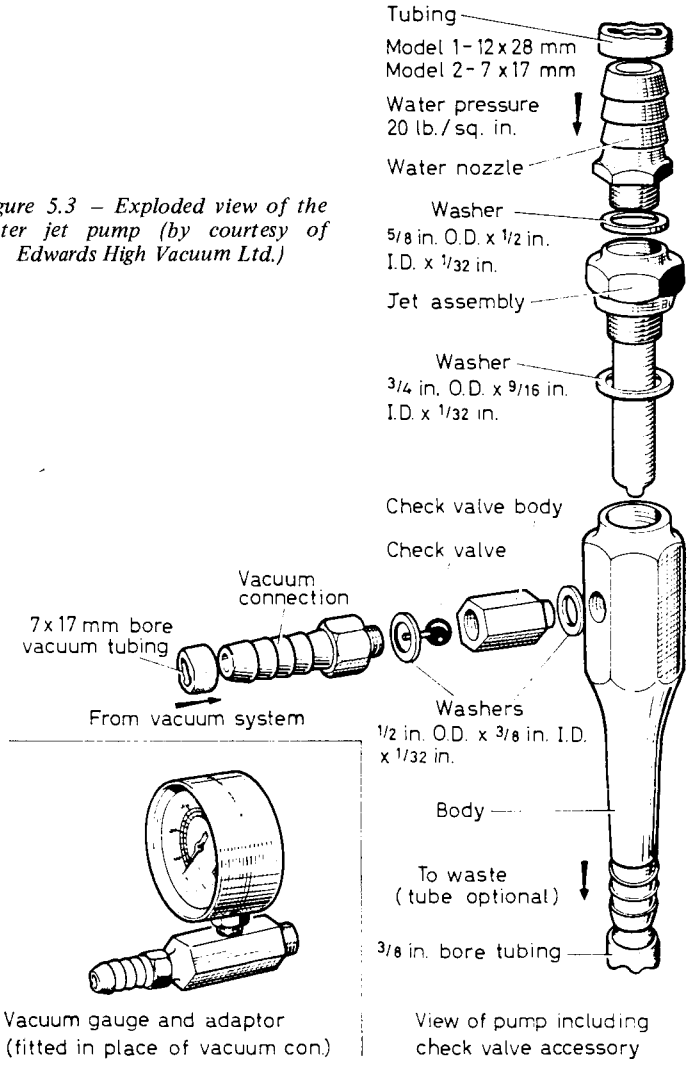


Figure 5.3 – Exploded view of the water jet pump (by courtesy of Edwards High Vacuum Ltd.)

chamber fits. The vacuum chamber is a circular, flat-bottomed brass vessel, which has a thick plate-glass lid resting on a thick rubber chamber, one being connected to the exhaust pump, the other being used to admit air to restore atmospheric pressure at the end of impregnation. To avoid contamination of the wax in the bath by the water or

oil used in the vacuum pump, the pump is connected to a thick-walled vessel (flask or bottle) which acts as a trap; this in turn is connected by a 'T' or 'Y' piece to the vacuum chamber and to a mercury manometer *(Figure 5.2)*. A type of water vacuum pump is available from Edwards High Vacuum Ltd. *(Figure 5.3)* fitted with a non-return valve and a reliable gauge, which obviates the necessity of a manometer. A glass stop-cock, or metal screw-clip must be inserted near the valve–if the valve itself is not fitted with one–to control the pressure within the chamber *(Figure 5.2)*.

Technique

(1) The paraffin wax is put into the bottom of the vacuum chamber, or into separate containers within the chamber. The most economical use of space is made by the employment of special containers about 2 inches deep and about the diameter of the chamber, which are divided into eight compartments by walls of perforated metal, and may be stacked one on top of the other in the vacuum chamber.

(2) Tissue is removed from the clearing agent, laid on blotting paper for a few seconds and lightly blotted to remove excess clearing agent, and then placed in the molten wax. If the specimen has been in an automatic tissue processer it may be transferred to the vacuum embedding oven from the second wax bath, where it should remain for 30 minutes as a final change.

(3) The air valve is closed, and the vacuum pump started to reduce the pressure slowly to minus 400–500 mm/Hg.

(4) The stop-cock is closed (or the screw-clip between the vacuum pump and chamber tightened) and the vacuum pump stopped.

(5) When the tissue has been impregnated for a sufficient length of time the air valve is opened very slowly to admit air and restore the atmospheric pressure in the chamber.

(6) The glass lid is removed and the tissue either embedded or, if a change of wax is required, the whole process is repeated.

The stages of expulsion, and readmission of air to the vacuum chamber should never be hurried as this will lead to artefacts in the more delicate tissues; for example, rupture of the alveoli of the lung.

Casting or 'Blocking'

Tissue is blocked by transferring it from the final wax bath to a mould filled with molten wax, inverting the tissue to free the surface to

be cut from air bubbles and orientated so that this surface rests on the base of the mould. The block is then quickly cooled.

Type of Mould

Leuckhart's L pieces are simple in construction and use. They consist of two L-shaped pieces of metal, usually brass, which are laid on a metal or glass plate to form an oblong. By adjusting the L pieces the shape and size can be modified according to the tissue. Various sizes of L pieces are available, enabling almost any piece of tissue to be embedded by this method. When the wax has set, the L pieces are easily removed, and are ready for the next block, with a minimum of cleaning required.

Glass Petri Dishes are convenient embedding moulds, if they have been previously smeared with glycerin. They may be used to embed several pieces of tissue at one time, by placing tissues near the periphery and inserting the accompanying label in the wax at the edge of the dish. When the wax has set, it is turned out of the dish; if any difficulty is encountered gentle heat may be applied, but the use of glycerin will usually obviate this. The large block of wax is then cut allowing a margin of about ¼ inch all round each piece of tissue.

Metal Petri Dishes are available commercially, usually being made from aluminium. They are very strong and if treated with moderate care have a very long life. The final block of wax is easily removed due to its contraction during setting. The method of labelling used is as described above. The size of dish used will depend on the number of specimens received daily; those about 6 inches in diameter and about ½ inch deep will be found most useful.

Paper 'boats' make cheap and convenient moulds, in spite of the labour and the time involved in making them. They have the advantage that tissues so blocked may be stored, still in their paper boats, with the identifying name and number written on the projecting tag. One or more pieces of tissue may be blocked in each boat.

The method of making the paper boats is very simple; ordinary paper may be used but they are best prepared from glossy paper—old catalogues are suitable.

The paper is cut to allow 1 inch for each side of the block and 2 inches for each end; that is to say, if the finished mould is to measure 1 X 1½ inches at the base, then a piece of paper is cut measuring 3 X 5½ inches. It is then creased to show the size of the base of the mould by folding longways into three, and folding the resultant 1 inch strip 2 inches inwards from each end. The paper is then unfolded, the four sides are brought together and the four

triangular-shaped pieces of excess paper at each corner are folded back over each end and held in position by folding down the ends to the level of the sides

Watchglasses, which have been smeared with glycerin may be used as moulds for small pieces of tissue.

Test Tubes may be used for small fragments which have been processed in them throughout, for example, bone marrow, or fragments will collect at the tip of the tube, which concentrates them without the damage caused by orientation with forceps, but it has the disadvantage that it is often necessary to break the tube to remove the block (*see* Chapter 19).

Plastic Embedding Rings (Tissue-Tek)*

There are available plastic embedding rings, which together with a stainless steel mould, allow much more rapid embedding and cutting of tissue; they are simple to use. The tissue is placed in an appropriately sized base mould, and a plastic ring is laid on top and the wax poured in up to the top of the plastic ring. The wax is then cooled rapidly in running water, or the refrigerator. The base mould, which has been previously sprayed or dipped into a mould release solution, is then easily detached. This leaves the block of wax ready for cutting without trimming. The wax filled plastic ring functions as the block holder, fitting directly into the chuck of the microtome. After cutting the sections, the blocks are stored complete with the plastic ring. This saves deblocking and makes the recutting of tissues simple and quick. In two 5-day time trials at the Vancouver General Hospital, using the old technique (paper boats and fibre blocks), 763 specimens required 25 hours 15 minutes (average 3.9 minutes per specimen) to embed, block, cut and deblock for filing. Using the plastic embedding rings 910 specimens required 14 hours (average 1.8 minutes per specimen) to embed and cut; they are then ready for filing. There are two disadvantages to this technique: (1) the space required for filing is approximately double, and (2) the cost of the embedding rings. In our trial it was found that the saving in technicians' time compensated for the cost, but the space requirement may be a more difficult problem to solve.

Tissue-Tek II Embedding Rings

These are a modification of the original model (above). The Mark II have the advantage of providing a cassette to hold tissues during

*Fisher Scientific Supplies.

processing since a stainless steel cover clips on to the plastic cassette for this part of the process *(Figure 5.4)*. The cassette, which has a roughened surface on which to write identifying material (in pencil), forms the base for the embedded block; this information has therefore

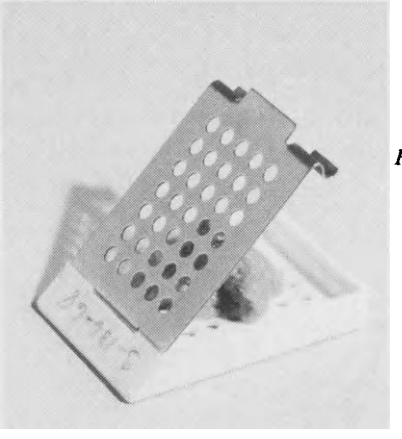


Figure 5.4 – Tissue-Tek II cassette and cover

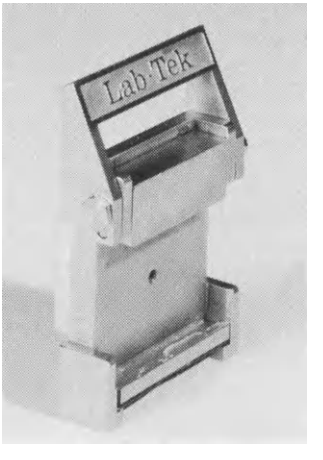


Figure 5.5 – Tissue-Tek II microtome chuck adapter

only to be written once. A special clamp has to be used on the microtome which makes the clamping of blocks a very simple and rapid operation since the lever has only to be lifted (to clamp), or lowered to free the block *(Figure 5.5)*. The metal base molds still form an integral part of the system.

The original Model II cassette was made of a plastic which softened in some clearing agents (and occasionally released the tissues) but this has now been overcome by using a different material. One of the few

disadvantages to this system is the relatively shallow cassette; however, provided thin pieces of tissue are taken (as they normally should be) this is of little importance.

There are also racks available which carry 24 cassettes on the automatic tissue processing machines.

Technique of Casting

(1) Fresh molten wax is poured from the stock jug or container into the mould. The wax touching the mould will quickly form a thin solid layer.

(2) With forceps previously warmed to prevent the wax setting on them, the tissue should be lifted from the final wax, and placed in the bottom of the mould. Gently press the surface of the tissue to be sectioned against the solid layer which will hold it in position; ensure that no air bubbles are trapped.

If there is likely to be difficulty in recognizing the surface to be cut, it is an advantage to thread a piece of horsehair through the opposite surface; the tissue is then embedded with the horsehair uppermost, the hair then being withdrawn. If several pieces are to be embedded this stage must be carried out rapidly, otherwise the wax in the mould will solidify. The skin which forms on the surface may be broken with the warmed forceps. With practice this operation presents no difficulty.

(3) Fix the label in position by pressing one edge against the solidifying wax, at one corner if a single mould, or about ¾ inch from the specimen if there are several specimens. In the latter case, care must be taken to ensure that there is no possibility of confusing the labels and specimens.

(4) When the block has cooled sufficiently to form a skin on the surface it should be immersed in cold water to cool it rapidly; failure to do this will often cause crystallization of the wax. A large flat enamel tray containing about ½ inch of water is convenient for this purpose and may be kept permanently on the embedding bench.

(5) The blocking wax, having set quite hard, can be removed from mould or paper boat, care being taken to ensure that it has set properly throughout. It may then be trimmed ready for cutting, or stored.

After cutting such blocks should have their labels attached by pressing them on the side with a hot searing iron (or old scalpel handle) so as to embed them slightly in the block when they may be stored.

Automatic Tissue Processers

The overall length of time required to fix, dehydrate, clear, and impregnate tissues with wax during a series of normal working days has

long been the obstacle to more rapid diagnosis. Tissues which are laid at the bottom of a container may require up to 4–5 hours in each reagent. It follows, therefore, that only two such changes may be made during a normal working day; furthermore, schedules must be arranged so that tissues are not left overnight in fluids which will unduly harden them.

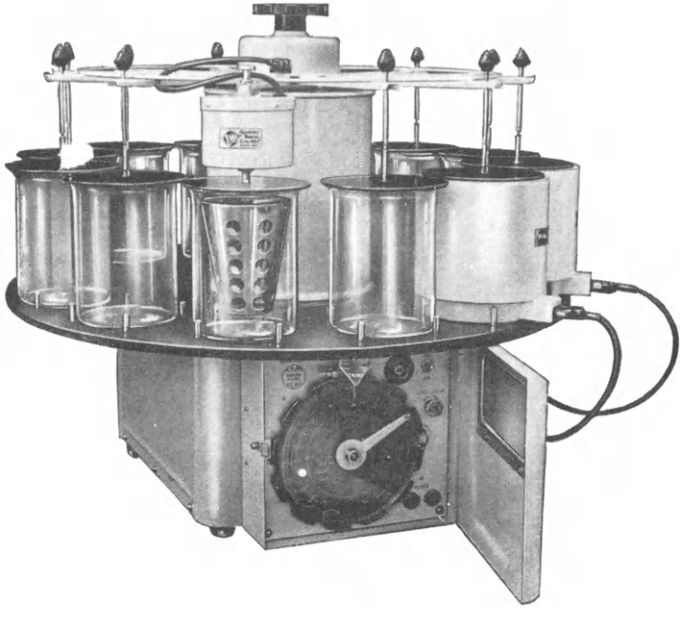


Figure 5.6 – The Histokinette bench model automatic tissue processer (by courtesy of Hendrey Relays Ltd.)

Automatic tissue processers have two advantages. In addition to transferring the tissues mechanically from reagent to reagent both by day and by night, they also reduce the time required in each fluid by the action of continual agitation. They are equipped with a 1-hour clock in addition to a 24-hour clock, and so can be used for rapid processing of tissues, or, by using a special rack to carry 25 slides, for staining sections; a 7-day clock is now available for longer processes.

This method eliminates the possibility of human error and forgetfulness, and may be trusted to the most junior member of the staff. As

with all complex automatic machines the possibility of faults must be recognized but it is felt that the advantages of such a machine far outweigh the disadvantages.

Figure 5.6 illustrates one such machine. They are available either for use on a bench, or with a pedestal.

While the detailed operation of each make of machine will vary slightly, they are, in the main, very similar, and consequently only one such technique will be described.

Tissue Containers

It is usual for 24 containers to be supplied with a machine, and each of these may be sub-divided into two compartments by means of a special clip fitting. If the pieces of tissue are small three may be placed in each container, using suitable partitions. Special containers may be obtained for very small fragments of tissue, such as curettings.

The tissue to be processed is placed in an appropriate container, together with a label, and the lid snapped on. These containers are placed in the tissue basket in which they remain throughout the whole process.

Beakers and Wax Baths

Most machines are equipped with ten beakers and two wax baths thermostatically controlled to 56°C ± 3°C. The beakers are filled with appropriate fluids (*see* processing chart at end of section) and wax (melting point 56°C or less) is placed in the wax baths after ensuring that the main switch is on in order to keep the wax in a molten state.

All twelve positions on the beaker platform are positively located by four small chromium pegs to ensure alignment with the arms of the operating assembly.

Stirring Mechanism

One of the arms supports the stirring mechanism, where there are 3 pegs on which the tissue basket is clipped. The basket will then rotate at about 1 revolution per minute. The remaining arms support covers to protect the remainder of the beakers from dust, and undue evaporation. The whole lifting assembly may be raised manually, and be locked in the upper position by being turned slightly in a clockwise direction to a central position between any two beakers, allowing the tissue basket to be fitted, or beakers to be set up.

Timing Mechanism

The timing mechanism consists of a 1-hour, 24-hour, or 7-day electric clock which has a time disc instead of hands. The disc most commonly employed revolves once in 24 hours and in so doing the notches which are cut in it come under a cam switch which energizes the lifting, turning and lowering mechanism. The timing notches are marked and cut on a special template supplied with the machine, remembering that the disc rotates in a clockwise direction. If a first change is required after 2 hours the notch will be cut in the position of 10 o'clock, and not 2 o'clock, assuming the time disc to be an ordinary clockface. It must be remembered that two types of discs are supplied for use with cycles of 1 hour and 24 hours and it is important to use the correct disc, and the correctly shaped notch for each.

The first notch should be cut at the correct distance back from 'Start'. and the second notch placed the appropriate distance back from the first notch cut. Continue until a notch has been cut for each reagent except the last; for example 11 notches when using the full range of 10 beakers and 2 wax baths. It is customary to allow the tissue to remain in the last wax until ready for embedding, or transfer to a vacuum embedding oven. A twelfth notch may be marked with pencil and used when setting the stopping mechanism.

The timing disc is fitted to the control panel with the stop indicator set in the appropriate position. The tissue basket is put manually into the first reagent, and the cycle of operations commenced by switching on the mechanism.

When it is desired to leave the machine unattended for periods longer than 24 hours, as at weekends, it can be set for continuous running. This means that after 24 hours the clock will have completed a cycle and certain or all of the notches will be used a second time; this must be remembered when cutting them. In the author's laboratory specimens are allowed to process in each reagent for 4 hours (*see* page 106). Alternatively, the machine may be set for a delayed start of a predetermined period, after which the tissue is given the routine 24-hour processing.

Normally the machine has only one basket rotor, which plugs into the main head, but it is possible to fit a second one and use them simultaneously with fewer stations, thus doubling the output.

RAPID PARAFFIN PROCESSING OF TISSUES

In an attempt to shorten the time of tissue processing in routine surgical pathology departments, we investigated the minimum time

required in each of the following four stages of paraffin processing when using an automatic tissue processor:

(1) Fixation (formol calcium, SUSA and Carnoy's fluid, *see* Chapter 3).
(2) Dehydration (through graded and absolute ethanol)
(3) Clearing (removal of dehydrating agent and replacement with a clearing agent that is miscible with wax).
(4) Impregnation (with paraffin wax or substitute waxes).

Table 5.1 illustrates a time schedule known to be more than adequate for routine tissue processing. The time for each stage was sequentially reduced within an otherwise unaltered timetable, for example, the time of each of the alcohols was reduced to 60, 30, 15 and 5 minutes with the rest of the timetable unchanged. It was hoped that this would allow us to determine the minimum time required for each stage.

Tissues

Liver, kidney, skin and spleen from human surgical or autopsy specimens and laboratory rats were used for the experiments. Each tissue was trimmed to approximately 20 X 10 X3 mm in thickness, the last measurement being the most important. The eventual time chosen was the one that gave good sections of each tissue.

Fixation

Tissues were fixed in formol calcium for 24, 12, 6, 3 and 0 hours; SUSA fixative for 12, 6, 3, 2, 1, 0 hours; or Carnoy's fluid for 12, 6, 3, 2, 1, 0 hours.

For the initial processing experiments, tissues fixed overnight in formol calcium were used. When minimum times were established the fixation experiments were carried out using both the long and short schedules.

Dehydrants

Ethanol was used throughout for dehydration. Anhydrous copper sulphate was present in the 100 per cent ethanol baths II and III.

Clearing Agents

Chloroform was used throughout for clearing; it was realized that the use of xylene or benzene might result in shorter times but they

TABLE 5.1

A Rapid Processing Schedule for Tissues on Automatic Tissue Processors
(Tissues approximately 3 mm in thickness)

Stage	*Long routine time*	*Shortest time (determined by experiment)*
Dehydration		
70% Ethanol	1 hour	15 minutes
90% Ethanol I	1 hour	20 minutes
90% Ethanol II	2 hours	20 minutes
100% Ethanol I	1 hour	20 minutes
100% Ethanol II	2 hours	20 minutes
100% Ethanol III	2 hours	25 minutes
Totals	9 hours	2 hours
Clearing		
Chloroform I	3 hours	30 minutes
Chloroform II	4 hours	30 minutes
Chloroform III	4 hours	30 minutes
Totals	11 hours	1½ hours
Wax impregnation		
Paraffin wax I	1 hour	40 minutes
Paraffin wax II	1 hour	40 minutes
Paraffin wax III	2 hours	40 minutes
Totals	4 hours	2 hours
Or		
Paraplast I	1 hour	25 minutes
Paraplast II	1 hour	25 minutes
Paraplast III	2 hours	25 minutes
Totals	4 hours	1¼ hours
Grand Totals	24 hours	5½ or 4¾ hours

were not used because of the possibility of over-hardening the tissue and also because of the fire hazard, due to their low flashpoints.

Embedding Agents

A variety of commercially available waxes were tested within the time experiment. They were also evaluated for their ability (containing tissue) to section and/or ribbon at 1, 2, 3 and 5 micron thicknesses, and to produce flat, wrinkle-free sections. Tissues were embedded in Tissue-tek embedding rings, cooled rapidly and placed in a refrigerator to set hard.

Evaluation of Sections

Sections were stained by haematoxylin and eosin (1), P.A.S./Alcian blue technique (1) and Weigert and Van Giesen (1). Sections were examined for: (1) cracking and loss of staining in central areas (particularly in spleen and liver); (2) spaces between cells and basement membranes (particularly in kidney); and (3) spaces in connective tissue and epidermis in skin.

Results

Table 5.1 shows the minimum times that were determined by the method described above. The minimum times for each stage were actually slightly less than those in the Table because it was found that, for an unknown reason, the absolute minimum times for each stage did not produce satisfactory sections (or blocks) when incorporated into a total schedule. Those times given in the Table represent the minimums when incorporated into a total schedule for an automatic tissue processing machine (with its accompanying agitation of the tissue throughout).

Fixation

To our complete surprise, *completely unfixed tissue gave excellent sections when processed by the long schedule (24 hours),* and even on an overnight (18 hour) schedule. It then became obvious that unfixed tissues or inadequately fixed tissues entering such a processing schedule are fixed in the ethanol during dehydration. *This may have some bearing on histochemical results where tissues labelled formalin-fixed are in fact alcohol-fixed.* When using the short processing schedule the shortest fixation times that could be used are those shown in *Table 5.2.*

TABLE 5.2
Fixation Times for Routine Processing
(Tissue 3 mm in thickness)

Fixative	*Routine times*	*Short time (determined by experiment)*
Formol calcium	12 hours	5 hours
SUSA fixative	6 hours	3 hours
Carnoy's fluid	6 hours	2 hours

Wax Impregnation

As will be seen in *Table 5.1,* Paraplast gave appreciably faster impregnation than the other waxes tested. It was the only wax that cut consistently at one micron thickness, with good ribbons at a thickness of 2 microns.

Effect of Vacuum and Heat

The effect of processing under vacuum with and without heat were studied. It was not felt that processing *in vacuo* produced a shorter time. The application of heat did increase the speed of processing, but above 40°C there were indications of tissue damage.

A short processing schedule is described above which will allow fixed tissue to be processed in 4¾ hours.

ESTER WAX EMBEDDING

Embedding in ester wax, described by Steedman (1947), is said to combine certain advantages of both paraffin and celloidin. Ester wax is much harder than paraffin wax, although it has a lower melting point (46°– 48°C), it is similar to celloidin in that it can be compressed, and is therefore less likely to crumble when one is cutting hard tissue. Cutting is very similar to that of tissue in paraffin wax: thin sections (1–2μ) are more easily cut and ribbons present difficulty. The author considers this inferior to Paraplast (*see* page 83).

Technique

Tissue should not be transferred directly from the clearing agent to ester wax, but into a mixture of clearing agent and wax where it is left for 3–6 hours according to size.

At least 3 changes of wax should be used during impregnation, and 4 or even 5 for large pieces of tissue. Tissue should be left in each change for 3–6 hours.

For embedding, the wax is best heated to 68°–70°C, and poured into a mould as for paraffin wax embedding. The tissue is put in and orientated with warm forceps to ensure cutting in the correct plane. The block should be rapidly cooled in water, but since contraction occurs, and it may be necessary to fill the central depression with more wax to avoid exposure of the tissue, it is not advisable to submerge the block.

When completely solid, the block is freed from the mould and trimmed. Because of the hardness of the wax, sections from these blocks need to be cut slowly, with a very sharp knife. The microtome used should be of a heavy type, preferably one of the sledge variety, and should be rigidly secured. Failure to fulfil these conditions will result in sections of uneven thickness.

Sections should be flattened in the usual way, by floating on warm water at a temperature of 35°–40°C; they are then picked up on clean plain or albuminized slides, and dried in an oven at 37°C. *They must not be dried at room temperature* or sections will crumble. Following de-waxing, sections are stained as are the paraffin sections. Owing to the water tolerance of this wax, sections may be stained with the wax still present by floating on staining reagents, with a slight increase in staining times. The sections are then fixed to slides, de-waxed and mounted.

WATER-SOLUBLE WAXES

Water-soluble waxes are solid polyethylene glycols having the great advantage of not requiring the tissues to be dehydrated and cleared before infiltration with them. They have the additional advantage, probably due to the avoidance of dehydrating and clearing reagents, that the degree of shrinkage of tissue is considerably less than that caused by the paraffin wax technique *(see below)* Several types of water-soluble wax are available commercially.

These waxes have been used with success for the demonstration of lipids (Hack, 1951), and to preserve enzymatic activity in plants (McLane, 1951) after freeze-drying or special fixation.

Miles and Linder (1952) compared the tissue shrinkage caused by a water-soluble wax (Nonex 63B) and paraffin. They found the shrinkage was 11 per cent using paraffin wax, and only 4.7 per cent using water-soluble wax. The technique they employed, which they now use routinely for hard tissues, is given below with slight modifications.

Technique

(1) Wash tissue well to remove all traces of fixative. If a large amount of fat is present remove it by treatment with acetone.

(2) Transfer the tissue to 50 per cent polyethylene glycol 900 in distilled water, and leave until it sinks (about 10–15 minutes).

(3) Transfer to four successive changes of molten polyethylene glycol 900 at 28–30°C, allowing 45 minutes in each bath.

(4) Transfer to a mixture of equal parts of polyethylene glycol 900 and Nonex 63B at 39°C for 30–40 minutes.

(5) Transfer to a mixture of three parts of Nonex 63B and one part of polyethylene glycol 900 and leave for 15 minutes. (In stages 4 and 5 a layer of glass wool on the bottom of the containers will keep the tissue off the bottom where the polyethylene glycol 900 tends to accumulate.)

(6) Transfer the tissue to the first of three changes of Nonex 63B at 39°C, and leave it in each change for 30–45 minutes. At the end of the third change it is ready for embedding.

(7) Embed in a paper boat as for paraffin wax embedding (*see* page 88), or, as Miles and Linder suggest, in a paraffin wax boat made by pouring molten paraffin wax into L pieces adjusted to a convenient size, allowing the outside layer of wax to set, and then pouring out the molten centre. This leaves a thin wax embedding mould which, when the Nonex 63B has set, can easily be broken away.

Blocks should be stored temporarily in a desiccator, and coated with paraffin wax for permanent storage.

Cutting

Sections are cut in the usual manner. On microtomes with a tilt adjustment this should be greater than is used for cutting paraffin sections.

Fixing to Slides

The floating of these sections is probably the most difficult part of the technique, since if they are floated on water violent diffusion currents are set up which cause the sections to fragment. These currents may be minimized by the addition of a trace of soap or a few drops of Teepol to the water: alternatively 10–20 per cent of polyethylene glycol 900 may be added to the water. The wax-free sections thus

flattened may be floated on slides in the usual manner, and stained; or they may be treated in the manner described for frozen sections (*see* page 197).

GELATIN EMBEDDING

Gelatin embedding is used when frozen sections of friable or partially necrotic tissue, or numbers of small fragments such as uterine curettings, are required.

Following embedding, the block may be immersed in 10 per cent formalin to convert the gelatin to an irreversible gel.

The usual method, employing formalin to harden the gelatin, is as follows.

Technique

(1) Tissue is fixed in formol saline, and then washed in running water for 6–12 hours to remove the formalin.

(2) Tissue is transferred to 10 per cent gelatin in 1 per cent phenol (to prevent the growth of moulds) for 24 hours at 37°C.

(3) Tissue is transferred to 20 per cent phenol–gelatin for 12 hours at 37°C.

(4) Tissue should now be embedded in 20 per cent gelatin, using a mould as for paraffin wax embedding (page 87).

(5) Having been allowed to set, preferably in the cold room, excess gelatin should be trimmed, leaving a margin of approximately 3 mm around the specimen, and as little as possible on the surface to place on the freezing microtome stage.

A minimum amount of gelatin remaining, consistent with adequate support of the specimen, is essential as the gelatin tends to inhibit freezing. For the same reason, pieces of tissue for inclusion by this technique should not exceed 2–3 mm.

(6) The trimmed block is then immersed in 10 per cent formalin for 12–24 hours to harden.

(7) Frozen sections can now be cut in the usual way; it is preferable to cut them thin to avoid undue background staining caused by the gelatin.

The disadvantage of this method is that when staining techniques are applied the gelatin tends to hold the stain, giving an indifferent background to the section. Some workers recommend avoiding stage 6 *(above)*, and after fixing sections to the slide remove the gelatin with warm water. The difficulty with the latter technique lies in floating out and fixing the section to the slide, but this may be

overcome to some degree by floating out on very cold water, and transferring immediately to albuminized slides. The slides are then drained on the bench, gently heated two or three times (except when demonstrating lipids) to coagulate the albumin, and placed in warm water to remove the gelatin.

CELLOIDIN EMBEDDING

Celloidin is a purified form of collodion, or nitro-cellulose, and is used as an embedding medium for tissue requiring special treatment, particularly exceptionally hard tissues. As an embedding mass it has the advantage of not requiring heat at any stage of processing, and it has a rubbery consistency which gives support to hard tissues in circumstances where paraffin wax would crumble. Because of this consistency it is possible to cut sections of mixed hard and soft tissue of even thickness, while preserving the relationship of cell layers. An example of such tissue is the eye, from which it is difficult to prepare even sections due to the extreme hardness of the sclera. Sections other than those prepared from celloidin usually show displacement of the retina.

Celloidin embedding was once the favoured method for cutting sections of large pieces of tissue, for example, whole kidneys or breasts, but it has now been shown that paraffin wax sections of such blocks can not only be cut, but are thinner and easier to process and stain than celloidin sections. This is particularly true of Paraplast.

Disadvantages of the Method

(1) It is diificult, if not impossible, to cut thin sections. They may be cut at 10 microns, but 15 microns is the normal thickness.

(2) Serial sections are difficult to prepare, and then only by a tedious process. This difficulty may be partially overcome by double embedding, which is dealt with on page 109.

(3) Celloidin processing is very slow, taking several weeks.

(4) Blocks and sections must be stored in 70 per cent alcohol otherwise they become discolored, dry and shrunken.

Preparation of Solutions

Celloidin is supplied as celloidin wool, damped with absolute alcohol. This wool is used direct from the container and dissolved in equal parts of absolute alcohol and ether, making an allowance for the absolute alcohol present in the wool.

Thick celloidin	8 per cent
Medium solution	4 per cent
Thin solution	2 per cent

These solutions, particularly the thick solution, take some time to dissolve, and should be made long before they are required and kept in stock. It is recommended that the shreds be soaked in absolute alcohol for 4–5 hours, before the addition of the ether, to decrease the time taken to dissolve the shreds, but this is of doubtful value.

Solutions of celloidin (also those of Necoloidin and low viscosity nitro-cellulose) should be kept in well-stoppered jars, preferably of the ground-glass-stoppered variety, to avoid evaporation of the ether-alcohol solvent and to prevent contamination with water vapour. It must be remembered at all times that *ether is one of the most highly inflammable materials used in a laboratory and care should therefore be taken to ensure that naked lights are extinguished before it is used.* A fire has been started in a laboratory by an uncorked bottle of ether which was no nearer than 10 yards from a lighted Bunsen burner. The flame ignited the vapour and leapt across the 10-yard gap causing the bottle of ether to explode.

Necol (Necoloidin)

This material is similar in many respects to celloidin, and has completely replaced it in some laboratories. It is appreciably cheaper, and is supplied as a strong (8 per cent pyroxylin) solution.

The solutions generally used are:

Thick solution	16 per cent
Medium solution	8 per cent
Thin solution	4 per cent

Some workers recommend that the solution supplied be evaporated to dryness in a fume cupboard, weighed, and the strong (16 per cent) solution prepared using equal parts of ether and alcohol as a solvent. The author has not found this to be necessary and, as a routine, the solution supplied (8 per cent) is left open in the fume cupboard until approximately one-half of the volume has evaporated; it can then be used as the thick solution (16 per cent) without any further treatment – the medium solution (8 per cent), and the thin solution (4 per cent) being prepared from this stock. Occasionally, the 8 per cent solution supplied has been used as the thick solution (16 per cent) with almost as good results.

Necol blocks are generally easier to cut than comparable celloidin

ones and, in addition, thinner sections may be cut because the final block is slightly harder. They generally remain quite clear, and do not tend to become cloudy on keeping, as in the case of celloidin blocks. In all other respects Necol is used in exactly the same way as celloidin, and the time schedule is applicable to either.

Technique of Impregnation

Tissue for impregnation by the celloidin techniques requires thorough dehydration by the technique described for paraffin wax impregnation. Following treatment in successive grades of alcohol, and absolute alcohol (with copper sulphate), the tissue is transferred to a mixture of equal parts of absolute alcohol and ether for 24 hours. This preliminary treatment facilitates permeation by the successive celloidin solutions.

The size of the tissue will decide the period needed in each of the celloidin solutions; the times given below are for specimens of not more than 10 mm thickness. Any appreciable increase in this thickness will require a similar increase in the time of impregnation.

Alcohol–ether mixture	24 hours
2 per cent celloidin or 4 per cent Necol	5–7 days
4 per cent celloidin or 8 per cent Necol	5–7 days
8 per cent celloidin or 16 per cent Necol	2–3 days

Complete impregnation with the thick celloidin would require a considerable length of time. The shorter time recommended above is sufficient for impregnation of the outer surface which allows embedding in the stronger solution without the danger of the tissue later becoming detached from the surrounding mass.

Casting or 'Blocking'

Following impregnation in the thick solution, the tissue is placed in a mould containing 8 per cent celloidin. The mould must be at least 1–1¼ inches in depth to avoid exposure of the tissue as the celloidin contracts due to evaporation. It may be of the same contruction as that used for paraffin wax embedding (page 88), except that Leuckhart's L pieces cannot be used. Glass Petri dishes, about 2 inches in depth, with loose-fitting ground-glass lids, are particularly useful for this purpose. The celloidin block is hardened by evaporation, and if the latter is uneven a bad and uneven block will result. It is essential to prevent air bubbles from being trapped on the cutting surface of the block; tissue, with the surface to be cut uppermost, is placed in a mould full of thick

celloidin for 12 hours to allow the bubbles to come to the surface. During this procedure the block is kept in an air-tight container to prevent evaporation, either in a desiccator or under an air-tight bell jar. Using the Petri dish, as described above, a lead weight is palced on the lid. Following elimination of the air bubbles, the tissue is inverted so that the surface to be cut faces downwards. The solution is then thickened by sliding the desiccator lid to one side, or by raising one side of the bell jar. To ensure even evaporation the area of the mould directly exposed to the air is changed every 3 or 4 hours. At night it is best to close the container. This hardening process is continued until the celloidin is of a rubbery consistency so that the ball of the finger no longer leaves an imprint.

Hardening of celloidin may be accelerated by the use of chloroform vapour. A few drops of chloroform in a separate container under a bell jar, or in the bottom of the desiccator, will harden small blocks 2 cm square in about 12–24 hours; larger blocks take proportionately longer.

When the block is sufficiently hardened, the excess celloidin is trimmed off with a sharp knife, leaving a margin of $^3/_{16}$–¼ inch all round, with the exception of the surface to be cut which should not be trimmed at this stage. This celloidin block is then fixed to a vulcanite fibre or hardwood (teak) block of an appropriate size to fit the microtome chuck. The surface of the wooden or vulcanite block must be roughened, or serrated with a saw, followed by a liberal application of thin (2 per cent) celloidin; the celloidin block is then pressed on and a lead weight used to hold the surfaces in close contact for about 1 hour. The block is then hardened in 70 per cent alcohol for ½ an hour before cutting.

Celloidin, Necoloidin and low viscosity nitro-cellulose blocks and sections must be preserved and stored in 70 per cent alcohol.

LOW VISCOSITY NITRO-CELLULOSE (L.V.N.)

Low viscosity nitro-cellulose has taken the place of celloidin in some institutes because being of a low viscosity it allows higher concentrations to be used, with greater speed of impregnation. Since the tissues are impregnated with a higher concentration the final block is harder, allowing thinner sections to be cut.

It has a greater water tolerance than celloidin, and 95 per cent alcohol and ether have been recommended as a solvent to avoid the hardening effects of absolute alcohol. Subsequent times and solutions will give 100 per cent alcohol as the diluent, but if extra hard tissues are being dealt with, the final stage of dehydration in absolute alcohol may

Table of Tissue

PROCESS	ROUTINE PARAFFIN PROCESSING (MANUAL)	ROUTINE PARAFFIN PROCESSING (AUTOMATIC TISSUE PROCESSER)	WEEKEND PARAFFIN PROCESSING (AUTOMATIC TISSUE PROCESSER)
Tissue thickness	2–6 mm.	2–6 mm.	2–6 mm.
DEHYDRATION	*Hours*	*Hours*	*Hours*
50% alcohol			4
60% alcohol			
70% alcohol (with iodine)	2–4	2	4
80% alcohol			
96% alcohol	2–4	4 (2 changes)	12 (2 changes)
Absolute alcohol I	2–4	3	4
(with copper sulphate) II	2–4	2	4
Acetone			
CLEARING			
Chloroform *or* Carbon tetrachloride	6–12 (overnight)		
Xylene *or* Toluene *or* Benzene I		3	4
II		2	4
Cedar-wood oil			
IMPREGNATION Paraffin wax/clearing agent			
Paraffin wax I	2–4*†	2	4
Paraffin wax II	2–4*†	2	4
Paraffin wax III		1*†	1*†
CELLOIDIN, NECOLOIDIN, OR L.V.N. Ether/alcohol			
2% celloidin *or* 4% Necol			
5% L.V.N.			
4% celloidin *or* 8% Necol			
10% L.V.N.			
8% celloidin *or* 16% Necol			
20% L.V.N.			
TOTAL TIME TAKEN	18–36	21	45

* Impregnate in vacuum embedding oven.

† Not tissue from the central nervous system (*see* page 84).

Processing Times

Rapid Paraffin Processing	Very Rapid Paraffin Processing	Research or Delicate Tissue	Paraffin Processing of Giant Sections	Celloidin or Necoloidin Processing	L.V.N. Processing
2–4 mm.	2–3 mm.	2–8 mm.	10–20 mm.	4–6 mm.	4–6 mm.
Hours	*Minutes*	*Hours*	*Hours*	*Hours*	*Hours*
		2	24		
		2			
½		2	24	4	4
		2			
1 (1 change)		2	24	4	4
½		2	24	4	4
½		2	24	4	4
	60 (3 changes each of 20 mins.)				
		6–12 (overnight)	24		
¼		↑			
¼–⅜§		OR			
		↓			
		see page 80			
		1	overnight		
½*	20*	2*	3*		
½*	10*	2*	2*		
		2*	2*‡		
				Days	*Days*
				1	1
				5–7	
					3–5
				5–7	
					2–4
				2–3	
					1
3½–4½	90	27–33	7 *days*	14–19	8–12

‡ If the final wax has the slightest smell of clearing agent, the tissue should be transferred to a further (fourth) change of wax.

§ Leave in second xylol until tissue is translucent. 'Susa' fixed tissue does not become translucent and should be left for 45 minutes.

be avoided and the L.V.N. used dissolved in equal parts of 95 per cent alcohol and ether.

A great disadvantage of L.V.N. as an embedding medium is that sections have a tendency to crack during handling and staining. The incorporation of 1 per cent tricresyl phosphate or 1 per cent celloidin is said to minimize this tendency, but Moore (1951) recommends the use of 0.5 per cent oleum ricini (castor oil) which gives sufficient elasticity to the L.V.N. to allow moderately easy handling of the sections. Using this method first-class sections can be cut on a Cambridge rocker using an ordinary Heiffor knife.

This tendency of the sections to crack, and the fact that L.V.N. is sufficiently difficult during staining, are probably responsible for its general unpopularity.

It must be handled with extreme caution as it is highly explosive, and should under no circumstances be allowed to dry.

L.V.N. solutions generally used are: 5, 10, and 20 per cent in a mixture of five parts of absolute alcohol to six parts of ether, with either tricresyl phosphate, celloidin or castor oil as a plasticizer (*see* above).

Times of impregnation in these solutions are as follows.

5 per cent L.V.N.	3–5 days
10 per cent L. V. N.	2–4 days
20 per cent L.V.N.	1 day

The shorter times given are for pieces of tissue not more than 7 mm in thickness; any increase in thickness will require a proportionate increase in time of impregnation.

The technique of dehydration, embedding, trimming and cutting are as already given under the general heading of celloidin

THE DRY CELLOIDIN METHOD

This method was devised to overcome the disadvantages of the standard method, that is to say, that blocks must be stored in 70 per cent alcohol and the block and knife kept wet with 70 per cent alcohol during cutting.

Celloidin or Necoloidin blocks are prepared as already described, but instead of being fixed to vulcanite blocks are transferred to Gilson's mixture, which is equal parts of chloroform and cedarwood oil. Cedarwood oil is added twice daily, over a period of 3–10 days, until the mixture is composed of about 90 per cent cedarwood oil. The celloidin will gradually become quite transparent during this process, and should then be removed and exposed to the air until dry. When the block is dry

it is fixed to a vulcanite block in the usual way and sections are cut dry. Blocks should be kept in air-tight bottles.

DOUBLE EMBEDDING

The preparation of serial sections of celloidin-impregnated tissue is normally a tedious procedure since the sections will not adhere to one another in the same manner as paraffin embedded material. Double embedding is the name given to a technique whereby the tissue is first impregnated with celloidin, and subsequently blocked in paraffin wax. In this manner the advantages of both materials are, to some degree, combined.

While some workers simply transfer tissue directly from 8 per cent celloidin, following the routine celloidin technique, to two changes of chloroform, or benzene, and subsequently embed in paraffin wax as for ordinary tissue,the method of Peterfi, as given below, is recommended.

Peterfi's double embedding method

(1) After dehydration of the tissue in the usual manner, the tissue is transferred to celloidin-methyl benzoate mixture, which is prepared by dissolving 1 per cent dry celloidin in methyl benzoate in a stoppered bottle. The bottle should be shaken several times daily to assist solution of the celloidin, which may take several days.

(2) The celloidin solution is replaced daily for 2–3 days, depending on the size of the tissue. Complete impregnation is indicated by the tissue becoming transparent.

(3) The tissue is transferred to benzene and given 2 or 3 changes each of 6 hours. Should the tissue need 3 changes of the celloidin solution, it will require 3 changes of benzene.

(4) Transfer tissue to paraffin wax, and impregnate and embed in the usual manner.

(5) Sections are cut, as are ordinary paraffin sections, on any of the standard microtomes.

(6) If sections, after floating on water in the usual way, tend to curl off slides, they should be floated on 90 per cent alcohol which will soften the celloidin without dissolving it (Lillie, 1954).

Chapter 6

Section Cutting

Introduction

The cutting of good sections depends on (1) a thorough knowledge of the equipment used, and (2) practical experience. In no other field of laboratory work is there such a need for manual dexterity, and this can only be acquired by a great deal of practice. Students should first cut paraffin sections of soft tissue, such as liver or kidney, and only when they are capable of producing long ribbons of thin sections (4–6 microns in thickness) should the more difficult tissue such as skin, breast or decalcified bone, be cut. Once this skill is acquired, like riding a bicycle, it will never be lost; an early training which has been hurried and inadequate will reflect years afterwards, but a high standard achieved during training will be maintained throughout. Speed in itself must never be a primary object, it leads to badly cut, poorly mounted, and inadequately stained sections. The well-trained and practised technologist will produce first-class sections in a far shorter time than one who has always aimed primarily at speed.

MICROTOME KNIVES

The knife is probably the greatest single factor in producing good sections, since it is capable of causing a greater number of faults than any other piece of apparatus; yet a really sharp, well adjusted knife can make section cutting easy. If possible, every worker should have his own knives and care for them himself. The care and attention will be repaid over and over again.

Types of Microtome Knife

Microtome knives are of two main types:

(1) The Heiffor knife (used on rocking microtomes) with a fixed handle.

(2) Larger knives ranging from 8 cm in length for freezing microtomes, to the knife 24 cm in length used on base sledge microtomes; these have detachable handles which screw into one end.

In descriptions of knives the expressions 'heel' and 'toe' are often used to indicate which end of the cutting edge is referred to. The 'heel' of the knife is the angle formed by the cutting edge and the end of the knife nearest the handle; the 'toe' of the knife is the angle formed by the cutting edge and that end of the knife farthest from the handle.

Microtome knives are generally classified not by their size but by the manner in which they are ground *(Figure 6.1)* as follows.

Plano-concave

This type of knife is recommended for use on sledge-type microtomes and certain rotary microtomes *(Figure 6.1a)*

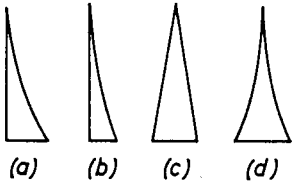


Figure 6.1 – Microtome knives: (a) plano-concave; (b) plano-concave (very concave); (c) plane-wedge; (d) bi-concave (from An Introduction to Medical Laboratory Technology, 2nd Ed. London; Butterworth)

Plano-concave (very concave)

This type of knife is used only for celloidin work, since the blade is comparatively thin and would vibrate if used to cut a harder material *(Figure 6.1b)*

Plane-wedge

Because of its rigidity the plane-wedge type of knife was originally designed for use in cutting frozen sections; it is now used in addition for giant and ordinary paraffin section cutting for this same reason *(Figure 6.1c).*

Bi-concave

The bi-concave knife is recommended for paraffin section cutting on rocking (Heiffor knife) and sledge-type microtomes *(Figure 6.1d)*. Most workers prefer the plane-wedge type of knife for sledge microtomes.

Choice of Knife

Although, as will be seen from the above descriptions, a certain amount of personal preference enters into the use of each knife for paraffin work, celloidin sections should always be cut with a plano-concave (very concave) knife. Frozen sections are best cut with a plane-wedge type, but can be cut with a Heiffor knife (bi-concave) if a wedge is not available.

With the smaller types of knife, it is an advantage to have two knives, one being used for rough work such as trimming the block prior to the actual cutting with the best knife. (The rough knife is usually an old one that has been ground down until it has insufficient width for correct siting of the bevel.) With the longer type of knife one end can be used for trimming and the other end reserved for cutting; there is also the advantage that they require less sharpening because when one part of the edge becomes blunt the knife can be moved along to bring a sharper part to bear on the block.

Sharpening Microtome Knives

The cutting edge of an ideal microtome knife would be the straight line formed by the intersection of two planes, the cutting facets. The angle between the planes is called the bevel angle and is greater than the wedge angle between the sides of the knife. Such an ideal edge is not possible because the inhomogeneous structure of the steel results in a slightly rounded edge. The radius of curvature of this edge should be between 0.1 and 1.0 μ (Schmeritz, 1932). Ardenne (1939) would limit the radius of curvature to 0.01 μ (C).

The cutting edge of a very sharp knife, when examined by reflected light under 100 magnifications, will appear as a very fine discontinuous line varying slightly in width. Von Mahl (1857) recommended sharpening the microtome knife until the two planes of the cutting facets come together to give a minimum reflection; his criterion of sharpness is still used. If the cutting edge is thicker than the cells, for instance, they will be destroyed rather than sectioned. Ssobolew (1909) emphasized the importance of proper hardness of the knife temper and that a fine edge could only be obtained by using fine honing material.

The proof of a sharp knife, according to Apathy (1912), was the ability to cut a paraffin ribbon at 2 μ (Paraplast, 1 μ) with no compression. Bensley and Bensley (1938) also recommended polishing the edge until no reflection can be seen from the actual cutting edge. They test with paraffin at 3 microns and advise the operator not to try the knife on hair or skin as these rather difficult tests may spoil the edge.

The general condition of the cutting edge may be determined by examining it with a microscope. Most microtomists recommend a magnification of 100 diameters for this purpose. While sharpening the knife, or at failure to section, examination of the condition of the edge with a microscope may save both time and material.

The size and general shape of the microtome knife have become established by use. The standard microtome knife has a wedge angle of about 15 degrees and the bevel angle (between the cutting facets) for knives of American manufacture varies between 27 and 32 degrees. The width of the two facets which make the cutting edge of the knife has been recommended from 0.1 to about 0.6 mm.

Honing

After prolonged use, or after cutting very hard tissue, the cutting edge of the knife will have become so damaged that stropping will not re-sharpen it; small pieces of metal may have been removed which will give a jagged edge, causing lines or even tears in the sections during cutting. A straight cutting edge and the correct bevel must be restored by grinding the knife on a hone.

There are various types of hone some of which are described below.

Belgian black vein. – The best hone obtainable is probably the Belgian black vein. It is a yellow stone about ½ inch thick, and is backed with a black stone of about the same thickness; only the yellow side should be used for honing. It is quite a fast hone and may be used for coarse grinding and finishing. By putting some Gumption (a mild abrasive slide cleaner) on the surface it can be made even faster, but such treatment should only be adopted on badly jagged-edged knives and should be confined to not more than 30 strokes in each direction.

Arkansas. – A very hard, pale yellowish-white stone which is not very fast, the Arkansas is normally used to finish a knife after honing on a coarse hone, such as carborundum.

Aloxite. – This is a composition, fairly fast but coarse and not good for finishing a knife.

Tam o' Shanter Scotch hone. – This is a good medium hone, which is very soft with a comparatively short life. It is cheap and can be used for the removal of jagged edges and for finishing.

Carborundum. – These hones can be obtained in a variety of grades, only the finest of which should be used, and even these only for coarse work, for example, removing large nicks in a badly damaged knife.

Plate glass. – Plate glass may be used as a hone by applying an abrasive, such as aluminium oxide, to the surface and then using in the same way as an ordinary hone. The advantage of such a hone is that it can be used for all types of honing by changing the abrasive powder or paste used. It is supplied in a case, complete with three grades of abrasive powder, coarse, medium and fine, the powder being made into a paste by adding water and spreading evenly on the plate glass.

The size of hone to use will depend on the type of knife to be sharpened; the smaller type, such as a Heiffor knife, is easier to hone on a stone measuring 8 X 2 inches, rather than on one measuring 12 X 3 inches which is intended for use with large sledge microtome knives.

Hones should be lubricated with a light oil or soap and water before use; oil is the better of the two. After use the hone should be thoroughly cleaned to remove the lubricant together with any small pieces of metal.

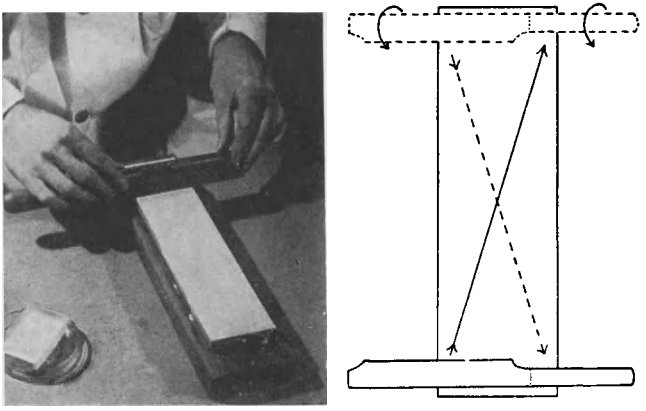
The better type of hone is supplied fitted and fixed in a box, preferably surrounded by a channel into which excess oil will flow. The cover should always be replaced immediately after use to prevent grit and dust from falling on the stone; such grit if not removed will damage the surface of the hone and can cause nicks in the edge of a knife being honed.

Method

(1) The hone is placed on the bench on a non-skid surface (a damp cloth laid on a shiny bench will usually suffice) since it is essential that it does not move during honing.

(2) A small quantity of light lubricating oil (or soapy water) is poured on the centre of the hone and lightly smeared over the surface.

(3) The knife, complete with handle and backing sheath, is laid



(a) (b)

Figure 6.2 – Photograph (a) and diagram (b), illustrating the honing of a mcirotome knife (from An Introduction to Medical Laboratory Technology, 2nd Ed. London; Butterworths)

on the hone with the cutting edge facing away from the operator *(Figure 6.2a)*, and the heel roughly in the centre of the nearest end of the hone.

Correct positioning of the fingers is important during honing to ensure an easy flowing movement, and this is achieved by holding the handle of the knife between the thumb and forefinger with the cutting edge facing away from the operator (so that the thumb is on the back). When the knife is on the hone the tips of the finger and thumb of the other hand (which placed together form a V) rest on the other end of the knife, ensuring an even pressure along the whole edge of the knife during honing. The V prevents the fingers from slipping on the knife when it becomes greasy with the oil; only light pressure is applied during both honing and stropping.

(4) The knife is pushed forward diagonally from heel to toe *(Figure 6.2b)*, turned over on its back and moved across the hone until the heel is in the centre with the cutting edge leading, and then brought back diagonally. It is then turned over on its back and moved across the hone to its original position, thus completing a figure of 8 movement. Although this is the traditional and more correct technique the writer uses the method of laying the knife on the nearest end of the hone with the toe in the middle, pushing away diagonally using only a light pressure on the knife, turning it over on

its back, and bringing it back diagonally from heel to toe. This method is simpler, faster, and just as efficient. Practice will bring speed of action and a sharp knife, but to aim at speed too early in training will result in a blunt knife and cut fingers.

(5) The process is continued until all jagged edges have been removed; in the case of a very badly damaged knife both a coarse and fine hone should be used, the larger nicks being removed on the coarse hone and the remainder on the fine hone. The knife is then ready for stropping.

A knife well looked after, never laid on a bench when it should be placed in its box, not used for cutting obviously calcified tissue, will last a competent technologist for months without honing.

Stropping

Stropping is the process of polishing an already fairly sharp edge; a really blunt knife cannot be sharpened on a strop.

Types of strop. – The best strops are made from hide from the rump of the horse, and are usually marked 'shell horse.' Such a strop, properly cared for, will give many years of wear. They may be either flexible (hanging), or rigid.

The type of strop used will depend on personal preference; it is claimed by those who prefer the rigid strop that the hanging strop imparts a rounded edge to the knife, but this fault is generally due to the strop not being held sufficiently taut. The writer prefers the hanging type because of the easy flowing action that is possible with its use. The back of the strop is made of canvas and is intended to support the leather during stropping, although most technologists strop their knives on the back for a dozen or so strokes before using the leather. Strops should be kept soft by working a small quantity of vegetable oil into the back of the leather, and, like hones, they should be kept free from grit and dust. Strop dressings are available which incorporate jeweller's rouge and other mild abrasives, but if used they should be applied sparingly and the surface should not approach the sticky mess that has been observed in laboratories from time to time.

The rigid type may be either a single leather strop stretched over a wooden frame, to give a standard tension, or a block of wood about 12 X 2 X 2 inches in size having a handle at one end, with four grades of leather, or even a soft stone, cemented on each side. The sides of these strops are numbered, and the knife is first stropped on No. 1, then No. 2, and so on, finishing on the finest leather.

Technique. – The knife is laid on the near end of the strop with the cutting edge towards the operator (that is to say, in the opposite direction to that used in honing). The knife is held mainly with the forefinger and thumb (as for honing) to facilitate easy rotation at the end of each stroke. The action is the exact opposite of that used in honing, trying to make use of the full length of the strop and stropping evenly the whole of the blade. Care must be exercised in turning the knife over on its back at the end of each stroke to avoid cutting the strop.

Stropping may be necessary between the cutting of each block, or only once or twice a day, depending on the type of tissue being cut.

Knife Sharpening Machines

These machines, despite their high cost, are becoming popular because of the very great saving in time; a large microtome knife can be honed and ready for stropping in 10 minutes, compared with the 2 hours which might be required for manual sharpening of a badly damaged knife. They also have the advantage that with a short initial training, an unskilled junior can obtain a consistently good finish on a straight cutting edge, with a predetermined standard bevel. The disadvantage is that the feed mechanism for the knife movement is operated by hand so that the degree of speed and pressure may vary, although in those laboratories where the sharpener is in use this is not regarded as a serious handicap. Cleaning of the machine after use takes about 10 minutes and must be carried out thoroughly.

An early model of the machine had revolving glass wheels against the edge of which the knife was ground, using a lapping compound, but these were not completely satisfactory and have been replaced by cast iron wheels.

Coarse lapping compound composed of alumina, suspension fluid and water is used first, followed by a compound containing a finer grade of alumina. Lastly, the suspension fluid is used alone to polish the edge. If the knife is not badly damaged the coarse abrasive need not be used, and sharpening can commence with the fine grade. A built-in pump ensures that the lapping compound is circulated continuously.

The angle of the bevel is set by adjusting the cutting angle indicator and the cutting angle control to a predetermined figure so that all knives will have the exact bevel required.

It is essential that the operator *does not apply any pressure* when holding the knife against the sharpening wheel, for such pressure will result in burning of the edge of the knife with consequent loss of 'temper' of the steel. The knife should be moved forward slowly and

evenly, resting against the right-hand wheel. The time taken to traverse the whole of the cutting edge of a large knife should be about 2 seconds, the same time being taken to bring it back against the left-hand wheel; about 30 strokes in each direction should suffice with each grade of lapping compound. It should be remembered that the machine will require cleaning between each grade of abrasive.

Following sharpening on such a machine, the knife should be stropped in the manner described.

Occasionally it will be found impossible to obtain or maintain a sharp edge to a particular knife; this is probably due to a fault in tempering and such a knife should be returned to the suppliers.

Automatic microtome knife sharpeners

Knife sharpening machines are now available which are completely automatic, that is to say, the actual honing process is automatic. There are now several excellent models available, the one described below is the only one with which I have experience. Over the past 7 years it has given good results with no problems.

*Automatic Spencer microtome knife sharpener**. – It is designed to provide a precise, efficient and safe method for honing knives and assuring a proper edge without subsequent honing. In use, the knife is locked in knife holder, at the proper fixed angle to high-frequency vibrating honing plate. A small amount of special abrasive is placed on the plate, and the hinged transparent Plexiglass cover is brought down to cover the entire working system. The electrical timing device is set to the desired time and the switch turned on. After four exactly equivalent strokes on one side, the knife is automatically flipped over for four strokes on the other side, this stroking cycle being repeated continuously for the duration of the electrical time setting. After the selected time, the stroking process stops and the knife holder is held aloft for convenient removal of the knife. Abrasive for both coarse and fine housing is supplied. Two interchangeable glass honing plates are included. These can be dressed by manually stroking against each other, with hone glass compound between the plates. The over-all dimensions are 10¾ X 16½ X 12 in. It weighs approximately 20 lb.

MICROTOMES

Microtomes are mechanical devices for cutting thin uniform slices of tissue (sections). The tissue is supported in hard paraffin wax, or some

*Available from most laboratory suppliers.

similar material, and is moved automatically towards the knife, between cuts, by the thickness desired for the section: the latter mechanism is usually graduated in 1 micron (μ) stages. When cutting paraffin wax embedded tissue each section sticks to the previous one by its edge, and a ribbon of sections is produced.

There are five basic types, named according to the mechanism – rocking, rotary, sledge, sliding and freezing microtomes.

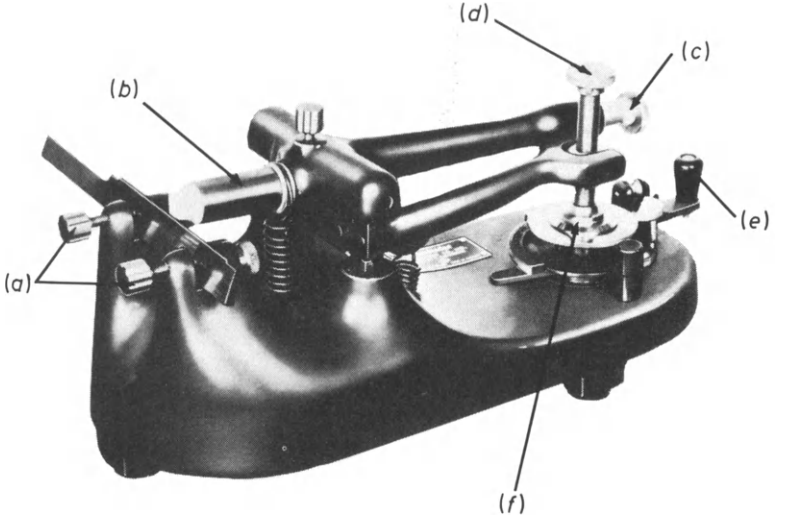


Figure 6.3 – The Cambridge rocking microtome: (a) knife clamps, (b) block-holder, (c) tension adjustment, (d) micrometer screw, (e) operating handle, (f) feed mechanism

The Rocking Microtome

In the Cambridge rocking microtome *(Figure 6.3a)* the knife is fixed and the block of tissue moves through an arc and strikes against the knife; between strokes the block is moved towards the knife for the required thickness of the sections, by means of a micrometer thread operated by a ratchet. The name of the apparatus comes from the rocking action of the cross arm. It was for many years a familiar sight in almost every histological laboratory in Great Britain, and although the sledge-type microtome has replaced it in a great many laboratories for routine work, it is still favoured by some – including the writer – for

cutting sections from small blocks of tissue of any type. The mechanism is simple and rarely, if ever, gives trouble, and the machines literally last a lifetime.

A disadvantage is that the size of block that can be cut is limited, although a recent model has been designed to overcome this. Moreover, since the block moves through an arc when cutting, the sections are cut in a curved plane; this, however, seems to be more of a theoretical than a practical disadvantage. The Unicam is similar to the Cambridge but is designed to cut perfectly flat sections, the block moving through an arc at right angles to the edge of the knife.

The rocking microtome was designed primarily for cutting paraffin wax sections, but, in an emergency, it may be adapted for frozen sections by inserting in the chuck a simple wooden block, on which the tissue is frozen with an ethyl chloride spray; this method, however, is not to be recommended except where no other apparatus is available and a frozen section is required urgently.

In view of the lightness of this type of microtome it is advisable either to fit it into a tray which is screwed to the bench, or to place it on a damp cloth to avoid movement during cutting. The movement of the cutting arm should depend on the type of tissue to be cut; normally a steady forward and backward movement of the handle will give ribbons of good sections, but with difficult tissues there are two alternative movements worth trying: (1) pulling the handle forward and releasing it from this position, allowing the spring to pull it back sharply, or, if this does not produce a good section, (2) pulling the handle forward and letting it back very slowly. One of these methods will usually result in a ribbon of good sections.

The Rotary Microtome

The rotary microtome is so called because a rotary action of the hand-wheel actuates the cutting movement. The block-holder is mounted on a steel carriage which moves up and down in grooves, and is advanced by a micrometer screw; it therefore cuts perfectly flat sections *(Figure 6.4)*.

It has the advantage of being heavier and therefore more stable than the rocking type, and is ideal for cutting serial sections; consequently it is more often used in teaching establishments for cutting large numbers of sections from each block. Larger blocks of tissue may be cut on this machine, and the cutting angle of the knife (tilt of knife) is adjustable. Since a heavier and larger knife is used with this type of microtome there is less likelihood of vibration when cutting exceptionally hard tissue. The first machine of this type was designed by Professor Minot,

and is sometimes known as the Minot Rotary. By using a special holder to set the knife obliquely it may be used for cutting celloidin-embedded sections.

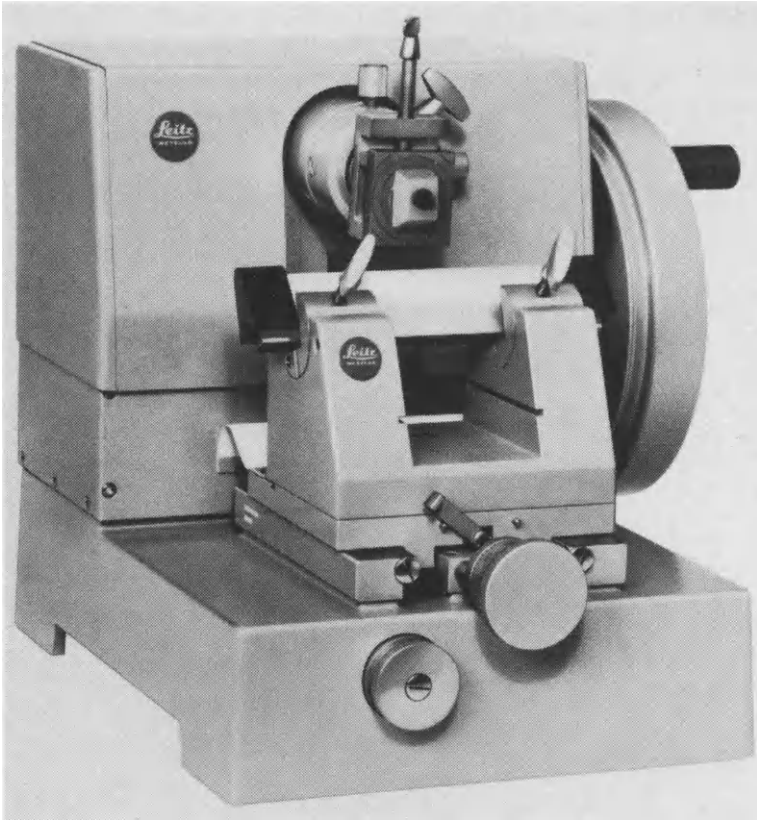


Figure 6.4 – The rotary microtome 1500 (by courtesy of Ernst Leitz, Ltd.)

The Sledge Microtome

Originally designed for cutting sections of very large blocks of tissue (for example, whole brains), the sledge microtome has become a popular machine for routine use since World War II. Within very wide limits the size of the block is of no account. The block-holder is mounted on a steel carriage which slides backwards and forwards on guides against a fixed horizontal knife *(Figure 6.5)*. This microtome is heavy and consequently very stable and not subject to vibration; the

knife used is a large one (24 cm in length) and usually wedge-shaped, which again reduces the possibility of vibration and requires less honing. The knife-holding clamps are adjustable and allow the tilt and the angle (slant) of the knife to the block to be easily set.

A variety of stages is obtainable for use with blocks of various sizes, either mounted on wood or directly fixed with molten paraffin.

A criticism often heard about the routine use of this instrument is that it is very much slower in use than a rocker or rotary microtome: this is true only when a change from one instrument to another is first made, for with practice, sections from routine paraffin blocks can be cut as quickly on the sledge as on any other type of microtome. Moreover, there is no restriction on size when selecting pieces of tissue for sectioning.

Because of the adjustable knife holder, this machine may be used for cutting celloidin sections by setting the knife obliquely, and in some

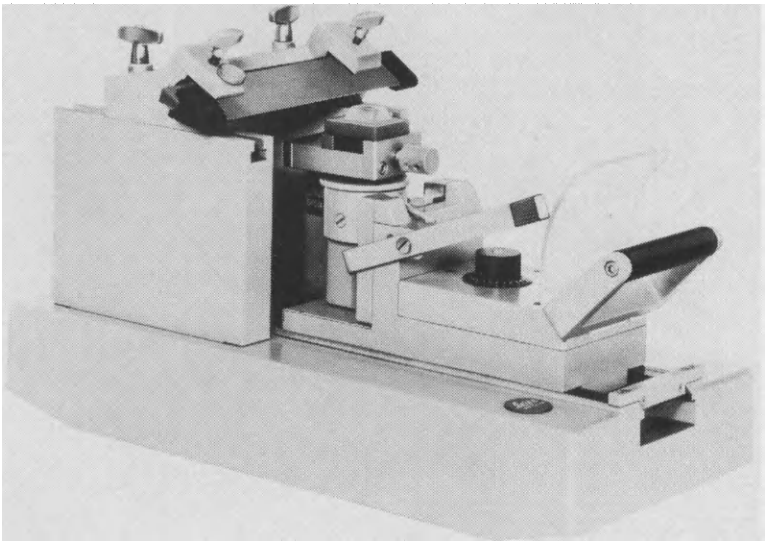


Figure 6.5 – The base sledge microtome 1400 particularly suitable for extensive or hard specimens (by courtesy of Ernst Leitz, Ltd.)

laboratories the knife is left permanently in this position because it is felt that even paraffin wax embedded sections are more easily cut in this manner.

A freezing stage is available on this machine also, but in the writer's experience is inferior to the type illustrated in *Figure 6.7*.

The Sliding Microtome

In this type, the knife is moved horizontally against a fixed block which is advanced against it up an inclined plane.

The sliding microtome *(Figure 6.6)* was designed for cutting celloidin embedded sections, and is probably the best type if sufficient celloidin sections are cut to justify its purchase. It can also be used for paraffin wax embedded sections.

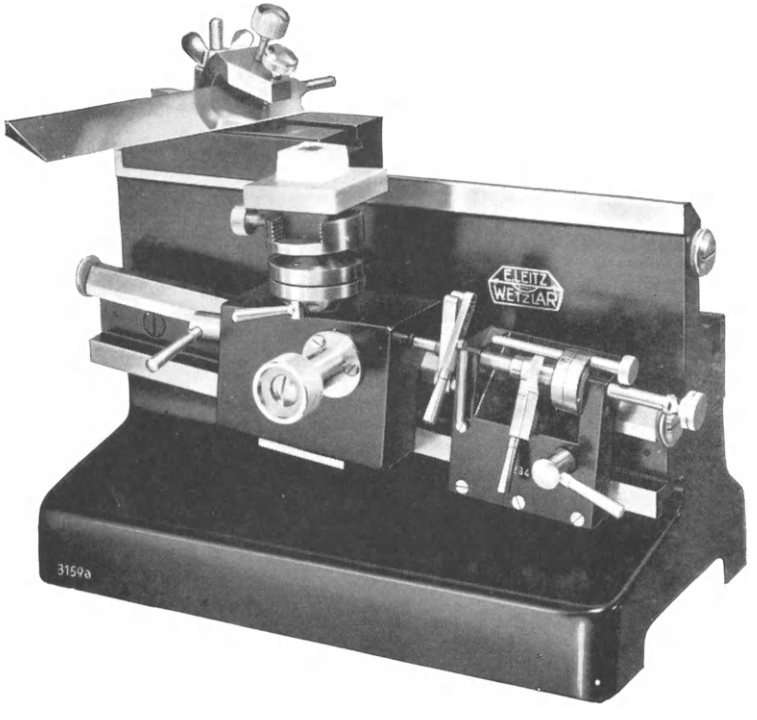


Figure 6.6 – Sliding microtome with inclined plane for paraffin and celloidin embedded section (by courtesy of Ernst Leitz, Ltd.)

The Freezing Microtome

Although other microtomes can be modified for cutting frozen sections, this type *(Figure 6.7)* will give the best results and is used

almost universally. It can be fitted with a block-holder for paraffin sections.

The machine is clamped to the edge of a bench and is connected to a cylinder of CO_2 by means of a specially strengthened flexible metal tube. Some care is necessary with regard to the position of the cylinder for this work: liquid CO_2 must reach the valve in the chuck, therefore

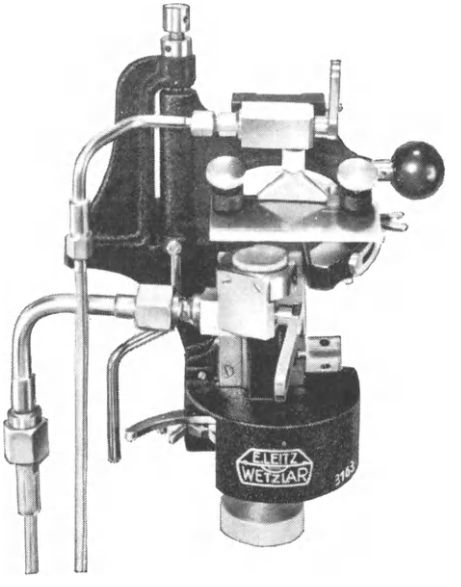


Figure 6.7 – The freezing microtome for CO_2 frozen section with low-temperature knife cooling (by courtesy of Ernst Leitz, Ltd.)

ordinary cylinders must be held upside down in a special holder, or blocks placed under them to ensure that (*a*) the cylinder valve is the lowest point of the cylinder; and (*b*) that the cylinder valve is above the level of the chuck of the microtome. A special cylinder fitted with a central tube is available and this type may be used in a floor-stand with the valve uppermost, since the pressure inside the cylinder (about 1,000 lb. per square inch) will force liquid CO_2 up the tube from the bottom of the cylinder. At one time it was advised that a very short tube be used between the cylinder and the microtome, but with the type now available this is not important and the normal length supplied may be used with complete safety. Serious accidents can happen if connexions

are made hurriedly, or the machine is dismantled before closing the valve on the cylinder. In practice, the connexions between the cylinder and the machine should be checked before turning on the gas, and the cylinder valve should be opened only during the time of actual cutting.

A knife-freezing attachment is supplied with most machines; that is to say, a separately controlled flow of CO_2 on to the edge of the knife. This is not generally used, but is intended to delay the thawing of the sections on the knife and make it possible to transfer them directly from the knife to slides.

The feed mechanism is similar to that of the other types except that the section thickness gauge is graduated in units of 5 microns instead of single microns.

CUTTING OF PARAFFIN WAX EMBEDDED SECTIONS

Trimming The Block

When cutting sections on the rocking or rotary types of microtome, blocks must first be trimmed and fixed to wooden fillets. This is not necessary if Tissue-Tek embedding rings are used (*see* page 89). Wax is removed with a sharp knife until ⅛ inch remains on all sides of the tissue. Great care is necessary to avoid exposing the tissue at any point, and the edges of the block should be made parallel so that an even ribbon of sections results. The surface of the block which is to be cut should be trimmed on the microtome during the preliminary cutting; if the tissue was positioned correctly when the block was being cast there should be very little spare wax on this surface.

Only small flakes of wax should be trimmed at a time; attempts to trim large pieces sometimes lead to splitting of the block and exposure of the tissue; this will entail reblocking in fresh wax.

Blocks to be cut on the sledge microtome can be trimmed in the same way, but when cutting single sections for routine diagnosis it has become the practice in some institutes not to trim these blocks but to attach them directly to the platform of the microtome.

Fixing the Block on the Microtome

When using a microtome with a flat metal stage on which the block can be fixed directly, a wooden handled spatula, or an old metal scalpel which has been inserted into a wooden handle, is heated in a Bunsen burner. When hot, but not red hot, this is applied to the underside of the block and to a layer of wax on the stage until the two wax surfaces

melt, when they are quickly and firmly pressed together.

The surface of a wooden fillet is first serrated with a saw or scalpel and coated with hot wax; the block of tissue is then attached to the wood in the manner described for the flat metal stage. It is advisable always to ensure a good joint by running the hot iron around the base of the paraffin block. The wooden fillet is then mounted in a clamp-type holder on the microtome.

Cutting Technique

(1) Fix the block in the block-holder on the microtome in such a position that it will be clear of the knife when this is in position.

(2) Turn back the feed mechanism on the microtome almost as far as it will go.

(3) Insert the appropriate knife in the knife-holder and screw it tightly in position; check that the tilt of the knife is set at the correct angle (if this is adjustable). If it is intended to use the knife obliquely, the movable knife-holder should be adjusted in the desired position.

(4) Move the block-holder forward or upward, or adjust the feed mechanism until the wax block is almost touching the knife. Ensure that the whole surface of the block will move parallel to the edge of the knife, and that the leading edge of the block is parallel to the edge of the knife in order to ensure a straight ribbon of sections; the means of achieving this vary with different microtomes.

(5) Tighten all the adjusting screws on the microtome; faults in section cutting are more frequently due to looseness of the block or knife in the early stages of section cutting than to any other reason.

(6) To trim the block set the section-thickness gauge to about 15 microns and, with the rough knife – or one end of a large knife which is kept for trimming – in position, operate the microtome until complete sections of the tissue are being cut.

(7) Replace the rough knife by a sharp one, or move the knife to a new position; check that it is screwed tightly in position. Rack the feed mechanism back a little to allow for slight differences which always exist in different knives, and even in different parts of the same knife. Apply ice to the surface of the block for a few seconds, and wipe the surface of the block free of water; this is optional, but makes flat even sections easier to cut.

(8) Set the thickness gauge. For routine work 6 microns will give moderately thin sections with ease of cutting. It should be remembered that dense tissue, such as spleen, should be cut thinner than open tissue, such as lung.

(9) The microtome is now operated until complete sections are

being cut; with practice an easy rhythm is achieved with the right hand operating the microtome and the left hand holding the sections away from the knife, either with a needle or forceps. Between each section it will be found an advantage to breathe on the block, particularly if the tissue is difficult to cut. Gentle breathing on the sections already cut will remove many of the creases and result in long, flat, even ribbons. Successful section cutting is really a set of reflex actions with the breathing regulated to the speed of cutting. The formation of the ribbon is said to be due to the slight heat generated during cutting which causes the edges of the sections to adhere. If difficulty is experienced in forming the ribbon, it is sometimes overcome by rubbing one of the edges of the block with a finger.

(10) Routine sections may be laid in small cardboard boxes, or directly floated on to slides. It is a useful routine to have a stock of small cardboard boxes on which the number or name of the tissue is marked in pencil, and to keep a few sections in them for a short time. If subsequent sections are required, these may be used instead of having to set up the microtome and cut the blocks again. Such sections may be kept until the case is reported, when they may be discarded. The box, after details of the specimen have been erased, may be used again.

(11) Cutting is normally only continued until sufficient perfect sections have been produced. It is sometimes necessary to mount and examine all the sections from a block (or every fifth or tenth section) in the order in which they are made: these are known as serial sections. The free end of each ribbon is supported either with fine forceps or with the fingers until it is about 12 inches in length; the ribbon is then freed from the knife by bringing a dissecting needle up under the last section – it must not be allowed to touch the cutting edge of the knife. The sections are then laid on black paper and should be fixed in position by gentle pressure with a finger at each end of the ribbon to avoid them being scattered by stray draughts. The author remembers the look of dismay on the face of an examination candidate whose serial sections were draped around the water tap after a door had been opened, a catastrophe which could have been prevented by a little care. Serial numbers are marked on the black paper to indicate the order of the ribbons.

Fixing The Sections to Slides

During cutting, paraffin wax embedded sections become slightly compressed and creased. Before being attached to slides these creases must be removed and the section flattened, and this is achieved by floating them on warm water by one of the following three methods.

Waterbath Method

Thermostatically controlled baths are now available with the inside coloured black. These baths are controlled at a temperature 5–6 degrees below the melting point of the paraffin wax. If a thermostatically controlled bath is not available an enamel or glass bowl may be used and filled almost to the brim with water of the correct temperature. It is easier to see creases in the sections if the inside of the receptacle is painted black, although glass dishes laid on black paper give equally good results. Air bubbles which form on the bottom of the dish must be dislodged with the fingers, or by vibration.

The sections are divided with a scalpel into lengths convenient to go on a slide, usually single section or 3–4 small sections. It must be remembered that room must be left at one end of the slide for a label, and that sections must not reach the extreme edge of the other end because of the difficulty of examining them under a microscope. It is preferable to leave ¾ inch at one end for the label, and at least ½ inch at the other.

The section, or sections, are lifted off the bench and on to the surface of the water by inserting the point of a dissecting needle into the wax at one end, care being taken not to damage the tissue during this operation.

Should the creases in the section not disappear almost immediately after contact with the water, dissecting needles should be used to tease them out by applying one to each side of a crease with very gentle pressure; should the section become even slightly damaged during this operation it should be discarded. With practice the floating of sections and the removal of creases becomes almost automatic. If the creases are very difficult to remove it may be because the block was not sufficiently cold when the sections were being cut and ice should have been applied, or because the wax was compressed by a blunt knife, a poor bevel on the knife, or an incorrectly tilted knife.

A clean or albuminized slide (*see below*) is half submerged in the water, and with a dissecting needle the section is brought into contact with it; the slide is then withdrawn, bringing the flattened section with it. With the needle the section is then orientated on the slide; it is important that only the wax edge be touched with the needle, and that this operation be carried out while there is still water on the slide.

The slide is then set in an upright position to drain. Special racks are available commercially for this purpose, but are easily made by making a series of saw-cuts in a block of wood of a convenient size, the slides being placed on end in the saw-cut to drain and the rack placed in the incubator to allow the slides to dry. The mounted sections may then be

left in the incubator at a temperature of 37°C overnight to dry, and are then ready for staining. Should albuminized slides be used, the water is drained from beneath the section, and they may then be placed in the oven at 60°C to dry and to coagulate the albumin; after 2 hours they are ready for staining.

Hot stage method

Hot stages (*Figure 6.8*) are available commercially and consist of a specially shaped metal top heated by gas or electricity to maintain the temperature of the stage at 45° – 50°C. Most workers use a controllable low temperature electric hotplate.

A clean, or albuminized slide is laid on the hot stage, flooded with distilled water, and the section or sections are laid on the surface of the

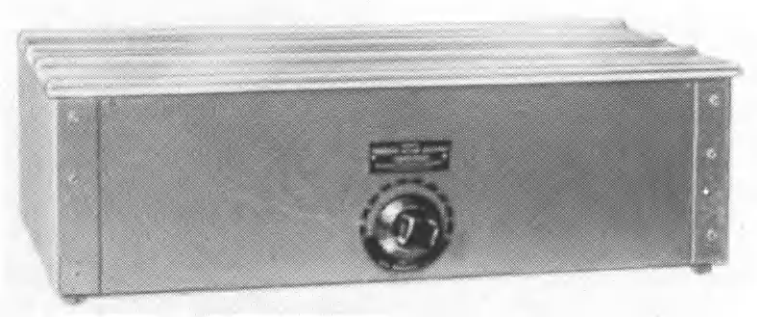


Figure 6.8 – The hot stage for flattening and drying sections (by courtesy of Hospital and Laboratory Supplies, Ltd.)

water. Any major creases should be teased out with needles or forceps, and the slide left for a few moments to get warm. Under the influence of the heat the small creases disappear and the section will flatten out. When it is completely flat the slide is removed from the hotplate, the excess water is drained off, and the section orientated into the correct position. It is then returned to the stage, section downwards to prevent dust settling on it, and left for 30 minutes (in an emergency 10 minutes will usually suffice). The section is then ready for staining.

Warmed Slide Method

The forerunner of the hot stage method is still used in some small laboratories, the technique being very similiar. A clean or albuminized slide is flooded with distilled water, sections are laid on the surface and,

with the major creases removed, the slide is held over a Bunsen burner for a second or two to slowly warm the water. If necessary a second heating may be given, but the wax must not be melted during this operation otherwise the section may be damaged. The creases will flatten out during this process, following which the excess water is drained off, and the slide put into the oven at a temperature of 37° or 56°C to complete drying. The section is then ready for staining.

The Use of an Adhesive

Provided the sections have been thoroughly dried on the slides an adhesive is not strictly necessary in the case of routine haematoxylin and eosin staining; it is essential, however, for techniques which employ certain reagents such as ammonia, particularly for the reticulin group of methods, and where the technique involves long periods of immersion in dyes or reagents. The main criticism of adhesives is that the albumin used as an adhesive retains some of the stain and gives a 'dirty' background. A dirty background occurs with albuminized slides only when using a too highly concentrated solution of albumin, or when using old albumin from an unstoppered bottle which has evaporated, and contains dust.

A thymol-resistant organism growing in the adhesive has been known to cause confusion in a Gram-stained section. The turbid appearance of the adhesive, and the disposition of the bacteria in the preparation (in a single plane below that of the section) should make the diagnosis obvious, and a Gram-stained film of the adhesive alone will confirm it.

An alternative adhesive which gives results superior to albumin is starch paste. It not only gives a completely clear background with all stains and silver techniques, but is also a more effective adhesive. Large brain sections mounted by this method remain attached to slides after quite long periods of immersion in most fluids. *It is P.A.S. positive and cannot be used for sections to be stained by this technique.*

An excellent adhesive for sections likely to become detached from albuminized slides is chrome gelatin (0.1 per cent chrome alum in 1 per cent gelatin).

Preparation of Albuminized and Starched Slides

Starch Paste

Mix thoroughly 1 g of powdered starch (soluble starch may be used) in 10 ml of cold water. Pour the mixture into 20 ml of boiling water, add 2 drops of N/1 hydrochloric acid and boil for 4–5 minutes, stirring

well. Add a large crystal of thymol, or several small ones, and put into containers described below.

Albumin Solution

Albumin solution is prepared by mixing equal parts of glycerin, distilled water, and white of egg (or reconstituted dried egg albumin). It is then filtered through either several layers of butter muslin, or coarse filter paper and a crystal of thymol added to prevent the growth of moulds.

Method

A piece of 5 mm glass rod which has had both ends rounded over a Bunsen burner is kept in the stock bottle as an applicator; a convenient method is to bore a hole through the cork into which the rod fits, thus keeping the starch paste or albumin solution free from dust and ready for instant use.

These slides may be prepared as required – which is the more popular method – or prepared in large numbers, dried off in an incubator and stored in a dust-free container.

The clean, grease-free slides are laid in a row and a drop of starch paste or albumin solution is placed on each with the applicator and smeared on the surface of the slide with the ball of the finger. One dip of the applicator into the stock bottle will be sufficient for 5 or 6 slides.

When using a hotplate an alternative method is to use a 2 per cent albumin solution in distilled water to float the sections on to the slides, sufficient albumin remaining after drainage to hold the section.

CUTTING PARAFFIN WAX EMBEDDED SECTIONS OF DIFFICULT TISSUE

At this point it should be made clear to the student that 99.9 per cent of tissues will cut reasonably well provided they are properly fixed, decalcified if necessary, completely dehydrated, cleared, and impregnated with paraffin wax, and if a sharp knife and properly adjusted microtome are used when cutting. There are rare occasions, however, when in spite of correct technique it will prove difficult, if not impossible, to produce a good section from a piece of tissue such as skin, bone, or blood clot (*see* page 134).

These difficulties may be divided into two groups: (1) where the

tissue is too hard to cut; and (2) where fragmentation of the tissue occurs as it is cut. The following techniques will usually overcome these difficulties.

Hard Tissue

The problems associated with hard tissue are dealt with on page 72. Where difficulty in cutting is encountered in spite of, or in the absence of, early precautions, the block should be soaked in Mollifex (obtainable from British Drug Houses) overnight and cut in the usual way the next morning. Although the surface will have a soapy consistency after this treatment sections cut and stain quite well.

Fragmentation of Tissue

If sections tend to break down in spite of the application of ice to the surface of the block – this is most likely to happen when there is a large amount of blood in the tissue – the block should be coated with celloidin between sections. The surface of the block should be wiped dry, and painted with a camel-hair brush which has been dipped in 1 per cent celloidin. After allowing a few seconds for the celloidin to dry a section is cut in the usual way. The process must be repeated for each section.

It must be remembered that when floating the sections to remove the creases, the celloidin layer must be uppermost, and the water should be a little hotter than usual to counteract the effect of the celloidin. The sections are floated on the slides and dried in the usual way. Following drying, the celloidin is removed with equal parts of ether and alcohol before removing the wax with xylol.

CUTTING SERIAL SECTIONS

As has been described under the cutting of paraffin wax embedded sections, serial sections are best cut in ribbons of about 10–12 inches in length and laid on black paper (usually obtainable from the x-ray department).

Every section must be saved during this process, and if any are lost or damaged the appropriate number of spaces should be left in the ribbons with a mark to this effect (L). The surface of the block is not trimmed in the normal way, and the serial sections should commence with the very smallest piece of tissue. This same rule is especially applicable to small biopsy specimens, where the only piece of malignant tissue present may be in the first few sections.

Such sections should always be mounted correctly, with the lower

surface fixed to the slide. The two surfaces may easily be distinguished by the shine of the lower one and the dullness of the upper one.

TABLE 6.1

Faults in Paraffin Section Cutting

FAULT	REASON AND REMEDY
(1) Sections scored (or cut) vertically.	(*a*) Knife edge is damaged (has small nicks in it) and needs honing. (*b*) Knife is dirty and needs cleaning.
(2) Sections curl or ' roll up.'	(*a*) Knife is blunt. (*b*) Tilt of knife is too great.
(3) Sections are alternately thick and thin, or each have thick and thin zones (' chatters ').	(*a*) Microtome adjusting screws need tightening. (*b*) Tissue is very hard and needs treatment with a softening agent (page 132). (*c*) Tilt of knife is too great.
(4) Sections crumble on cutting.	(*a*) Knife is blunt. (*b*) Wax is too soft and needs ice applied to its cutting surface. (*c*) Wax is crystallized due to slow cooling, or contamination with water or clearing agent.
(5) Ribbon of sections curved.	(*a*) Block edges are not parallel to each other. (*b*) Block edges are not parallel to the knife edge.
(6) The breadth of sections is less than the breadth of the block and, consequently, creases cannot be removed without splitting the surrounding wax.	Caused by compression of the block; this is due to loss of bevel on the knife which needs to be honed to restore it.

Note.—Almost all the faults encountered in cutting paraffin sections are due to either: (*a*) a blunt or damaged knife edge; or (*b*) the block or knife are not being held sufficiently firm by the adjusting or locking screws. An experienced microtomist therefore avoids most of these faults by double checking both (*a*) and (*b*) before commencing the cutting of sections.

CUTTING OF CELLOIDIN EMBEDDED SECTION

The Wet Method

The vast majority of celloidin embedded sections are cut by the wet method and it should therefore be regarded as the standard technique.

CUTTING OF CELLOIDIN EMBEDDED SECTION

The sections are best cut on a sliding microtome *(see Figure 6.6)*, but the ordinary sledge microtome does almost as well, and a rotary microtome may be used, with a special attachment to hold the knife obliquely.

The block, knife, and sections have to be kept 'wet' throughout with 70 per cent alcohol. A camel-hair brush dipped in the alcohol is used to wet the knife and block between each section and to remove sections from the knife. If they are not to be stained immediately they should be put into a glass-stoppered or screw-capped bottle containing 70 per cent alcohol.

Technique

(1) The vulcanite or fibre block together with the embedded tissue (page 104) is fixed in the block-holder of the microtome.

(2) The feed mechanism on the microtome should be turned back as far as possible.

(3) A plano-concave (very concave) knife is fixed in the knife-holders with only a very slight tilt and set obliquely to the edge of the block (an angle of 30–40 degrees). The screws holding the knife are tightened to clamp it securely.

(4) Move the block-holder, or knife, depending on the type of microtome used, and adjust the feed mechanism until the celloidin block is almost touching the knife. With the adjustments on the microtome, ensure that the whole surface of the block will move parallel to the cutting edge of the knife; thoroughly tighten the adjustments.

(5) Set the section-thickness gauge at about 15 microns. By means of the camel-hair brush flood the surface of the block and the knife with 70 per cent alcohol and operate the microtome until complete sections of the tissue are being cut.

(6) Adjust the section-thickness gauge to an appropriate thickness; celloidin embedded sections cannot be cut as thinly as paraffin wax embedded sections, and although with care sections of 8–10 microns can be cut, for routine work the gauge should be set at 12–15 microns.

(7) Flood the surface of the block and the knife with 70 per cent alcohol and operate the microtome slowly; the section so cut will slide up on the knife blade. Some workers prefer to use a jerking action to cut celloidin embedded sections, while others find that better sections are produced by cutting halfway through the block, moving the knife or block back a little, then going forward to complete the cutting of the section. The writer has found a slow smooth action the better, but if any difficulty is encountered either or both of the other techniques are employed.

(8) The section is removed from the knife either with the brush,

the finger, or with a pair of fine forceps, and placed in 70 per cent alcohol in the prepared container. An alternative method, particularly useful for serial sections, is to prepare beforehand small squares or oblongs of tissue paper (toilet paper is quite good) a little larger than the sections, numbered with Indian ink if serial sections are required (every tenth piece numbered will suffice); when such a piece of paper is dropped on to the wet section it will adhere to it, and the paper is placed with forceps in the container where subsequent sections will form a pile, all in order. The sections are easily detached from the paper in 70 per cent alcohol.

The Dry Method (see page 108*)*

Sections are cut as described in the previous section, but without keeping the block and the knife wet with alcohol. The sections must, however, be stored in 70 per cent alcohol.

Serial Sections

Ribbons of celloidin embedded sections cannot be cut, therefore successive sections must be cut and stored in order of cutting; this is best done as detailed above using tissue paper; an alternative method is to mark the number of the section each time on the face of the block with Indian ink, after wiping it free of alcohol. The method of staining celloidin serial sections is given on page 202.

Fixing Celloidin Embedded Sections On Slides

Celloidin embedded sections are generally, and more satisfactorily, stained by carrying them loose through the staining reagents, and attaching them to slides immediately before mounting. Should a method of staining on the slide be preferred a section may be attached in the following manner:

(1) Transfer the section from 70 to 95 per cent alcohol for 1–2 minutes.

(2) Float it on a slide, and orientate in the correct position, allowing to drain for a few seconds to remove the excess alcohol; if necessary blot lightly to flatten the section, but do not let the section dry.

(3) Pour ether vapour over the sections, taking care that only vapour is poured and not the liquid ether. This will partially dissolve the celloidin and cause it to adhere to the slide.

(4) Place the slide in 80 per cent alcohol for 5 minutes to harden the celloidin, and finally in running tap-water for 10 minutes. The section is now ready for staining.

FROZEN SECTIONS

Tissue may be frozen (embedded in ice) and sections cut on a freezing microtome. This technique is useful for two special purposes: (1) the demonstration of fats, lipids, and special tissue components; and (2) for the rapid preparation of sections for diagnostic purposes.

Fats are removed from tissue during the normal process of paraffin or celloidin embedding, but not by the frozen section technique. There is usually no urgency for either paraffin or celloidin embedded sections and tissue may be fixed for the optimal time.

The preparation of a routine paraffin or celloidin embedded section (using an automatic tissue processer) takes about 24 hours, and the shortest time in which it can possibly be prepared (*see* page 106) is about 1½–2 hours. If the tissue is frozen and sectioned while supported by the ice, sections may be cut and stained and ready for examination within 5–15 minutes of receipt in the laboratory. These sections are not as satisfactory as paraffin embedded sections for critical examination, but when examined by an experienced observer they enable a rapid diagnosis to be made.

Fixation

Formol Saline

If the utmost speed is required it is possible to cut sections of frozen unfixed tissue, and in some institutes biopsies from the operating theatre for immediate diagnosis are cut unfixed as a routine. A short period of fixation before freezing, however, makes section cutting easier and usually yields better sections; this is particularly so in the case of tissue containing much fat or mucin, of which it is difficult to cut good frozen sections even when properly fixed.

Small pieces of such tissue should be immersed into preheated 10 per cent formol saline for 10 minutes at 60°C, which will have a hardening and stabilizing effect on them. Another technique which the writer has seen used with success is to drop small pieces of tissue in boiling formol saline, leave for 5 minutes, then section in the usual way. The former technique is preferred for any rapid frozen section if time will allow.

The fixative of choice for frozen sections is 10 per cent formol

saline; it gives a consistency to the tissue which is ideal for this technique, and does not interfere with its rapid freezing. Some text-books advocate the washing of formalin-fixed tissue in running water before sectioning, but the writer has not found this to be necessary; there appears to be no difference between comparable sections from washed and unwashed formalin-fixed tissue. Tissue which has been fixed routinely in formol saline may be transferred directly to the stage of a freezing microtome.

Fixatives Containing Mercuric Chloride

These tend to make sectioning difficult because of their over-hardening effect. Thin tissue so fixed should be put into iodine alcohol for 30 minutes, followed by sodium thiosulphate for 15 minutes, then washed in running water for several hours.

Chrome-osmium Fixatives

These tend to over-harden the tissue, and generally make sectioning almost impossible. Such tissue should be washed overnight in water, and soaked in gum syrup solution for 2–3 hours before sectioning.

Alcohol-containing Fixatives

Alcohol inhibits freezing and tissue so fixed must, therefore, be well washed – overnight if possible – before sectioning.

Friable Sections

Friable material should be embedded in gelatin (page 101).

Gum Syrup

Immersing the tissue in a thick aqueous solution of gum arabic or gum syrup for 1–12 hours prior to cutting is thought by some workers to make sectioning easier; when such tissue is frozen, instead of solid ice forming in and around the tissue, the gum imparts a hard, rubbery consistency. It is recommended that tissue be pre-treated with gum syrup for one minute, and that it be used on the microtome stage to support the tissue; the tissue is slightly easier to cut than when using water. It should be remembered, however, that many institutes use water routinely to get first-class results.

Cutting of Sections

(1) Set up the microtome and CO_2 cylinder as described on page 124.

(2) Check the connexions between the CO_2 cylinder and the microtome, and ensure that there is no leak. Open the cylinder valve and lift the CO_2 operating lever on the microtome to ensure the gas is flowing freely.

(3) If gum syrup is to be used, apply a coating with a brush to the microtome stage and place the tissue in the desired position; some workers prefer the knife edge to cut obliquely (from one corner of the tissue), but unless the tissue is difficult to cut this is not of as much importance as in celloidin section cutting. A piece of filter paper, soaked in water, should be laid on the stage to hold the tissue in position during cutting.

(4) Clamp the knife in position and rack up the stage by means of the coarse adjustment at the bottom of the microtome until the upper surface of the tissue is almost level with the edge of the knife.

(5) Lift the CO_2 control lever for short bursts of 1–2 seconds with a pause of 3–4 seconds between each; when the gum or water on the stage is frozen (it turns white) apply more gum or water with the brush and build it up around the side of the tissue. Using gum syrup, this building-up process is continued until the tissue is completely surrounded to a thickness of $\frac{1}{8}$ inch, but with water it is advisable only to build a base to support the tissue during cutting. The brush should not be allowed to touch the frozen material or it will adhere. Continue freezing until the tissue is quite hard.

(6) Ensuring that the knife is well clear, rub the top surface of the tissue with the finger until it has a firm rubbery consistency – this must be judged in the light of experience. Set the section-thickness gauge at 20 microns and operate the microtome until complete sections are being cut.

(7) Set the section-thickness gauge at the appropriate mark; for routine diagnosis sections may be cut at 8–10 microns, while for fat staining 15 microns is the normal setting.

(8) In operating the microtome some workers prefer to catapult the sections forward with a jerky cutting action, and to catch them in a bowl of water held down on the front left of the microtome; others, with the tissue a little softer, collect the sections from the blade of the knife with a wet brush, or finger, and put them into a jar of water.

Fixing Sections on Slides

While many techniques are carried out by floating frozen sections through the various reagents and floating on slides immediately before

mounting, for rapid diagnosis and routine haematoxylin and eosin staining, the mounting of sections on slides before staining is recommended.

Floating on Slides

Floating on slides may be done by orientating the section in a dish of clean water, inserting a slide (clean or albuminized) beneath it and slowly withdrawing the slide even if a few creases are present (the section must not be twisted). Allow the excess water to drain off, then put the slide back in the water so that only one half of the section is submerged or floating; the creases are then easily removed and the slide taken out. The excess water is drained off, the slide reversed and the other half of the section is freed of creases.

An alternative method is to transfer sections with a glass hockey stick (a thin glass rod, the last 2 cm of which have been bent at right angles) to a small container of 70 per cent alcohol for a few seconds, and then transfer them to the dish of water. The difference in surface tension between the two fluids will cause the section to flatten on the surface of the water for a few seconds (after which it slowly sinks), when it may be quickly transferred to the surface of a clean slide.

A method of floating and fixing frozen sections on slides for rapid diagnosis has been devised by J. F. Wilson (personal communication) and is as follows.

(1) Pieces of fine filter paper are cut slightly larger than the sections to be mounted.

(2) The sections are placed in 70 per cent alcohol for a few seconds and then transferred to a dish of clean water. The dish must be at least 9 inches in diameter otherwise the filter paper will sink.

(3) The sections will flatten on the surface of the water, and if any creases are present they should be put back to the 70 per cent alcohol for a few seconds and then returned to the water.

(4) With a pair of fine forceps lightly place a filter paper square or oblong on the section, and hold a starched or albuminized slide (page 131) in the water close to the section. By a gentle pushing motion the section with its filter paper covering is brought in contact with the slide, which is then withdrawn. The filter paper and the section should, of course, be in the centre of the slide. Ensure that the section is beneath the paper, it occasionally sinks while the paper is still floating.

(5) The slide is then blotted firmly between filter paper and held over a Bunsen flame for a second; when the filter paper

becomes white it is easily removed from the slide without damaging the section. Although such heating is theoretically bad it has not, using formol-fixed tissue, produced any gross artefacts. Sections so fixed to slides are very secure and will withstand quite rigorous treatment.

For rapid sections the writer recommends this technique for beginners as well as for advanced technologists as one which, with little practice, is simple, effective and rapid.

Attachment to Slides

There are three basic methods of fixing frozen sections to slides in addition to the one described above. These involve using: (1) albuminized or starched slides; (2) gelatinized slides; and (3) celloidinization.

Albuminized or Starched Slides

Sections are floated on to prepared slides (page 131) and the excess water is drained off without allowing the section to dry. It is then blotted gently but firmly with fine filter paper and a mixture of equal parts of clove and aniline oil is poured on the slide and allowed to remain for 3 minutes to coagulate the albumin. Xylol is then poured on the slide, followed by alcohol, and the section may be stained in the usual way.

Gelatinized Slides

These are clean, grease-free slides having a thin layer of glycerin jelly (page 180) spread on the surface; the jelly is melted in hot water, a drop placed on one end of the slide and spread over the whole surface with the edge of another slide. Such slides should be dried in an incubator and stored in a dust-free container. In use the section is floated on to a prepared slide and the excess water is drained off before being placed into a covered glass container (Coplin jar), at the bottom of which is put a piece of cotton-wool soaked in formalin. The formalin converts the gelatin to an irreversible gel which holds the section in position. After 30 minutes the section is washed in running water for 10 minutes and is then ready for staining.

Celloidinization

The section, having been floated on to a clean, grease-free slide, is drained of excess water and blotted gently but firmly with fine filter paper. Fresh filter paper is then laid on the section to which is added a few drops of alcohol, and the section blotted with the alcohol-soaked paper. The slide is then transferred to a container of 1 per cent celloidin, left for 5 minutes and, after wiping the back, transferred to 70 per cent alcohol for 10 minutes to harden the celloidin.

The section is then ready for staining, the times of which should be increased slightly. After staining, the celloidin should be removed by immersion in equal parts of alcohol and ether, or by blotting with alcohol-soaked filter paper. The latter is more rapid, but there is a risk in that the section may adhere to the blotting paper.

Frosted Slides

A new slide, marketed in Vancouver by Becton-Dickinson and called Gold Seal Cytology Microslides, has been tested by us and seems to be very useful for cytology smears. We have also used these slides for cryostat sections, which do not detach during subsequent staining; furthermore, we have found them to be of great use with paraffin sections in histochemistry when employing a series (sometimes as many as 7) of blocking techniques, for example, methylation and saponification. Problems with the slides are the retention of stain, and of water in the crevices, but an extra dip in alcohol and xylol usually overcomes these problems. It should be noted that sections stored (mounted on the slides) show the apparent effect of alkali upon them, which may modify subsequent staining reactions. Stored sections of colon will give an increased P.A.S. reactivity (*see* K.O.H./P.A.S. technique on page 286).

CRYOSTAT CUT SECTIONS

The introduction of fluorescent antibody staining techniques by Coons, Creech and Jones in 1941 led to a need for thin sections (3–5 microns) of fresh frozen tissue free of ice crystal defects.

To satisfy these criteria such tissue must be quick frozen at a very low temperature (**Quenching** *see* page 55), and sections cut without allowing the tissue to thaw.

In addition to being suitable for fluorescent antibody staining, such sections are ideal for many histochemical techniques, particularly enzyme methods.

Types of Apparatus Available

Harris Microtome Cryostat

Linderstrom-Lang and Mogensen designed the first cryostat in 1938. Coons and his colleagues redesigned it in 1951 when it was produced commercially by the Harris Refrigeration Company*. It consists of a microtome of any type but preferably rustproof, which is enclosed and operated within a deep freeze cabinet. The cabinet is fitted with a

Figure 6.9 – Harris Microtome Cryostat

double glass window, and a door through which material may be passed in and out. *Figure 6.9* shows a later model, which was in use successfully in the author's laboratory from 1958–65.

The cabinet is equipped with a fluorescent light and a fan to ensure

*Harris Manufacturing Company, Inc., Cambridge, Mass.

the circulation of cold air, the latter being controlled by a foot switch to disengage it during the actual cutting of sections.

The temperature may be regulated between $-10°C$ and $-40°C$.

We have used a conventional Spencer rotary microtome, oiled with a special low temperature oil (Shell 'Tellus Oil 15'). The machine was defrosted every 4–5 months, rusting of parts was minimal and did not prove to be a practical disadvantage.

Figure 6.10 – International Harris Microtome

International Harris Microtome

This latest model* *(Figure 6.10)* permits operation of the microtome through an open top giving direct control over the cutting operation.

*Available from International Equipment Ltd., Boston, Mass., or A. Gallenkamp & Co. Ltd., London, E.C.2.

This unit operates on the 'open top–cold box' principle, and the temperature remains constant with the cover open or closed.

The microtome supplied with the unit is an International Minot rotary which is completely rust proof. It is mounted at an angle of 45 degrees which makes for easy manipulation of sections during cutting *(Figure 6.11)*. This model we now use routinely.

Any cryostat can be used as an alternative to a freezing microtome

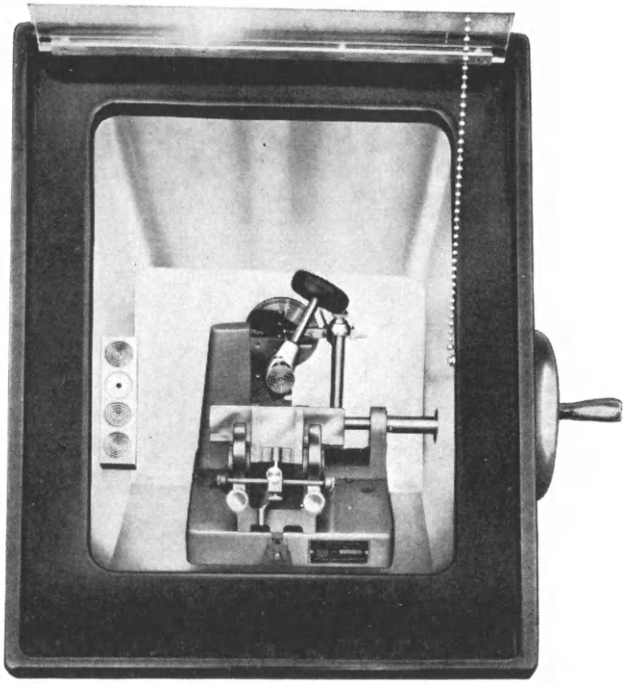


Figure 6.11 – Harris International Cryostat, shown with top open ready for use. Note the 'quick freezing block' on left-hand side

for rapid sectioning (Nummally and Abbott, 1961). The International has been designed for this purpose by incorporating an internal Freon quick freeze stage. This stage holds four block holders which, after the tissue is frozen on them, are transferred to the microtome. Because the sections adhere firmly to warm slides, staining and mounting may be carried out more rapidly.

The cabinet temperature may be varied between $-10^{\circ}C$ and $-20^{\circ}C$. The microtome may be adjusted to cut sections from 2 to 16 microns, in 2 micron steps.

The Pearse–Slee Cryostat

This cryostat was designed by Everson-Pearse and is produced commercially by the South London Electrical Equipment Company Limited*. It consists of a deep freeze cabinet in which a standard Cambridge rocking microtome is installed, all controls of which may be operated externally. There is available a stainless steel dry ice box for attachment to Heiffor pattern knives to give knife temperatures between $-30^{\circ}C$ and $-60^{\circ}C$ for thin serial sections; except for sections of skin the author has not found this necessary for serial sections at 5 microns.

This cryostat, like other models, may be used for rapid diagnosis of biopsies.

Operation of the Cryostat

The operation of a cryostat calls for only a little practice by a competent histopathological technician. It is most important that a very sharp knife is used, as the majority of difficulties encountered in cutting are due to the knife not being sufficiently sharp.

Attachment of Tissues

Tissues for rapid diagnosis are usually frozen directly on to a metal block holder. Unattached frozen tissue is attached as follows: a small piece of filter paper is placed on the surface of the block holder, a drop of water placed on it with a Pasteur pipette, followed by the tissue. The water freezes almost instantly holding the tissue firmly in place.

Temperature of Cabinet

For the cutting of thin sections of most tissues this should be kept at $-15^{\circ}C$ and $-20^{\circ}C$. Thick sections (30–40 microns) of brain, and so on, will tend to crumble unless the temperature is raised to $-5^{\circ}C$.

*Hither Green Lane, London, or Allied Scientific Co., Toronto 12, Ontario.

Cutting

The knife is placed in the cabinet at least 15 minutes before cutting is commenced.

Sections may be cut using an antiroll device, which is available commercially, the section as it is cut remaining flat between the coverslip (glass or plastic) and knife *(Figure 6.11)* The adjustment of these devices is critical, but properly adjusted they give good flat sections. The top of the plastic coverslip should project 0.5–1.0 mm above the cutting edge of the knife. When the section is cut, the antiroll device is swung back and the section picked up. The author has found a camel hair brush a useful alternative to this device, particularly when cutting a variety of tissues. The brush is used to prevent the section from curling at the beginning of the cutting stroke, it then holds the section flat during the rest of the cutting stroke.

Handling of Sections

Sections may be attached directly to warm slides or coverslips (at room temperature) by simply touching the slide (or coverslip) against the section. When the section is air dried, it will remain on the slide throughout most staining techniques. Coverslips may be held by a suction device, a rubber suction disc connected through a metal or plastic tube with a rubber bulb; a light touch on the bulb being sufficient to pick up or release the coverslip.

Sections may be placed directly into a substrate, or into a fixative (cold or warm ether/alcohol for fluorescence technique). Slides with attached sections may be fixed either before or after air drying.

For rapid diagnosis, sections are usually air dried, fixed for one minute in acetic alcohol (Clarke's fluid page 50) and stained with Polychrome methylene blue or any of the techniques detailed on page 206. This technique results in a section which is superior in most respects to that produced by the standard freezing technique.

For fluorescent antibody techniques sections are used either directly after air drying on slides or following post fixation in 95 per cent alcohol after air drying.

Chapter 7

Theory of Staining

The object of staining tissue sections is to impart colour and therefore contrast to specific tissue constituents. Consequently, it is important to understand why a dye molecule appears a certain colour and why it becomes attached to a specific site.

Colour is seen by the eye as a result of the effect of certain electromagnetic waves on the rods and cones of the retina. These waves, which have a varying length, will determine the colour that is seen. White light being composed of all the colours of the visible spectrum varies in wavelength from 4,000 Å to 8,000 Å *(see Figure 32.2)*

If light of a specific wavelength is absorbed from white light the resultant light will then be coloured, the colour being dependent upon the particular wavelength that has been removed. Colour filters function in this manner by the absorption of specific wavelengths (or colours), as can be seen in *Table 7.1.*

Stained materials act in a similar manner to filters in that, by virtue of their resonance systems, they absorb certain components of the white light illuminating the specimen and therefore appear coloured.

The theory of staining is a very complex subject and is not fully understood. It is not surprising, therefore, that many theories have been put forward that will explain all the facts as we know them. It is not intended to enter here into a full discussion of the subject, and only a very brief review will be given of the basic theories. Those interested in the subject are recommended to read the paper by Marcus Singer (1952) and Conn (1961).

Physical Theories

All staining reactions as we know them at present may be explained by the physical theories that have been advanced, either separately or

together. These theories depend on: (1) simple solubility; and (2) adsorption.

TABLE 7.1

Colour of Filter	*Absorbs*	*Transmits*
Yellow	Blue	Green and red
Blue-green (Cyan)	Red	Green and blue
Blue-red (Magenta)	Green-yellow	Blue and red

Solubility

Examples of this method of staining are the fat stains which are effective simply because the stain is more soluble in the fat than in the 70 per cent alcohol or other solvent in which it may be dissolved. That this may be applicable to other stains, although less obviously, is possible but not very probable according to the weight of informed opinion.

Adsorption

This is the property by which a large body attracts to itself minute particles from a surrounding medium, and is a phenomenon well known to chemists. Since this action may be selective under certain circumstances (for example, it is affected by the pH of both substances), it seems to be the most likely physical explanation of the actions of stains as we at present know them. Bayliss (1906) developed this particular theory and called it an electrical theory of staining.

Chemical Theories

The early workers in the field of staining held the theory that an actual chemical combination of dye and tissue component takes place. While it is generally true that acid dyes stain basic elements (cytoplasm) and basic dyes stain acidophil material (nucleus), this is far from being the complete truth: indeed haematoxylin, which is an acid dye, does

not stain the cytoplasm, but (in the presence of a mordant) is one of the most widely used nuclear stains. The point made in the previous edition that, if staining were purely a chemical reaction it would be expected to continue until one of the reagents were exhausted, yet no matter how dilute a stain is used the solution is never completely decolorized by the tissue (Conn, 1946), is not a good argument against chemical action. In fact, many chemical reactions take place which stop before either component is exhausted, when it is assumed that equilibrium has been reached. Further, since tissues take up relatively larger quantities of dye from dilute than from concentrated solutions would be an argument in favour of a chemical reaction.

The specific information we have on the binding of dyes to tissue components reinforces the opinion of Mann in 1902 that 'dyes and tissue molecules can adhere chemically to one another by their side-chains'.

Most dyes are classified, and react as either acid or basic dyes and combine with basic or acid tissue components respectively. Furthermore, their action is modified by the pH of the solution in which they are employed since although the nucleus may be considered as an acid tissue component (and cytoplasm as basic) they both exhibit amphoteric characteristics (*see* page 225).

The critical nature of the pH of the dye bath may be seen, and usefully employed, in the differentiation of CooH and sulphate groups using aqueous alcian blue at pH 1.0 and pH 2.5, since below pH 2.5 the ionization of the CooH groups is suppressed; and it has been shown that at pH 1.0, only the sulphate groups are stained (*see* page 275).

Therefore, staining should be considered as being functionally, if not completely, chemical in nature.

STAINS AND DYES

The early microscopists suffered a tremendous handicap because they worked before the use of dyes in biology had been discovered; nowadays the majority of laboratory workers would be at a complete loss without the very large range of biological stains and reagents that is available.

Leeuwenhoek was probably the first person to use a dye as a biological stain when, in 1714, he applied saffron (crocus in wine) to muscles. Carmine was introduced by Goppert and Cohn in 1849, and haematoxylin by Waldeyer in 1863; but the major advance was the introduction of aniline dyes in 1856, which were at that time being

used in the textile industry. In 1891, Heidenhain devised the iron haematoxylin technique which is still in common use today.

Biological stains are basically no different from commercial dyes, but they have been adapted for use in biology. Special care and greater control is used in their manufacture, and generally they are purer. On occasions, difficulty has been experienced from using too pure a form since one of the apparent impurities has, in fact, acted as a mordant and therefore been a necessary component. Laboratory workers who remember the ease and regularity of first-class staining obtainable with Grübler's dyes before World War I have been heard to say that it was not just that he supplied the best dyes, but that he included the best impurities.

METHODS OF USE

Before discussing the chemistry of various stains and reagents and their uses in demonstrating tissue elements, it is important to define the various methods of employing them.

Impregnation

Impregnation is the deposition of the salts of heavy metals over certain selective cell and tissue structures and processes. It cannot be classified as staining, having the following main characteristics:

(1) Structures so demonstrated are usually rendered *opaque,* and black.

(2) The colouring matter is *particulate.*

(3) The deposit is *on* or *around* but not *in* the element so demonstrated.

In spite of these differences the wide use of impregnation, either alone or in combination with staining techniques, is sufficient reason for its inclusion in a chapter devoted mainly to staining.

Staining

An element may be broadly defined as stained when, following treatment with a reagent or series of reagents, it acquires colour; usually, no particles of dye are seen and the stained element is transparent.

Staining techniques may be sub-divided as follows.

Vital staining. – This involves the staining of structures in living cells, either in the body *(in vivo)* or in a preparation *(in vitro)*–for

example, Janus green is taken up by living cells and stains the mitochondria.

Vital staining by phagocytosis. – Particles of coloured matter are taken up, by phagocytic cells, from colloidal suspensions. These particles are seen in the cytoplasm, but do not demonstrate specific structures (for example, recticulo-endothelial cells take up trypan blue or Indian ink). This type is included here for convenience; it is not truly staining as defined above.

Histochemical reactions. – These are predictable reactions which indicate the chemical composition of elements, the reaction sometimes taking place between a colourless fluid and the tissue (for example, Feulgen reaction for nucleic acid).

Fat stains. – In these the colouring agent is more soluble in the element to be demonstrated than in the vehicle (or solvent) in which it is applied (for example, Sudan III for neutral fats).

Histological stains. – These stain killed or other non-living tissue elements by methods that may appear to be specific for a particular tissue element, or group of elements, but where the mechanism of staining is not understood.

PRINCIPLES OF DYE CHEMISTRY

Although the bulk of the earlier dyes adapted to biology were aniline dyes, this name has now been applied to a much larger group which has no real connexion with aniline but are also derivatives of coal tar; these should be called by the more correct title of 'coal-tar dyes'.

All coal-tar dyes are derivatives of benzene, and a brief study of the structure of this compound assists greatly in understanding modern dye chemistry.

Benzene

The benzene molecule is formed by six carbon atoms and six hydrogen atomes which are so arranged that the carbons form a ring, and each carbon atom has a hydrogen atom attached to a free valency *(Figure 7.1a)* For convenience the benzene ring is usually drawn as shown in *Figure 7.1b.* As a carbon atom is considered to have four valency bonds, the hexagon arrangement will allow each carbon atom to have a double bond on one side, and a single bond on the other (that

is to say, alternate single and double bonds). When two of the hydrogen atoms are replaced by oxygen *(Figure 7.1c)* a readjustment of the double bonds takes place and a new compound, quinone ($C_6H_4O_2$), is formed, compounds from which are called quinoid compounds. The change

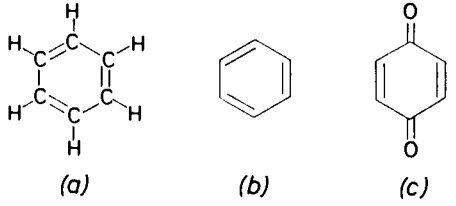


Figure 7.1 – Hydrocarbon benzene: (a) showing hydrogen atom attached to each free valency; (b) as usually illustrated; (c) quinone

from benzene to quinone is of importance, for whereas benzene derivatives absorb light only in the ultra-violet band and are colourless, quinoid compounds have absorption bands in the visible spectrum and are coloured.

Chromophores

Chemical structures that absorb certain wavelengths of light and therefore confer colour are known as chromophores. Chromophoric groups are usually a linear sequence of atoms along which there is an uninterrupted alternation of double and single bonds. The linear sequence may be simple, branched or follow a closed ring (*see* quinone, above). Such a sequence is called a resonance system. The longer this system the longer the wavelength at which maximum absorption occurs. The quinoid arrangement *(Figure 7.1c)* is a chromophore and the presence of one quinoid ring in a compound containing several rings will confer the property of colour on that compound. The nitro group (NO_2) is also a chromophore, and when three nitro groups replace three hydrogen atoms in a benzene molecule *(Figure 7.2a)* the result will be trinitrobenzene, which is yellow. Such a compound, containing a chromophoric group, is called a chromogen. These chromogens, although coloured, are not dyes since they have no affinity for cloth fibres or tissue constituents, and although such material placed in a chromogen may appear coloured by the substance it will be easily and quickly removed.

Auxochromes

To be a dye, a compound must contain, in addition to a chromophoric group, another group which gives to the new compound the power of electrolytic dissociation (a salt-forming property); such groups are known as auxochromes. If to the chromogen trinitrobenzene *(Figure 7.2a)* is added an hydroxyl group (OH) which is an auxochrome, the chromogen will be converted to a dye—picric acid *(Figure 7.2b).* This is capable of forming a salt with a base and has an affinity for fibres and certain tissue constituents from which it is not easily removed.

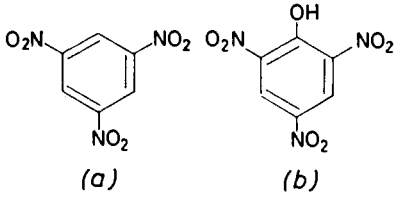


Figure 7.2 – (a) Trinitrobenzene; (b) picric acid

There are several auxochromic groups; some are acidic, such as the hydroxyl group, others are basic, such as the amino group (NH_2), and there are in addition a few chromophoric groups which may be slightly auxochromic in nature.

The Sulphonic Group (SO_3H)

The sulphonic group is important in dye chemistry because it forms acid salts. It is only slightly auxochromic and is used principally either to make an insoluble dye soluble in water, or to convert an otherwise basic dye into an acid dye. For example, basic fuchsin is converted to acid fuchsin by the introduction of a sulphonic group.

Leuco Compounds

The conversion of a dye into a colourless compound (leuco compound) is of importance in the modern laboratory, and an understanding of this conversion is, therefore, useful.

Although the chromophoric groups differ in many respects, they share the property of being easily reduced; that is, they have unsatisfied affinities for hydrogen; for example, the double bonds in the quinoid ring may break and the valencies become attached to hydrogen. When the chromophore is destroyed the compound loses its colour and the prefix leuco is applied to it; for example, leuco-fuchsin.

Certain dyes become colourless on removal of their acid radicle, as in the case of the triphenyl methanes (basic fuchsin) and the xanthenes (phenolphthalein) and, since this reaction is normally freely reversible, such dyes are useful indicators of the hydrogen ion concentrations (pH).

CLASSIFICATION OF DYES

Dyes are classified not, as might be imagined, by their colours, but by the chromophoric group present. The important groups are listed below, but the keen student of dye chemistry is recommended to read *Biological Stains* by H.J. Conn which gives a comprehensive survey of the chemistry, synonyms and uses of dyes.

(1) The nitroso group.
(2) The nitro group.
(3) The azo group.
(4) The thiazol group
(5) The anthraquinone group.
(6) The quinone-imine group.
(7) The phenyl-methane group.
(8) The xanthene group.

Natural Dyes

Although the great majority of dyes in use today are synthetic there are still a few natural dyes in use.

The natural ones in present-day use are: cochineal (carmine), orcein and litmus, and haematoxylin. Of this group the most important is haematoxylin and this will be dealt with in some detail. Indigo, another natural dye, is little used in modern techniques.

Carmine

Carmine, used in many routine techniques, is produced from cochineal after treatment with alum, the cochineal being extracted from the female cochineal insect *(Coccus cacti).* Carmine is only

slightly soluble in water of neutral pH, and is employed in strongly acid or alkaline solutions. It is of little use alone and must be employed with the salt of a metal as a mordant (page 161), unless being used to demonstrate tissues containing a metal.

Orcein and Litmus

Extracted from lichens, orcein and litmus are still commonly used, litmus as an indicator and orcein as a specific stain for elastic tissue.

Haematoxylin

This is, even today, the most commonly used stain in the histological laboratory. It is a constituent of the heart wood of the logwood tree *(Haematoxylon campechianum)* which grows in Campeche, Mexico.

Haematoxylin as supplied has no staining properties until it has been 'ripened' by oxidation into haematein. This ripening or oxidation of haematoxylin is achieved either by exposure of prepared solutions to the air for periods of up to 6–8 weeks, preferably in the sunlight or, alternatively, by the addition of an oxidizing agent such as sodium iodate, potassium permanganate or mercuric oxide. In this ripening process haematoxylin ($C_{16}H_{14}O_6$) loses two hydrogen atoms to become haematein ($C_{16}H_{12}O_6$), which has a quinoid arrangement in one of the rings (the lower ring in *Figure 7.3*).

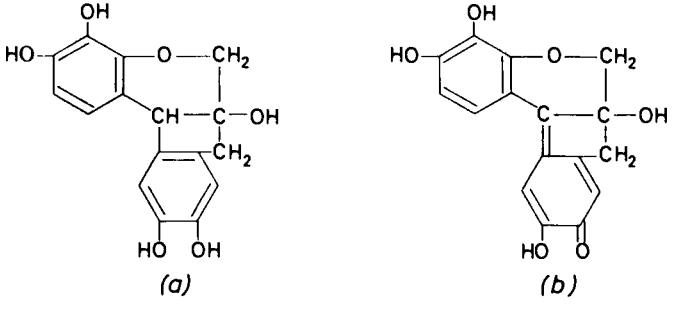


Figure 7.3 – (a) Haematoxylin; (b) haematein

Continued oxidation of haematein will result in the formation of a colourless compound, and this is one reason why some workers prefer the slow and gradual natural process of oxidation to the chemical

method. The latter coverts all haematoxylin to haematein immediately, and further natural oxidation produces the colourless compound, giving staining solutions so prepared a shorter life.

Even ripened haematoxylin is seldom used alone since it has no affinity for tissue structures. Except when being used to demonstrate a metal a mordant must be used (page 161). Such mordants are salts of metals which are used prior to the staining solution, or incorporated with it.

It is rarely appreciated how many tissue and cell elements may be demonstrated with this stain by the use of different mordants; some of these are detailed below.

Element	*Mordant (metal)*
Nuclei	Aluminium, iron, tungsten
Myelin sheaths	Chromium, copper, iron
Elastic fibres	Iron and iodine
Collagen	Molybdenum
Fibroglia, myoglia, neuroglia and epithelial fibrils	Tungsten
Axis cylinders	Lead
Mucin	Aluminuim
Fibrin	Tungsten
Mitochondria	Iron

Acid, Basic and Neutral Dyes

All ordinary stains are salts; they are composed of an acid and a base.

Acid Dyes

In these, the acid component is coloured and the base colourless; for example, acid fuchsin is composed of sodium, which is colourless, combined with rosaniline trisulphonic acid, which is coloured.

Acid dyes usually stain basic components, such as cytoplasm.

Basic Dyes

In these the base contains the colouring substance combined with a colourless acid; for example, basic fuchsin is composed of the base rosaniline, which is coloured, combined with an acid radicle Cl, which is colourless.

Basic dyes usually stain acid components, such as nuclei.

Neutral Dyes

The first neutral dyes were prepared by mixing methylene blue and eosin to make a one-step for blood films. Effectively, what followed was a combination of the coloured acid and basic radicles of the two dyestuffs which resulted in a precipitate of coloured material with the uncoloured acid and basic radicles being combined in a virtually colourless solution. This precipitate which was water insoluble was found to give the best results when dissolved in methyl alcohol and was the forerunner of todays Romanowsky stains (Wright's or Leishman's).

Since both the acid and basic radicles are coloured, it is a neutral stain, and since certain tissue elements have an affinity for the composite neutral stain these structures are termed 'neutrophilic'.

It is well known today that only modified methylene blue and eosin will combine in this fashion and they are known as 'azure eosin' and 'azure methylene blue'.

MORDANTS

If sections of tissue are put directly into simple aqueous or alcoholic solutions of certain dyes, such as the coal-tar derivatives, they become stained. Such staining is known as simple or 'direct' staining *(Figure 7.4a).* These stains, after washing in water or alcohol, may even be selective; for example, methylene blue will stain the nucleus and cytoplasm of a cell in different shades of blue.

Other stains, particularly haematoxylin, will not stain tissue in a simple solution, but only when a third element is present. This third element, which is known as a mordant, forms a link between the tissue and the stain. The combination of the dye and the mordant forms a compound which is sometimes called a 'lake', which is then capable of attaching itself firmly to the tissue *(Figure 7.4c).* Some lakes are unstable, and with such compounds tissue may be soaked in the mordant immediately before treatment with the stain, or even afterwards; for example, Heidenhain's iron haematoxylin stain and Gram's stain respectively.

The term mordant is only strictly applicable to salts and hydroxides of di-valent and tri-valent metals, and should not be used to indicate any substance that improves or aids staining in some other manner (*Figure 7.4b,* accentuators and accelerators). The mordants most commonly used in histological technique are salts, usually sulphates, of chromium, aluminium and iron. Generally double sulphates or alums are used.

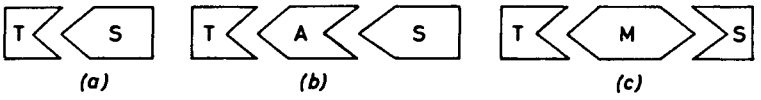


Figure 7.4 – Diagrammatic representation of types of staining: (a) simple staining, with direct union between stain and tissue; (b) use of accelerators, or accentuators, illustrating the point that while a union between tissue and stain as above is possible, a larger compound (to represent increased staining power or specificity) may be formed by use of a third element; (c) use of mordants, illustrating that a union between tissue and stain is not possible without the presence of a third element

ACCENTUATORS

Accentuators and accelerators were the names given to a group of substances which, while not acting as mordants and forming lakes with the dyes, or taking part in any obvious chemical union, increase the selectivity or staining power of the dyes which were already capable of staining without the accelerators. Common examples of accentuators are potassium hydroxide in Loeffler's methylene blue, and phenol in carbol thionin and carbol fuchsin. It will be obvious that the effect on staining of this group is due to the change in pH of the staining solution (*see* page 153).

Accentuators when used in the impregnation of the nervous system with metallic salts have been called *accelerators.* These are generally hypnotics but the reasons why this should be so is not understood; examples are veronal in Cajal's method for axis cylinders, and chloral hydrate in Cajal's method for motor end-plates *(Figure 7.4b).*

METACHROMASY

The majority of dyes stain tissues in differing degrees of intensity of the same colour; for example, acid fuchsin will stain tissue in varying shades of red, and light green in varying shades of green. There are, however, certain tissue components which in the presence of certain basic dyes of the coal-tar group will stain a colour other than that of the dye. Mucin will stain red with toluidine blue, while the rest of the tissue stains in shades of blue. Such a staining reaction is known as *metachromasy,* the tissue is said to exhibit *metachromasia*, and the dye is known as a *metachromatic* dye. Among the principal tissue components which exhibit metachromasia are mucin, cartilage and mast-cell granules. Everson Pearse (1968) defines metachromasia histochemically as 'the staining of a tissue component so that the absorption spectrum of the

resulting tissue-dye complex differs sufficiently from that of the original dye, and from its ordinary tissue complexes, to give a marked contrast in colour.' The most important metachromatic dyes are found in the thiazin group; for example, thionin, methylene blue and its azures, and toluidine blue. Methyl violet will stain amyloid deposits red and this is therefore considered to be metachromasia. Since, however, the methyl violet metachromasia of amyloid deposits depends on bonding of a different type from that associated with toluidine blue metachromasia of acid polysaccharides (Pearse, 1968), it should be considered in a separate category.

The explanation of metachromasia most generally accepted is that put foward by Michaelis, and supported by the work of Sylven. The change in colour of the dye is thought to be due to polymerization. Sylven has shown that the presence of high molecular weight substances with free anionic (acid) groups is essential. Sulphonate (SO_3H) groups are stronger chromotropes than are carboxyl (COOH) or phosphate (PO_4) groups. He introduced carboxyl groups into cellulose fibres and showed that metachromasy commences as soon as every second glucose unit in the chain is carboxylated. The introduction of one sulphate and one uronic group per disaccharide unit resulted in a stronger metachromasia. Kramer and Windrum (1954) have further shown that metachromasia could be induced in polysaccharides by sulphation, for example, treatment with sulphuric acid for 30–60 seconds. It would appear that a distance of less than 5Å between acid groups is necessary for weak metachromasia and an intercharge distance of less than 4Å together with the presence of sulphate groups for a strong stable metachromasia. The best example of the latter is mast cell granules, containing heparin which is highly sulphated.

The three types of metachromasia usually described are as follows.

Type	*Colour*	*Form of dye*
(alpha)	Blue	Monomeric (single molecules)
(beta)	Violet	Dimeric or trimeric (aggregates of 2 or 3 molecules) or a mixture of the alpha and beta forms
(gamma)	Red	Polymeric (long chain aggregates)

Sylven first stressed the necessity for the presence of water for appearance of metachromasia, it being postulated that a molecule of water was bound between each dye molecule in the polymer. It was

further thought that this fact explained why alcohol abolished metachromasia; however, Kramer and Windrum (1955) contended that alcohol never reverses metachromasia due to the presence of protein in an acid mucopolysaccharide-protein complex, and that the metachromasia of acid mucopolysaccharides (not in association with protein) is alcohol-labile. They also feel that the abolition of metachromasia by alcohol is not due to dehydration, since stained samples which had been dried *in vacuo* retained their metachromasia.

It should be remembered that the absence of metachromasia may be due to the fact that acid groups while present may be either bound and not free to react, or that they may not be present in sufficient density.

THE USE OF CONTROL SECTIONS IN THE IDENTIFICATION OF CHEMICAL STRUCTURES OR TISSUE ELEMENTS

It is sometimes possible to specifically remove or modify chemical groups, and/or tissue components from sections by chemical or enzymic action. For example, diastase removes glycogen; ribonuclease removes ribonucleic acid; methylation esterifies carboxyl groups (which renders them non-reactive to the stains employed for their demonstration) and removes sulphate groups; acetylation renders 1:2 glycols non-reactive to the P.A.S. reaction.

Such techniques are, in many cases, the only method available of specifically demonstrating the original presence of specific tissue entities. If the control method can be proven to be specific, it can be used in combination with a relatively non-specific technique as a 'histochemical procedure'.

If a section treated in this fashion is stained by a routine method in parallel with a similar but untreated control section, the absence of colour in an element in the treated section will serve to identify it in the control section. *It must be remembered that both sections should be treated as near identically as possible.*

Chapter 8

Preparation of Stains

The preparation of stains is a very important basic procedure in histological technique and therefore should not always be left to the most junior member of the staff. Careless preparation, such as inaccurate weighing, the absence of chemical cleanliness, and the use of tap-water instead of distilled water may all lead to great confusion at a later stage; and in those laboratories where a control section is not stained by each new batch of stain as a routine, it may lead to incorrect negative or even positive reactions. During the preparation of any stain or staining reagent the following basic rules should be rigidly adhered to:

(1) All glassware should be thoroughly cleaned and well rinsed in distilled water (of neutral pH) and dried. After drying, a clean cotton-wool plug should be inserted to prevent contamination by dust if the receptacle is not to be used immediately.

(2) The correct solvent should be used. Distilled water should be used unless tap-water is specified.

(3) Flasks and pipettes used for silver techniques should be marked and kept solely for this purpose.

(4) Silver and osmic acid solutions should always be kept in dark bottles, preferably in a cool dark place.

(5) Dilute ammonia used for staining solutions should preferably be freshly prepared, the stock of ammonia being kept in a refrigerator.

(6) Constituents of stains should be dissolved in the order given in the formulae; for example, haematoxylin should always be dissolved in alcohol before the remainder of the constituents are added.

(7) Alcoholic solutions of stains should be kept in glass-stoppered bottles or containers to avoid evaporation of the alcohol and consequent precipitation of the stain.

(8) All dyes used for the demonstration of bacteria should be filtered immediately before use.

SOLVENTS

Water

Water is the most common solvent for stains and, as noted above, distilled water should always be used unless the formula specifies tap-water. The distilled water should be checked periodically to ensure that it is at pH 7.

Ethyl Alcohol (Ethanol)

Alcohol in varying concentrations is a commonly used solvent. When 'alcohol' is specified it should be understood to mean absolute ethyl alcohol unless the context indicates otherwise. Substitutes for alcohol should not be used as a solvent for stains unless experiment has shown that they have no effect on the subsequent staining reaction.

Methyl Alcohol (Methanol)

Methyl alcohol, usually absolute, is used principally as a solvent for Romanowsky stains for which purpose, it is said, it must be free of acetone.

Acetone

Acetone is used alone or in combination with other fluids.

Phenol

Phenol is used as a 0.5–5.0 per cent aqueous solution.

Aniline Water

Aniline water is prepared by adding 5–10 ml of aniline oil to 500 ml of hot distilled water in a flask. This mixture is well shaken and allowed to cool on the bench. When cool it is filtered and is then ready for use. Although once popular as a solvent it is little used today.

TABLE 8.1
Solubility Chart

Solubilities, expressed as grammes per 100 millilitres of solvent, of the more common histological stains in water and ethyl alcohol at room temperature.

Stain	*Water*	*Alcohol*	*Stain*	*Water*	*Alcohol*
Acid fuchsin	18·0	0·3	Methyl violet 6B	4·2	6·2
Alizarin	nil	0·125	Methylene blue	2·5	1·5
Alizarin red S	5·3	0·15	Night blue	2·25	2·35
Auramine	0·35	7·0	Nile blue sulphate	1·0	1·0
Aurantia	1·3	0·3	Neutral red	3·2	2·0
Azo black	0·3	0·25	Oil red O *		
Basic fuchsin	0·4	7·6			
Bismarck brown	1·2	1·1	Orange G	7·1	0·3
Brilliant cresyl blue	2·2	0·5	Phloxine	39·4	8·0
Brilliant green	3·0	3·3	Picric acid	1·1	8·5
Carminic acid	8·3		Pyronin	11·0	
Congo red	4·5	0·8	Purpurin	nil	0·76
Crystal violet	1·5	7·0	Safranin	6·0	2·5
Eosin—water			Scarlet R	nil	0·2
soluble	40·5	3·5	Sudan black B	nil	0·23
Eosin—alcohol			Sudan II	nil	0·3
soluble	nil	0·45	Sudan III	nil	0·15
Erythrosin	11·0	2·0	Sudan III †		
Fluorescein	nil	2·1			
Gallamine blue	0·07		Sudan IV	nil	0·08
Haematoxylin	1·75	60·0	Tartrazine	11·0	0·13
Indigo carmine	1·1		Tartrazine ‡		
Janus green	5·3	1·1	Thionin	0·22	0·23
Jenner's stain		1·3	Toluidine blue	3·1	0·5
Light green	18·5	0·85	Trypan blue	10·4	
Martius yellow (Na)	4·7	0·16	Uranin (sodium		
Methyl green	9·2		fluorescein)	50	6·0
Methyl blue	10·4		Victoria blue 4R	2·0	18·4
Methyl orange	0·05	0·01			

* Oil red O: 0·1 per cent in isopropyl alcohol. † Sudan III: 0·2 per cent in propylene glycol.
‡ 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° and 25°C, also affects the solubility.

BUFFER TABLES

The use of buffers as solvents, differentiators, or controls is becoming increasingly important in the histological laboratory. A complete range of such buffers is outside the scope of a work such as this, and students

who wish to study the subject more deeply, or who want a special buffer, are referred to a standard chemical text-book. In the following pages details are given of the more useful and commonly used buffers. *Table 8.2* lists the chemicals used in their preparation.

TABLE 8.2
Molecular Weights of Reagents Commonly Used in Buffer Solutions

Reagent	*Molecular weight*
Acetic acid—$CH_3.COOH$	60·03
Borax (sodium tetraborate)—$Na_2B_7O_7 . 10\ H_2O$	381·43
Boric acid—$B(OH)_3$	61·84
Citric acid (anhydrous)—$C_3H_4(OH)(COOH)_3$	192·12
Citric acid crystals—$C_3H_4(OH)(COOH)_3 . H_2O$	210·14
Glycine—NH_2CH_2COOH	75·07
Hydrochloric acid—HCl	36·465
Potassium acid phosphate (potassium dihydrogen phosphate)—KH_2PO_4	136·09
Potassium hydroxide—KOH	56·104
Sodium acetate (anhydrous)—CH_3COONa	82·04
Sodium acetate crystals—$CH_3COONa . 3\ H_2O$	136·09
Sodium acid phosphate (sodium dihydrogen phosphate)—$NaH_2PO_4 . H_2O$	138·01
Sodium citrate crystals—$C_3H_4OH(COONa)_3 . 5\frac{1}{2}\ H_2O$	357·18
Sodium citrate, granular—$C_3H_4OH(COONa)_3 . 2\ H_2O$	294·12
Sodium chloride—NaCl	58·46
Sodium hydroxide—NaOH	40·0
Sodium phosphate, dibasic—Na_2HPO_4	141·98
Sulphuric acid—H_2SO_4	98·082

Walpole's Sodium Acetate–Hydrochloric Acid Buffer (pH Range 0.65–5.2)

Solutions Required

M/1 sodium acetate, prepared by dissolving 82.04 g (anhydrous), or 136.09 g crystals in distilled water, and making up to 1 litre in a measuring flask.

M/1 *hydrochloric acid*, prepared by diluting concentrated hydrochloric acid which is approximately 10M, and titrating against a normal alkali.

TABLE 8.3

pH	M/1 *Sodium acetate* (*ml.*)	M/1 *Hydrochloric acid* (*ml.*)
0·65	100	200
0·91	100	160
1·09	100	140
1·24	100	130
1·42	100	120
1·71	100	110
1·99	100	105
2·32	100	102
2·72	100	99·5
3·09	100	97
3·29	100	95
3·5	100	92·5
3·79	100	85
3·95	100	80
4·19	100	70
4·58	100	50
4·76	100	40
4·92	100	30
5·2	100	20

Method

To prepare buffer solution, take the appropriate amount of each solution *(Table 8.3)* and make up with distilled water to 500 ml.

Sodium Citrate–Hydrochloric Acid Buffer (pH Range 1.1–7.5)

Solutions Required

0.1M sodium citrate, prepared by dissolving 35.718 g (crystals) or 29.412 g (granular) in distilled water, and making up to 1 litre.

0.1M hydrochloric acid, prepared by diluting concentrated acid (approximately 10M), and titrating against a normal alkali.

TABLE 8.4

pH	*0·1 M Hydrochloric acid*	*0·1 M Sodium citrate*
1·1	100	0
1·35	90	10
1·85	80	20
2·5	76	24
3·6	70	30
4·51	60	40
5·1	50	50
5·49	40	60
5·75	30	70
6·0	20	80
6·4	10	90
6·73	4	96
6·99	2	98
7·5	0	100

Walpole's Acetic Acid–Sodium Acetate Buffer (pH Range 3.6–5.6)

Solutions Required

0.1 *N acetic acid,* prepared by diluting glacial acetic acid (Mol. wt. 60.03) and titrating against 0.1 N alkali.

0.1 *N sodium acetate,* prepared by dissolving 8.204 g (or 13.6 g of crystals) in distilled water, and making up to 1 litre in a measuring flask.

Method

To prepare buffer solution take the appropriate amount of acetic acid and sodium acetate solutions.

TABLE 8.5

pH	*0·1 N Acetic Acid (ml)*	*0·1 N Sodium acetate (ml)*
3·6	185	15
3·8	176	24
4·0	164	36
4·2	147	53
4·4	126	74
4·6	102	98
4·8	80	120
5·0	59	141
5·2	42	158
5·4	29	171
5·6	19	181
6·0	10	190
6·3	5	195
6·8	0	200

Sörensen's Phosphate Buffer (pH Range 5.3–8)

Solutions Required

M/15 *sodium phosphate dibasic* (Na_2HPO_4), prepared by dissolving 9.465 g of the salt in distilled water and making up to 1 litre in a measuring flask.

M/15 *potassium acid phosphate* (KH_2PO_4), prepared by dissolving 9.07 g in distilled water, and making up to 1 litre.

Method

To prepare M/15 buffer solution take the appropriate amount of each solution in millilitres, as given below in *Table 8.6*. The resulting

100 ml of buffer solution may be diluted with distilled water to 1,000 ml for certain staining techniques.

TABLE 8.6

pH	M/15 *Sodium phosphate dibasic* (*ml.*)	M/15 *Potassium acid phosphate* (*ml.*)
5·3	2·5	97·5
5·6	5·0	95
5·91	10	90
6·24	20	80
6·47	30	70
6·64	40	60
6·8	49·6	50·4
6·98	60	40
7·2	72	28
7·38	80	20
7·73	90	10
8·04	95	5

Michaelis's Veronal–Hydrochloric Acid Buffer (pH Range 4.5–9.2)

Solution Required

Veronal acetate solution

Sodium acetate . . . . . 1.943 g
Veronal (sodium barbiturate) . . . . . 2.943 g
Distilled water . . . . . to 100 ml

Method

To prepare a buffer solution add to 5 ml of the veronal acetate solution the amount of M/10 hydrochloric acid and distilled water given in *Table 8.7.*

TABLE 8.7

pH	*Veronal acetate solution (ml.)*	M/10 *Hydrochloric acid (ml.)*	*Distilled water (ml.)*
4·5	5	11	9
4·95	5	9	11
5·3	5	8	12
6·1	5	7	13
6·75	5	6·5	13·5
7	5	6	14
7·25	5	5·5	14·5
7·4	5	5	15
7·66	5	4	16
7·9	5	3	17
8·2	5	2	18
8·6	5	1	19
8·7	5	0·75	19·25
8·9	5	0·5	19·5
9·2	5	0·25	19·75

Tris(Hydroxymethyl)Aminomethane–Maleic Acid (Gomori's Buffer) (pH Range 5.08–8.45)

Solutions Required

M/1 maleic acid ($C_4H_4O_4$). Dissolve 116 g in distilled water and make up to 1 litre in a measuring flask.

M/1 tris(hydroxymethyl)aminomethane $(CH_2OH)_3C{-}NH_2$. Dissolve 121 g in distilled water and make up to 1 litre in a measuring flask.

M/2 sodium hydroxide (NaOH). Dissolve a little over 20 g in 1 litre of distilled water and titrate against a normal acid.

Method

To prepare the buffer solution take the appropriate amounts of

each solution in millilitres as given in *Table 8.8*. The resulting 50 ml of buffer solution may be diluted up to 250 or 500 ml for certain techniques.

TABLE 8.8

pH	M/1 *Maleic acid* (*ml.*)	M/1 *Tris(hydroxymethyl)aminomethane* (*ml.*)	M/2 *Sodium hydroxide* (*ml.*)	*Distilled water* (*ml.*)
5·08	5	5	1	39
5·30	5	5	2	38
5·52	5	5	3	37
5·70	5	5	4	36
5·88	5	5	5	35
6·05	5	5	6	34
6·27	5	5	7	33
6·5	5	5	8	32
6·86	5	5	9	31
7·20	5	5	10	30
7·5	5	5	11	29
7·75	5	5	12	28
7·97	5	5	13	27
8·15	5	5	14	26
8·30	5	5	15	25
8·45	5	5	16	24

Gormori's 0.2 M 'Tris' Buffer (pH Range 7.2–9.1)

Solutions Required

0.2 *M tris (hydroxymethyl) aminomethane* (Mol. wt. 121.14)
0.1 *N hydrochloric acid*

Method

To prepare buffer solution take the appropriate amount of each solution and make up with distilled water to 100 ml.

TABLE 8.9

pH	0·2 M *tris* (*ml.*)	0·1 N *Hydrochloric acid* (*ml.*)
7·19	25	45·0
7·36	25	42·5
7·54	25	40·0
7·66	25	37·5
7·77	25	35·0
7·87	25	32·5
7·96	25	30·0
8·05	25	27·5
8·14	25	25·0
8·23	25	22·5
8·32	25	20·0
8·41	25	17·5
8·51	25	15·0
8·62	25	12·5
8·74	25	10·0
8·92	25	7·5
9·10	25	5·0

Holmes's Boric Acid–Borax Buffer (pH Range 7.4–9.1)

Solutions Required

M/5 boric acid, prepared by dissolving 12.368 g of boric acid in distilled water in a 1 litre measuring flask, and making up to 1 litre with distilled water.

M/20 *borax (sodium tetraborate)*, prepared by dissolving 19.071 g of borax in distilled water in a 1 litre measuring flask, and making up to 1 litre with distilled water.

Method

To prepare buffer solution mix the appropriate amounts of each solution, as given in *Table 8.10.*

TABLE 8.10

pH	M/5 *Boric acid* (*ml.*)	M/20 *Borax* (*ml.*)
7·4	90	10
7·6	85	15
7·8	80	20
8·0	70	30
8·2	65	35
8·4	55	45
8·7	40	60
9·0	20	80

TABLE 8.11

pH	0·1M *Glycine in* 0·1M *NaCl* (*ml.*)	0·1M *NaOH* (*ml.*)
8·45	95	5
8·79	90	10
9·22	80	20
9·56	70	30
9·98	60	40
10·32	55	45
10·9	51	49
11·14	50	50
11·39	49	51
11·92	45	55
12·21	40	60
12·48	30	70
12·66	20	80
12·77	10	90

Sörensen and Walbum's Glycine–Sodium Chloride–Sodium Hydroxide Buffer (pH Range 8.45–12.48)

Solutions Required

0.1 *M glycine in* 0.1 *M NaCl,* prepared by dissolving 5.846 g of NaCl in a quantity of distilled water in a 1 litre measuring flask. Add 7.507 g of glycine and more distilled water to dissolve it; make the resultant solution up to 1 litre with distilled water.

0.1 *M NaOH,* prepared by dissolving 4 g of NaOH in distilled water, and making up to 1 litre with distilled water. This solution should be checked by titrating against a decimolar acid.

Method

To prepare a 0.1 M buffer solution mix the appropriate amounts of each solution as given in *Table 8.11.*

Chapter 9

Mountants

Histological sections which need to be examined for any length of time, or to be stored, must be mounted under a coverslip. The media in which the sections are mounted will depend on varying factors, but in general fall into two main classes: (1) aqueous media; and (2) resinous media. The former will be used for material which is unstained, stained for fat, or metachromatically stained; the latter are used for most of the routine staining techniques with the exception of those where the element or the dyes used in its demonstration are soluble in the dehydrating or clearing agents.

The methods of mounting various types of section are dealt with in the next chapter.

AQUEOUS MOUNTING MEDIA

Most of these media have a low refractive index (1.4–1.42), although higher levels are obtainable with certain of the syrups. Occasionally single reagents, such as pure or dilute glycerin, are used, but generally these media are of three types: (*a*) the syrups; (*b*) gelatin media; and (*c*) gum arabic media. In the last two media glycerin is usually incorporated to prevent cracking and splitting on drying; all three are best preserved by coating with a ringing medium (*see below*).

Some of the metachromatic stains tend to diffuse from the section into the mounting media shortly after mounting: this may be prevented by using a fructose syrup, or by mounting in potassium acetate gum syrup. About 20 per cent by weight of potassium acetate, or 50 per cent by weight of sugar, is necessary (Report of the Committee on Histological Mounting Media, 1953).

The setting qualities of simple syrups may be improved by the addition of 12 per cent gelatin.

All aqueous mounting media should contain a bacteriostatic agent such as a crystal of thymol, 25 per cent phenol, or sodium merthiolate, 0.025 per cent, to prevent the growth of moulds.

Glycerin Jelly (Refractive Index 1.47)

This is usually regarded as the standard mountant for fat stains.

Formula

Gelatin	10 g
Distilled water	60 ml
Glycerin	70 ml
Phenol	0.25 g

Dissolve the gelatin in the distilled water in a conical flask in a water bath or steam sterilizer, using just sufficient heat to melt the gelatin; add the glycerin and phenol, mix well, and transfer to containers. One ounce screw-capped bottles make suitable containers for all the aqueous mounting media, one of the caps (which can be changed to the bottle in use) being drilled to take a piece of glass rod which has been rounded at each end to serve as an applicator.

For use melt in a water bath, hot water or wax oven; avoid shaking to speed this process otherwise the mountant will be full of air-bubbles; 0.025 per cent sodium merthiolate may be substituted for phenol as a preservative.

Apathy's Medium (Refractive Index 1.52)

This medium is used when an aqueous medium of higher refractive index is required. It is one of the most useful aqueous mountants for fluorescent microscopy, being virtually non-fluorescent.

Formula

Gum arabic	50 g
Cane sugar	50 g
Distilled water	50 ml
Thymol	0.05 g

Dissolve the ingredients with the aid of gentle heat. As this mountant sets by evaporation it must be kept in a well-stoppered bottle, or screw-capped container.

Highman's Modification of Apathy's Medium (Refractive Index 1.52)

This medium is recommended for use with metachromatic stains.

Formula

Gum arabic	20 g
Cane sugar	20 g
Potassium acetate	20 g
Sodium merthiolate	10 ml
Distilled water	40 ml

Dissolve the ingredients with the aid of gentle heat, and keep in an air-tight container.

Farrant's Medium (Refractive Index 1.43)

This medium, being liquid, is more convenient than glycerin jelly for mounting, but has the disadvantage that it takes much longer to set, and air-bubbles sometimes form during the setting process.

Formula

Gum arabic	50 g
Distilled water	50 ml
Glycerin	50 ml
Arsenic trioxide	1 g

Dissolve the gum arabic in the distilled water with gentle heat, add glycerin and arsenic trioxide.

The addition of 50 g of potassium acetate will give a neutral medium (pH 7.2) instead of an acid one (pH 4.4), and raises the refractive index to 1.44. Sodium merthiolate (0.025 per cent) may be substituted with advantage for the arsenic trioxide as a preservative.

Fructose (Laevulose) Syrup (Refractive Index 1.47)

This syrup is useful as a temporary or special mountant, but is not recommended for routine use.

Formula

Fructose (laevulose)	75 g
Distilled water	25 ml

A high concentration of sugar such as this will require some time to dissolve in the 60°C oven or water bath, and unless the high refractive index and more viscid solution is required Mallory's formula of 30 g of fructose with 20 ml of distilled water is easier to prepare. Gelatin (10 g) may be added so that it sets; alternatively, it may be used as a

permanent mount by using a ringing medium (page 185). The advantage of fructose over the other sugars used in mounting media is that it does not crystallize in the preparation.

RESINOUS MOUNTING MEDIA

At one time the great majority of stained preparations were mounted in Canada balsam as a routine. Today there is available a wide range of natural and synthetic resins which are used both routinely and for special purposes.

In general these media are composed of a resin (natural or synthetic) either in its natural solvent, or dissolved in a solvent such as xylol until sufficiently liquid to allow easy mounting compatible with fairly rapid drying and hardening. The ideal viscosity of these media will vary according to personal preference; they should be sufficiently liquid to allow trapped air-bubbles to be removed easily, and to flow freely between coverslip and section, yet sufficiently viscid to avoid the formation of air spaces under the coverslip during drying.

Selection of a Resinous Mounting Medium

A mounting medium should be chosen that will not fade the particular stains used; for example, basic aniline dyes should be mounted in non-acid-containing mountants; preparations showing the Prussian blue reaction should be mounted in non-reducing media. The medium should have the correct refractive index; unstained tissue shows best in a medium having a very low or very high refractive index, while stained preparations are most transparent when the mounting media has a refractive index of 1.54.

NATURAL RESINS

Canada Balsam (Refractive Index 1.52)

Canada balsam, from the Canadian fir tree (*Abies balsamea*), is a solid resin and is composed of terpenes, carboxylic acids and their esters; it is usually dissolved in xylol. Haematoxylin and eosin stained slides are fairly well preserved, but basic aniline dyes tend to fade, and Prussian blue is slowly bleached.

Canada balsam is dissolved in xylol to 55–70 per cent by weight; the actual amount used will vary according to personal preference and a 55 per cent solution is a suitable strength for routine use. This is a very messy reagent to prepare, since it must be ground in a pestle and mortar

with the solvent until free of lumps, and it is more economical to purchase the ready-made mountant.

Dammar Balsam

Dammar balsam is similar to Canada balsam. It is rarely used today because of the dirt and impurities usually present, and the difficulty of filtering prepared mountant.

Colophonium Resin

This is occasionally used as a mountant, when it is dissolved in xylol or turpentine. In alcoholic solution it may be used as a differentiating agent.

SYNTHETIC RESINS

There are a great number of synthetic resins either made in the laboratory or prepared commercially; those in most common use are the polystyrenes, such as Kirkpatrick and Lendrum's mountant, Gurr's Depex and H.S.R.

Plasticizers, such as tricresyl phosphate or dibutylphthalate, should be incorporated with the solution of polystyrene in xylol, otherwise air spaces appear under the coverslip as the preparation dries; alternatively, higher boiling solvents, such as technical dimethylbenzene, or technical diethylbenzene, may be used. The report mentioned on page 179 recommends a polystyrene dimethylbenzene as the best mountant for preserving the Prussian blue reaction.

New Unimount (Refractive Index 1.50)*

This mountant which was marketed as a universal mountant (Unimount) had a tendency to trap air bubbles if not mounted with some care. The use of a new solvent has obviated this problem and it is now available as New Unimount. Since it contains neither oxidizing nor reducing contaminants, it is recommended for use as a routine mountant with all those staining techniques suitable for mounting in a resinous mountant. During preparation it is tested to ensure that it does not decolorize or change the absorption spectra of most of the standard methods. Culling (1967) in *Nature* stated it to be the only mountant available to give permanent preparations of fluorescent antibody

*Obtainable from Sherwood Medical Industries, Inc., 1831 Olive St., St. Louis, Missouri, U.S.A., 63103.

preparations (*see* page 631). We now have a large number of such preparations (stained with fluorescent anti-IgG, anti-IgM, anti-IgA, anti-β1C, and so on) which are over 4 years old in which the fluorescence shows little or no fading. Kamamura (1969) comments that the best fluorescent antibody mountant (polyvinyl alcohol-buffer-glycerin) shows 30 per cent fading overnight and 50 per cent fading after a week, but since it is not mentioned it is assumed he has not tested Unimount.

Kirkpatrick and Lendrum's D.P.X. (Refractive Index 1.52)

Distrene 80	10 g
Dibutylphthalate	5 ml
Xylol	35 ml

This is the most commonly used routine mountant preserving, as it does, most routine stains. It has the great advantage over balsam that slides can be cleaned of excess mountant simply by stripping it off after cutting around the edge of the coverslip.

Harleco Synthetic Resin (H.S.R.) and Gurr's Depex are also in general use and give excellent results.

OTHER MOUNTING MEDIA

Euparal (Refractive Index 1.48)

Euparal is a semi-synthetic mountant composed of sandarac resin dissolved in a mixture of eucalyptus, paraldehyde, and camsal (a liquid composed of camphor and phenyl salicylate). It is of use when a lower refractive index is required, and has the advantage that sections may be mounted from xylol, or from graded alcohol of 90 per cent upwards. There is a green variety containing copper salt which is said to preserve haematoxylin stains.

Neutral Mounting Medium (Gurr's) (Refractive Index 1.52)

This medium, probably a mixture of coumarone and other resins dissolved in eucalyptol, is specially prepared for mounting blood films and sections stained by Romanowsky methods. Blood films are mounted dry, as are sections from alcohol or xylol.

Other Mountants

While there are many more mountants available commercially, particularly from American sources, the range given above will suffice for all routine work, and the majority of special techniques.

RINGING MEDIA

Mounting media which powder or develop air-bubbles due to evaporation should be coated at the edges of the coverslip with a non-porous ringing* medium.

A ringing iron is needed to spread the solid type of medium around the coverslip. This is easily made from a T-shaped piece of copper which should be about one-eighth of an inch in thickness with the bottom of the upright piece embedded in a rubber bung, or wooden handle. The cross-piece should be about 1 inch in width. During use the cross-piece is heated in the flame of a Bunsen burner, touched onto the surface of the ringing agent and then applied to the edges of the coverslip. With a little practice ringing may be simply, quickly and cleanly completed.

Paraffin Wax

Paraffin wax is applied with a ringing iron and is satisfactory as a temporary ringing agent.

Du Noyer's Wax – Colophonium Resin Mixture

Du Noyer's wax is prepared by heating 10 parts of paraffin wax in an evaporating dish or an enamel mug, and dissolving in it 40 parts of colophonium resin which has been powdered in a pestle and mortar. It is then allowed to set in either paper moulds or in the enamel container. It is applied in a similar manner to paraffin wax, but is a more permanent mount.

Cement

Any of the plastic type adhesives (such as Durofix) may be applied as a ringing medium direct from the collapsible tube in which it is supplied. These types of cement make permanent mounts.

Asphaltum Varnish

Any of the plastic type adhesives (such as Durofix) may be applied as a ringing medium direct from the collapsible tube in which it is supplied. These types of cement make permanent mounts.

*The term 'ringing' originated because round coverslips were then used, and the coating applied in the form of a circle or 'ring'.

Chapter 10

Basic Staining and Mounting Procedures

EQUIPMENT

The most satisfactory lay-out of equipment in a histological laboratory is one that allows separate benches, or parts of benches, for the cutting of specimens, processing, embedding, staining and mounting of sections. The bench for cutting specimens should be near the door, and the other benches should be arranged in the order in which the procedures are discussed below.

Staining Bench and its Equipment

The staining bench should be 6–8 feet in length, and should be equipped with a sink, preferably at one end. The sink should be shallow, and large enough to: (*a*) contain a slide-washing tray capable of carrying 20 per cent more than the average number of slides stained daily; (*b*) take a staining rack composed of two glass rods about 1¾ inches apart (either fitting into grooves cut into the wooden sides of the sink top, or held at the correct distance apart by rubber tubing at each end); and (*c*) still provide space into which unwanted fluids may be poured. It should be fitted with at least two cold water taps, one being permanently connected to the slide-washing tray.

A gas point is necessary for a Bunsen burner which should be kept permanently in position.

Sufficient shelf space should be available to carry stocks of stains and drop bottles of less frequently used reagents.

A well-lighted bench is essential, and it is preferable to have a window at the back of the staining bench, supplemented by a fluorescent warm white tube 6 feet in length (or a pair of 3 feet tubes) for use during evening work or when natural lighting is poor.

An adequate supply of distilled water should be available, stored in a 5 or 10 litre aspirator jar (glass or polythene) resting on a shelf attached to the wall above the sink. From the outlet of the jar a length of rubber tubing controlled by a pinch-clip should allow slides on the staining rack to be rinsed. When not in use the nozzle should be just above the sink; careless use of the pinch-clip will then only waste the distilled water and not flood the bench. If a large jar is not available an 8-ounce polythene container fitted with a glass or polythene nozzle may be used for distilled water.

A staining microscope is essential, equipped with a 16 and 4 mm. objective and, if available, a 1/7 inch water immersion objective. Since this is to be used for examining wet slides the stage should permit easy and rapid cleaning; for this reason a mechanical stage is likely to prove more of a hindrance than a help unless it is of the type which moves the complete stage top.

Stock stains and reagents should be kept in glass-stoppered, or screw-capped containers. Reagents which require protection from the light should be kept in dark bottles; all such stains and reagents are best kept in dark cupboards, and kitchen cupboard units are ideal for this purpose and may be fitted to the wall above the bench.

Stains and reagents in constant or fairly frequent use are best kept in drop bottles, Coplin jars, or wide-mouthed screw-capped jars. The choice of containers for particular stains depends on their speed of evaporation, the frequency of use, the length of exposure of sections to their action, and the need for easy access; for example, Leishman's stain and methylene blue are kept in drop-bottles, haematoxylin in a Coplin jar, alcohol and xylol in wide-mouthed screw-capped jars.

Developing dishes are especially suited for staining giant sections, since reagents may be easily poured back into stock bottles after use, using the one dish for a series of stains and reagents.

The author recommends the following standard equipment for a routine staining bench, laid out as shown in *Figure 10.3.*

Wide-mouthed Screw-capped Bottles

About 3 inches high, and 2½ inches wide, these bottles are convenient for the storage of xylol, absolute alcohol and 90 per cent alcohol.

Coplin Jars

Coplin jars may be of the upright type with a capacity of 10 slides back to back, or of the flat variety with room for 20 slides back to

back, dependent on the number of slides dealt with at one time. It is generally more convenient to use the upright type if possible. More recently, a new type of polythene jar has been introduced *(Figure 10.1*

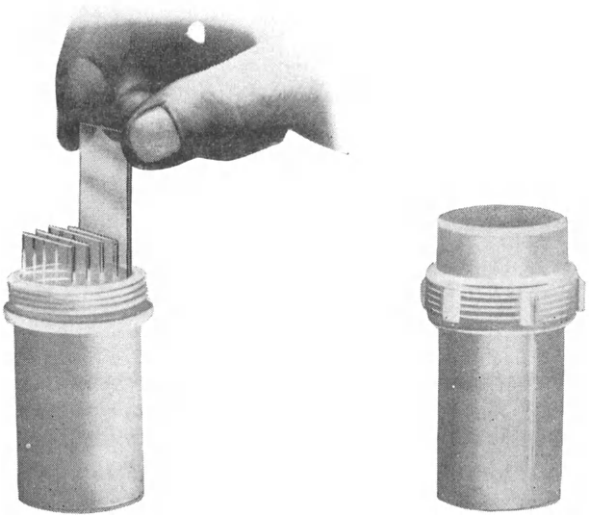


Figure 10.1 – Wide-mouthed screw-capped polythene staining jar, capable of holding 10 slides (by courtesy of G. T. Gurr, Ltd.)

a and *b)* with the slides projecting from the top of the jar during staining, thus almost completely obviating the staining of the fingers in removing them. In spite of the fact that it holds 10 slides (back to back) a relatively small amount of stain is required, and the screw-on water-tight lid enables it to be used to transport sections or smears in solutions from one laboratory to another. The jar is unbreakable and has a much longer life than the glass variety, but it has the disadvantage that, being made of polythene, only aqueous, alcoholic, or certain other solutions may be put into it with safety. Moreover, the polythene tends to take up a certain amount of stain and becomes discoloured which virtually restricts the use of each jar to any one stain.

For routine work the following are kept in Coplin jars:xylol (as a reservoir before mounting); 1 per cent eosin; Ehrlich's haematoxylin; iodine alcohol; and 3 per cent sodium thiosulphate.

Wide-mouthed Glass-stoppered Bottles

About 3 inches high and 1½–2½ inches wide, these are used for acid alcohol. Since this is in constant use but evaporates easily, a glass-stoppered container offers easy access with rapid control of evaporation.

Drop Bottles (60 ml Capacity)

These are used for storing the following:

Celloidin, 1 per cent.
Aniline-xylol.
Ammonia (0.880).
Acetone
Toluidine blue, 0.1 per cent.
Methylene blue (Loeffler's).
Carbol fuchsin.
Gram's stains (methyl violet, Lugol's iodine, neutral red).
Van Gieson's stain.

Coverslips and Ancillary Instruments

Coverslips may be kept in 1 per cent acid alcohol in separate containers (when they are wiped this usually gives a clean, grease-free coverslip), or dry in a Perspex coverslip holder. The latter can be made to hold coverslips of as wide a range of sizes as desired and for use the dry coverslips may be dipped into acid alcohol and then wiped clean. The most useful range is: ⅞ inch squares, 1 × ⅞ inch, 1¼ × ⅞ inches, 1½ × ⅞ inches, and 2 × ⅞ inches – other sizes should be available in a drawer. Such coverslips may be No. 2 thickness, but the thinner No. 1 coverslips should be used when oil immersion objectives are likely to be used in examining the section.

Dissecting needles (to adjust coverslips during or after mounting), forceps, scalpel, ringing iron, ringing media and rubber teats should be available on the bench or in a drawer immediately below it.

The mountants in routine use (D.P.X., Canada balsam, glycerin jelly, and so on) should be available in the covered pots with glass applicators which are specially made for this purpose commercially.

Slide-washing Tray

Several types of tray are available, from the simple developing dish with a lead sheet resting in the centre (leaving a margin of about 1½ inches on all sides), to the more complex Perspex type designed by Crawford.

The author has designed a simple type (*Figure 10.2*) which permits slides to be arranged around the centre as well as peripherally. It can easily be made from Perspex.*

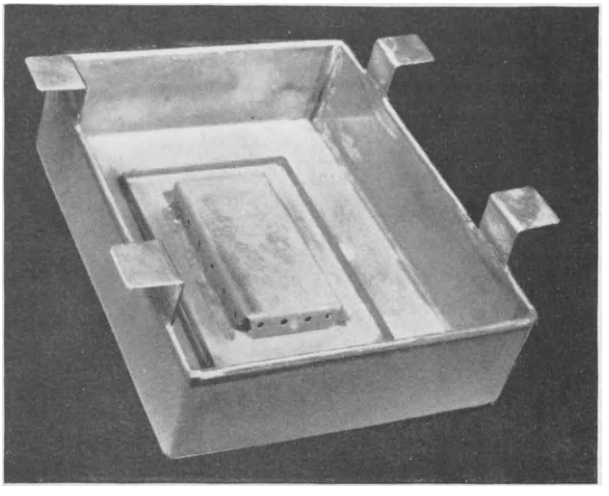


Figure 10.2 – This slide-washing tray, made of copper, was copied from the Perspex original. The centrepiece should have been raised to the same height as the sides to enable slides to be rested against it. The water supply is connected to a copper pipe underneath the centre of the tray. The four L pieces fit the edges of the sink in which it is used, although a stand may be used to support it in a large sink

The criteria of a good slide-washing tray are:

(1) Rapid change of water content without jets of water as these are likely to detach sections from slides.

(2) Complete replacement of *all* the water in the tray. Water running from the tap, with a simple spill-over, does not change the water at the bottom of the tray: the water supply should feed the tray from the bottom, have baffles to ensure an even steady distribution of pressure, and have an overflow pipe near the surface.

(3) The ridge on the bottom of the tray should be of sufficient height to prevent slides from being easily dislodged.

*Obtainable from Chimo Labs, Ltd., 1200 West 6th Avenue, Vancouver, B.C., Canada.

The efficiency of a slide-washing tray may be tested by filling it with slides, adding a few drops of eosin and turning on the water supply. The eosin should quickly spread throughout all the water in the tray and then completely disappear within a minute or two.

It will be noted that the majority of the equipment listed above is for use when staining paraffin sections, for these usually form 80–90 per cent of the routine work in a histological laboratory. Special equipment used in the staining of frozen and celloidin sections will be dealt with under the appropriate headings.

THE APPLICATION OF HEAT DURING STAINING PROCEDURES

A reduction in the staining time of certain procedures may usually be effected by the application of heat; this is generally achieved by immersing the sections or smears in stain – for example, Heidenhain's haematoxylin – in a Coplin jar which is then put into a 37° or a 56°C oven for the required time.

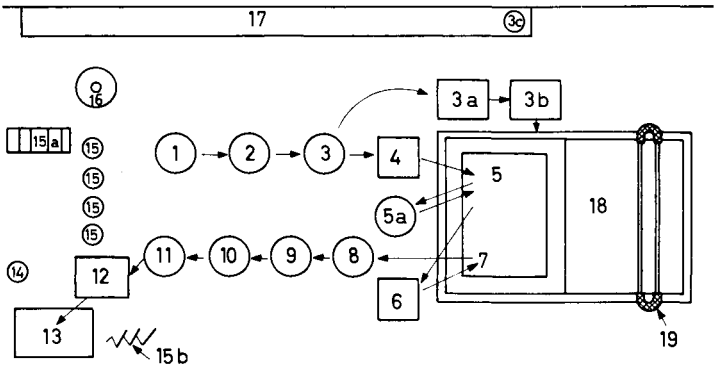
Where the technique requires boiling, or near boiling, stain to be applied, such as in the Ziehl-Neelsen technique, the stain may be boiled in a test-tube and poured on to the slide; or the slide may be flooded with stain and direct heat applied beneath the slide. This direct heating causes evaporation of the solvent and may lead to precipitation of the stain. This can be avoided by covering sections with a square or oblong of filter paper before applying the stain. Washing in water after the section is stained will easily remove the filter paper without damage to the section.

PARAFFIN SECTIONS

The basic steps in staining and mounting paraffin sections are as follows.

(1) Removal of wax with xylol.
(2) Hydration with graded alcohols.
(3) Staining.
(4) Dehydration through graded alcohols.
(5) Clearing in xylol.
(6) Mounting under a coverslip.

The steps given in this technique are the routine ones for haematoxylin and eosin and will apply (apart from the actual staining) to all methods given in this book, unless an alternative method is specifically given. The superior figures given in parenthesis apply to the diagram (*Figure 10.3*).



1. Xylol in screw-capped wide-mouthed jar.
2. Absolute alcohol in screw-capped wide mouthed jar.
3. 90 per cent alcohol in screw-capped wide-mouthed jar.
3*a*. Iodine alcohol in flat-type Coplin jar.
3*b*. 3 per cent sodium thiosulphate in flat-type Coplin jar.
3*c*. Picric alcohol in drop bottle.
4. Ehrlich's haematoxylin in Coplin jar.
5. Slide-washing tray.
5*a*. Acid alcohol in glass-stoppered jar.
6. 1 per cent eosin in Coplin jar.
7. Slide-washing tray.
8. 90 per cent alcohol in screw-capped wide-mouthed jar.
9. Absolute alcohol (I) in screw-capped wide-mouthed jar.
10. Absolute alcohol (11) in screw-capped wide-mouthed jar.
11. Xylol (1) in screw-capped wide-mouthed jar.
12. Xylol (II) in flat-type Coplin jar.
13. Blotting paper.
14. Mountant.
15. Coverslips in separate containers (in acid alcohol).
15*a*. Coverslips in Perspex coverslip holder* (dry).
15*b*. Cleaned coverslips on corrugated filter paper (end view).
16. Bunsen burner.
17. Row of drop bottles, either at back of bench or on shelf at the back of the bench.
18. Sink.
19. Staining rack (glass rod and rubber tubing type).

Figure 10.3 – Layout of bench for routine haematoxylin and eosin staining of paraffin sections

Technique

Removal of Wax

(1) Sections are placed in xylol [1] for 1–2 minutes to dissolve the wax.

Hydration

(2) The section is taken out of xylol (it should appear quite clear) and is transferred to absolute alcohol [2] for 1 minute, when it will become opaque.

Note. – If all the wax has not been removed clear patches will be seen, and such sections should be returned to the xylol [1] for a

*Obtainable from Chimo Labs, Ltd., 1200 West 6th Avenue, Vancouver, B.C., Canada.

further minute. Very rarely, sections coming from the xylol pot will show white patches; these slides should be first put into alcohol for 1 minute, then returned to the xylol for a further minute. The white patches, which are probably caused by water being trapped under or in the section, will now have disappeared and the slide is left in alcohol for a minute and then treated routinely.

(3) The section is removed from the absolute alcohol [2], drained, and placed in 90 per cent alcohol [3] for 1 minute.

If the sections were fixed in a mercury-containing fixative, the deposit should be removed at this stage with iodine-sodium thiosulphate treatment.

(*a*) The section is taken from the 90 per cent alcohol, drained, and placed in iodine alcohol (0.5 per cent iodine in 80 per cent alcohol) or Lugol's iodine for 3 minutes [3a].

(*b*) The slide, after draining and rinsing in water, is transferred to 3 per cent sodium thiosulphate [3b], and left for 3 minutes, following which it is placed in the slide-washing tray for a few minutes, rinsed in distilled water and the routine method from stage 4 is continued.

If sections are known to contain formalin pigment, they are treated as follows.

(*c*) The section is removed from the alcohol, placed in picric alcohol [3c] (saturated solution of picric acid in alcohol) for 5–10 minutes, followed by washing in the slide-washing tray for 10 minutes. The sections are then transferred to 90 per cent alcohol [3] for 2 minutes, and the routine method from stage 4 is continued.

Staining

(4) Slides are transferred from 90 per cent alcohol [3], after draining, to haematoxylin [4], where they are left for 10–40 minutes.

Note. – This is a regressive staining method, in which sections are first overstained, and the stain then selectively removed in acid alcohol. The advantage of this method is that the degree of staining is controlled (some tissue elements retaining more stain than others), and a perfectly clear cytoplasm and background can be obtained.

(5) Slides, after draining off excess haematoxylin, are transferred to the slide-washing tray, and washed until the sections are blue (when

first removed from the haematoxylin they are pink). This change of colour has caused this stage to be known universally as 'blueing sections,' and takes about 10 minutes in tap water of pH 8. (Lithium carbonate may be added to water which is not sufficiently alkaline.)

(6) Sections are dipped into acid alcohol [5a] where they are agitated for a few seconds and then returned to the slide-washing tray until blue again. They should then be examined under the low power of the staining microscope to ensure that they are sufficiently differentiated. With practice this microscopic control may be omitted.

(7) Should a section be under-differentiated it is returned to the acid alcohol [5a] for a short period; if it is over-differentiated (nuclei too pale) the section, after rinsing in distilled water, may be returned to the haematoxylin [4] for 10–15 minutes, after which it is again differentiated.

(8) Sections which have been differentiated and 'blued' are transferred to 1 per cent eosin [6] for 2–4 minutes to counterstain them.

(9) Sections are transferred from the eosin to the slide-washing tray [5,7] for 3–4 minutes; this will differentiate the eosin.

This stage has not been correctly carried out unless muscle fibres, keratin, cytoplasm, connective tissue and red blood cells, are easily identified by varying shades of red and pink.

Dehydration

(10) After draining, sections are transferred from the slide-washing tray [5,7] to 90 per cent alcohol [8] where they are agitated for 10–15 seconds.

(11) From 90 per cent alcohol they are transferred to absolute alcohol I [9], where they are agitated for 10–15 seconds.

(12) They are then taken from absolute alcohol I to absolute alcohol II [10] for 30 seconds.

Clearing

(13) Sections should be transferred from absolute alcohol II to xylol I [11] and left until completely clear. This should take about 15 seconds, and they should be tested for clarity by being held against a dark background with the light striking them, when any patches containing water will have a milky appearance. Such sections should be returned to absolute alcohol II for a further 30 seconds, and again placed in xylol I until clear.

(14) Sections when clear are transferred to xylol II [12] from which they may be mounted.

Mounting

(15) A sufficient number of coverslips of appropriate sizes for the section to be mounted are wiped with a soft, fluffless glass cloth. Such coverslips may be conveniently laid in rows on a piece of filter paper folded like a concertina [15b], which allows them to be picked up by the edges. Alternatively, they may be cleaned singly as required and laid on the pad of blotting paper [13].

(16) A coverslip is laid on the blotting paper; the section is removed from the xylol, the surplus xylol is removed by wiping the back of the slide and around the section, leaving a margin of about one-eighth of an inch – this stage should be quickly completed to avoid the section drying.

(17) One or two drops of Canada balsam (depending on the size of the coverslip) are placed on the section; being laid along the middle of the section to reduce the likelihood of trapping air bubbles.

(18) The slide is quickly inverted over the coverslip, one end is placed on the blotting paper and the other end slowly lowered until the balsam touches the coverslip. The balsam quickly spreads under the coverslip, and the slide, with the coverslip attached, is again quickly inverted, and the coverslip guided into place with a dissecting needle. This whole operation will, with practice, be complete in 5–10 seconds.

An alternative method is to put the mountant on the section as described, place one end of the coverslip on the slide (clear of the section) and, with the aid of a dissecting needle, slowly lower the coverslip into position. The author has employed both methods over a period of years and prefers the former.

The quantity of mountant to be used can only be assessed with experience; too little will cause air bubbles, and too much a messy slide. The latter is the better of the two evils since it can be cleaned afterwards, but an experienced technologist should be able to judge the correct amount to use every time and avoid the tedious job of removing excess balsam. D.P.X. should always be used more liberally than other mountants because of the danger of retraction on drying; this disadvantage is minimized since it is easily stripped off with a scalpel blade after setting.

Air bubbles. – While an odd air bubble may be expressed by gentle pressure on the coverslip with a dissecting needle, the practice of chasing bubbles under a coverslip is a time-wasting procedure, and will almost certainly damage the section. When there is more than one air bubble in the mountant it is quicker to put the slide back into xylol

(which will remove the coverslip) and remount the section. Such a practice will soon lead to the mounting of sections without bubbles every time.

FROZEN SECTIONS

Frozen sections may be stained by either of the two following methods.

(1) *Attachment to slides.* – After frozen sections have been attached by one of the methods described on page 141, they are stained by the technique for paraffin sections given in the preceding pages. This method obviously cannot be used for the demonstration of lipids.

(2) *Floating through reagents.* – This entails the transference of loose sections from one reagent to the next by means of a 'hockey stick', the section being mounted on a slide after staining. This method will be given in detail in the following pages.

Equipment for 'Floating Through' Method

Dishes and Containers

The following dishes and bottles will be required.

Small, loose-covered dishes (2–2½ inches in diameter and ¾–1 inch in height).

Large, loose-covered dishes (3–4 inches in diameter and 2–2½ inches in height).

One or two very large glass, or enamel, containers (8–10 inches in diameter and 3–4 inches in height).

Glass-stoppered, wide-mouthed bottles (1–2 inches in diameter and 2–3 inches in height); these are for reagents and stains which are very volatile, and are particularly useful for fat stains which will quickly precipitate on sections if the solvent (70 per cent alcohol) is allowed to evaporate.

Painting the initials of the fluid contained within on the bottom of the dish (for example, A–alcohol, S–spirit [90 per cent alcohol]), will be found a great asset during staining; alternatively, small slips of paper may be laid under each, marked with the type and strength of reagent.

Glass 'Hockey Sticks'

Glass rods 3–4 mm in diameter will be found most convenient for making hockey sticks, although the actual diameter used is a matter of personal preference.

A 6 inch length of rod, rounded in a Bunsen flame at both ends, is heated about 1 inch from the end. When softened the last inch is allowed to fall down at right angles (90 degrees) to the remainder of the rod, although some prefer a slightly larger angle (about 108 degrees) like a real hockey stick.

Dissecting Needles

These are used for the manipulation of sections and coverslips.

Floating Through Technique

The steps given in this technique are for haematoxylin and eosin staining, and will apply (apart from the actual staining process) to any staining method described in this book, unless an alternative method is specified (for example, fat staining). Frozen sections, which are not attached to slides, stain much more rapidly than those which are attached, and therefore diluted stains are used, or the time of staining reduced.

It must be remembered that frozen sections are very fragile, and careless handling in staining and processing will result in a poor section. To pick up a section, put the bent end of the hockey stick into the dish and gently agitate the fluid above the section, causing it to float off the bottom. Slide the end of the hockey stick under the middle of the section and lift it out; the section should be draped over the glass rod evenly and without creases. The section should now be lowered into the next reagent, and the hockey stick gently slid out from underneath it. Care must be taken to ensure that sections lie absolutely flat in reagents, as creases will result in uneven staining.

The superior numbers in the following description refer to *Figure 10.4*, which shows the layout of the bench.

(1) The sections, which will have been received in distilled water after cutting, are transferred to a dish of fresh distilled water [1]. Sections which have been stored in formol saline are washed in tap-water (two changes) and then transferred to the distilled water.

> *Note.* – Occasionally it is necessary to remove fatty material, which would otherwise interfere with staining, by a preliminary dehydration and clearing (stages 2–8).

Stages 2–8 are optional.

(2) Transfer sections to 70 per cent alcohol [2] for a few seconds.
(3) Transfer to 90 per cent alcohol [3] for a few seconds.

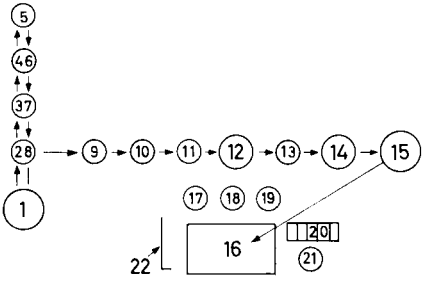


Figure 10.4 – Layout of bench for routine haematoxylin and eosin staining of frozen sections (for details of method see page 198)

1. Distilled water in a large container (8 inches in diameter).
2 and 8. 70 per cent alcohol in a small Petri dish.
3 and 7. 90 per cent alcohol in a small Petri dish.
4 and 6. Absolute alcohol in a small Petri dish.
5. Xylol in a small Petri dish.
6, 7, and 8. *(See above).*
9. Haematoxylin (diluted equal parts with distilled water) in a small Petri dish.
10. Tap-water, to which a few drops of lithium carbonate have been added, in a large Petri dish.
11. 1 per cent acid alcohol (1 per cent HCl in 96 per cent alcohol) in a small Petri dish.
12. Tap-water, with a few drops of lithium carbonate added, in a large Petri dish.
13. 1 per cent eosin in water, in a small Petri dish.
14. Tap-water in a large Petri dish. Sections may be mounted from this dish, or from the next, but a better eosin differentiation is possible from 70 per cent alcohol.
15. 70 per cent alcohol in a large container (6 inches in diameter).
16. Pad of blotting paper or filter paper.
17. 90 per cent alcohol in a drop bottle.
18. Absolute alcohol in a drop bottle.
19. Xylol in a drop bottle.
20. Coverslips, which may be kept dry in a container, or in various sizes in pots of acid alcohol.
21. H.S.R. or D.P.X. (glycerin jelly would be used to mount the section direct from water).
22. Glass 'hockey stick'.

(4) Transfer to absolute alcohol [4] for a few seconds.
(5) Transfer to xylol [5] for a few seconds.
(6) Transfer to absolute alcohol [6] for a few seconds.
(7) Transfer to 90 per cent alcohol [7] for a few seconds.
(8) Transfer to 70 per cent alcohol [8] for a few seconds.
(9) Trnasfer to haematoxylin diluted 50 per cent in distilled water [9] for 15 minutes.
(10) Transfer to tap-water [10] to which a few drops of saturated solution of lithium carbonate have been added, and leave until section is blue.
(11) Transfer to 1 per cent acid alcohol [11] for a few seconds, gently agitate the fluid.
(12) Transfer to fresh tap-water [12] (with lithium carbonate added) until blue. Staining may be controlled by floating the section on to a slide (page 140), and examining to ensure that only nuclei are stained. If sections are not sufficiently differentiated, they should be returned to acid alcohol for a few seconds, blued, and re-examined under the microscope.

(13) Transfer sections to 1 per cent eosin [13] for 1 minute.

(14) Transfer to tap-water [14] for 2 minutes, gently agitating the fluid periodically.

(15) Transfer to a large dish of 70 per cent alcohol [15], from which section should be attached to a clean, grease-free slide.

Although some workers prefer to mount from alcohol, or even xylol, it is in these reagents that gross shrinkage of the section is likely to take place, and it is therefore better to attach them to slides from dilute alcohol or water.

(16) The section is now drained, but not allowed to dry, laid on the pad of blotting paper [16], and blotted firmly with fine filter paper.

(17) Without removing the slide from the pad of blotting paper, pour 90 per cent alcohol [17] from a drop bottle on to the section, leave for a second or two, and blot with filter paper.

(18) Repeat the above stage but with the use of absolute alcohol [18].

(19) Without moving the slide, pour on xylol from a drop bottle [19], leave for a second or two, blot with fresh filter paper, and pour on more xylol. The section should now be completely clear. If any milky patches are present, they may usually be removed by blotting once more, followed by fresh xylol. Should patches still persist, stages 18 and 19 should be repeated.

(20) Wipe excess xylol from the back of the slide and around the section, and mount as described for paraffin sections on page 196.

CELLOIDIN SECTIONS

Celloidin sections are stained in a manner very similar to that used for frozen sections in that they must be floated through reagents and are only attached to a slide after staining is completed. They have an advantage over frozen sections in that they are not so fragile, but it is still important to remember that damage done to sections while staining cannot be repaired.

Since the tissues are impregnated with celloidin, which decreases the rate of penetration by reagents, the times of staining should be increased. Sections when stained are mounted without removing the celloidin, and this factor makes them more difficult to clear (*see* stage 12, page 202). Mounting is also made more difficult because the celloidin surrounding the section tends to corrugate, and unless care is taken these corrugations may trap air bubbles.

Technique

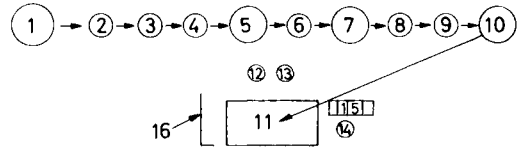
The equipment listed for frozen section staining is also suitable for celloidin section staining, including the glass hockey stick.

The steps given below are those used routinely for haematoxylin and eosin, but they will apply (apart from the actual staining technique) to all methods described in this book unless an alternative technique is specifically given.

The superior numbers refer to the key of *Figure 10.5.*

(1) Transfer the sections from the container of 70 per cent alcohol, into which they have been received after cutting, or in which they have been stored, to the large dish of 70 per cent alcohol [1]. Leave in this dish for 2–3 minutes.

(2) Transfer to haematoxylin in a small Petri dish [2] for 40 minutes.



1. 70 per cent alcohol in a large container (6–8 inches in diameter).
2. Haematoxylin in a small Petri dish.
3. Tap-water in a small Petri dish.
4. 1 per cent acid alcohol (1 per cent HCl in 70 per cent alcohol) in a small Petri dish.
5. Tap-water in a large Petri dish.
6. 1 per cent aqueous eosin, in a small Petri dish.
7. Tap-water in a large Petri dish.
8. 70 per cent alcohol in a small Petri dish.
9. 90 per cent alcohol in a small Petri dish.
10. Absolute alcohol in a large Petri dish (96 per cent alcohol may be used to float section on slide at this stage, and the previous stage of 90 per cent alcohol omitted).
11. Pad of blotting or filter paper.
12. Carbol-xylol (or carbol-cresol-xylol) in a drop bottle.
13. Xylol in a drop bottle.
14. Mountant (H.S.R. or D.P.X.).
15. Coverslips.
16. Glass 'hockey stick'.

Figure 10.5 – Layout of bench for routine method of staining celloidin sections with haematoxylin and eosin

(3) Transfer to tap-water in a large Petri dish [3] with several changes of water until sections are 'blued'.

(4) Transfer to acid alcohol (1 per cent HCl in 70 per cent alcohol) in a small Petri dish [4] for a few seconds to differentiate, gently agitating the fluid.

(5) Transfer to fresh tap-water in a large Petri dish [5] (to which a few drops of saturated aqueous lithium carbonate may be added). Examine the sections to ensure that they are correctly differentiated (page 195).

(6) Transfer to 1 per cent aqueous eosin in a small Petri dish for 2 minutes [6].

(7) Transfer to a large dish of tap-water [7], gently agitating the fluid occasionally until the eosin is differentiated, usually about 4–5 minutes, or longer.

(8) Transfer to 70 per cent alcohol in a small Petri dish for 1–2 minutes [8].

(9) Transfer to 90 per cent alcohol in a small Petri dish for 1–2 minutes [9].

(10) Transfer to absolute alcohol in a large Petri dish [10], from which the sections are attached to clean, grease-free slides (as described for frozen sections on page 140).

Note. – Sections may be mounted from 96 per cent alcohol (methylated spirit) if preferred; when this is done the previous step of 90 per cent is omitted. The reason for mounting from absolute alcohol is that the celloidin is softened in this reagent, and therefore is less likely to corrugate. Beginners are advised to mount from 96 per cent alcohol, and only when they are reasonably proficient with this reagent, to mount from absolute alcohol.

(11) Flood the slides with carbol-xylol [12] (saturated solution of phenol in xylol) to clear the section, and then blot again, this process being repeated until the section is clear.

The reason for using an intermediate between alcohol and xylol is because of the difficulty of clearing sections by the routine method.

(12) Flood the sections with xylol [13], blot, flood again and blot with fresh filter paper to remove the phenol and creosote.

(13) Place the mountant of choice [14] on to the sections, a generous amount being used, and lay one end of a clean coverslip of an appropriate size on the slide about one-eighth of an inch from the section. Place the point of a dissecting needle under the other end, and slowly lower it on to the section. The trapping of air bubbles should be avoided as the coverslip is lowered, but any that are trapped may be expressed from the sides of the coverslip by pressure with the dissecting needle on the centre of the coverslip. The use of thicker balsam for mounting celloidin sections will make mounting easier. When the coverslip has set (the following morning) the excess balsam is wiped away and the slide cleaned with a cloth which has been moistened with xylol.

STAINING SERIAL SECTIONS OF CELLOIDIN-IMPREGNATED MATERIAL

Serial celloidin sections may be stained by either of the following methods.

(1) Singly, after numbering by one of the methods described on page 136.

(2) In strips or groups of sections. Of this second type of method the author prefers that of Obregia which is given below in detail.

It will be found that, unless large numbers of serial sections are to be stained regularly, the method of single staining is easier.

Equipment and Solutions

A sheet of opal glass, or white Perspex, of a convenient size (12 X 8 inches) to hold the largest number of serial sections dealt with is essential.

Celloidin, 1 per cent, in equal parts of alcohol and ether is required.

Obregia's Gum Solution (Anderson's Modification)

This is prepared by dissolving 45 g of dextrin in 150 ml of syrupus simplex by boiling until a clear solution is obtained, then slowly adding 150 ml of 80 per cent alcohol, shaking between additions. This solution should be well shaken before use.

Technique

(1) Wash the sheet of opal glass, or Perspex, in a solution of Teepol (or other detergent), rinse in hot water and dry thoroughly.

(2) Coat the sheet with Obregia's solution, making sure that the plate is completely and evenly covered with the solution by using a camel-hair brush (which should be washed immediately after use), or by smearing it over the surface with the tips of the fingers.

(3) Dry the plate in the oven or incubator, taking care to protect it from dust. When the surface is hard and smooth place it on the bench ready to receive the sections in rows in order from left to right. Mark the plate so that the order of the sections can easily be seen (such as filing or cutting off that corner of the plate where the first section is laid).

(4) The sections must be kept moist during the whole of the procedure, and this is achieved most simply by using the toilet paper slip method of cutting. The first paper is then lifted out of the dish (with the section still attached) and laid in position on the plate (section downwards), smoothed out with a camel-hair brush moistened with 70 per cent alcohol, and the paper lifted off with forceps without disturbing the section. If the section is in the correct position replace the paper with its edge overlapping the edge of the plate (Anderson, 1929).

(5) Position the next section in the same way, and replace the paper so that it overlaps the paper of the preceding section; continue arranging sections and papers until all the sections are on the plate. If there is a large number of sections it will be advisable to moisten the papers of the sections already plated with 70 per cent alcohol (using a camel-hair brush) from time to time.

(6) Remove the papers by lifting the overhanging edge of the first paper which will, in turn, lift the next one and so on; this method enables the papers to be removed without damaging or moving the sections.

(7) Blot all the sections firmly and carefully into position with filter paper which has been moistened with 70 per cent alcohol.

(8) Pour on to the plate a 1 per cent celloidin solution, spreading it evenly by tipping the plate backwards and forwards until every section is covered. Drain off the excess celloidin.

(9) Leave on a perfectly level bench until the celloidin has hardened; this is tested by running the finger gently over one side of the plate. When the celloidin does not rise it is sufficiently hard.

(10) Flood the whole plate with 96 per cent alcohol (methylated spirit) for a few minutes.

(11) With a sharp scalpel, cut round the outside edge of the plate without damaging the sections, also between the rows of sections. Since the strips are to be stained collectively they should be of a convenient size, and it may therefore be necessary to cut each long strip into two or three shorter strips.

(12) Pour off the spirit and place the whole plate in a dish of warm water; the gum will now dissolve and the strips of sections will float off. If difficulty is likely to be experienced in recognizing the order of the strips a system of dots with Indian ink may be employed during the hardening stage of the celloidin (stage 9).

(13) The strips may be stained immediately or stored in 70 per cent alcohol until required.

The method of staining is that already described for celloidin sections. Staining may be carried out in developing trays to accommodate large strips, using the one dish and tipping reagents into and out of the tray in their correct sequence. The sections may be mounted in strips, or cut with a sharp scalpel and mounted singly.

RAPID FROZEN SECTIONS FOR EMERGENCY DIAGNOSES

Demand will occasionally be made for a very rapid section, usually in a case of suspected malignancy. The patient may be in the operating

theatre under an anaesthetic, and the operation may be suspended until a diagnosis on a biopsy specimen is given by the pathologist.

These emergency diagnoses may sometimes be made from a Terry slice: a thin slice of tissue (½–1 mm) is cut with a sharp scalpel, laid on a slide and the upper surface painted with a polychrome methylene blue or Field's stain 'A' which will stain the top layer of cells. The stain is washed off, a coverslip laid on the upper surface, and it is then examined under the microscope. While the author has seen this technique used with success by one pathologist it demands a great deal of experience and generally a frozen section is preferred; in the case quoted, a frozen section was always cut to confirm the findings of the Terry slice.

Sections may be cut in a cryostat as described on page 142, or by the frozen section technique (page 137). When a warning has been given that a frozen section will be required at an approximate time, all the apparatus likely to be required – staining solutions, and so on – should be set up in a laboratory (or ante-room) near the theatre. This will ensure that no time is lost once the specimen is received from the theatre.

Ensure that the cylinder of CO_2 to be used in freezing the tissue is not exhausted; if there is any doubt use a fresh one, for nothing can be more irritating to the pathologist and to the surgeon (who will probably be present) than the sight of a technoligist with the specimen half frozen having to spend 10 minutes or more obtaining a new cylinder and changing over the connections from the microtome. A mental dress rehearsal, about an hour before the specimen is due to arrive, is sound practice. Imagine the specimen coming in, then go through all the steps necessary to cut, stain and mount it, to ensure that everything is ready and in the right position. Prepare for accidents by having available a spare microtome knife, spare glass hockey sticks, and so on.

Technique

Fixation

Tissue may be frozen unfixed or fixed. The choice is a matter of personal preference, both in cutting by the technologist, and in diagnosis by the pathologist. Fresh unfixed tissue is more difficult to cut than fixed tissue, and presents a slightly different appearance under the microscope. Fixation prolongs the processing, but the time is probably well spent, and a tube of boiling formalin should be at hand into which the tissue is immersed for 1 or 2 minutes before sectioning.

Cutting

Sections are cut as already described on page 139.

Attachment of Sections to Slides

Although some technologists prefer to carry sections through solutions, as described on page 197, it is recommended that the sections be attached to slides immediately after cutting, and then stained. Any of the methods given on page 140 may be used, but Wilson's technique, which was devised for rapid work, is probably the most efficient. Three sections should be attached to slides to guard against damage to one of them during staining.

Choice of Staining Technique

The stain or stains to be employed are again a matter of personal preference, and will depend largely upon the experience of the pathologist. A single stain will quite often suffice to show malignancy, and simple toluidine blue is often used first as a routine because of its speed of action. Further sections may be stained by a double stain such as haematoxylin and eosin, or the phloxine-methylene blue method of Thomas. The latter is exceptionally good for staining sections of unfixed tissue which occasionally gives a hazy picture with haematoxylin and eosin; it is also quicker and gives a better nuclear definition than a rapid haematoxylin and eosin. A toluidine blue stain is recommended, followed by phloxine-methylene blue for rapid frozen section staining.

Toluidine Blue Staining

Method

(1) Slides should be placed in 90 per cent alcohol for a second or two.

(2) Transfer to absolute alcohol for a second or two.

(3) Transfer to xylol and agitate the slide until the section is clear (about 2 seconds).

(4) Transfer to absolute alcohol for a second or two.

(5) Transfer to 90 per cent alcohol for a second or two.

(6) Transfer to the slide rack, flood with 1 per cent toluidine blue, and leave for ½–1 minute.

(7) Rinse rapidly in water, transfer to a pad of filter paper, and blot firmly.

(8) Flood with 90 per cent alcohol and blot firmly.

(9) Flood with absolute alcohol and blot firmly.

(10) Flood with xylol, blot firmly and, if the section is clear, mount under a coverslip with balsam or D.P.X. If the section is not completely clear after the first application of xylol, flood the slide with xylol and blot a second time, and repeat until the section is clear, when it is mounted.

The whole staining process should take 3–4 minutes.

Phloxine-methylene Blue (Thomas)

Solutions required

A	Phloxine	0.5 g
	Acetic acid	0.2 ml
	Distilled water	to 100 ml
B	Methylene blue	0.25 g
	Azure B	0.25 g
	Borax	0.25 g
	Distilled water	to 100 ml

Method

Stages 1–5 are as described above for toluidine blue staining. During these stages two slides are held together, back to back, after which separate staining techniques are employed for each slide.

(6) Transfer the slide to the slide rack, flood with water and drain.

(7) Flood with solution A for 1 minute.

(8) Wash in water for 10 seconds, and drain.

(9) Flood with solution B, and leave for ½ a minute.

(10) Wash with 0.2 per cent acetic acid in distilled water until clouds of excess stain cease to flow from the section (about 20–30 seconds).

(11) Give three washes with 96 per cent alcohol (methylated spirit) to differentiate the section.

(12) Flood with absolute alcohol and blot firmly.

(13) Flood with xylol, blot firmly and, if the section is clear, mount in balsam or D.P.X.

Recommended Staining Methods

ELEMENT	ROUTINE METHODS	OTHER METHODS
Adrenaline	Cramer's reaction	
Amyloid	Alkaline Congo red	King's silver technique
	Thiaflavin T	Lendrum's methyl violet
Argentaffin cells	Diazo method	Silver method
		Schmorl's ferric ferricyanide
Bismuth	Brucine-iodide	
Bone marrow	Cappell-Leishman	Maximow's stain
		Unna-Pappenheim
Bone	Weigert-van Gieson	Ground bone section
	Schmorl's picro-thionin	Schmorl's thionin
Calcium	Von Kossa's	Lillie's oxalic acid
Chromaffin tissue	Chromaffin reaction	
Connective tissue	Weigert-van Gieson	
	Trichrome stains	
Copper	Mallory's haema-toxylin	
Deoxyribonucleic acid (D.N.A.)	Feulgen reaction	Feulgen N.A.H.
		Unna-Pappenheim
D.N.A. and R.N.A. (Ribonucleic acid)	Unna-Pappenheim (enzyme controlled)	Fluorescent micro-scopy
Elastic tissue	Gomori's aldehyde fuchsin	Sheridan
	Verhoeff	Hart-Weigert
Eleidin	Picro-nigrosin	
Fibrin	Mallory's P.T.A.H.	Weigert's fibrin stain.
	Lendrum's acid picro-Mallory.	
Glycogen	P.A.S. (with enzyme control).	Best's carmine
		Iodine
Golgi apparatus	Da Fano Cajal	
	Baker's Sudan black B	Ludford-Weigl
Haematoidin (Bilirubin)	Gmelin's	Stein
Haemoglobin	Lison's patent blue	
Haemosiderin	Tirmann-Schmelzer's Turnbull blue.	Perl's Prussian blue
Iron	Tirmann-Schmelzer's Turnbull blue.	Perl's Prussian blue
Lipids (all)	*See* chart	

Recommended Staining Methods—*contd.*

Element	Routine Methods	Other Methods
Lipofuscin	Schmorl's ferric-ferricyanide	Sudan black B
Lead	Mallory's haema-toxylin	
Melanin	Masson-Fontana	
Mitochondria	Altmann's acid fuchsin	Champy-Kull Heidenhain
Mucin	Alcian blue aldehyde fuschin Mucicarmine	P.A.S. Thionin Weigert-Pal
Myelin (normal)	Luxol fast blue Loyez	
Myelin (degenerate)	Swank-Davenport	Marchi
Nervous system (general)	Haematoxylin and eosin Mallory's P.T.A.H.	Morris's rapid method
Nerve cells and axons (neurones)	Bielschowsky Glees and Marsland	Gros-Schultz
Neutral fats	Chiffelle and Putt's propylene glycol Lillie's isopropanol	Govan's gelatin-dye
Neutral red vacuoles	Vital staining	
Neuroglia fibres	Mallory's P.T.A.H.	Anderson's Victoria blue
Neuroglia astrocytes	Scharenberg's triple impregnation	Cajal's gold sublimate
Neuroglia, microglia and oligodendroglia	Penfield's silver carbonate Weil-Davenport	
Nissl substance	Unna-Pappenheim	Gallocyanin Thionin
Nuclei	Heidenhain's haematoxylin Toluidine blue	Romanowsky stain
Pancreas	Trichrome stains	Gomori's aldehyde fuchsin
Paneth cell granules	Lendrum's phloxine-tartrazine	
Pituitary (pars anterior)	Trichrome stains	P.A.S. orange G. acridine orange
Plasma cells	Unna-Pappenheim	
Reticulin fibres	Gomori's	Foot's Gordon and Sweet
Suprarenal glands	Haematoxylin and eosin Chromaffin reaction	Giemsa Toluidine blue

Results

Nuclei and bacteria	Blue
Collagen and muscle	Bright red
Erythrocytes	Bright scarlet

The staining process should be completed in 3–4 minutes.

Rapid Haematoxylin and Eosin

Method

Stages 1–5 are as described for toluidine blue staining.

(6) Transfer the slide to a Coplin jar of haematoxylin for 2 minutes.

(7) Rinse in tap-water and dip in 1 per cent acid alcohol; wash immediately in tap-water to which a few drops of saturated aqueous solution of lithium carbonate have been added, and leave in this solution until the section is blue (about 20–30 seconds).

(8) Transfer the slide to a Coplin jar containing 1 per cent eosin, leave for 10–15 seconds and then rinse in water.

Stages (8), (9) and (10), as described for toluidine blue staining, are carried out to dehydrate, clear and mount the section.

The time taken to complete the staining process is approximately 5 minutes.

Chapter 11

Routine Stains

A good routine stain for histological diagnosis must stain selectively not only cell nuclei and cytoplasm, but also connective tissue. A properly differentiated haematoxylin and eosin stained section will distinguish these structures, the nuclei appearing blue and the cytoplasm and connective tissue fibres shades of pink; for that reason it is the most popular routine stain. Some pathologists prefer a preparation in which the connective tissue fibres are stained various colours instead of differing shades of one colour; certain connective tissue stains have therefore been included in this chapter (*see also* page 412).

HAEMATOXYLIN AND EOSIN

Haematoxylin and eosin is the most popular routine stain in Great Britain and, whilst there are many formulae for haematoxylin, that devised by Ehrlich is the most popular because of its durability, easy differentiation and comparative permanency. It has the disadvantages that it requires 1–2 months to ripen after preparation and, when used as a regressive stain, sections need 30 minutes immersion to stain adequately. Other haematoxylins (listed below) may be used to overcome these disadvantages, but it should be remembered that each of them has its own peculiar advantages and disadvantages.

As a counterstain 0.5–1 per cent aqueous solution of water-soluble eosin is still generally preferred, although in the United States of America, and more recently in Great Britain, phloxine (0.55–1 per cent aqueous solution) has been used. The latter gives a more vivid and brilliant red stain, but eosin, properly differentiated, gives a more informative picture. Orange G, Biebrich scarlet, and Bordeaux red have also been used as counterstains.

Staining Technique

The staining technique has been fully described, with varying types of embedding media, in Chapter 10; the only changes with the various haematoxylins are in the staining times and these will be mentioned together with the advantages and disadvantages of each stain, in the methods which follow.

HAEMATOXYLINS

Ehrlich's Alum Heamatoxylin

Staining time. – Thirty to forty minutes.

Haematoxylin	6 g
Absolute alcohol	300 ml
Distilled water	300 ml
Glycerol	300 ml
Glacial acetic acid	30 ml
Potassium alum	in excess

Preparation

The haematoxylin should be dissolved in the alcohol, in which it is readily soluble, and the other components added in the order given. Finally, potassium alum is added while the solution is shaken until there is a deposit of alum crystals on the bottom of the stock container. This is one of the haematoxylins where the mordant (potassium alum) is combined with the stain. The incorporation of glycerol is said to give more even and precise staining, to stabilize the stain against over-oxidation, and to prevent rapid evaporation.

Ehrlich's haematoxylin retains its staining power for years, and lasts for months in a Coplin jar.

Ripening

After preparation of the stain it must be kept in a loosely plugged bottle in a warm, light place–for example, on the window sill–until oxidation of the haematoxylin to haematein has taken place (the process known as ripening); this will take from one to two months. A fresh stock solution of this stain should be prepared well in advance, and one bottle should be ripening while another is in use.

Delafield's Alum Haematoxylin

Staining time. – Fifteen to twenty minutes.

(*a*)	Ammonium alum	55 g
	Distilled water	600 ml
(*b*)	Haematoxylin	6 g
	Ethyl alcohol	50 ml
(*c*)	Glycerol	150 ml
	Ethyl or methyl alcohol	150 ml

Preparation

Prepare solutions (*a*), (*b*) and (*c*) separately. Mix (*a*) and (*b*) and leave overnight, pass the mixture through filter paper and add (*c*). Delafield's original formula called for methyl alcohol but ethyl alcohol gives equally good results.

Ripening

Like Ehrlich's stain, Delafield's needs to be stored for 1–2 months in a light warm place to ripen; it will keep almost indefinitely.

Harris's Alum Haematoxylin

Staining time. – Fifteen to twenty minutes.

Haematoxylin	2.5 g
Absolute alcohol	50 ml
Ammonium or potassium alum	50 g
Distilled water	500 ml
Mercuric oxide	1.5 g
Glacial acetic acid	20 ml

Preparation

Dissolve the haematoxylin in the absolute alcohol, and the alum in the water–using heat if necessary– and mix the two solutions together. Heat the mixture to boiling point and add the mercuric oxide; cool rapidly by plunging the flask into cold water. The solution is ready for staining as soon as it is cool. Although optional, it is preferable to add

the glacial acetic acid before use as this gives more precise and selective nuclear staining.

Harris's alum haematoxylin has the advantage of being ready for use as soon as it is cool.

Ripening

The addition of the mercuric oxide ripens the stain immediately.

Although this haematoxylin is said to have good durability, the selectivity and speed of staining decreases after two or three months; it is advisable, therefore, to prepare small quantities each month, rather than a large stock. A precipitate forms during storage which may be filtered off without detriment to the stain, although this is an indication that the stain is deteriorating.

Mayer's Haemalum

Staining time. – As a progressive stain – 15 minutes. As a regressive stain – 40–60 minutes.

Haematoxylin	1 g
Distilled water	1,000 ml
Ammonium or potassium alum	50 g
Sodium iodate	0.2 g
Citric acid	1 g
Chloral hydrate	50 g

Preparation

Dissolve the haematoxylin in the distilled water, using gentle heat if necessary. Add the alum, shaking to dissolve but again using heat if required; when the alum is dissolved add the sodium iodate followed by the citric acid and chloral hydrate.

This modification by Mayer of his early haemalum uses haematoxylin instead of haematein and is recommended because of its improved durability.

Mayer's haemalum is very precise for nuclei, and is one of the few haematoxylin stains still used progressively, usually as a counterstain (for example, after mucicarmine or glycogen staining). It is particularly good for the demonstration of amoebae in sections if diluted with an equal quantity of distilled water and differentiated after 12–24 hours.

Weigert's Iron Haematoxylin

Staining time. – Twenty to thirty minutes.

(*a*)	Haematoxylin	1 g
	Absolute alcohol	100 ml
(*b*)	30 per cent aqueous solution of ferric chloride	4 ml
	Distilled water	100 ml
	Hydrochloric acid	1 ml

Preparation

Mix equal parts of (*a*) and (*b*) immediately before use. When mixing in a test-tube it is convenient to put solution (*a*) into the tube first, then add solution (*b*), when they will mix easily; if (*b*) is put into the tube first the solutions are much more difficult to mix. The staining solution should be violet-black in colour.

Ripening

Solution (*a*) must be allowed to ripen and should be prepared about a week before it is required, or it may be prepared from a stock solution of 5 per cent haematoxylin in absolute alcohol. The latter is useful for preparing both this stain and Heidenham's haematoxylin and, after 4–5 weeks, has the advantage of being ripened ready for use at all times.

Since this is an iron haematoxylin it stains nuclei black, resists removal by counterstains containing differentiating agents such as picric acid (van Gieson), and will not be decolorized by light; it is, therefore, a more permanent stain than the alum haematoxylins.

Heidenhain's Iron Haematoxylin

Staining time. – Thirty to forty-five minutes at 56°C will give adequate results with formol saline, Susa and Bouin fixatives, and usually with chrome fixatives such as Zenker, but the last named sometimes inhibits staining and slightly longer times may be necessary.

Material fixed in chrome-osmium fixatives, which inhibit most haematoxylin stains, will need 12–24 hours at room temperature for adequate staining.

Principle

This method stains tissue jet black and, by selective differentiation *(see below)*, may be used to demonstrate a great variety of tissue components in shades of black and grey; it is, therefore, ideal for photography. It is permanent, provided that the alum is properly removed, and is applicable after any fixative.

It is only used regressively (over-stained and then differentiated), and experience is required to obtain the best results. Beginners should differentiate in half-strength iron alum until they are familar with the technique.

The technique of Heidenhain's iron differs from the other haematoxylin techniques in two respects: (1) the mordant is employed separately from the haematoxylin; and (2) the mordant is employed as a differentiating agent.

Iron alum, in addition to being a mordant, is a powerful oxidizing agent, and it oxidizes the haematoxylin into a colourless soluble compound which diffuses into the alum solution.

Staining Solutions Required

Iron Alum Solution

Ferric ammonium sulphate (violet crystals)	5 g
Distilled water	100 ml

Haematoxylin Solution

Haematoxylin	0.5 g
Absolute alcohol	10 ml
Distilled water	90 ml

This solution must be kept for 4–5 weeks to ripen.

or

5 per cent stock (ripened) alcoholic haematoxylin	10 ml
Distilled water	90 ml

This solution is ready for immediate use.

Staining Technique

(1) Bring sections to 90 per cent alcohol as described on page 192.

(2) Transfer to a Coplin jar containing iron alum solution for 30–45 minutes at 56°C, or at room temperature for 12–24 hours.

(3) Rinse rapidly in water.

(4) Transfer to a Coplin jar containing the haematoxylin solution and leave for the same time and at the same temperature as in iron alum.

(5) Rinse rapidly in water.

(6) Differentiate in iron alum solution, either the 5 per cent used above, or a 2 per cent solution which will give a slower and more easily controlled differentiation. Two-thirds saturated picric acid (6 per cent) in 96 per cent alcohol, which is slower than 5 per cent alum, gives a slightly better differentiation of muscle striations.

(7) Wash the slide in running water for 5 minutes to remove the iron alum. Dehydrate, clear, and mount in D.P.X. or Canada balsam, as described on page 195 (stages 10–18).

Results

Provided that the method of fixation has preserved them, the following tissue elements may, by the degree of differentiation employed, be demonstrated: mitochondria, cross-striation of muscle fibres, ground cytoplasm, nuclear membrane, yolk, chromosomes, chromatin, nucleoli and centrioles.

The section will be jet black when removed from the stain, and successive short washes in the differentiating solution will demonstrate the above tissue elements in the order given. Differentiation of Heidenhain's stain must be rigidly controlled at all stages under the microscope. At any stage of differentiation, it is possible to return the section to haematoxylin to restain an element which has been over-differentiated.

It is possible to use a connective tissue counterstain such as van Gieson's, but as this may detract from the main stain it is not commonly used.

Mallory's Phosphotungstic Acid Haematoxylin (P.T.A.H.)

Staining time. – Twelve to twenty-four hours.

Haematein	1 g
Phosphotungstic acid	20 g
Distilled water	1,000 ml

Mallory's haematoxylin is a good routine stain for use with tissues of the nervous system. It entails a bleaching technique at the beginning (stages 2–5) which aids differential staining, and is known as the Mallory bleach.

Preparation

Dissolve the haematein and the phosphotungstic acid separately in distilled water, using gentle heat. When cool, combine the solutions, and make up to one litre.

Ripening

This is accomplished immediately by adding 0.177 g of potassium permanganate, which is the more common practice, or by exposing to light and warmth for 5–6 weeks.

Staining Technique

(1) Bring section to water and treat with iodine and sodium thiosulphate (stages 1–3*b* on page 193).
(2) Place in 0.25 per cent aqueous potassium permanganate for 5 minutes.
(3) Wash in water for 2 minutes.
(4) Rinse in distilled water.
(5) Place in 5 per cent oxalic acid for 10 minutes.
(6) Rinse in distilled water.
(7) Wash in water for 5 minutes and rinse in distilled water.
(8) Stain in P.T.A.H. for 12–24 hours.
(9) Dehydrate through 95 per cent and absolute alcohol. This must be done rapidly as alcohol removes the red staining.
(10) Clear in xylol and mount in Canada balsam or D.P.X.

Results

Nuclei, centrioles, fibroglia, myoglia and neuroglia fibres, fibrin and cross-striation of muscle fibres	Blue
Collagen, reticulin, and ground substance of bone	Yellow to brick red

COUNTERSTAINS ROUTINELY EMPLOYED WITH HAEMATOXYLIN NUCLEAR STAINS

Of the single counterstains employed, eosin is preferred for the reasons given at the beginning of this chapter. Details of the technique have

been given in the section on haematoxylin and eosin staining (page 195).

Phloxine, orange G, Biebrich scarlet and Bordeaux red are used in a similar manner to eosin.

van Gieson's Stain

Saturated aqueous solution of picric acid (approximately 1 per cent)	100 ml
1 per cent acid fuchsin	10 ml

The above quantities may be prepared and kept as a stock solution, but it is better to use a freshly prepared solution containing 5 ml of saturated aqueous solution of picric acid and 0.75 ml of 1 per cent acid fuchsin which gives more precise and sharp staining.

Although 1 per cent ponceau S may be used in place of acid fuchsin to give a more permanent stain, it is not so precise and the original method is preferred.

As the picric acid component quite rapidly removes alum haematoxylin, this counterstain is usually employed after an iron haematoxylin (usually Weigert's), which it removes more slowly.

Staining Technique (Weigert and van Gieson)

(1) Bring section to water.

(2) Stain with Weigert's haematoxylin for 40 minutes on the staining rack.

(3) Blue in tap-water.

(4) Differentiate in 1 per cent acid alcohol.

(5) Blue in tap-water.

(6) Place the slide on the rack and flood with van Gieson's stain, leave for 3 minutes.

(7) Rinse rapidly in tap-water to differentiate the fuchsin.

(8) Dehydrate in 90 per cent and absolute alcohol which a few drops of saturated picric acid in alcohol have been added, rinse quickly in fresh absolute alcohol, blot and clear in xylol (picric acid is removed rapidly by alcohol, and the addition of picric acid to the dehydrating bath prevents this).

(9) Mount in acid balsam (to preserve the counterstain) or D.P.X.

Results

Nuclei	Blue black
Collagen	Bright red
Cytoplasm, muscle, red blood cells	Yellow

EOSIN-AZURE STAINS (ROMANOWSKY STAINS)

These methods (described on page 406) are sometimes used in place of haematoxylin and eosin stains since they give better cytoplasmic differentiation. Lillie in the United States America uses them almost exclusively, but such a practice is still uncommon in Great Britain. The ease of staining and the better nuclear picture obtained with haematoxylin and eosin probably accounts for this.

SINGLE STAINS

Single stains such as toluidine blue are occasionally employed, usually for a rapid diagnosis (*see* page 206).

Chapter 12

Proteins

INTRODUCTION

The histochemical differentiation of proteins requires a knowledge of their characteristics, as well as the types and reactive groups of the amino acids. The time spent on this aspect will enable the student of histochemistry to utilize the right technique, and the appropriate blocking methods, if they are presently available.

The proteins are a group of complex organic nitrogenous compounds, widely distributed in plants and animals, which form the principal constituents of the cell protoplasm. They are essentially combinations of α-amino acids and their derivatives. It is perhaps appropriate that the word 'protein' is derived from the Greek word *proteios* meaning 'of first importance'.

CHEMISTRY

Proteins are macromolecules and, like nearly all biological macromolecules, are polymers. There is no definitive number of monomeric units required to make a protein. The lower boundary for the molecular weight of proteins is about 5,000, but those commonly occurring in living material have molecular weights very much higher.

The Peptide Bond

Proteins have been likened to strings of beads, each bead being an amino acid, the amino acids being held together by the peptide bond;

In 1902, Fischer and Hofmeister independently suggested that proteins were formed by the splitting out of water (H_2O) from the α-amino (or α-imino) group (NH_2) of one amino acid and the terminal carboxyl (COOH) of the adjacent amino acid *(see Figure 12.1)*, the

resulting linkage is called 'the peptide bond'. Experimental evidence over the past 60 years has confirmed the theory of the peptide bond which is now considered to be the principal type of linkage.

Proteins may be classified into *simple proteins,* yielding only α-amino acids on hydrolysis, and conjugated proteins which yield α-amino acids and one or more groups of a non-protein nature. The latter are known as prosthetic groups (*gr. prosthesis,* an addition).

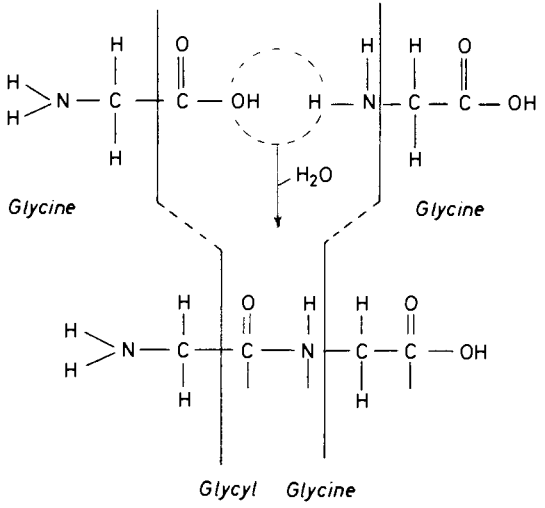


Figure 12.1 – The peptide bond

Simple Proteins

(*a*) *Albumens.* These are very soluble proteins which can be precipitated from an aqueous solution by saturating with an acid salt such as ammonium sulphate, or by saturating with a neutral salt such as sodium sulphate in slightly acid solution.

(*b*) *Globulins.* These are insoluble in water but soluble in dilute salt solutions, for example, 5 per cent sodium chloride solution, and are precipitated by half saturation with ammonium sulphate. They occur in serum and tissue; antibodies are found in the globulin fraction of serum.

(*c*) *Schleroproteins (albuminoids).* These are the fibrous proteins having a supporting or protective function in the animal organism. Sub-members are the *collagen* of the skin, tendons and bones, the *elastins* of elastic tissue, and the *keratins* from hair, nail and horn.

TABLE 12.1

		Isoelectric point (pI)
Neutral Amino Acids		
1.	Glycine	5·97
2.	Alanine	6·02
3.	Valine	5·97
4.	Leucine	5·98
5.	Isoleucine	6·02
6.	Serine	5·68
7.	Threonine	6·53
Sulphur-containing		
8.	Cysteine	5·02
9.	Cystine	5·06
10.	Methionine	5·75
Aromatics		
11.	Phenylalanine	5·98
12.	Tyrosine	5·65
13.	Thyroxine	
14.	Tryptophan	5·88
Imino acids		
15.	Proline	6·10
16.	Hydroxyproline	5·83
Basic Amino Acids		
17.	Lysine	9·74
18.	Hydroxylysine	9·15
19.	Argenine	10·76
20.	Histidine	7·58
Acidic Amino Acids		
21.	Aspartic acid	2·87
22.	Glutamic acid	3·22

(*d*) *Histones.* These are soluble proteins which are basic and are precipitated by the addition of ammonium hydroxide. Very few examples of this class of proteins are known; they are probably limited to the nuclei of cells. Thymus histone is a classic example.

(*e*) *Protamines.* These are water-soluble proteins which are very basic in nature and are not coagulated by heat. Protamines form crystalline salts with mineral acids, and insoluble salts with other more acidic proteins. An example of a protein salt is the commonly used drug, protamine-insulin. Protamines are the simplest proteins.

Conjugated Proteins

(*a*) *Nucleoproteins.* These are proteins combined with nucleic acids.

(*b*) *Glycoproteins.* These are proteins combined with carbohydrates, for example, mucin.

(*c*) *Lipoproteins.* These are proteins combined with lipids, for example, serum lipoprotein.

(*d*) *Chromoproteins.* These are proteins combined with pigments, for example, haemoglobin.

(*e*) *Phosphoproteins.* These are proteins combined with phosphoric acid, for example, casein of milk.

Amino Acids

The most commonly occurring 22 amino acids are listed in *Table 12.1* with their isoelectric points, and have been grouped as neutral, basic or acidic; the sub-groups indicating structural differences, and so on.

A protein with a high proportion of amino acids with two carboxyl radicals (for example, aspartic) will be an acid protein and one that has a high proportion of basic amino groups (for example, histidine) will be a basic protein. Those that are mainly composed of amino acids with one amino and one carboxyl radical, or have balanced amounts of acid and basic amino groups will be neutral proteins.

IDENTIFICATION OF PROTEINS

In view of the fact that tissue sections are composed mainly of proteins and that there are many commonly used stains (both acid and basic) which will stain a section in its entirety, it follows that proteins will stain with relative ease. However, when the conditions of staining are

carefully controlled proteins can be shown to be either acid, basic or neutral. Their ability to bind each stain is due to the fact that they are amphoteric in nature; that is, they have the capacity to ionize as acids or bases, depending upon the medium immediately surrounding them.

TABLE 12.2

Reactive Groups in Amino Acids

Group	*Formula*	*Chemical nature*	*Distribution*
Amino	$-NH_2$	Primary amine	All amino acids except the prolines
Imino	$-N-H-$	Secondary amine	Proline, hydroxyproline
Carboxyl	$-COOH$	Acid carboxyl	Aspartic, glutamic acids
Hydroxyl	OH	Alcoholic hydroxyl	Serine, threonine hydroxyproline
β-thio	$-S-S-$	Disulfide	Cystine
β-thiol	$-S-H$	Sulphydryl	Cysteine
δ-guanidine	$H_2N-C(=NH)-N(H)-$	Strongly basic group	Arginine
β-hydroxyphenyl	HO-C$_6$H$_4$-	Phenol (aromatic –OH) derivative	Tyrosine
Indole		Aromatic heterocycle	Tryptophan

An acid protein will therefore stain with an acid dye if the dye solvent is sufficiently acid and *vice versa.*

Proteins are ubiquitous components of all living tissue and, because of their complexity, the infinite nature of their variety and the fact that their constituent molecular chains may fold, spiral or cross-link with other chains, complete histochemical identification is extremely difficult, if not completely impossible. This is true because the multiplicity of reactive groups available to us for identification *(Table 12.2),* are not specific for any one amino acid, much less for any one type of protein.

Some of them, however, do have certain features which, when combined with available blocking procedures and micro-anatomical localization, will enable group and occasionally individual identification. It should be remembered that immunohistochemical methods using fluorescent tagged antibodies can be used to give specific information as to the nature of individual proteins. Enzymes are also a special group of proteins which can be identified specifically. They are considered separately in Chapter 15.

In spite of the foregoing, it is often of importance to identify material as being proteinaceous in nature and to demonstrate increases in certain types of amino acids in particular areas.

Fixation

The type of fixative used will depend upon the reactive groups involved in the method to be employed; obviously, methods for amino groups (NH_2) are unlikely to be positive after fixation with aldehydes, since they intereact; similarly, mercuric chloride containing fixatives are to be avoided when sulphydryl (SH, thiol) groups are to be demonstrated. The recommended method of fixation is given for each technique.

GENERAL METHODS FOR PROTEIN

Determination of Proteins (by insolubility at their isoelectric points)

This method is based upon the fact that a protein will be least soluble at its isoelectric point. Frozen dried sections are treated in a selected buffer solution at room temperature for 1–2 hours. Sections are then fixed in alcohol (general proteins), formalin (mucoproteins) or Carnoy's fluid for nucleoproteins for 4–12 hours. They are then stained by an appropriate method.

Millon Reaction (Modified by Baker, 1956)

This reaction for tyrosine may be used as an oversight method for most proteins (but not collagen). It depends upon the hydroxyphenyl group (only found in tyrosine in tissue) being converted into a nitrosophenol by the sodium nitrite to which the mercury links to form a red compound.

Fixation is not critical – formalin, alcohol, and so on, may be used.

Solutions Required

(1) *Mercuric sulphate solution*

Sulphuric acid (conc)	10 ml
Distilled water	90 ml
Mercuric sulphate	10 g

Add acid to water, then add mercuric sulphate. Stir and heat to dissolve. Allow to cool and make up to 200 ml

(2) *0.25 per cent Aqueous solution of sodium nitrate*

Method

(1) Bring paraffin sections to water.

(2) Place the sections in a small beaker containing 30 ml of mercuric sulphate solution and 3 ml of sodium nitrate solution. Heat beaker gently until solution boils and leave sections for 30 seconds.

(3) Remove sections and rinse in three changes of distilled water (1–2 minutes each).

(4) Dehydrate, clear and mount in a synthetic resin.

Results

Tyrosine containing proteins (most proteins) are stained red, pink or yellow-red.

Oxidized Tannin-Azo Technique (OTA)
(Dixon, 1959, 1962)

This method is dependent upon the attachment of tannic acid to the tissue proteins; it is thought to react with the amino groups since there is a marked diminution of staining after deamination in nitrous acid (page 241). Following the reaction of the tannic acid with protein, it is oxidized by periodic acid to a 1:2-quinone, which is coupled with diazotized O-dianisidine to produce a salmon-red azo dye (azoquinone).

Fixation – Carnoy's fluid gives excellent results (Dixon, 1962).

Solutions Required

(1) *Tannic-Hcl*

Tannic acid	10 g
Distilled water	225 ml
N/l hydrochloric acid	25 ml

(2) *0.5 per cent Aqueous periodic acid (pH 4.0).*

(3) Buffered diazotized O-dianisidine (pH 4.0) (ice-cold solutions should be used in preparation).

Fast blue B	100 mg
0.2 M acetic acid	82 ml
0.2 M sodium acetate	18 ml

Method

(1) Bring sections to distilled water.
(2) Transfer sections to Coplin jar of Tannic-Hcl and leave for 10 minutes.
(3) Wash well in distilled water (3 changes).
(4) Oxidize with periodic acid for 5 minutes (sections darken).
(5) Wash well in distilled water (3 changes).
(6) Transfer to ice-cold distilled water for 2 minutes.
(7) Place in ice-cold buffered diazotized O-dianisidine solution in the refrigerator and leave for 20 minutes.
(8) Wash in running tap-water.
(9) Dehydrate, clear and mount in a neutral synthetic resin.

Results

Tannophilic proteins are salmon pink.

Control Sections

Stain an extra section omitting steps (4) and (5); this section should be either colourless or a faint pink colour.

Oxidized Tannin-oxazine Technique (OTO) (Dixon, 1962)

This is a variant of the above (OTA) method and differs in that the 1:2 quinone is reacted with freshly prepared 6-amino 3-dimethylamino-phenol into a blue-grey oxazine.

Solutions Required

(1) *Tannic-Hcl solution (as above).*
(2) *0.5 per cent Aqueous periodic acid.*
(3) *Freshly prepared aminophenol solution*

6-nitroso 3-dimethyl aminophenol (I.C.I. Ltd.) . . .	500 mg
Glacial acetic acid	50 ml

Dissolve 500 mg of 6 nitroso- 3 dimethyl aminophenol (ICI) in 50 ml of glacial acetic acid. This dark orange solution is cooled until crystals begin to form and then an excess of zinc dust is added. The orange colour disappears and the mixture is filtered free of zinc dust into a 100 ml cylinder, until 34 ml of filtrate is obtained; this is made up to 100 ml with glacial acetic acid. In contact with air, the solution becomes blue. It should be used immediately.

Method

(1) Steps (1) to (5) as for OTA above.
(6) Wash with three changes of glacial acetic acid.
(7) Place in aminophenol solution at 37°C for 20 minutes.
(8) Wash in glacial acetic acid.
(9) Dehydrate in alcohol, clear in xylol and mount.

Results

Tannophilic proteins are stained blue-grey (including nuclear chromatin and what appears to be mitochondria).

Acrolein-Schiff Technique

According to van Duijn (1961) the double bond of acrolein will react with SH, NH_2, NH and imidazoles leaving a free aldehyde to react with Schiff reagent. Acrolein is toxic and lachrymatory and should be handled in a fume hood.

Fixation – not critical.

Solutions Required

(1) *5 per cent Acrolein in 95 per cent ethanol in screw-capped Coplin jar.* The acrolein must be fresh and should not be discoloured.
(2) *Schiff reagent* (*see* page 229).

Method

(1) Bring sections to 95 per cent alcohol.
(2) Place in 5 per cent acrolein for 15–60 minutes (usually 15 minutes).
(3) Wash in three changes of 95 per cent ethanol.
(4) Wash in distilled water.
(5) Place in Schiff reagent for 10–30 minutes.
(6) Wash in running water for 1 minute.
(7) Dehydrate, clear and mount.

Result

Sites of protein reactive groups (*see above*) are coloured red.

Dinitro-fluoro-benzene Technique (DNFB) (Danielli, 1953)

This technique is dependent upon the reaction of the aromatic hydroxyl of tyrosine, SH, free α-amino groups (and possibly the imidazole of histidine) with DNFB to give a colourless product; this product is reduced with titanous chloride, diazotized and then coupled with 'H-acid' to give a red-purple colour.

Fixation in Carnoy, alcohol, and so on.

Solutions Required

(1) *Alkaline DNFB solution*
DNFB . . . . . 1 ml
Absolute ethanol . . . . . 99 ml
N/1 sodium hydroxide . . . . . 0.2 ml
(2) *Titanous chloride solution*
15 per cent Titanous chloride . . . . . 2 ml
0.5 M Sodium citrate buffer (pH 4.5) . . . . . 8 ml
(3) *Freshly prepared nitrous acid*
5 per cent Sodium nitrite . . . . . 8 parts
N/1 Sulphuric acid . . . . . 1 part
(4) *'H-acid' solution*
Saturated solution of 'H-acid' (8 amino-1 naphthol-3-6-disulphonic acid) in veronal acetate -Hcl buffer at pH 9.0.

Method

(1) Remove wax with light petroleum (if necessary).
(2) Wash sections with absolute ethanol.
(3) Incubate in alkaline DNFB (at 22°C) for 2–20 hours (usually 2–4 hours).
(4) Wash four times in 90 per cent alcohol, and rinse in distilled water.
(5) Reduce in titanous chloride solution at 37°C for 15–30 minutes.
(6) Wash in 0.5 M sodium citrate buffer (pH 4.5), followed by distilled water.
(7) Diazotize in fresh nitrous acid at 4°C (in refrigerator) for 5 minutes.
(8) Wash in distilled water.
(9) Couple in 'H-acid' solution (pH 9.0) in refrigerator at 4°C for 5 minutes.
(10) Wash in running water.
(11) Dehydrate, clear and mount in a synthetic resin.

Result

Proteins (*see* above) will be coloured red-purple. Appropriate blocking methods can be used to make reaction specific for tyrosine.

Biebrich Scarlet Technique for Basic Protein (Spicer and Lillie 1961; Lillie, 1965)

This method is based upon the principle of selective dye uptake at various pH levels (*see* above).

Fixation – buffered mercuric chloride, Carnoy, alcohol, but *not aldehydes* (6 per cent $HgCl_2$ in 1.25 per cent sodium acetate).

Solutions Required

Spicer and Lillie (1961) used 0.01 per cent Biebrich scarlet in glycine NaOH buffers at pH 8, 9.5 and 10.5. Lillie prefers using 1 ml of 1 per cent Biebrich scarlet in 49 ml of each of the appropriate buffer solutions (0.02 per cent) for a shorter period.

Method

(1) Bring sections to water, treat with iodine-hypo sequence if mercury fixative is used (*see* page 40).

(2) Stain for 30–90 minutes in each of the 0.01 per cent staining solutions, or stain for 20 minutes in Lillie's 0.2 per cent solutions.

(3) Without rinsing in water, dehydrate with 95 per cent then 100 per cent alcohol.

(4) Clear in xylene, and mount in synthetic resin.

Results

At pH 9.5 basic proteins stain strongly, above and below this pH the staining is appreciably lighter.

METHODS FOR SPECIFIC GROUPS OR AMINO ACIDS

It should be remembered that very few of these methods are specific alone, they must always be controlled by the appropriate blocking methods.

Tyrosine

The Millon reaction (page 226) is probably the best and most reliable, almost equally so is this diazotization coupling technique. The technique is based upon the fact that tyrosine bearing proteins develop a yellow colour on treatment with nitrous acid (diazotization), this compound is then coupled with 'S-acid' to give a red compound. The urea (Lillie) or ammonium sulfamate (Glenner) is added to the coupling reagent to decompose excess HNO_2.

Fixation – Neutral formalin, Carnoy, and so on.

Diazotization – Coupling Technique for Tyrosine (Glenner and Lillie, 1959)

Solutions Required

(1) *Diazotizing agent*

Sodium nitrite	4 g
Distilled water	56 ml
Dissolve the nitrite in the water, then add:	
Glacial acetic acid	3.4 ml

(2) *Alkaline coupling reagent*

70 per cent Alcohol	50 ml
8-amino-1-naphthol-5-sulphonic acid (S-acid)	500 mg
Potassium hydroxide	500 mg
Either Urea	1 g
or Ammonium sulphamate	500 mg

Dissolve in order and chill to 3°C in refrigerator.

Method

(1) Bring sections to water.

(2) (Optional) Geyer (1962) recommends pre-treatment of sections in 10 per cent iodine in alcohol for 1–6 hours to accelerate and intensify the reaction.

(3) Diazotize overnight at 3°C in strict darkness (or for 1–6 hours after iodine treatment).

(4) Rinse in four changes of distilled water at 3°C for 5 seconds each.

(5) Place in alkaline coupling reagent for 1 hour at 3°C also in the dark.

(6) Wash in 3 changes of 0.1 N hydrochloric acid for 5 minutes in each.

(7) Wash in running water for 10 minutes.

(8) Dehydrate, clear and mount.

Results

Tyrosine sites are coloured red-purple to pink. Hair cortex, soft keratin, the inner sheath cells and medulla of the root zone of hairs show a strong reaction.

DMAB-Nitrite Method for Tryptophan

This method is based upon the reaction of the DMAB (dimethylaminobenzaldehyde) with the indole to form a β-carboline, which is converted to a blue pigment by nitrate oxidation. The structure of the pigment (carboline blue) is unknown.

Fixation – short formalin (6–12 hours), 70 per cent methanol, 10 per cent aqueous sulphosalicylic acid.

Solutions Required

(1) *DMAB solution* 5 per cent *p*-dimethylaminobenzaldehyde in concentrated hydrochloric acid (sp.G. 1.18).

(2) *1 per cent Soaium nitrite in concentrated hydrochloric acid.*

Method

(1) Sections should be firmly affixed to slides (albuminized or coated with chromate gelatin), dry in 37°C oven for 2–3 days.

(2) Bring slides to alcohol, and coat with celloidin if there is doubt about their ability to withstand treatment (*see* page 142).

(3) Immerse sections in DMAB solution for 1 minute.

(4) Transfer to 1 per cent sodium nitrite-Hcl solution for 1 minute.

(5) Wash in water for 30 seconds.

(6) Rinse in 1 per cent acid-alcohol.

(7) Dehydrate, clear and mount.

Results

Tryptophan containing proteins are coloured deep blue, for example, fibrin, Paneth cell granules, and so on.

Sakaguchi Dichloronaphthol Hypochlorite Technique for Argenine (Deitch, 1961)

This method is based upon the linking of the dichloro-α-naphthol to the guanadine group of argenine; colour is produced by alkaline hypochlorite treatment.

Fixation – Carnoy's fluid, acetic ethanol.

Solutions Required

(1) *Reaction mixture*

Prepare and mix the following solutions in order, immediately before use:

(*a*) 4 per cent Aqueous barium hydroxide (filtered) 25 ml

(*b*) 20 per cent Commercial 5 per cent sodium hypochlorite (Chlorox) in distilled water (1:4) . . . . . 5 ml

(*c*) Dissolve 75 mg 2, 4-dichloro-α-naphthol in 5 ml *tert* butanol . . . . . 5 ml

(2) *Dehydrant*

5 per cent Tributylamine in *tert* butanol (aniline in place of tributylamine is almost as good (Lillie, 1965).

(3) *Clearing agent*

5 per cent Tributylamine in xylene (or 5 per cent aniline).

(4) *Mountant*

Shillaber's oil plus 10 per cent tributylamine *or* cellulose caprate (resin 50 g: xylene 50 ml) to which is added 10 per cent tributylamine (or aniline) (Lillie, 1965).

Method

(1) Bring slides to distilled water, blot dry.

(2) Place sections in freshly mixed reactant solution in Coplin jar for 10 minutes at room temperature (22°C).

(3) Take sections through three changes of dehydrant of 5 seconds each change, agitating vigorously in each change.

(4) Clear in two changes (30 seconds each) of clearing agent.

(5) Drain and mount in Shillaber's oil or cellulose caprate (above).

Results

An orange-red colour at sites containing argenine (this colour will fade in 7 days to 1–2 months, depending upon the mountant).

METHODS FOR SULPHYDRYL GROUPS (SH)

It is most important to remember that *-SH groups* are sensitive to oxidation, and oxidation of undeparaffinized sections or of previously cut paraffin blocks of tissue can take place in atmospheric air which in days and/or weeks can make a previously positive section negative, it is obviously of similar importance to avoid oxidant fixatives. *Disulphide groups (-SS-) can be reduced to sulphydryl (-SH) with alkaline reagents* (Lillie, 1965).

Dihydroxy-dinaphthyl-disulphide (DDD) technique for SH and SS Groups (Barnett and Seligman, 1954; Pearse, 1968)

I have found this technique both simple and reliable and we use it routinely. In this method the reagent splits and forms a protein-naphthyl disulphide by combining with the protein SH. After washing in acid

distilled water to convert the reagent and the other reaction product to free naphthols they are then removed by washing first in alcohols then ether. The sections are then treated with a diazonium salt (fast blue B salt); this combines with the naphthol to form an azo dye.

Fixation – Carnoy, formalin, and so on.

Solutions Required

D.D.D. reagent

Dihydroxy-dinaphthyl-disulphide (DDD)	25 mg
Absolute alcohol	15 ml

Dissolve DDD in alcohol, and add to 35 ml of 0.1 M veronal-acetate buffer (pH 8.5).

Fast blue B salt solution

Fast blue B salt	50 mg
0.1 M phosphate buffer (pH 7.4)	50 ml

This must be *freshly prepared*.

Method

(1) Bring sections to water.
(2) Incubate in DDD reagent for 1 hour at 50°C.
(3) Cool to room temperature, and rinse in distilled water.
(4) Wash for 10 minutes in two changes of distilled water acidified to pH 4 with acetic acid.
(5) Remove the free naphthols by washing in 70 per cent, 80 per cent, 95 per cent and 100 per cent alcohol, followed by two washes in absolute ether for 5 minutes each.
(6) Rinse in distilled water.
(7) Immerse for two minutes (at room temperature) in fast blue B salt solution.
(8) Wash in running tap-water.
(9) Dehydrate, clear and mount.

Results

Blue staining indicates a high concentration of SH groups; red staining may indicate areas of lower concentration although elastic

tissue (which may non-specifically bind free naphthols) and collagen may stain pink.

-SS- Groups

If the reaction is intended to demonstrate -SS- and -SH groups together, then pre-treat sections with thioglycollate (*see* page 243).

Mercury Orange Technique for SH Groups (Bennett and Watts, 1958)

This technique uses the ability of mercurials to 'block' or react specifically with sulphydryl groups. The mercury is attached to an azo dye and therefore acts as a marker for SH groups. This method rarely gives a strong reaction.

Fixation – Carnoy's (Lillie, 1965) 1 per cent trichloracetic in 80 per cent alcohol and formalin (Mescon and Flesch, 1952) have all been recommended.

Solutions Required

Mercury orange solution

Mercury orange . . . . . 1 mg
Toluene . . . . . 100 ml
Alternatively 3 mg in 100 ml in absolute alcohol may be used (Mescon and Flesch, 1952).

Method

(1) Bring sections to alcohol.
(2) Immerse in mercury orange solution for 1–3 hours in alcoholic solution, or overnight in toluene.
(3) Dehydrate, clear and mount.

Results

Orange-red indicates the presence of sulphydryl groups.

Ferric-Ferricyanide Reaction for -SH Groups

This reaction is considered by some to be the most sensitive for -SH groups, the method will be found on page 386, as Schmorl's technique.

It will be seen that it is not specific and must be used in combination with a sulphydryl (-SH) blocking method.

METHODS FOR DISULPHIDE GROUPS (-SS-)

The disulphide (-SS-) groups in cystine may be demonstrated directly by use of the performic acid-alcian blue which is very specific, but not very sensitive; or indirectly with the DDD method by first blocking the -SH groups (*see* page 242), then reducing the -SS- groups to -SH by treatment with potassium cyanide. Pearse's performic acid-Schiff (*see* page 374) may also be used with appropriate controls.

Performic Acid-Alcian Blue Technique for SS Groups (Adams and Sloper, 1956)

This method is based upon the conversion of cystine to cysteic acid by oxidation with performic acid (or peracetic acid). The cysteic acid is then stained preferentially by alcian blue in 2 N sulphuric acid (pH 0.2); sections should be well affixed to slides for this method.

Fixation – formalin, Carnoy, and so on.

Solutions Required

(1) *Performic acid (see page 374)*

(2) *Alcian blue solution*

Alcian blue 8 GS	3 g
2 N Sulphuric acid (approximately 5.4 per cent conc. H_2SO_4).	100 ml

Heat to 70°C to dissolve dye and filter when cool. This solution has an approximate pH of 0.2.

Method

(1) Bring sections to water, remove excess water by blotting lightly.

(2) Immerse in fresh performic acid reagent for 5 minutes.

(3) Wash gently in several changes of distilled water for 5–10 minutes.

(4) Stain in alcian blue solution for 1 hour.

(5) Wash in water for 5 minutes.

(6) Counterstain if preferred (with tartrazine, and so on).

(7) Dehydrate, clear and mount.

Results

Structures containing 4 per cent or more of cystine appear as a dark steely blue, lesser amounts are pale blue (Pearse, 1968). Neurosecretory substance and hair keratin stain bright blue. Nuclei may stain faintly.

METHODS FOR CARBOXYL GROUPS (COOH)

Mixed Anhydride Method for Protein-Bound Side-Chain COOH (Barrnett and Seligman, 1958)

This method is based upon conversion of the COOH groups into amido ketones by treatment with acetic anhydride in pyridine. The ketones condense with 2-hydroxy-3 naphthoic acid hydrazide (NAH), this compound is coupled with fast blue B to give a highly coloured product.

Fixation – Formalin, Carnoy, and so on.

Solutions Required

NAH Reagent

2 Hydroxy-3 naphthoic acid hydrazide	50 mg
Glacial acetic acid	2.5 ml
50 per cent Alcohol	47.5 ml

Dissolve the hydrazide in warm glacial acetic acid, then add the alcohol.

Method

(1) Remove wax from sections with light petroleum.
(2) Allow to dry, and immerse in glacial acetic acid for 2 minutes.
(3) Incubate in equal parts of acetic anhydride and anhydrous pyridine (redistilled over barium oxide) for 1 hour at 60°C.
(4) Rinse in glacial acetic acid and wash in absolute alcohol.
(5) Incubate in NAH reagent for 2 hours at room temperature.
(6) Wash in 3 changes of 50 per cent alcohol, 10 minutes each.
(7) Immerse in 0.5 N hydrochloric acid for 30 minutes.
(8) Rinse in distilled water and then in 3 changes of 1 per cent sodium bicarbonate.
(9) Rinse in several changes of distilled water.
(10) Transfer to equal parts of absolute ethanol and 0.06 M phosphate buffer (pH 7.6) containing 1 mg/ml fast blue B salt for 5–6 minutes.

(11) Rinse in distilled water
(12) Dehydrate, clear and mount in resinous mountant.

Result

Protein-bound side-chain COOH groups – red-purple.

C-Terminal Carboxyl (COOH) Groups (Stoward, 1964; Stoward and Burns, 1967)

This method is based upon the conversion of the COOH groups to amido-ketones by treatment with acetic anhydride in pyridine. These ketones interact with salicyloyl hydrazide to give an intense blue fluorescence. Treatment with zinc acetate quenches the fluorescence of mucosubstances. This method appears to be specific.

Fixation – Formalin, alcohol, and so on.

Solutions Required

(1) *Acetic anhydride-pyridine*
Equal parts of acetic anhydride and anhydrous pyridine (redistilled over barium oxide).
(2) *1 per cent Salicyloyl hydrazide in 5 per cent glacial acetic acid*
(3) *P.A.F. solution*
A fresh dilute solution of pentacyano-amine-ferroate (tri-sodium salt).
(4) *1 per cent Aqueous zinc acetate solution*

Method

(1) Remove wax from sections with light petroleum.
(2) Allow to dry, wash in glacial acetic acid for 2 minutes.
(3) Treat with acetic anhydride-pyridine in a Coplin jar at 60°C for 1 hour.
(4) Wash in two changes of 95 per cent alcohol, then water.
(5) Transfer to salicyloyl hydrazide in a Coplin jar, and leave at room temperature for 30–40 minutes.
(6) Rinse in distilled water.
(7) Treat for 2 minutes in P.A.F. solution to remove excess hydrazine.
(8) Rinse well in distilled water.
(9) Treat with zinc acetate solution for 5–10 minutes.
(10) Rinse in distilled water, dehydrate and clear in xylene.
(11) Mount in non-fluorescent mountant such as Unimount or Fluormount.

Results

C-terminal COOH groups give an intense blue fluorescence.

BLOCKING METHODS FOR PROTEINS

While the specific demonstration of many proteins is not yet possible with the methods available, a greater degree of specificity may be conferred by the use of appropriate blocking methods for specific groups. It should be remembered that while many of these are not universally accepted as being completely specific, in combination with the above methods many proteins can be identified with a degree of certainty.

Amino Group Blockade (Deamination)

This has been achieved by treatment with nitrous acid alone, but Stoward (1963) showed that the following deamination method is much more specific.

Method

(1) Bring sections to water.

(2) Immerse in fresh nitrous acid (1 g of sodium nitrite in 30 ml of 3 per cent sulphuric acid) for 48 hours at the refrigerator at 0–5°C in the dark.

(3) Wash in distilled water.

(4) Treat for 4 hours at 60°C in either (*a*) water, or (*b*) alcohol.

(5) Stain sections by the appropriate method for the protein in question, absence of staining after treatment in a previously stained area is a reliable indication *of the presence of amino groups before treatment.*

Carboxyl Groups (COOH) Blockade

Methylation Technique

Method

(1) Bring sections to alcohol

(2) Treat with 0.1 N hydrochloric acid in absolute methanol at 37°C for 4–48 hours (*mild methylation*), or at 60°C for 4–24 hours (normal methylation). *We routinely use* 4 hours at 60°C with a control section to ensure that methylation is complete.

(3) Rinse in alcohol.

(4) Stain by appropriate method.

'Mild Methylation' is said to block protein carboxyls, but not primary amines (Pearse, 1968). Normal methylation desulphates in addition to its methylating action (*see* page 290).
Methylation is reversed by treatment with KoH/alcohol (saponification, *see* page 292), *the loss of sulphate groups is, of course, irreversible.*

Persulphate Block for Tryptophan

The persulphate is thought to block reactions for tryptophan by breaking the pyrrole ring.

Method

Sections are brought to water, then incubated in 2.5 per cent potassium persulphate in 0.5 N potassium hydroxide, for 16–18 hours (overnight) at room temperature.

N-haloamide Bromination Block for Tyrosine and Tryptophan

Proteins containing tyrosine and tryptophan are split by N-haloamide bromination and this can be regarded as a specific method for tyrosine provided that it is not required that tryptophan remains reactive (Pearse, 1968).

Method

Sections are brought to absolute alcohol and then transferred to 0.02 per cent N-bromosuccinimide ($(CH_2CO)_2$ NBr) in 50 per cent alcohol at pH 4.0.

Sulphydryl Group Block

These may be blocked by the iodoacetate or maleimide methods.

Iodoacetate Method

Bring sections to water and treat with 0.1 M (approximately 2 per cent) aqueous sodium iodoacetate at pH 8.0 (with NaOH) for 20 hours at 37°C.

Maleimide Method

(1) Bring sections to water.

(2) Treat in 0.1 M (1.25 per cent) N-ethyl maleimide in 0.1 M phosphate buffer at pH 7.4 for 4 hours at 37°C.

(3) Rinse in 1 per cent acetic acid then with tap-water.

Disulphide Reduction Method (-SS to SH)

Lillie (1965) recommends the use of a freshly prepared 10 per cent sodium thioglycolate solution, adjusted with sodium hydroxide to pH 9.5, for 10 minutes at room temperature. Pearse (1968) specifies a freshly prepared 0.5 M thioglycollic acid solution at pH 8.0 (with 0.1 N NaOH) for 4 hours at 37°C. In spite of pre-celloidinization, the latter is much more likely to remove sections.

Chapter 13

Nucleoproteins–Deoxyribonucleic Acid and Ribonucleic Acid

Nucleoproteins are combinations of basic protein with nucleic acids. In the nucleus we find mostly deoxyribonucleic acid, with some ribonucleic acid: in the cytoplasm of most cells there is abundant ribonucleic acid concentrated mainly in the ribosomes.

DEOXYRIBONUCLEIC ACID (DNA)

Friedrich Miescher in 1869 isolated a substance from the nuclei of cells which he called nuclein. We now know nuclein to be deoxyribonucleic acid (DNA). Robert Feulgen in 1914 first demonstrated his colour test for DNA in a test tube, but not until 1924 did he describe his method of staining cells to demonstrated that DNA is located in the chromosomes in the nucleus. The work of innumerable biochemists, biologists and biophysicists, particularly in the past twenty years, has shown that all genetic information is carried in the DNA, and it may now be postulated how this could be achieved. Chemists have shown that the molecule consists of a long, unbranched chain, the backbone of which is made up of alternate five-carbon sugar (deoxyribose) and phosphate groups; a nitrogenous base being attached to each sugar group. There are four nitrogenous bases found in most DNA's; the purines (adenine and guanine) and the pyrimidines (thymine and cytosine). The chain may be subdivided into units, or nucleotides, each consisting of a phosphate–sugar–base; polynucleotide chain is the term used to describe a series of such units.

The work of Watson and Crick, using x-ray crystallography, enabled a model to be constructed of the DNA molecule. They showed that it

consists, not of a single polynucleotide chain, but of two chains intertwined into a double helix, held together by hydrogen bonds *(Figure 13.1)*. Some idea of its structure may be gained by thinking of it as a ladder structure, the cross struts being the sugars and nitrogenous bases, which has been twisted into a spiral *(Figure 13.1)*. Although the bases on the cross struts are never identical, they are specifically complementary with adenine always linked to thymine and guanine to cytosine.

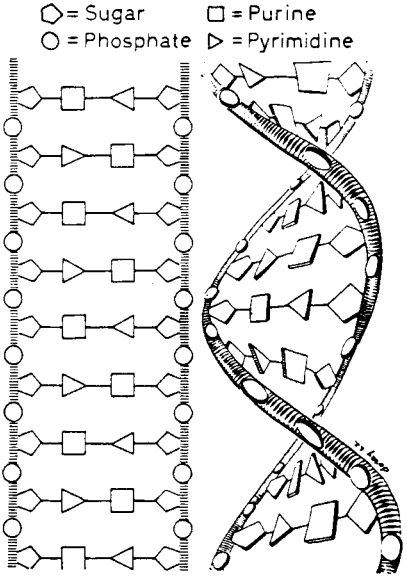


Figure 13.1 – Diagram to illustrate the schematic structure of DNA

This base pairing accounts for the ability of DNA to be self-replicating, since when DNA primes the making of more DNA the double helix uncoils and separates, and the sequence of bases on each chain will select an exact counterpart of the chain from which it has separated (for example, adenine to thymine and guanine to cytosine). It has been shown experimentally that a single synthetic polynucleotide made up of only thymine bases will only bind a complementary chain made up exclusively of adenine bases. Arthur Kornberg of Washington University has since shown that in a cell-free substrate containing rhe four nucleotides and the enzyme polymerase, together with some DNA to prime the reaction, synthetic DNA could be prepared. He has shown that some DNA has to be used to prime the reaction, and furthermore

that the synthetic DNA so prepared replicates the DNA used as a primer. Using the same substrate with a number of different DNA's (from bacterial, virus and animal sources) the synthetic DNA was always identical to that used to prime the reaction.

The variation that could be achieved, by differential grouping of base pairs, is almost infinite, and is believed to function as a genetic code. Inherited DNA, being self-replicating, is eventually passed on to the next generation, genetic information is passed from cell to cell, each gene being derived from a pre-existing gene.

RIBONUCLEIC ACID (RNA)

In RNA the sugar is ribose instead of deoxyribose, and the bases, adenine, guanine, cytosine and uracil (instead of thymine), they are also linked by phosphate groups to form a polynucleotide chain. It can be seen therefore how DNA in addition to being self-replicating can in a similar manner form RNA having the same genetic information, which can pass from the nucleus to the cytoplasm. This nuclear RNA being seen in the nucleolus (*see* page 6), cells which are actively synthesizing protein are known to be rich in RNA, this RNA being concentrated in the ribosomes of the endoplasmic reticulum. The DNA in this way predetermines the complete function of the cell.

Demonstration of Nucleic Acids (DNA, RNA)

The demonstration of nucleic acids is dependent on the nucleotide structure consisting of phosphate radical, sugar (deoxyribose or ribose) and the nitrogenous bases *(vide supra).*

Phosphate Radical

This being acid will combine with basic dyes, and is thought to be the mechanism of nuclear staining by basic dyes.

Deoxyribose, Ribose

Backler and Alexander have described a modification of the Turchini reaction but in view of the excellent Feulgen methods for DNA and other methods for RNA it is not generally used.

Nitrogenous Bases

There is no reliable histochemical technique available for these groups.

ROUTINE STAINING

Simple staining of the nucleus and nucleoli may be effected by any of the routine stains described in Chapter 11.

The regressive technique with haematoxylin, particularly with iron haematoxylin, may be employed to demonstrate chromosomes during mitosis, and is selective for chromatin in a resting nucleus when properly differentiated. The basic coal-tar dyes, such as toluidine blue, thionin in aqueous solution, or safranine and neutral red employed in a 0.5–1.0 per cent aqueous acetic acid, may be employed in a similar manner.

Staining of the nuclei of living cells is generally regarded as impossible, but de Bruyn (1953) describes their staining with fluorescent dyes.

TECHNIQUES SPECIFIC FOR DEOXYRIBONUCLEIC ACID (DNA)

The Feulgen Reaction

The reaction is based upon the cleavage of the purine–deoxyribose bond by mild acid hydrolysis to expose a reactive aldehyde group. The aldehydes may then be detected by the use of a Schiff reagent, the classical reagent leucofuchsin gives a reddish-purple colour in their presence, owing to the formation of a quinoid compound. In practice, those structures in a section which contain DNA are stained red by this technique.

Acid Hydrolysis

The time of hydrolysis is important. Increasing the time at first results in a stronger reaction, but there is an optimum time beyond which the reaction may weaken and even become negative. This may happen in bone specimens which have been decalcified over a long period. The type of material may occasionally affect the time of hydrolysis, but the most important single factor is the method of fixation.

Bauer (1932) gives the following as optimum times of hydrolysis after various fixatives *(Table 13.1)*.

Immersion in cold N/1 hydrochloric acid before and after treatment at 60°C is sometimes advised, but this has no apparent advantage.

Fixation

Provided the correct time of hydrolysis is given *(see above)*, most fixatives will give reasonable results with this technique. It is obviously

preferable to use a nuclear fixative, such as Carnoy, if only the nuclei are to be studied, but perfectly good results are obtainable after formol saline fixation. For smears of tissues the use of methyl alcohol, or Clarke's fixative (page 50) is preferred.

TABLE 13.1

Fixative	*Minutes*
Champy	25
Chrome-acetic	14
Flemming	16
Helly	8
Regaud	14
Zenker	5
Susa	18
Zenker formol	5

Schiff Reagents

The conventional Schiff reagent is prepared by treating basic fuchsin with sulphurous acid (aq.SO_2), this results in the loss of the quinoid structure (*see* page 156) with the consequent loss of colour; in the presence of an aldehyde the quinoid structure (and colour) is restored.

Ornstein and colleagues (1957), Kasten (1958, 1959) and Culling and Vassar (1961) have shown that many dyes other than basic fuchsin can be utilized as Schiff reagents.

Kasten has shown that any basic dye, lacking acid groups, with at least one primary amine group can be utilized as a Schiff-type reagent after treatment with sulphurous acid. There are many such dyes, *Table 13.2* containing those recommended by him.

All the dyes listed below are not decolorized and sections may need to be treated with acid-alcohol to remove unreacted dye (*see* page 616); similarly they are not all stable when prepared. Their usefulness may lie in the variety of colours available for the histochemical demonstration of DNA and P.A.S. positive material in double staining methods. We have utilized an acriflavine-Schiff as a fluorescent reagent for the demonstration of DNA, carbohydrates, and the L.E. cell phenomenon (*see* page 615 and *Figure 13.2*).

Preparation of Schiff Reagents

Although the method of de Thomasi was formerly recommended, using sodium metabisulphite, the reagent of Barger and De Lamater is

found to be simple to prepare, reliable, and more stable. Equally good is the reagent of Itikawa and Oguru who simply bubble SO_2 gas slowly through a solution of 0.5 per cent basic fuchsin. The author finds it simpler to be able to add a reagent (thionyl chloride) to a solution than to set up the apparatus for the SO_2 gas technique.

TABLE 13.2

Dye	C.I. No.	Colour in Nucleus after Feulgen-type reaction
Acid fuchsin	692	Violet
Acridine yellow	785	Yellow-green
Acriflavine hydrochloride	790	Yellow-green
Azure A		Blue
Azure C		Blue
Bismark brown R	332	Yellow-brown
Bismark brown Y	331	Yellow-brown
Brilliant cresyl blue	877	Blue-grey
Celestine blue B	900	Blue-green
Chrysoidine 3R	21	Yellow
Chrysoidine Y extra	20	Yellow
Cresyl violet		Blue-violet
Crystal violet	681	Blue-violet
Gentian violet (methyl violet)	680	Violet
Methylene blue	922	Blue-green to blue
Neutral red	825	Yellow-brown
Neutral violet	826	Red-violet
Phenosafranin	840	Red-violet
Phosphine GN	793	Yellow-green
Proflavine	790	Yellow
Safranin O	841	Red-violet to red
Thionin	920	Blue
Toluidine blue O	925	Blue
Toluylene blue	820	Rust-brown

Reagents

de Thomasi Schiff Reagent

(1) Dissolve 1 g of basic fuchsin in 200 ml of boiling distilled water in a stoppered 1 litre flask.

(2) Shake for 5 minutes.

(3) Cool to exactly 50°C, filter, and add 20 ml of N/1 hydrochloric acid to the filtrate.

(4) Cool further to 25°C, and add 1 g of sodium (or potassium) metabisulphite.

(5) Store for 18–24 hours in the dark, add 2 g of activated charcoal and shake the mixture for 1 minute.

(6) Remove the charcoal by filtration and store the solution in the dark at 0–4°C.

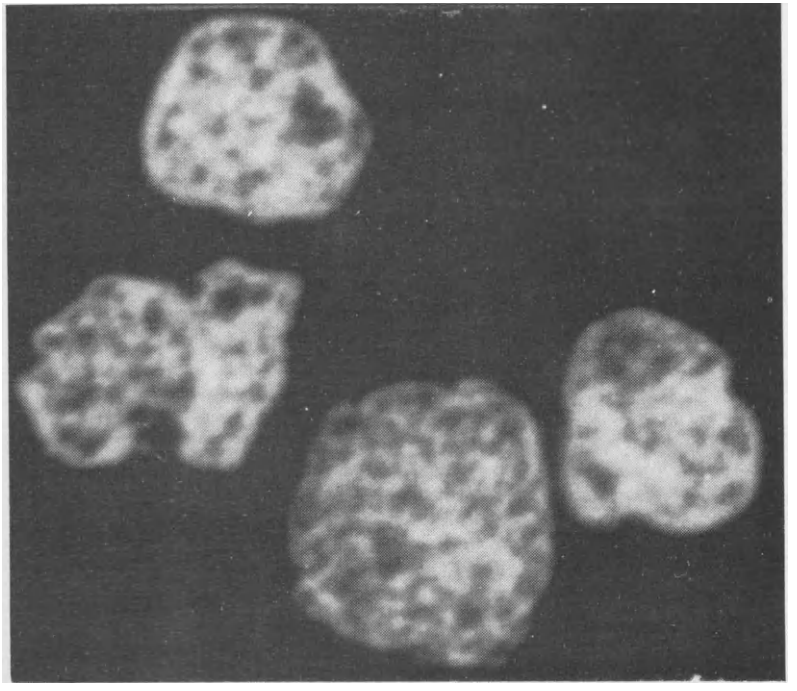


Figure 13.2 – Fluorescent Feulgen staining of bone-marrow cells; only the nuclei fluoresce. Note the presence in the nuclei of nucleoli, which appear as holes (unstained)

Barger and De Lamater's Schiff Reagent

(1) 1 g of basic fuchsin is dissolved in 400 ml of distilled water, using heat if necessary.

(2) Add 1 ml of thionyl chloride ($SOCl_2$), stopper the flask and, after shaking, allow to stand for 12 hours.

(3) Add 2 g activated charcoal, shake, and immediately filter. This will keep several months in a well-stoppered dark bottle in the refrigerator.

Sulphite Rinses

Sulphite rinses must be freshly prepared each day by adding 7.5 ml of 10 per cent potassium metabisulphite, and 7.5 ml of n/1 hydrochloric acid to 135 ml of distilled water; these amounts will amply fill 3 Coplin jars.

Method

(1) Bring sections to water.
(2) Rinse in cold N/1 hydrochloric acid (optional).
(3) Transfer to N/1 hydrochloric acid at 60°C and leave for optimal time of hydrolysis *(see above).* As a routine, with all but SUSA and chrome-osmic fixatives, a period of 10 minutes will suffice.

Note. – The hydrochloric acid must be pre-heated to 60°C.

(4) Rinse in cold N/1 hydrochloric acid (optional) and rinse briefly in distilled water.
(5) Transfer section to Schiff's reagent for 30–90 minutes.
(6) Transfer to the first of three sulphite rinses, leave for 1 minute.
(7) Transfer to the second sulphite rinse, leave for 2 minutes.
(8) Transfer to the third sulphite rinse, leave for 2 minutes.
(9) Rinse well in distilled water.
(10) Counterstain, if desired, with 1 per cent aqueous light green or tartrazine in Cellosolve.
(11) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

DNA	Red–reddish purple
Other constituents	Green or yellow, depending on the counterstain used

Naphthoic Acid Hydrazine Reaction (Feulgen NAH)

Although the Feulgen reaction is used routinely as a specific method of demonstrating DNA, doubts as to its absolute specificity have been raised in recent years on the grounds that the aldehyde-leucofuchsin compound which is formed, being diffusible, may become attached to other protein components. This objection may be overcome by the use

of the Feulgen and the Feulgen NAH reaction, since the latter utilizes a different reaction to demonstrate the aldehyde groups released by acid hydrolysis; material giving a positive reaction by both techniques may therefore be certainly described as DNA. The use of both techniques should be confined to those occasions when a critical assessment of DNA content is to be made.

Special Reagents Required

(1) *NAH solution*

2-hydroxy-3-naphthoic acid hydrazine	0.1 g
Ethyl alcohol	95 ml
Acetic acid	5 ml

(2) *Fast blue B salt solution*

Fast blue B salt	0.1 g
Veronal acetate buffer (pH 7.4)	100 ml

Method

(1) Bring sections to water, and rinse in cold N/1 hydrochloric acid.

(2) Transfer to N/1 hydrochloric acid at 60°C (pre-heated) and leave for the same time as for Feulgen reaction.

(3) Rinse in cold N/1 hydrochloric acid.

(4) Rinse in 50 per cent alcohol.

(5) Transfer to NAH solution in a Coplin jar at a temperature of about 22°C for 3–6 hours.

(6) Rinse in 3 changes of 50 per cent alcohol, leaving sections for 10 minutes in each change.

(7) Rinse in water.

(8) Transfer to fast blue B salt solution at 0°C (which as been pre-cooled) for 1–3 minutes.

(9) Wash in water.

(10) Counterstain if desired.

(11) Dehydrate, clear, and moutn in D.P.X.

Results

DNA	Bluish-purple
Cytoplasmic and other proteins	Pinkish-red

TECHNIQUES SPECIFIC FOR RIBONUCLEIC ACID (RNA)

Acridine Orange Technique for RNA

This method is to be preferred for the demonstration of RNA in smears or alcohol fixed tissues. It has, however, the great disadvantage of not being permanent.

The Unna-Pappenheim (Methyl Green–Pyronin) Stain

Following treatment of the methyl green component with chloroform, and the use of buffered solutions, this stain has regained its popularity. Methyl green is regarded by many as being specific for DNA and the pyronin, if controlled by ribonuclease extraction, specifically demonstrates RNA. The type of fixative employed is important, and the best results with the methods given below are obtainable after formol saline, formol alcohol, Carnoy, or Zenker fixation.

Each batch of stain should be tested by staining known positives, and, if possible, a section which has been treated with ribonuclease, before being used for routine purposes.

The methods preferred are those of Trevan and Sharrock for formalin-fixed material, and that of Jordan and Baker for tissues fixed in Zenker's fluid. A 2 per cent aqueous solution of methyl green is washed with chloroform in a separating funnel to extract methyl violet (a breakdown product of methyl green). The extraction is continued until the washings are colourless; methyl green should then stay reasonably pure for 6–9 months.

Pyronin Y gives more selective staining than pyronin B. Twenty-four different published modifications of this stain were tried, using pyronin Y or pyronin B before and after ribonuclease extraction; in every case the staining with pyronin Y was less diffuse and better differentiated than with pyronin B.

Rapid differentiation in acetone, followed by acetone-xylol, and clearing in xylol gave the more consistent results.

Special Reagents Required

(1) *Trevan and Sharrock's methyl green–pyronin*

		Final concentration of stain (per cent)
5 per cent aqueous pyronin Y . . .	17.5 ml	0.16

2 per cent aqueous methyl green (washed)	10 ml	0.036
Distilled water	250 ml	

Dilute with an equal quantity of acetate buffer pH 4.8 (page 170) before use. (In the original technique orange G was added)

(2) *Jordan and Baker's methyl green–pyronin*

		Final concentration of stain (per cent)
0.5 per cent aqueous pyronin Y	37 ml	0.185
0.5 per cent aqueous methyl green (washed)	13 ml	0.065
Acetate buffer (pH 4.8)	50 ml	

The prepared stains are said to have a life of 2–4 months, but smaller amounts, made up from the stock solutions weekly, appear to give more precise staining.

The same staining technique may be used with either of the above solutions.

Method

(1) Bring section to water.

(2) Pour on prepared staining solution and leave for 15–60 minutes (15 minutes will usually suffice). If several sections are to be stained a Coplin jar should be used.

(3) Rinse quickly in distilled water, and blot on non-fluffy filter paper.

(4) Flood slide with acetone, leave for a second or two, and flood again with acetone.

(5) Flood slide with acetone-xylol (equal parts).

(6) Flood with xylol; leave until clear. Should this take unduly long, the section may be blotted and fresh xylol poured on.

(7) Mount in neutral mountant or D.P.X.

Results

Deoxyribonucleic acid (DNA)	Green
Ribonucleic acid (RNA)	Red

Gallocyanin–Chrome Alum Technique

This method stains both types of nucleic acid but may be used in conjunction with deoxribonuclease or ribonuclease extraction for the specific demonstration of either acid.

Special Reagent Required

Einarson's gallocyanin–chrome alum

(1) To 100 ml of a 5 per cent aqueous solution of chrome alum (K_2SO_4 $Cr_2SO_4)_3$ 24 H_2O) is added 0.15 g of gallocyanin.

(2) Shake well, bring slowly to the boil, and allow to boil for 5 minutes.

(3) Cool, filter, and make up the volume of filtrate to 100 ml by pouring distilled water through the filter paper.

Note.–This staining solution will have a pH of 1.64, and will keep for 4–5 weeks.

Method

(1) Bring sections to water.

(2) Stain in gallocyanin–chrome alum in a Coplin jar for 48 hours at room temperature.

(3) Wash in water for a few seconds.

(4) Dehydrate, clear, and mount in D.P.X.

Results

Nucleic acid	Deep blue
Cartilage	Red (at pH 1.64)

Note. – The non-specific staining of structures other than nucleic acids varies with the pH of the staining solution. It is very slight at low pH values (0.83), and increases with the pH reaching a maximum value of 3.3–3.5. The staining of the nucleic acids does not vary with the pH. The addition of up to 10 ml of N/1 hydrochloric acid (for pH levels 0.33–1.64), and up to 5 ml of N/1 sodium hydroxide (for pH levels 1.64–3.76) may be employed to eliminate or accentuate non-specific staining.

EXTRACTION OF NUCLEIC ACIDS

Deoxyribonuclease Extraction

Deoxyribonuclease, a very expensive reagent, should be used to positively identify deoxyribonucleic acid. One of a pair of duplicate sec-

tions is treated with deoxyribonuclease *(see below)*, and the two sections are then stained. Any structure which is coloured in the control section, but is not coloured in the enzyme-treated section, is composed of deoxyribonucleic acid.

The method described by Kurnick (1952) has been employed with success. The enzyme is used in a concentration of 2 mg per 100 ml of Gomori's tris(hydroxymethyl)aminomethane buffer at pH 7.6 which has been diluted 1:5 with distilled water (*see* page 173). Alcohol-fixed or Carnoy-fixed material was used, and sections were treated at 37°C for 3 hours (Kurnick recommends 2 hours at 37°C or 24 hours at 21°C). As with all enzyme extractions, a buffer control section which has been treated in the buffer solution alone at 37°C for 3 hours together with one fresh untreated section, should be stained and examined in parallel; these two controls should give identical results.

The Feulgen reaction or the methyl green-pyronin stain may be used to stain the extracted sections.

Ribonuclease Extraction

Ribonuclease, which is thermostable, is usually prepared from pancreas; it is available commercially in crystalline form, or boiled acid extract of fresh pancreas may be used.

The dilutions at which the crystalline form should be used are variously given from 1:100,000 to 1:1,000 in glass-distilled water. Better results were obtained with the latter dilution, in formol saline, formol alcohol, and alcohol fixed smears and sections.

A section is treated at 37°C for 1 hour, a control section being placed in distilled water at the same temperature and for the same time; in addition, a fresh uncontrolled section is also stained.

Following extraction, all three sections are stained by one of the methods given above. The ribonuclease-treated section is compared with the two controls: the absence of a structure which is present in the controls indicates that this structure was composed of ribonucleic acid.

Perchloric Acid

Perchloric acid (Erickson, Sax and Ogur, 1949) may be used to extract either RNA alone, or RNA and DNA by varying the time and temperature of exposure. To remove RNA alone, sections are treated with 10 per cent perchloric acid in distilled water at 4°C for 12–18 hours. To remove both nucleic acids, sections are treated with 5 per cent perchloric acid in distilled water at 60°C for 20–30 minutes.

Following extraction, sections are neutralized in 1 per cent sodium

carbonate for 1–5 minutes, washed in running tap-water, and stained with appropriate techniques. The author has used perchloric acid routinely and finds it a reliable technique.

Bile Salts

Bile salts (Foster and Wilson, 1947) are the only chemical compounds that have been described as selectively removing RNA alone. Continual oxygenation during extraction is essential for good results, and is best achieved by using a small aerator pump such as is used in small fish tanks.

The sections are immersed in 2 per cent aqueous sodium tauroglycocholate for 24–48 hours, the solution being constantly aerated during the whole process.

Following extraction, sections are washed, and stained with 1 per cent toluidine blue, Gram's stain, or methyl green–pyronin.

Trichloracetic Acid

Treatment with 4 per cent trichloracetic acid (Schneider, 1945) at exactly 90°C for 15 minutes will remove both types of nucleic acid (RNA and DNA). After exposure to the acid, sections are washed in distilled water and stained with methyl green–pyronin or toluidine blue.

Chapter 14

Carbohydrates

The histochemical differentiation of polysaccharide–protein complexes in normal and pathological tissue is of ever increasing importance in the investigation of normal and disease processes.

Although this chapter is entitled Carbohydrates, it is concerned with the study of polymers of carbohydrates, most of which are linked covalently to protein. The protein moiety may constitute a majority or a minority of the residues present and this factor has, at times, been used as one basis for their classification. To the histochemist such a classification has no meaning, since even the demonstration of both protein and carbohydrate at the same site is no proof that they are part of the same molecule.

Whether a reaction is given by a series of long chain carbohydrate polymers attached to a small core of protein (for example, nucleus pulposa in intervertebral discs), or a series of short chain carbohydrate polymers attached to a large protein core (for example, salivary glycoproteins), cannot be determined histochemically. The only information that can be derived is based upon the reactive groups that are demonstrable in carbohydrates, namely 1:2 glycols (vicinal diols), carboxyl (CooH) and ester sulphate; with the additional information to be gained from enzyme hydrolysis with diastase, hyaluronidase, sialidase and various chemical procedures (blocking, and so on.)

It is for this reason that, in spite of the many (and complex) classifications of carbohydrates that are currently in use, the following has been adopted because it is based upon their histochemical differentiation and can easily be interpreted within the scope of any of the other existing chemical classifications (Meyer, 1953; Jeanloz, 1960; Davidson, 1964; Spicer and colleagues, 1965; Pearse, 1969 and Zugibe, 1970).

CLASSIFICATION OF NATURALLY OCCURRING POLYSACCHARIDES

Group I: Neutral Polysaccharides (Non-ionic Homoglycans)

(1) *Glucose-containing;* Glycogen (starch, cellulose).
(2) *N-acetyl-glucosamine-containing* (chitin).

Group II: Acid Mucopolysaccharides (Anionic Heteroglycans)

All in this group are thought to be attached to protein, even though the word protein is not embodied in their name. These are found in connective tissues and are P.A.S. negative.

(1) *Carboxylated;* Hyaluronic acid – connective tissues, umbilical cord.
(2) *Sulphated (OSO_3H)*

(*a*) Containing hexuronic acid (COOH).

(*i*) Chondroitin sulphate A (chondroitin-4-sulphate).
(*ii*) Chondroitin sulphate C (chondroitin-6-sulphate), found in cartilage, chondrosarcomas, cornea and blood vessels.
(*iii*) Chondroitin sulphate B (dermatan sulphate), found principally in skin, also in connective tissue, aorta and lung.
(*iv*) Heparin, found in mast cells, and intima of arteries (Zugibe, 1970).

(*b*) Not containing hexuronic acid (COOH-free).

(*i*) Keratosulphate, in human aorta and bovine cornea.

Group III: Glycoproteins (Mucins, Mucoids, Mucoproteins)

These are found in epithelial mucins, but some may occur in connective tissue. They are potentially, but not necessarily, P.A.S. positive. Group 2 (containing sialic acid) is potentially sialidase labile, but for reasons not completely understood, may show no loss of alcianophilia after sialidase (*see* page 285).

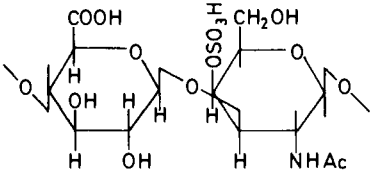
(1) *Neutral;* Ovimucoid (egg white), mucin in stomach, paneth cell granules.
(2) *Carboxylated;* Sialoglycoproteins (containing sialic acid, but no sulphate).

(*i*) Sialomucins, found in submaxillary gland mucins (Gottschalk, 1960), small intestine mucins (Gad, 1969), foetal mucins (Lev, 1968), upper part of colonic crypts (Felipe, 1970), human sublingual gland (P.A.S. positive, sialidase-resistant even after KOH).

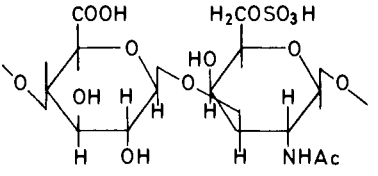
(*ii*) Serum glycoproteins.

(*iii*) Blood group substances.

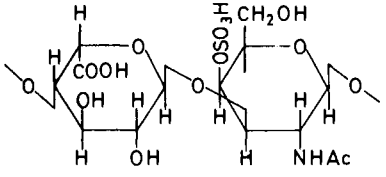
(3) *Sulphated;* Sulphated sialoglycoproteins (containing sialic acid and sulphate).
Colonic mucins of sheep (Kent, 1962), colonic mucins of man (Lev, 1968).



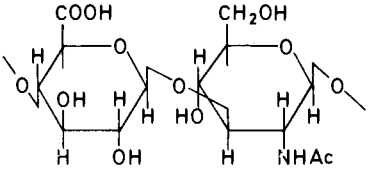
Chondroitin 4-sulphate



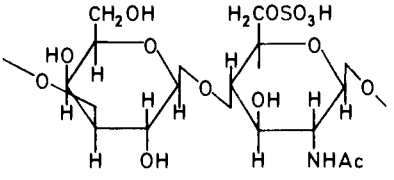
Chondroitin 6-sulphate



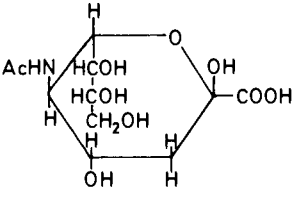
Dermatan sulphate



Hyaluronic acid



Keratosulphate



N-Acetyl neuraminic acid
(sialic acid)

Group IV: Glycolipids

The members of this group have a fatty residue bound to a carbohydrate structure.

Cerebrosides, in central nervous system and other tissues.

Phosphatides are a group of non-carbohydrate-containing lipids, which include lecithin, cephalin and sphingomyelin, which are only mentioned in this chapter because of their P.A.S. positivity (*see* page 374).

IDENTIFICATION OF CARBOHYDRATES

Table 14.1 is intended as an aid to the identification of carbohydrates in tissues. It will be seen that differentiation between neutral mucopolysaccharides and muco- or glycoproteins is not possible histochemically although quite often histological localization will permit an 'informed guess'. It must also be remembered that almost invariably polysaccharides occur in the body as a mixture.

Because sialic acid contains a carboxyl (COOH) group in addition to adjacent 1 : 2 glycol groups. it will react with the P.A.S. technique and also give a positive reaction for acid mucopolysaccharides.

PERIODIC ACID–SCHIFF PROCEDURES

The histochemistry of carbohydrates was for a long time largely based on the P.A.S. technique and it remains today, if properly controlled, the most useful single technique.

The use of Schiff reagent for the demonstration of aldehydes produced after hydrolysis with hydrochloric acid (the Feulgen reaction) has been dealt with on page 248. This reaction was first described as long ago as 1924. McManus (1946) described a periodic acid oxidation-Schiff (P.A.S.) method for the demonstration of mucin, and in 1947 he extended its use to the demonstration of reticulum and glycogen.

Other oxidation-Schiff procedures such as Bauer's chromic acid-Schiff (for glycogen), Rossman and Casella's potassium permanganate-Schiff, and Crippa's lead tetra-acetate-Schiff (both for mucins), have been described. Although they are positive with most P.A.S. positive material, they are not usually employed since the reaction is not as intense and may even be negative with material giving a P.A.S. positive. The use of performic or peracetic acid with Schiff reagent for the demonstration of unsaturated lipids is dealt with on page 374.

The P.A.S. reaction is now accepted as a routine method. It is used with various controls, blocking procedures, and supplementary staining techniques, which are necessary to distinguish between the wide variety of tissue elements that are demonstrated.

The reaction is based on the fact that certain tissue elements are oxidized by the periodic acid (or other oxidant), one of the reaction products being an aldehyde; such aldehydes are then demonstrated with a Schiff reagent.

The chemical basis of the reaction is that periodic acid will cleave the carbon–carbon bonds where these carbon atoms have adjacent hydroxyl (— OH) groups (1:2 glycol groups) (formula *a*), or adjacent hydroxyl and amino (— NH_2) groups (1:2 amino, hydroxy groups) (formula *b*).

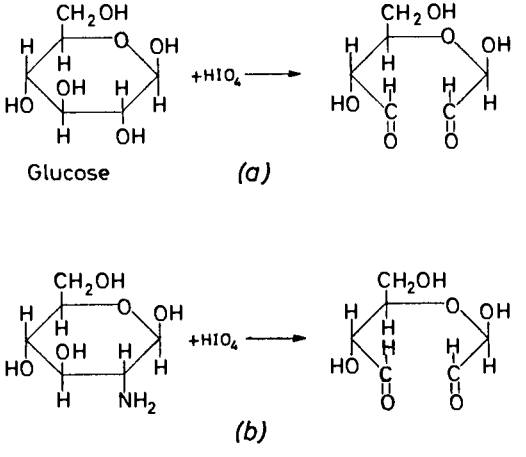
Any substance that satisfies the following criteria will give a positive result with the P.A.S. reaction (Hotchkiss, 1948).

(1) The substance must contain the 1:2 glycol grouping, or the equivalent amino or alkyl-amino derivative, or the oxidation product CHOH–CO.

(2) It must not diffuse away in the course of fixation.

(3) It must give an oxidation product which is not diffusible.

(4) Sufficient concentration must be present to give a detectable final colour.



Formation of dialdehydes after periodic acid treatment.

TABLE 14.1

Class	Type and Reactive Groups Present	Technique / Substance / Page No.	Schiff Reaction 298	P.A.S. 267	Acetyl/P.A.S. 290	Diastase/P.A.S. 300	AB2·5 275	Halc 278	AB1·0 275	HID 279	A.F. 283	M/S AB2·5, (Hale) 291	M/S AB 1·0, (A.F.) (HID) 291	T/Hy/AB2·5 (Hale) 293	T.Hy/AB1·0 (HID, AF) 293	S.Hy/AB2·5 (Hale) 294	S.Hy/AB1·0 (HID, AF) 295	SIAL/AB2·5 (Hale) 295	SIAL/AB1·0 (HID, AF) 295	Pur. Ac/Mx. DIAM 282	SUDAN BLACK 360	Bromine/P.A.S. 375
I	Neutral (1:2 Glycol)	Glycogen	I	+	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	Y	I	+
Acid Mucopolysaccharides II	Carboxylated (COOH)	Hyaluronic Acid	–	I	I	I	+	+	I	–	I	+	I	I	I	I	I	+	I	P	I	I
	Sulphated (COOH and OSO_3H)	Chondroitin Sulphate A and C	I	I	I	I	+	+	+	+	+	R+	I	I	+	+	+	+	+	P	I	I
		Chondroitin Sulphate B	I	I	I	I	+	+	+	+	+	R+	I	+	+	+	+	+	+	P	I	I
		Heparin	I	I	I	I	+	+	+	+	+	R+	I	+	+	+	+	+	+	P	I	I
	Sulphated (OSO_3H)	Keratosulphate	I	I	I	I	+	+	+	+	+	I	I	+	+	+	+	+	+	P	I	I

TABLE 14.1 *cont.*

Glycoproteins III	Neutral (1:2 Glycol)	Mucins	−	+	−	+	−	−	−	−	−	−	−	−	−	−	−	−	−	Y	−	+
	Carboxylated (1:2 Glycol and COOH)	Sialomucins	−	*+	−	*+	+	+	−	−	−	+[+]	−	+	−	+	−	−	−	(P)	−	*+
	Sulphated (1:2 Glycol, COOH and OSO_3H)	Sulphated-Sialomucins	−	*+	−	*+	+	+	+	+	+	+[+R]	−	+	+	+	+	+	+	P	−	*+
IV	Glycolipids (1:2 Glycol and Lipid)	Cerebrosides	−	+	+[R]	+	−	−	−	−	−	−	−	−	−	−	−	−	−	Y	+	+[R]
Unsaturated Lipids (Double Bonds)			−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	−	(Y)	+	−
Aldehyde Groups			+	+	(+)	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+

R = positive reaction is reduced
* = these mucins may occasionally be P.A.S.-ve (*see* page 263)
\+ = methylation may remove sialic acids resulting in negative reaction (*see* page 285)
0 = sialic acid present may be sialidase resistant (*see* page 294)
\# = reaction not always predictable

P = Purple
Y = Yellow-Brown

Acetyl/P.A.S. = Acetylation/P.A.S.
AB 2·5 = Alcian blue pH 2.5
Hale = Hale's colloidal iron
AB 1·0 = Alcian blue pH 1·0
H.I.D. = High iron diamine
Ald. Fuch. (AF) = Aldehyde fuchsin
M/S = Methylation/saponification
T/Hy = Testicular hyaluronidase digestion
S/Hy = Streptococcal hyaluronidase digestion
SIAL = Sialidase digestion
Per. Ac/Mx Diam. = Periodic acid/mixed diamine
Sudan Black = Sudan black B staining
Bromine/P.A.S. = Bromine block (for double bonds)/P.A.S.

The acid mucopolysaccharides (hyaluronic acid, the chondroitin sulphates, and so on) all contain 1:2 glycol groups (vicinal diols) (*see* page 261) but have been shown to be P.A.S. negative (Braden, 1955; Hoogh-Winkel and Smits, 1957). These groups are known to oxidize only slowly (7–16 hours, Zugibe, 1970). It is presumed that this is due to the presence of a highly charged group in the molecule (COOH or OSO_3H) which interferes with the periodic acid oxidation. That this is probably true was shown by Scott and Harbinson (1968) who suppressed the electrostatic field of the COOH groups of hyaluronic acid, by increasing the ionic strength of the periodate solution, when it became P.A.S. positive.

Sialic acids, however, in spite of their COOH groups (*see* page 261) were shown by Montreuil and colleagues (1959) and Montreuil and Biserte (1959) to be oxidized by periodic acid (in 20 minutes) and be P.A.S. positive, however, Spicer and colleagues (1960, 1967) states that 'sialic acid in epithelial mucins has not, in our experience, contributed to the P.A.S. reactivity'. In spite of this statement, perhaps because some of the sugar residues found in acid glycoproteins (sialomucins) are periodate reactive, the latter are usually, but not invariably, P.A.S. positive.

FIXATION OF POLYSACCHARIDES

The main problem, in the fixation of carbohydrates in tissue, is to ensure their total preservation, without diffusion. The main obstacle is that most carbohydrates are water-soluble, and for this reason aqueous fixatives have not been recommended. It has been shown, however, that many carbohydrates are covalently bound to protein (Engfeldt and Hjertquist, 1967) and that the fixation of the protein will in turn bind the polysaccharides. Even in the case of glycogen, which is not protein-bound, it is thought that the fixation of the tissue proteins forms a lattice around the glycogen, with its consequent preservation. The degree of preservation of carbohydrates reported for various fixatives is discussed under fixation on page 34. There is little agreement on which method of fixation is ideal; with the possible exception of formaldehyde vapour fixation of freeze-dried tissue. Even this method, however ideal it may be histochemically, can hardly be utilized in a routine laboratory. The use of cetylpyridinium chloride (CPC) in 10 per cent formalin (Scott, 1955), while recommended by many workers for mucopolysaccharides, was thought by Sochor (1965) to interfere with staining presumably by binding with reactive groups. A literature survey will give a similar division of opinion on most other fixatives.

In our laboratory, we routinely use formol-calcium fixation for the reasons given above (Culling, Reid and Dunn, 1971), and we use

Carnoy's or Newcomers' fixatives for specific problems (for example, the demonstration of basic protein in mucins). We are currently investigating carbohydrate fixation methods both chemically and histochemically to determine at which stage the losses, if any, take place.

P.A.S. TECHNIQUE

Oxidation

Sections must first be oxidized with periodic acid to convert substances with 1:2 glycol grouping to dialdehydes in order that they will react with the Schiff reagent (recolour it). Hotchkiss (1948) recommends an alcoholic buffered periodic acid wherever there is the possibility that the substances to be demonstrated are soluble in water (for example, glycogen). However, a simple aqueous solution of periodic acid will give equally good, if not better, results. The time of exposure to periodic acid must be kept to a minimum and should not exceed 15–20 minutes at room temperature; heat should never be used because of the chemical changes that may occur.

It should be realized that the specificity of the P.A.S. technique is dependent upon in its short oxidation time. Longer exposure to periodic acid will cause proteins and (with times of 7–24 hours) the acid mucopolysaccharides to become P.A.S. reactive.

Choice of Schiff Reagent

Most of the accepted Schiff formulae (*see* page 249) give equally good results, but the author prefers the Schiff reagent of Barger and de Lamater, which gives a brilliant reaction.

Reducing Rinses

Sulphite rinses (*see* page 252) were recommended for the purpose of removing uncombined leucofuchsin following the Schiff reagent. Most laboratories have found that simple washing in running water does not result in section staining with recoloured fuchsin, the sulphite rinses have therefore been omitted from the recommended method. Hotchkiss suggested using a reducing rinse between periodic acid and Schiff reagent to remove iodate or periodate remaining combined in the tissues, since these restore the colour of the Schiff reagent.

Lillie has shown that the reducing rinse may combine with the aldehydes and reduce the intensity of the reaction. Hale (1957) has shown that a five-minute wash in water is adequate to prevent recolorization of Schiff by iodates or periodates.

Recommended Technique

Method

(1) Bring sections to water.
(2) Oxidize for 5–10 minutes in 1 per cent aqueous periodic acid.
(3) Wash in running water for 5 minutes, and rinse in distilled water.
(4) Treat with Schiff reagent for 10–30 minutes.
(5) Wash for 10 minutes in running water.
(6) Counterstain with haematoxylin and tartrazine in Cellosolve (optional).
(7) Dehydrate, clear and mount in D.P.X. or H.S.R.

Results

P.A.S. positive substances	Bright red
Nuclei	Blue
Other tissue constituents	Yellow

Hotchkiss' Buffered Alcoholic P.A.S. Technique

Certain of the cheaper substitutes for alcohol cannot be used in this technique since they give false positive results.

Solutions required

(1) *Alcoholic periodic acid*

Periodic acid	0.8 g
Distilled water	20 ml
M/5 Sodium acetate solution (2.72 per cent)	10 ml
Pure ethyl alcohol	70 ml

(2) *Acid-reducing rinse*

Potassium iodide	2 g
Sodium thiosulphate	2 g
Distilled water	40 ml
Pure ethyl alcohol	60 ml
N/1 Hydrochloric acid	2 ml

(3) *Schiff reagent* (*see* page 249)

Method

(1) Bring section to 70 per cent alcohol.
(2) Treat with alcoholic periodic acid for 5–10 minutes.
(3) Rinse in 70 per cent alcohol.
(4) Treat with acid-reducing rinse for 1 minute.
(5) Rinse in 70 per cent alcohol.
(6) Wash in running water until free of alcohol.
(7) Treat with Schiff reagent for 10–30 minutes.
(8) Wash in running tap-water for 10 minutes.
(9) Counterstain if desired as in the preceding method.

A section for histochemical assessment should be examined without counterstaining.

(10) Dehydrate, clear and mount in D.P.X. or H.S.R.

Results

P.A.S. positive material	Bright red
Other tissue constituents	Dependent on counterstain

Periodic Acid – Phenylhydrazine – Schiff Method (P.A.P.S.) (Spicer, 1961)

Sialomucins differ in their reactivity to the periodic acid-phenylhydrazine-Schiff technique (Leppi and Spicer, 1967). This is useful in the identification of some mucosubstances since phenylhydrazine blocks the Schiff staining of periodate-induced aldehydes from neutral mucosubstances; or when the neutral and acid moieties in the same mucin are sufficiently distant from each other that the reaction of the phenylhydrazine with anionic groups does not prevent its condensation with periodate-induced aldehydes. Conversely, when the neutral and acid moieties are close the aldehydes will be coloured.

Method

(1) Bring sections to water.
(2) Oxidise in fresh 1 per cent periodic acid for 10 minutes.
(3) Wash in running water for 5 minutes.
(4) Block aldehydes *(see above)* in 0.5 per cent aqueous phenylhydrazine hydrochloride for 30 minutes.

(5) Wash in water.
(6) Immerse in Schiff reagent for 10–15 minutes.
(7) Wash in running water for 5 minutes.
(8) Counterstain with haematoxylin if desired.
(9) Dehydrate, clear and mount in resinous mountant.

Results

Phenylhydrazine blocks the staining of neutral polysaccharides and glycoproteins; some sialo-acid and sialo-sulphated glycoproteins are stained red *(see above)*.

Gomori's Periodic Acid–Methenamine Silver Technique (Grocott's Modn.)

Gomori utilized a silver solution to demonstrate aldehydes exposed by periodate treatment; the method cannot however be used in a Feulgen technique.

While it is not generally recommended as a substitute for Schiff reagent, it does give very good results with basement membranes (particularly in kidney) and with fungi in tissue sections. This modification, in which the stock silver methenamine solution is diluted in equal parts with distilled water, avoids the grossly overstained sections which sometimes resulted with the original method.

Technique

Sections should be as thin as possible.

Solutions Required

Gomori's Silver Methenamine Solution

Add 5 ml of 5 per cent silver nitrate to 100 ml of 3 per cent methenamine (hexamethylene tetramine) to prepare stock solution. For use add 3 ml of 5 per cent borax to 25 ml of stock silver and 25 ml of distilled water.

Differentiator

0.5 per cent sulphuric acid in 0.2 per cent ferric sulphate.

Method

(1) Bring sections to water.
(2) Oxidize in 5 per cent chromic acid for 1 hour.
(3) Wash in running water for 1–2 minutes.
(4) Treat with 2 per cent sodium bisulphite for one minute to remove chromic acid.
(5) Wash in running water for 5 minutes.
(6) Place in methenamine–silver solution at 60° for 1–3 hours. Examine sections microscopically at 10 minute intervals after the first 30 minutes. Do not use metal forceps. Sections should be rinsed in distilled water and if stain is too light may, after a further rinse in distilled water, be returned to the silver solution. If they are too dark they may be treated briefly in differentiator.
(7) Wash well in distilled water.
(8) Tone in 0.2 per cent gold chloride solution for 2 minutes, then rinse in distilled water.
(9) Place in 3 per cent sodium thiosulphate for 2 minutes, then wash in running water for 2–3 minutes.
(10) Sections may be counterstained if desired with haematoxylin or 0.2 per cent light green in 0.2 per cent acetic acid.

Results

P.A.S. positive structures	
Basement membranes, fungi, mucin, etc. .	Black-brown
Background	Light yellow or green

Light and Electron Microscopic Demonstration of P.A.S. Positive Substances with Thiosemicarbazide (Stastna and Travnic, 1971)

The principle of this method is the oxidation of tissue to convert 1:2 glycols to dialdehydes; these are reacted with thiosemicarbazide, which binds to them by its hydrazine group to give a thiosemicarbazone. This is treated with osmium tetroxide which binds with the thiocarbamyl groups to yield a final product which can be seen equally well by light (semi-thick sections) or electron microscopy (ultra-thin sections). Controls are not oxidized in periodic acid.

Method A (Semi-thick Sections)

(1) Tissues are fixed in the usual manner in glutaraldehyde and osmium tetroxide (OsO_4), and after dehydration, embedded in Epon 812. Semi-thick sections are cut and placed upon slides.
(2) Oxidize in fresh 1 per cent periodic acid for 45 minutes.
(3) Rinse in water.
(4) React in 2.5 per cent thiosemicarbazide in 5 per cent acetic acid for 3 hours.
(5) Rinse well in water.
(6) Treat with 1 per cent aqueous OsO_4 for 90 minutes.
(7) Rinse in distilled water.

Results

P.A.S. positive substances . . . . . Dark brown/black.

Method B (Ultra-thin Sections)

(1) Fix tissues in formaldehyde in 0.15M phosphate buffer at pH 7.4 in a refrigerator for 4 hours (glutaraldehyde is not recommended by the authors). Tissues must not exceed $1 mm^3$.
(2) Wash tissues in 0.5M glucose in 0.15 phosphate buffer (pH 7.4) for 60 minutes.
(3) Wash tissues in 0.15M phosphate buffer (pH 7.4) for 60 minutes with three changes.
(4) Oxidize in 0.05M sodium periodate in 0.15M phosphate buffer at pH 4.2 for 15–60 minutes.
(5) Wash in 0.15M phosphate buffer at pH 4.2 for 45 minutes, with three changes.
(6) React with a saturated solution of thiosemicarbazide in 0.28M acetic acid for 1–3 hours (time varies with different tissues).
(7) Wash in 0.15M phosphate buffer at pH 4.2 for 45 minutes, with three changes.
(8) Wash in 0.15M phosphate buffer at pH 7.4, with three changes.
(9) Post-fix in 1 per cent osmium tetroxide in pH 7.4 buffer for 60 minutes.
(10) Dehydrate, embed in Epon 812/Araldite.

Sections may also be contrasted with uranyl acetate and lead citrate if preferred (*see* page 667).

Results

P.A.S. positive substances are electron dense.

Methods for the Demonstration of Acid Mucopolysaccharides and Glycoproteins (COOH and OSO_3H Groups)

As will be seen in the following pages, there is a multiplicity of methods available for the demonstration of acid groups. Many of these methods have been included to satisfy individual preferences, some because of their specificity and yet other because they are good histological methods (for example, colloidal iron method).

All of these methods have been tried and I have indicated those we prefer in our laboratory, and their relative specificity. As has been pointed out absolute specificity is difficult to achieve even with available chemical and enzymic controls. For general purposes the P.A.S. Alcian blue will demonstrate mucin and the degree to which it is composed of neutral (red) and acid (blue) components; similarly, the Alcian blue/aldehyde fuchsin (or Alcian blue/high iron diamine) will stain acid mucins and demonstrate the degree to which they are carboxylated (blue) and/or sulphated (purple or black). The more specific techniques are the Alcian blue at H 1.0 and/or 2.5, the diamine methods, Scotts CEC method, metachromasia with Azure A at controlled pH, together with methylation, saponification, enzymic digestion techniques and so on.

Metachromatic Staining

The mechanism of staining is discussed on page 162. The question of alcohol stability is still in dispute and has not yet been resolved. The method described below avoids the use of alcohol, and it is of interest that colonic mucin (known to contain sulphate), which is metachromatic after clearing in xylol, loses most of its metachromasia if treated subsequently with alcohol.

The technique recommended for the demonstration of metachromasia is that of Vassar and Culling (1959). This gives a permanent preparation.

Fixation

Formalin does not react with polysaccharides but it reacts with proteins to bind protein bound polysaccharide,therefore a formalin fixative such as formol–calcium or formol–alcohol is recommended.

Staining Solution

Dissolve 0.25 g of toluidine blue in 100 ml of Michaelis's Veronal acetate–hydrochloric acid buffer at pH 4.5 (*see* page 172).

Method

(1) Bring sections to water.
(2) Stain in buffered toluidine blue for 10 seconds.
(3) Rinse in distilled water.
(4) Blot section with fluffless filter paper, allow to dry and clear in xylol. If section does not completely clear, blot dry and immerse in fresh xylol.
(5) Mount in a resinous mountant such as D.P.X. or H.S.R.

Metachromasia with Azure A at Controlled pH. (Spicer, 1960; Gad, 1969).

This technique is said to differentiate strongly acidic sulphomucins, which are metachromatic at pH 1.5 and 3.0 from weakly acidic mucosubstances which are metachromatic at pH 3.0 only. Sections are stained in 1:5000 Azure A in 0.1M phosphate–citrate buffer (or Walpole's buffer (page 168) for 30 minutes, dehydrated through acetone and mounted in xylene cellulose caprate (Lillie, 1964). A full range of buffered stains from pH 0.5 to 5.0 may be prepared and used in a similar manner.

Alcian Blue Methods

Alcian blue is a water soluble copper thalocyanin; its exact staining mechanism is not known although it is thought to stain by salt linkage to acidic groups. It must be used in acid solution, and has been shown to have a greater affinity for sulphate groups when used at a pH less than 2.0. Spicer has shown that it has a greater affinity for acid than for sulphated mucopolysaccharides. Although there is fairly general support for the above mechanism, Palladini and Lauro (1968) feel that the specificity of the dye is due to its inability to stain in the presence of a specific protein. In their opinion, this protein is soluble in various salt solutions and in 1M sulphuric acid, but is insoluble in 3 per cent acetic acid: it is also removable by peptic digestion. It is of interest to note that Quintarelli, Scott and Dellovo (1964) obtained increased staining with Alcian blue after pepsin treatment which they assumed was due to

the fact that the removal of the protein had made the reactive tissue radicals accessible to the dye. There are available Alcian green 3 BX or Alcian green 2 GX in place of Alcian blue 8 GS, but there is little advantage to be gained from their employment.

Each batch of Alcian blue 8 GS should be tested when it is received by staining known control sections (large and small intestine). The sections should be examined for depth of staining of mucins, and for non-specific background staining; in our experience there is considerable variation between different batches, some being so poor that they were useless.

Alcian blue was combined with chlorantine fast red by Lison (1954) to give a selective connective tissue stain. This is described on page 305, as a method for mucin, but should not be used as a histochemical method.

Lev and Spicer (1964) used Alcian blue at pH 1.0 and 2.5 to distinguish between acid and sulphated mucopolysaccharides or glycoproteins. At pH 1.0, carboxyl (COOH) groups are not ionized and do not stain, whereas sulphate (OSO_3H) groups are demonstrated. At pH 2.5 COOH groups stain well, while sulphated mucins may stain poorly (Spicer and colleagues, 1967).

We routinely use these methods in conjunction with Spicer's high diamine, methylation and saponification procedures.

Standard Alcian Blue Method (pH 2.5) for Acid Groups (COOH and OSO_3H)

Method

(1) Bring sections to water.

(2) Stain in freshly filtered 1 per cent Alcian blue 8 GX in 3 per cent acetic acid (pH 2.5) for 30 minutes.

(3) Wash in water.

(4) Dehydrate, clear and mount in resinous mountant.

Results

Acid polysaccharides . . . . . Deep blue
(nuclei may stain faint blue)

Alcian blue (pH 1.0) for sulphate groups

Method

(1) Bring sections to water.

(2) Stain in 1 per cent Alcian blue 8 GX in 0.1 N hydrochloric acid for 30 minutes. Rinse briefly in 0.1 N Hc1.

(3) Blot dry with fine filter paper to prevent the staining which sometimes occurs after dilution with water (which will change the pH) in washing.

(4) Dehydrate in alcohol, clear in xylol and mount in resinous mountant.

Results

Sulphated mucosubstances stain blue.

Alcian Blue/P.A.S. Technique

Steps (1)–(3) of standard Alcian blue method at pH 2.5 above, followed by steps (2)–(7) of the P.A.S. technique (page 268), dehydration, clearing and mounting. This technique is sometimes useful for the differentiation of neutral and acid mucopolysaccharides.

Alcian Blue/Aldehyde Fuchsin Stain

Steps (2) and (3) of Aldehyde fuchsin technique (page 283) are interposed between steps (3) and (4) of standard Alcian blue technique at pH 2.5.

Results

Acid M.P.S.	Blue
Sulphated M.P.S.	Purple
Mixed acid and sulphated	Violet-purple

Alcian Blue – Alcian Yellow Method

Carlo (1964) recommended this method, also used by Staple (1967), to differentiate between sulphated (blue) and acid (yellow) mucopolysaccharides and glycoproteins. Sorvari and Sorvari (1969) investigated this method and disputed its specificity. We have not found this method to be useful, it is included for the sake of completeness.

Method

(1) Bring paraffin sections fixed in Helly or formaldehyde to water.

(2) Stain for 30 minutes to 1 hour with Alcian blue 8 GX 300 0.5 per cent buffered at pH 0.5 (or in 0.1 N HCl).

(3) Wash for 10 seconds in buffer pH 0.5 (or 0.1 N HCl).

(4) Wash well in water.

(5) Stain 30 minutes to 1 hour with Alcian yellow GX 0.5 per cent buffered at pH 2.5 (or in 3 per cent acetic acid).

(6) Wash in water.*

(7) Counterstain 5 minutes in Kernechtrol (optional).

(8) Wash in water, dehydrate, clear in xylene, mount in Canada balsam.

Results

Sulphate groups are coloured blue, carboxyl groups yellow; sites containing both carboxyl and sulphate groups are coloured blue-green.

Alcian Blue – Ruthenium Red Method (Yamada, 1969)

This method, also included for completeness, was used to differentiate between sulphated (blue) and acid (red) mucopolysaccharides and glycoproteins.

Method

(1) Bring sections to water, and rinse twice in 0.1 N hydrochloric acid at pH 1.0

(2) Stain for 30 minutes in 0.5 per cent Alcian blue 8 GX in 0.1 N hydrochloric acid at pH 1.0.

(3) Rinse in 0.1 N HCl, and then twice in 3 per cent acetic acid at pH 2.5.

(4) Stain in 0.5 per cent ruthenium red in 3 per cent acetic acid (pH 2.5) for 5–20 minutes. Staining should be stopped when there is replacement of Alcian blue by ruthenium red.

(5) Rinse twice with 3 per cent acetic acid and then twice with water.

(6) Dehydrate, clear and mount.

Results

Sulphate groups	Blue
Carboxyl groups	Red

*P.A.S. technique may be applied here.

Many mucins stain purple presumably indicating the presence of both acid (COOH) and sulphate (OSO_3H) groups.

Alcian Blue – Safranin (Spicer and Colleagues, 1967)

This method has become popular in some laboratories as an alternative to the aldehyde fuchsin technique. It is used for the demonstration of mast cells and can be recommended for this purpose.

Method

(1) Bring sections to water.
(2) Stain in 0.5 per cent Alcian blue 8 GX in 3 per cent acetic acid for 30 minutes.
(3) Wash in water for 5 minutes.
(4) Stain in 0.25 per cent Safranin in 0.125 N hydrochloric acid for 30 seconds.
(5) Dehydrate rapidly, clear and mount in resinous mountant.

Results

Strongly acidic (sulphated) mucins, mast cells	Red
Acid mucins (carboxylated)	Blue

Hale's Colloidal Iron Technique

Hale's technique is based upon the affinity of acid groups for colloidal iron at a low pH. The iron forms a chelate with the acid groups and may then be demonstrated by the Prussian Blue reaction.

While this method is excellent for the demonstration of acid mucins it is inferior to Alcian blue as a specific method for histochemical investigation.

Special Solutions Required

Dialysed iron (British Drug Houses or Merck's)	1 volume
2 M acetic acid	1 volume

Dialysed Iron Solution (Rinehart and Abu'l Haj)

Dissolve gradually 75 g ferric chloride in 250 ml distilled water, adding 100 ml glycerol, followed by the gradual addition of 55 ml of

28 per cent ammonia with constant stirring. This mixture is dialysed against regularly changed distilled water for 3 days.

Acid-ferrocyanide Solution

Equal parts of 2 per cent potassium ferrocyanide and 2 per cent hydrochloric acid.

Technique

(1) Bring sections to water.
(2) Flood with dialysed iron solution (either solution works well) for 10 minutes.
(3) Wash well with distilled water.
(4) Flood with acid-ferrocyanide solution for 10 minutes.
(5) Wash well in water.
(6) Counterstain lightly with 0.1 per cent neutral red or safranin.
(7) Rinse in water, and dehydrate rapidly.
(8) Clear in xylol and mount in D.P.X. or H.S.R. resin.

Results

Acid mucopolysaccharides	Bright blue
Other tissue constituents	Shades of red

Spicer's Diamine Methods for Acid Mucosubstances

Spicer (1961, 1965) made yet another major contribution to histochemistry when he published his high iron, low iron and mixed diamine methods. The methods are based upon the formation of salt complexes between the cationic staining entity and the acid groups in the tissue. These diamines will also react with periodate engendered aldehyde groups to give yellow to brown Schiff bases.

The most useful of the methods at this time is the high iron diamine. This appears to be specific for ester sulphate (Gad and Sylven, 1969; Reid and colleagues, 1972), and although there is some slight coloration of nuclei and background tissue, a positive result is quite unmistakable. Some confusion arose when this method was first published since, due to an error, *the concentration of ferric chloride given was 10 per cent instead of 40 per cent* (Sheahan and colleagues, 1970). It is unfortunate that this incorrect formula has been republished in the literature, since it gives a very indifferent result.

We use this high iron diamine method routinely in parallel with Alcian blue at pH 2.5 and pH 1.0.

The other diamine methods are described below with their expected results. It will be seen that their area of usefulness is in distinguishing between epithelial sialo-mucins and sulpho-mucins. Spicer has shown that some sialo-mucins are reactive, and others non-reactive, with the low iron diamine-Alcian blue pH 2.5 technique. The mixed diamine method distinguishes between periodate-reactive and periodate-unreactive acid mucosubstances (Leppi and Spicer, 1967).

High Iron Diamine Method (Spicer, 1965; Leppi and Spicer, 1967)

Special Reagent Required – Diamine Solution

N, N-dimethyl-*m*-phenylenediamine dihydrochloride	120 mg
N, N-dimethyl-*p*-phenylenediamine hydrochloride	20 mg

Dissolve the diamines in 50 ml of distilled water then add 1.4 ml of 40 per cent ferric chloride. The pH of the prepared solution should be 1.5–1.6. *This solution must be freshly prepared and used immediately.*

Method

(1) Bring sections to water.
(2) Stain in diamine solution in Coplin jar for 24 hours.
(3) Rinse rapidly in water.
(4) Dehydrate rapidly, clear and mount in resinous mountant.

Results

Sulphomucins	Grey-purple-black.

Non-sulphated acid mucins are unstained unless an Alcian blue at pH 2.5 (*see* page 275) step is interposed after step 3, when they will be blue (high iron diamine-Alcian blue method).

Low Iron Diamine-Alcian Blue Method (Spicer, 1965; Leppi and Spicer, 1967)

Special Reagent Required – Diamine Solution

N, N-dimethyl-*m*-phenylenediamine dihydrochloride	30 mg

N, N-dimethyl-*p*-phenylenediamine
hydrochloride . . . . . 5 mg

Dissolve diamines in 50 ml of distilled water, and add 0.5 ml of 40 per cent ferric chloride. *This solution must be freshly prepared and used immediately.*

Method

(1) Bring sections to water.
(2) Stain in diamine solution in a Coplin jar for 24 hours.
(3) Rinse quickly (in and out) in water.
(4) Stain in Alcian blue 8 GX (1 per cent in 3 per cent acetic acid) for 30 minutes.
(5) Rinse quickly (in and out) in water.
(6) Dehydrate, clear and mount in resinous mountant.

Results

Sulphated and many acid non-sulphated mucins stain black; few acid non-sulphated mucins stain blue. If demonstration of neutral polysaccharides is desired, oxidize an additional section in 1 per cent periodic acid and then wash in water for 5 minutes before step 2, when they will be stained purple-grey.

Mixed Diamine Method (Spicer, 1965; Leppi and Spicer, 1967)

This method is extremely good for acidic mucosubstances.

Special Reagent Required – Mixed Diamine Solution

N, N-dimethyl-*m*-phenylenediamine
dihydrochloride . . . . . 30 mg
N, N-dimethyl-*p*-phenylenediamine
hydrochloride . . . . . 5 mg

Dissolve diamine in 50 ml of distilled water, then adjust pH to between 3.4 and 4.0 with 0.2M Na_2HPO_4 (0.15–0.65 ml). *This solution must be freshly prepared.*

Method

(1) Bring duplicate sections to water.

(2) Hydrolyse both sections in preheated N HCl at 60°C for 10 minutes (Feulgen hydrolysis to remove interfering staining of nuclei).

(3) Wash in running water for 5 minutes.

(4) Oxidize one section in 1 per cent aqueous periodic acid for 10 minutes, then rinse in running water for 5 minutes.

(5) Stain both sections in mixed diamine solution for 20–48 hours (usually 24 hours).

(6) Rinse and dehydrate in two changes of 95 per cent alcohol.

(7) Treat with two changes of absolute alcohol, clear in xylene-alcohol and xylene.

(8) Mount in a resinous mountant.

Results

Periodate unreactive acidic mucosubstances	Purple
Periodate reactive neutral and acidic mucosubstances	Grey–grey-brown

Both types of acidic mucosubstances are purple in unoxidized sections.

Mixed Diamine-Sodium Chloride Method (Spicer, 1965; Leppi and Spicer, 1967)

The procedure for this method is identical to that of the mixed diamine method (above) except that 3–7 ml of 1M sodium chloride replaces an equal volume of water in the mixed diamine solution.

Results

The inclusion of sodium chloride in the mixed diamine solution decreases the reactivity of many sialomucins but increases that of some sulphated mucins, especially in the cornea,.overian follicles and some connective tissue.

Saunders Acridine Orange – CTAC Method (Pearse, 1968)

Saunders used CTAC-acridine orange staining followed by elution with different concentrations of sodium chloride to differentiate between hyaluronic acid, chondroitin sulphates and heparin.

Recommended Fixation and Processing of Tissue

(1) Fix small pieces of tissue in Newcomer's fluid (page 51) for 12–24 hours.

(2) Transfer to equal parts of Newcomer's fluid and *n*-butanol for 30 minutes.

(3) Transfer to three changes of *n*-butanol for 30 minutes each.

(4) Place in equal parts of *n*-butanol and wax (or Paraplast) for 30 minutes.

(5) Impregnate in three changes of wax or Paraplast for 30 minutes each.

(6) Embed tissue and cut 3–5μ sections.

Method

(1) Treat 3 slides (A, B and C) with 1 per cent cetyltrimethylammonium chloride (CTAC) for 10 minutes.

(2) Wash in running water for 10 minutes.

(3) Treat all 3 slides with ribonuclease (page 257) for 2 hours at 45°C.

(4) Treat slide A with CTAC (10 minutes); wash; 0.1 per cent aqueous acridine orange (pH 7.2) for 3 minutes; running water 10 minutes, air dry and mount in fluorescent-free mountant.

(5) Treat slide B with 0.1 per cent acridine orange in 0.01M-acetic acid (pH 3.2) for 3 minutes; rinse in running water and then differentiate in 0.3 M-NaCl in 0.01M acetic acid; wash in running water; air dry and mount in non-fluorescent mountant.

(6) Treat slide A as slide B but substitute 0.6M NaCl for 0.3M NaCl.

Results

Slide A	Red fluorescence due to hyaluronic acid
Slide B	Red fluorescence due to chondroitin sulphates and heparin.
Slide C	Red fluorescence due to heparin.

Gomori's Aldehyde Fuchsin Stain (Halmi's modn.)

The combination of basic fuchsin and aldehyde in the presence of a strong mineral acid was described by Gomori in 1950, who noted its

preferential staining for certain tissue constituents, for example, elastic tissue, mast cell granules, mucin, and so on. Abu'l Haj and Rinehart (1953) noting these were polysaccharide in nature, concluded that the dye reacts with sulphated mucopolysaccharides. Although there is some dispute as to its absolute specificity, it is reasonable to assume that sites which are Alcian blue and aldehyde fuchsin positive, which become aldehyde fuchsin negative following methylation, and remain negative after saponification are sulphated mucopolysaccharides. Elastic fibres have been reported as positive after methylation but, in our experience, provided that pure methyl alcohol (reagent grade) is used, elastic fibres become negative after 24–48 hours, at 0°C.

Non-sulphated acid mucins and neutral mucins colour purple with aldehyde fuchsin (and are metachromatic) after the Kramer-Windrum type of sulphation technique (page 288).

Staining solutions

Dissolve 0.5 g basic fuchsin in 100 ml of 60 per cent alcohol, then add 1 ml paraldehyde (fresh) and 1.5 ml concentrated hydrochloric acid. Allow to 'ripen' for 24 hours before use.

Method

(1) Bring sections to 70 per cent alcohol.

(2) Stain in fresh aldehyde fuchsin for 5–10 minutes. Older solutions may require a longer staining period, and give a less selective result.

(3) Rinse in 70 per cent alcohol.

(4) Counterstain* in 0.25 per cent light green in 70 per cent alcohol for 10 seconds.

(5) Rinse rapidly in 70 per cent alcohol.

(6) Dehydrate, clear and mount in D.P.X., H.S.R. or other synthetic resin mountant.

Results

Aldehyde fuchsin positive structures . . . . . Purple

Specific Methods for Sialic Acids

Although several methods have been described for the purpose of specifically identifying sialic acids, there are few, if any, that have been

*Counterstaining is optional.

substantiated. *Their idenification is still mainly dependent upon staining with Alcian blue (and P.A.S.), both before and after sialidase (and mild acid hydrolysis) treatments, and possibly methylation (page 290) which removes most of the sialic acid* from tissue sections (Quintarelli and colleagues, 1964; Schmitz-Moorman, 1969).

At the present moment it would seem that the only method that can be used to demonstrate the presence of sialic acid with any certainty, and also to determine whether or not it is labile to sialidase (*see* page 295), is the thiobarbituric acid assay technique of Warren (1959). It has been used (Spicer and Warren, 1960; Mohos and Skoza, 1970) to assay the enzyme solution (and section washings (X3)) after section treatment. They were also able to assay single sections, before and after enzyme treatment, by scraping them off slides into test tubes and then performing the assay after acid hydrolysis to release the sialic acid.

Sialomucins differ in their reactivity to the periodic acid-phenylhydrazine-Schiff (PAPS) technique (*see* page 269) and in their lability to sialidase (neuriminidase) treatment; furthermore, some sialomucins, which are stable to sialidase, become labile after saponification, and so on (*see* page 295). This would tend to indicate that sialic acid varies between animal glands (Leppi and Spicer, 1967) and may even vary within species.

The importance of the identification of sialic acid in the histochemistry of mucins lies in the generalization that epithelial acid glycoproteins contain sialic acid and/or sulphate and connective tissue acid mucopolysaccharides contain uronic acid and/or sulphate.

BIAL Reaction for Sialic Acids (Ravetto, 1964; Pearse, 1968)

Special Reagent Required

Orcinol (5-methylresorcinol)	200 mg
Concentrated hydrochloric acid	80 ml
0.1 M copper sulphate ($CuSO_4$)	0.25 ml

Dissolve the orcinol in the hydrochloric acid, add the copper sulphate solution and make up to 100 ml with distilled water. Allow to stand for 4 hours before using.

Method

(1) Spray cryostat or frozen dried formalin vapour fixed sections with above reagent.

(2) Place sections, face down, on a glass frame in a preheated container, which has on the bottom a thin layer of concentrated HCl, at 70°5 for 5–10 minutes.

(3) Dry sections in air.
(4) Clear in xylene and mount in resinous mountant.

Results

High concentrations of sialic acids . . . . .Red to reddish-brown (this colour will fade rapidly).

Scott's Critical Electrolyte Concentration Technique

Scott and his colleagues (Quintarelli and Dellovo, 1964; Dorling, 1965) showed that carboxylated acid carbohydrates (for example, hyaluronic acid, sialomucins) will not stain with Alcian blue in concentrations of magnesium chloride at or above 0.4 M, whereas sulphated mucins will stain at concentrations of 0.8 and above.

Our experience with this method confirms the figures given by Pearse (1968) that carboxylated carbohydrates alone do not stain in concentration of MgCl above 0.1 M.

Special Reagent Required

Dissolve 0.1 per cent Alcian blue 8 GX in 0.05 M sodium acetate buffer at 5.7, add magnesium chloride to give molar concentrations of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0.

Method

(1) Bring sections to water.
(2) Stain serial sections in Alcian blue in each of the $MgCl_2$ concentrations for 30 minutes.
(3) Wash in running water.
(4) Dehydrate, clear and mount in resinous mountant.

Results

See above.

KOH/P.A.S. Staining Method (Culling, Reid and Dunn, 1971)

This method has been shown to give greatly increased staining (com-

pared to routine P.A.S. method) in human and rat ileocaecal valve*, large intestine* and rectum*, and a moderate increase in the same areas in guinea-pig and rabbit, Brunner's glands becoming strongly P.A.S. positive from a faint reaction.

These changes were not due to fixation as was suggested (Hale, 1959). Preliminary studies suggest that this method, following after a borohydride blockade of aldehydes (*see* page 289), which abolishes normal P.A.S. reactivity, will assist pathologists in the identification of metastases from these specific areas (above*), since the only P.A.S. staining seen can probably be ascribed to mucin produced by malignant cells arising from epithelia of such areas. We have recently noted that myelin sheaths are stained more deeply with P.A.S. after KOH treatment.

Method

(1) Bring serial sections to 70 per cent alcohol*.
(2) Saponify one section (*see* page 292).
(3) Stain both sections by routine P.A.S. method.
(4) Counterstain lightly with haematoxylin (optional).
(5) Dehydrate clear and mount in resinous mountant.

Results

Compare sections under comparison microscope for increased staining in saponified section.

Use of Radioactive Isotopes (^{35}Sulphur) for the Specific Localization of Sulphated Mucosubstances

The incorporation of ^{35}sulphur is the only truly specific method of localizing sulphate esters in tissues or tissue cultures. This may be done *in vivo* by injection of radioactive sulphate into laboratory animals, or *in vitro* by inclusion of the isotope in media for tissue cultures, minces, and so on.

*To avoid use of a comparison microscope first treat both sections with borohydride aldehyde block (*see* page 297). Then stain one section with P.A.S. directly (as a negative control to ensure that borohydride blockade is effective and continue through steps (2) to (5) with parallel section. The only P.A.S. staining seen in the test section will be due to KOH treatment.

In Vivo Method. – Leppi and Spicer (1967) injected rhesus monkeys with 2 mc of carrier-free ^{35} sulphur ($Na_2{}^{35}S\,O_4$) intraperitoneally, the animals being sacrificed 6 hours later. Tissues were taken, fixed in formol calcium for 24 hours, and radioautographs prepared using Kodak AR–10 stripping film and Alcian blue/P.A.S. staining (*see* page 507 for technique).

In Vitro Method. – Felipe (1971) recommended that tissues to be investigated be minced with a razor blade, placed on a stainless steel mesh support in a Petridish, and tissue culture medium 199 containing 1 μc/ml carrier free ^{35} sulphur added until a thin layer was drawn over the surface of the tissue by capillary action. The specimens were then incubated in an oven at 37°C in an atmosphere of 95 per cent oxygen and 5 per cent carbon dioxide for periods of 1, 2, 3 and 4 hours and overnight (3 hours incubation was found to be the optimal time). Tissues were fixed in formol calcium and embedded in paraffin. Radioautographs were prepared as described above and on page 507.

Sulphation Technique (Kramer and Windrum, 1954)

This method was used by Kramer and Windrum to induce metachromasia in P.A.S. positive structures.

Special Reagents Required

Mix 35 ml acetic anhydride, 15 ml ether, then 50 ml fuming sulphuric acid drop by drop. Cooling the reagent in the refrigerator before mixing lessens the danger of 'boiling' of the mixture.

Method

(1) Bring section to water.
(2) Treat in reagent for two minutes.
(3) Stain by metachromatic method.

BLOCKING TECHNIQUES

A blocking technique is one that, although failing to give a colour reaction with a certain tissue element, will combine with that tissue element in such a manner as to prevent it from giving a colour reaction with other reagents. It may be used for the detection of those groups which react in this way.

Blocking of Aldehyde Groups

By blocking aldehyde groups in a periodic-acid-treated section, it can be shown that a positive Schiff reaction may be given by substances other than aldehydes, such as peroxide, betone, or ethylene oxide. Several blocking techniques have been devised, but those most commonly used are described below.

Aldehyde Blocking Technique (Lillie and Glenner, 1957)

After exposure of the sections to periodic acid, and washing, they are treated in the following solution for 30 minutes at room temperature:

Aniline	10 ml
Acetic acid	90 ml

The sections are then washed in distilled water and the P.A.S. technique continued at stage 4.

Results

Only non-aldehyde P.A.S. positive material (reducing lipid, and so on) will be stained red.

Borohydride/Aldehyde Block (Lille and Pizzolato, 1972)

This method of blocking aldehyde groups with sodium borohydride is simple, quick and effective; alternatively, use steps (2), (3) and (4) of the method on page 296 in place of step (4) below.

Method

(1) Bring sections to water.
(2) Treat with freshly prepared 1 per cent periodic acid.
(3) Wash in running water for 10 minutes.
(4) Treat with 0.1 per cent sodium borohydride in 1 per cent $Na_2 HPO_4$.
(5) Treat slide with Schiff reagent (page 249).
(6) Treat by appropriate method, or dehydrate, clear and mount.

Results

Sections (at step 5) are Schiff negative.

Spicer's Phenylhydrazine Blocking of Aldehydes
(Spicer, 1961; Lev and Spicer, 1965)

Sections are treated with an 0.5 per cent aqueous solution of phenylhydrazine hydrochloride for 30 minutes.

Blocking Technique for 1:2 Glycol Group (Acetylation)

A P.A.S. positive substance which, after treatment with acetic anhydride, gives a negative P.A.S. reaction, indicates that the original reaction was due to a 1:2 glycol grouping (carbohydrate). Since acetylation is reversible by treatment with potassium hydroxide (saponification), the following technique of McManus and Cason is recommended.

Method

(1) Bring 3 sections to water.
(2) Treat 2 sections (A and B) in the following solution for 1–24 hours at room temperature.

Acetic anhydride	13 ml
Pyridine	20 ml

Note. – One hour will usually suffice in this reagent, but longer periods may be required before a histochemical evaluation can be made.

(3) Wash sections A and B in water.
(4) Treat section B with 0.1 N potassium hydroxide for 45 minutes at room temperature (deacetylation).
(5) Wash section B in water.
(6) Treat all 3 sections by the P.A.S. technique.

Results

A positive result in a given structure in sections B and C only indicates that the reaction was due to a 1:2 glycol grouping, and not to preformed aldehydes in the element.

Methylation

This procedure has been classified by Spicer (1960) as 'mild methylation' when performed at 37°C for 4 hours, whereas the term 'active

methylation' is used when it is performed at 60°C for 4 hours. Spicer and colleagues (1967) state that 'mild methylation' eliminates basophilia of most COOH groups. Basophilia of all acid mucopolysaccharides is occluded after 4 hours at 60°C. Schmitz-Moorman (1969) and Quintarelli, Scott and Dellovo (1964) suggest that sialic acid is mostly removed by active methylation (60°C) and its staining cannot be restored by saponification. Active methylation (at 60°C) is thought to methylate carboxyl groups (COOH) to methyl esters (COOMe) which can be restored to COOH by saponification.

Vilter (1968) supported by Sorvari and Stoward (1970) have suggested that the abolition of basophilia by methylation is due to the lactonization of carboxyl groups, rather than their esterification. Methylation has also been shown to desulphate the sulphated mucopolysaccharides and glycoproteins; staining of sulphate groups cannot be restored by saponification. This may also cause an increase in P.A.S. staining similar to the KOH/P.A.S. reaction (*see* page 286).

Method

(1) Bring sections to alcohol.
(2) Place in preheated 1 per cent hydrochloric acid in methyl alcohol, for 4 hours at 60°C (at 37°C for mild methylation – *see* above).
(3) Rinse in alcohol.
(4) Stain by appropriate technique (parallel sections may be saponified *(see below)* then stained and compared).

Results

See above.

Thionyl Chloride Methylation (Stoward, 1967; Sorvari and Stoward, 1970)

For the reasons give below (*see* Results) we do not find this method useful in the routine laboratory. We have used it in our research together with the borohydride method of blocking carboxyl (COOH) groups (*see* page 296).

Method

(1) Bring sections to methyl alcohol.
(2) Treat in 2 per cent thionyl chloride in methyl alcohol for 4 hours at room temperature.

(3) Rinse twice in methyl alcohol and rinse in water.
(4) Stain by appropriate procedure.

Result

Basophilia due to COOH groups and RNA are said to disappear in 30 minutes and that due to sulphated mucopolysaccharides in 4 hours. It is, however, possible that in sulphomucins containing both COOH and sulphate groups that only the sulphate groups are esterified. A few sulphated mucins are desulphated completely (Pearse, 1968).

Saponification (Spicer, 1960)

Unless sections are firmly attached to slides they will become detached during this procedure. It is recommended that 0.1 per cent chrome alum in 1 per cent gelatin be used as an adhesive.

Method

(1) Bring sections to 70 per cent alcohol.
(2) Treat with 0.5 per cent potassium hydroxide (KOH) in 70 per cent alcohol for 30 minutes at room temperature.
(3) Rinse carefully in 70 per cent alcohol.
(4) Wash in slowly running tap-water for 10 minutes.

Results

This treatment will restore the basophilia (and alcianophilia) of COOH which have been esterified by methylation. It may increase P.A.S. reactivity of certain G.I. tract mucins (*see* page 286).

Hyaluronidase Digestion

The histochemical identification of hyaluronic acid, chondroitin sulphates A and C (chondroitin-4-sulphate and chondroitin-6-sulphate) and chondroitin sulphate B (dermatan sulphate) is mainly dependent upon digestion with hyaluronidase allied with the staining and blocking methods described above. *Table 14.2* illustrates their mechanism of action, although it should be remembered that the results obtained will only be accurate if the enzymes used are both pure and active, traces of protease activity can give misleading information. For the preparation of *Flavobacterium heparium* extracts *see* Zugibe (1962, 1970).

Serial sections should be used for this type of investigation and the test and control slides examined with a comparison microscope.

TABLE 14.2
Activity of the Various Hyaluronidases (after Zugibe, 1962)

Enzyme	Acid mucopolysaccharide hydrolysed
Streptococcal hyaluronidase	Hyaluronic acid
Testicular hyaluronidase	Hyaluronic acid Chondroitin sulphate-A Chondroitin sulphate-C
Flavobacterium heparinum Extract (adapted to Chondroitin sulphate-A	Hyaluronic acid Chondroitin sulphates A, B and C
Flavobacterium heparinum extract (adapted to heparitin sulphate)	Hyaluronic acid Chondroitin sulphate A Chondroitin sulphate C Heparitain sulphate, heparin

Methods

It should be remembered that a *control section should always be incubated in buffer alone* for the same time and at the same temperature as the test sections. *A positive control section (umbilical cord, and so on) should also be treated with the test sections to ensure that the enzyme is active.*

Testicular Hyaluronidase Technique

Method

(1) Bring sections to water.

(2) Treat slides for 2–6 hours in pre-warmed (37°C) hyaluronidase solution (50 mg/100 ml of acetic-acetate buffer pH 6.0 (page 170)) at 37°C; place control slides in buffer alone for same time period.

(3) Rinse slides in buffer alone.

(4) Rinse in water.

(5) Stain with Alcian blue at pH 1.0 and/or 2.5 (*see* page 275).

Result

Loss of staining (by comparison with buffer control) is due to removal of hyaluronic acid and/or chondroitin sulphates A and/or B.

Streptococcal Hyaluronidase Technique (Zugibe, 1962)

Positive and negative control sections should be carried through with the test sections as described above.

Method

(1) Bring formol-fixed cryostat or paraffin sections to water.
(2) Rinse sections in acetic-acetate buffer at pH 5.0 (*see* page 170).
(3) Incubate sections in acetic-acetate buffer (pH 5.0) containing streptococcal hyaluronidase (1,500 TRU/100 ml), 0.1 M NaCl and 0.05 per cent gelatin, for 24 hours at 37°C.
(4) Rinse in water.
(5) Stain with Alcian blue at pH 2.5 (*see* page 275).

Results

Loss of staining (by comparison with buffer control) is due to removal of hyaluronic acid.

Sialidase Digestion

In the second edition I stated that sialidase (neuraminidase) specifically removed sialic acid, it has now been shown that some sialic acids are stable to sialidase treatment. Methods for enhancing their digestibility (described below) have all been found to be efficient in a given situation; however, it seems there are still some sialic acids (extracted from tissues after treatment) that are completely stable; these cannot be identified histochemically (*see* page 284). I recommend sialidase alone, and sialidase following potassium hydroxide treatment (as in *(a)* below).

Methods of Enhancing Digestibility of Sialic Acids Before Treatment with Sialidase (Gad, 1969)

(*a*) Pre-treatment with 1 per cent potassium hydroxide in 70 per cent ethanol for 5 minutes (Spicer and Duvenci, 1964).

(*b*) Trypsin 1/1000 in M/100 phosphate buffer at pH 8.0 for 5 minutes to 4 hours at 37°C (Lev and Spicer, 1965).

(*c*) Crystalline pepsin 2 mg/ml of 0.02 N sodium acetate Hcl buffer at pH 2.5 for 2 hours at 37°C (Quintarelli, 1963).

Sialidase Technique (Quintarelli, 1961; Gad, 1969)

Method

(1) Bring two (A and B) cryostat or paraffin sections of formol-fixed tissue to water, or air dry (Gad, 1969).

(2) Section A is incubated in sialidase (neuraminidase) 100 units per ml in 0.05 M acetate buffer at pH 5.5 (containing approximately 0.1 per cent calcium chloride) (section B in buffer alone) for 24 hours at 37°C.

(3) Rinse in water.

(4) Stain in Alcian blue pH 2.5 (*see* page 275).

Result

Loss of staining (by comparison with control B) is due to removal of sialic acids.

Acid Hydrolysis to Remove Sialic Acid (Quintarelli, 1961)

Since sialic acid is invariably a terminal group, it may be removed by mild acid hydrolysis (Quintarelli, 1961). As is noted on page 284, methylation is also said to remove most of the sialic acid from sections. This method, combined with pretreatment in KoH (see above) is used routinely in our laboratory.

Method

(1) Bring sections to water.

(2) Treat sections in pre-heated 0.02 N sodium acetate-HCl buffer pH 2.5 (*see* page 168) at 75°C for 2 hours (or in 0.1N H_2SO_4 at 80°C for 1 hour.

(3) Rinse in distilled water.

(4) Stain sections by Alcian blue/P.A.S. technique (page 276).

(5) Dehydrate, clear and mount in resinous mountant.

Result

Removal of sialic acid is shown by loss of Alcian blue staining.

Reduction of Uronic Acid Esters (CooMe)
Reid, Culling and Day, 1970)

Frush and Isbell (1956) described a method for the reduction of lactones and uronic acid esters (CooMe) to the corresponding primary alcohols; we have adapted this method for use in histochemistry.

This modification is based upon the esterification (by methylation) of the carboxyl groups (COOH), with the resultant uronic acid methyl esters (CooMe) or the lactones of Vilter (1968) and Sorvari and Stoward (1970) being reduced to primary alcohols with buffered sodium borohydride.

It had been intended to use this method together with the methanolic thionyl chloride (Stoward, 1967; Sorvari and Stoward, 1970) method, of esterification of COOH groups without de-sulphation, as a specific technique for sulphate esters. Unfortunately, our experience, similar to that of Stoward (1967b), was that partial desulphation took place.

Special Reagents Required – Buffered Borohydride Reagent

Solution A

Boric acid (H_3BO_3)	2.45 g
Distilled water	100 ml

Dissolve boric acid in distilled water. This solution may be prepared and kept in a refrigerator until needed.

Solution B

Sodium borohydride ($NaBH_4$)	1.89 g
Distilled water	167 ml

Immediately before use dissolve the borohydride in distilled water. This should be done in a fume hood.

Method

(1) Methylate (thionyl chloride method) sections as described on page 291 or by preferred method.

(2) Place slides in glass (or stainless steel) holder in a container that will hold approximately 300 ml, but narrow enough that slides will be covered by 100 ml. of ice-cold boric acid solution (solution A) that is first poured in. This container should be placed in crushed ice in a fume hood.

(3) Over a 30-minute period, add solution B to solution A. Leave sections in buffered borohydride in ice bath for 1 hour.

(4) Wash sections in running water for 10–15 minutes.

(5) Stain sections by Alcian blue pH 2.5 (*see* page 275) or other appropriate method.

(6) Dehydrate, clear and mount in resinous mountant.

Result

Only sulphate esters (surviving methylation) are stained blue. A control section should be saponified (*see* page 292) after step (4) to ensure that reduction of esters has taken place, strongly positive alcianophilia (with Alcian blue at pH 2.5) after this step will indicate the absence or incompleteness of methylation or reduction.

Smith Oxidation Hydrolysis (Reid, Culling and Day, 1970)

This procedure (Goldstein and colleagues, 1965) was adapted by us to abolish existing P.A.S. staining in tissue sections when investigating the KoH/P.A.S. phenomena (*see* page 286). The method is based upon the oxidation of 1:2 glycols (vicinal diols) with periodic acid to dialdehydes, which are then reduced to primary alcohols with buffered borohydride (Lillie and Pizzolato, 1972). Subsequent hydrolysis with hydrochloric acid should cleave residues which have been oxidized with periodate, thus fragmenting the polymer into small diffusable molecules. The degree of fragmentation will depend upon the position of the oxidized residues.

Method

(1) Bring sections to water.

(2) Treat in freshly prepared 1 per cent periodic acid for 10 minutes.

(3) Wash in running water.

(4) Treat with 0.1 per cent sodium borohydride in 1 per cent Na_2HPO_4 for 5 minutes.

(5) Immerse sections in 0.1 N hydrochloric acid for 6 hours at room temperature.

(6) Wash in running water.

(7) Perform P.A.S. reaction (page 268).

Result

Sections will be P.A.S. negative after reduction, following hydrolysis, however, certain structures may develop P.A.S. positivity due to the oxidation of 1:2 glycols which were previously blocked

(1) *A control section* should be stained with Schiff reagent after step (4) to ensure that aldehydes have been blocked (converted to primary alcohols).

(2) *Following step (6)*, treatment of sections of human or rat large intestine with methylation (*see* page 290) or alcoholic KoH (saponification page 286) will result in areas of P.A.S. positivity (*see* page 292).

GLYCOGEN

Glycogen, in colloidal solution, is found in the cytoplasm of certain cells. In adult life it may be demonstrated principally in the cells of the liver, but in foetal life it has a very wide distribution. After fixation it is seen as fine granules or an amorphous mass, dependent on the type of fixation employed.

It is a polysaccharide, which is derived from, and within 1 hour of death breaks down into, sugar. Consequently, tissues must be fixed while fresh, or frozen until fixation is possible. It is readily soluble in water before fixation, and tissues must not be rinsed or washed in water or saline solution prior to fixation.

Fixation

The original method by Best (1906) specified celloidin embedding following alcohol fixation. The celloidin was thought to be essential to prevent diffusion of the glycogen from the tissues, but Lillie (1947) and Vallance-Owen (1948) have shown that tissues may, after fixation, be washed in running water for 24 hours without any appreciable loss of glycogen.

Fixatives containing a high concentration of picric acid (for example, Gendre's fluid, page 49) are thought by some to give the best results. In recent years, however, it has been shown (Vallance-Owen, 1948) that glycogen is as adequately fixed by formol saline as by alcohol and alcohol-picric acid fixatives.

In routine laboratory work it will be found that formol saline gives adequate results, but for histochemical studies the freeze-drying technique should be used.

The latter is undoubtedly the best available method of preserving glycogen; it would appear to preserve 100 per cent of the glycogen, and effectively prevents streaming of the glycogen to one pole of the cells

(polarization). See also pages 34 and 266 on the fixation of carbohydrates.

Embedding Medium

Although celloidin was originally described as the method of choice, paraffin wax embedding gives just as good results. Frozen sections are not generally as satisfactory.

Method of Demonstration

Methods of demonstration fall into the four following groups.

(1) Iodine staining
(2) Carmine staining
(3) Acid hydrolysis – Schiff methods
(4) Silver impregnation

Since the methods used may demonstrate other cell constituents, it is necessary to employ enzymic control to obtain specific demonstration of glycogen, and this will therefore be discussed before the methods of demonstration are described.

Enzymic Control for Specific Demonstration of Glycogen

Glycogen is destroyed by the enzyme diastase (or amylase), which may be obtained from saliva or malt. Malt diastase is available commercially and is more reliable than saliva, but the latter is suitable for routine purposes and is readily available. If diastase is allowed to act on a section containing glycogen the glycogen present will be removed, and subsequent staining of this section and of a second untreated section will reveal those places where material has been removed by diastase. Diastase removes a variety of material other than glycogen (for example, ribonucleic acid), but since this material is not P.A.S. positive, or shown by Best's carmine, this is not a great disadvantage.

If sections are to be treated with celloidin it is essential that treatment with diastase be undertaken first. A celloidin film prevents the complete removal of glycogen by diastase for quite long periods, whereas treatment for 20–30 minutes at 37°C before applying celloidin will remove all the glycogen present.

An enzyme-treated slide and known positive control should always be stained in parallel with the test slide; comparison between these slides will show specifically the presence of glycogen.

Technique for Digestion (Stages 1–3) and Celloidinization (Stages 4–7)

Method

(1) Bring sections to water.
(2) Digest with diastase (human saliva, or 1:1,000 malt diastase in distilled water) for 30 minutes at 37°C.
(3) Wash in water for 5–10 minutes.
(4) Wash with 90 per cent alcohol, then absolute alcohol for ½ minute.
(5) Transfer to a stoppered container of 1 per cent celloidin for 2 minutes.
(6) Drain off the excess of celloidin, and transfer to 80 per cent alcohol for 5 minutes to harden.
(7) Wash in running tap-water for 1–2 minutes.
(8) Stain by any desired technique.

Note. – Test sections, and known positive controls which are not to be digested, are brought through xylol and alcohol directly to stage 4 above.

Iodine Technique

Methods employing iodine are not as specific as Best's carmine, or P.A.S., nor are they permanent; they are, however, simple and rapid in operation.

Method

(1) Bring sections to water.
(2) Place slide on rack, flood with Lugol's iodine and leave for 2–3 minutes.
(3) Drain, and flood with 1–2 per cent iodine in absolute alcohol.
(4) Blot with filter paper.
(5) Place 1–2 drops of origanum oil on the section and lower a coverslip on to the oil, avoiding air bubbles.
(6) Seal the edges of the coverslip with paraffin wax or similar compound. After a few moments the origanum oil will clear and differentiate the section.

Results

Glycogen	Dark brown
Tissue	Yellow

Best's Carmine Technique (1906)

This gives a fairly permanent preparation, and is specific when controlled by enzyme digestion.

Solutions Required

(1) *Alum haematoxylin*

(2) *Staining solution*

Best's stock solution *(see below)*	12 ml
Fresh concentrated ammonia (.880)	18 ml
Methyl alcohol	18 ml

Filter before use.

The amounts given immediately above are sufficient for one Coplin jar, but smaller amounts may be used, and poured on to the slide.

Best's stock solution is made as follows:

Carmine	2 g
Potassium carbonate	1 g
Potassium chloride	5 g
Distilled water	60 ml

The reagents, in a 250 ml flask, should be gently boiled until the colour deepens (3–5 minutes); the deeper the colour at this stage the deeper the staining of the glycogen. Cool, and add 20 ml of fresh concentrated ammonia (.880). This stock solution, in a tightly stoppered bottle, must be stored in a refrigerator (0–5°C), but even then it will only keep for 6–8 weeks.

(3) *Best's differentiating fluid**

Absolute alcohol	20 ml
Methyl alcohol	10 ml
Distilled water	25 ml

*Methyl alcohol alone may be used in place of this differentiating fluid for paraffin sections.

Method

(1) Celloidinize, or digest sections (for technique *see above*).

(2) Place in alum haematoxylin (Ehrlich or Harris) for 10–15 minutes to stain nuclei.

(3) Rinse rapidly in 1 per cent acid alcohol to clear the background.

(4) Wash in running water for 1 minute to remove acid (sections will be 'blued' by the ammonia in the carmine stain).

(5) Stain in a Coplin jar, or on a slide rack, with the staining solution for 10–15 minutes.

(6) Wash slide with Best's differentiating fluid until stain ceases to pour out, this usually takes only a few seconds: or wash rapidly with methyl alcohol if working with paraffin sections.

(7) Flood with alcohol or acetone to remove celloidin film (alcohol/ether mixture may be used if the film is difficult to remove).

(8) Clear in xylol and mount in Canada balsam or D.P.X.

Results

Glycogen	Red
Nuclei	Blue

Acid Hydrolysis – Schiff Methods

This group of methods (*see* page 268) is popular for the demonstration of glycogen, having the advantage that solutions do not require special preparation and, used in conjunction with enzyme digestion, are specific.

Schiff reagent, following oxidation with periodic acid, chromic acid, or potassium permanganate, gives a first-class demonstration of glycogen.

Following digestion of the control, celloidinize the control, test section and the positive control, and treat the three sections as detailed on page 300.

Silver Impregnation

Several methods of silver impregnation have been reported. Mitchell and Wislocki (1944) and Pritchard (1949) describe an ammoniacal silver technique. Gomori (1946) describes a methenamine silver method, which also colours mucin and melanin.

In view of the ease of demonstration by the methods already described, the silver techniques, rarely specific, are not popular.

MUCIN

(MUCOPOLYSACCHARIDES AND MUCOPROTEINS)

The term mucin is used to describe an intracellular secretion formed in a variety of cells. It is not a single entity and cells from different parts of the body secrete slightly differing substances having similar macroscopic appearances. Their chemical and histochemical differentiation is dealt with on page 260. This section will deal with their common characteristics and general demonstration, but although not listed below, the P.A.S. technique (page 267) is commonly used for this purpose.

In general, mucins have the following properties.

(1) They stain intensely with basic dyes.

(2) They are metachromatic, and therefore stain red to reddish-blue with thionin or toluidine blue.

(3) They are precipitated by acetic acid (except gastric mucin).

(4) They are soluble in alkaline solutions.

Fixation (*see* page 266)

Choice of Staining Method

Mucin may be demonstrated in frozen, paraffin or celloidin sections by any of the methods described below.

The traditional methods used are the metachromatic techniques, mucicarmine and mucihaematein; of these the most popular is Southgate's mucicarmine. The histochemical methods on pages 267–287 will demonstrate mucins histologically and give more information.

Metachromatic Staining

Metachromatic staining (page 273) is a simple method of demonstrating mucin. The following method gives good results, but the buffered toluidine blue method is slightly more permanent.

Feyrter's Enclosure Technique

This method was developed by Feyrter to demonstrate myelin sheaths in frozen sections but, for the rapid demonstration of mucin, either frozen or paraffin sections may be used.

Staining Solution

Freshly prepared 1 per cent thionin or toluidine blue in 0.5 per cent aqueous solution of tartaric acid is used. In practice, keep a stock bottle of 0.5 per cent tartaric acid, and add a few crystals of stain to 5 ml of the acid when required.

Method

(1) Bring section to water (frozen section mounted on slide).

(2) Filter a few drops of stain on to the section, and lower a coverslip on to the stain and section, taking care not to trap air bubbles.

(3) With blotting paper remove excess stain from the edge of the coverslip until the section can be seen.

(4) Ring the coverslip with paraffin wax or Vaseline and leave for a few minutes before examining.

Results

Metachromatic substances	Clear red
Other tissue	Shades of blue

The red will remain for a few days

Southgate's Mucicarmine Method

This modification of Mayer's original method (which did not contain aluminium hydroxide) gives more consistent results. Lauren and Sorvari (1969) investigating this stain showed that mucicarmine stains acidic mucins, especially those of epithelial origin, and that such staining is abolished by methylation. They found that at pH 0.5 only sulphated mucins were stained; however, we were unable to repeat this latter result in our laboratory.

It demonstrates epithelial mucin well.

Carmine	1 g
Aluminium hydroxide	1 g
50 per cent alcohol	100 ml

These constituents are mixed by shaking and to them is added

aluminium chloride (anhydrous)	0.5 g

Boil in water-bath for 2½–3 minutes. Cool, make up to original volume with 50 per cent alcohol, and filter. The stock solution is stable for several months.

Method

(1) Bring section to water.
(2) Stain nuclei with haematoxylin.
(3) Differentiate in acid-alcohol and blue in tap-water.
(4) Stain for 30 minutes in the staining solution given above.
(5) Rinse in distilled water, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Mucin	Red
Nuclei	Blue

Mayer's Mucihaematein

Mayer's mucihaematein stains connective tissue mucin well, but not gastric mucin (Laskey, 1950).

Staining Solution

Dissolve 1 g of haematoxylin in 100 ml of 70 per cent alcohol; add 0.5 g aluminium chloride, and 5 ml of 1 per cent aqueous sodium iodate to ripen the solution immediately. The volume is then made up to 500 ml and the solution is ready for use. It remains stable from 4 to 6 months.

Method

(1) Bring sections to water, and wash well with distilled water.
(2) Stain with mucihaematein for 5–10 minutes.
(3) Wash in three 5-minute changes of distilled water.
(4) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Epithelial mucins	Deep blue-violet
Connective tissue mucins and cartilage matrix	Lighter violet

Lison's Alcian Blue – Chlorantine Fast Red Stain

Steedman (1950) reported the selective staining of mucin by Alcian

blue: Lison combined it with chlorantine fast red to give, in addition, selective connective tissue staining.

Staining Solutions

(1) *Alcian blue solution*

Alcian blue 8G, 1 per cent aqueous	50 ml
Acetic acid 1 per cent aqueous	50 ml
Filter and add:	
Thymol	10–20 mg

(2) *Phosphomolybdic acid (1 per cent)*

(3) *Chlorantine fast red 5 B (0.5 per cent in distilled water)*

Method

(1) Bring sections to water.
(2) Stain in Ehrlich's haematoxylin for 10–15 minutes.
(3) Wash in tap-water until blue (the sections are differentiated by the succeeding steps).
(4) Stain in Alcian blue solution for 10 minutes.
(5) Rinse in distilled water for a few seconds.
(6) Treat for 10 minutes in 1 per cent phosphomolybdic acid.
(7) Rinse in distilled water.
(8) Stain for 10–15 minutes in chlorantine fast red.
(9) Rinse in distilled water.
(10) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Mucin, granules of mast cells, ground substance of cartilage, and some types of connective tissue fibres	Bluish-green
Nuclei	Purplish-blue
Collagen fibres and ossein	Cherry-red
Cytoplasm and muscle	Pale yellow

Iron Methods

Hales colloidal iron technique (page 278) may be used to demonstrate epithelial and connective tissue mucins. It is preferred by some workers because it stains more deeply than Alcian blue.

AMYLOID

Amyloid usually arises as a sequel to chronic suppurative and inflammatory conditions (such as tuberculosis) when it is known as secondary or 'typical' amyloidosis. It is then found most commonly in the spleen ('sago spleen'), liver, kidneys, adrenals, lymph nodes and pancreas, although other tissues may be affected. It may also arise as a primary condition, not associated with an obvious predisposing disease, and is then known as primary or 'atypical' amyloidosis (para-amyloidosis) (Symmers, 1956). Experimental amyloidosis has been produced in animals by a variety of techniques. These techniques all cause abnormal stimulation of the protein synthesizing mechanism of the reticulo-endothelial system, by repeated injections of foreign protein (for example, casein) with or without adjuvants such as Freund's, Pertussis vaccine, and so on.

Primary amyloid deposits are usually found in the cardiovascular system, but may be found in other organs including the skin. The finding of amyloid in a skin biopsy may be the first pathological indication of primary amyloidosis (Vassar and Culling, 1959). Amyloidosis may also be associated with multiple myeloma.

Although the exact chemical nature of amyloid has been in dispute for some years, it is now believed to be composed of a glycoprotein-sulphated mucopolysaccharide complex. That the mucopolysaccharide component is sulphated has been established by Kennedy (1962) using radio-autographic techniques with labelled sulphate.

Recent work has also partly explained some of the staining reactions of amyloid. Gottschalk (1960) has shown that the glycoprotein component contains sialic acid and this probably accounts for its staining, even although lightly, with both Alcian blue and P.A.S. (*see* page 276). Braunstein and Buerger (1959) have pointed out that the probable reason for amyloid not staining metachromatically with toluidine blue, (or thionin) is that, although it contains chondroitin sulphate, the anionic binding sites of the acid mucopolysaccharides are probably blocked by combination with the glycoprotein, since they can be demonstrated metachromatically after peptic digestion.

The demonstration and identification of amyloid has been the subject of numerous papers since the last edition of this book. It would seem that the only universally acceptable method of identifying amyloid is to demonstrate its peculiar fibrillar ultrastructure by electron microscopy (Cohen and colleagues, 1959, 1960 and 1965). These fibres were shown to be 75–100 Å in length and 50–300 Å in width, the fibril being composed of 1–8 laterally aggregated filaments, each about

75 Å in width and showing beading with a periodicity of 100 Å *(Figure 14.1).*

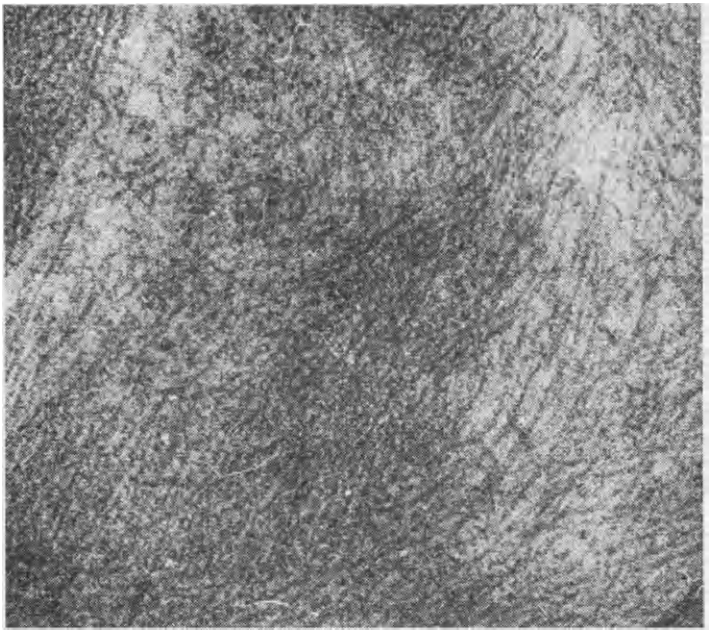


Figure 14.1 – The fibrillar ultrastructure of amyloid deposits in kidney as seen by electron microscopy. Note the discrete interlacing fibrils which are characteristic (X 120,000) (reproduced by courtesy of Dr. W. H. Chase)

However, most pathologists are faced with the problem of making a diagnosis using light microscope techniques. The thioflavine T technique (Vassar and Culling, 1959) has been recommended as the most specific and sensitive by Hobbs and Morgan (1963), Saeed and Fine (1967) and Schwartz (1969); the most sensitive with satisfactory specificity by Pearse (1968) and as very sensitive but not specific by Cooper (1969) and Zugibe (1970): Pearse (1968) finding fluorescence with phosphine 3R, and Cooper (1969), Congo red or Sirius red dichroism and DMAB-nitrite (page 233) the most specific. The danger of false positive staining with Thioflavine-T was emphasized by McAlpine and Bancroft (1964) and Wolman (1971) who both quote false staining of hyaline material, the latter encountered the same problem with thyroid colloid, keratin, and so on. In our experience, this apparent non-specific staining has been due to the use (Klatskin, 1969) of a fluorescence

microscope fitted with a BG 12 (exciter filter) and a yellow (OG^4 or Euphos) barrier filter, in which case blue-white autofluorescence will (because of the yellow barrier filter) appear yellow. Provided that ultraviolet light (UG1 or 2 exciter filter) is used in combination with a *colourless U.V. barrier filter* hyaline material will fluoresce an intense blue-white in contrast to amyloid which fluoresces yellow. This is only true if there are no pathological changes, for example, myeloma casts stain yellow (Vassar and Culling, 1962). Lipid granules and mast cells may fluoresce with an innate yellow fluorescence but morphologically these are easily differentiated.

Congo red staining, which has also been criticized and recommended over the past few years, has now been said to be specific if examined with polarized light for dichroism (Missmahl, 1957, 1959; and Heller and colleagues, 1964), when red stained amyloid gives a green anisotropic colour. Wolman and Bubis (1965) showed that this indicated that the green polarization colour depends upon the near perfect alignment of the dye molecules and, further, that the green colour was not present in too thin or too thick sections (5–10 μ being correct) since a retardation of approximately half a wavelength (red light) is required for interference between the fast and slow rays (Wolman, 1971). Klatskin (1969) stated that normal tissues, especially when not fixed in formalin, gave false positives with Congo red dichroism; however, he recommended parallel sections using the Congo red and Thioflavine-T techniques for routine use.

The DMAB-nitrite method for tryptophan (page 233) has been used to demonstrate amyloid (Cooper, 1969).

Wolman (1971), in an excellent review on amyloid, described a new toluidine blue polarized light method which is extremely simple to perform, but which is less sensitive than thioflavine-T or Congo red (polarization).

Routine Staining

Gross amyloid deposits may be recognized in a haematoxylin and eosin stained slide by its homogeneous pale pink colour, but needs a more selective method to be demonstrated specifically.

We routinely use the fluorescent thioflavine-T technique (Figure 14.2) and for confirmation examine a parallel congo red stained section by polarized light (see page 633*)* for the specific colour (dichroism).

Iodine Staining

Iodine staining is suitable both for sections and slices of tissue (page 552).

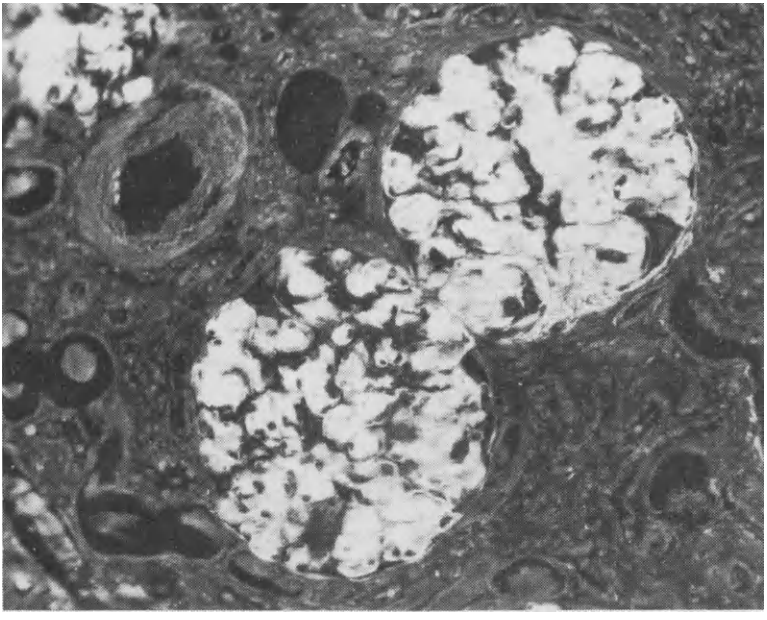


Figure 14.2 – Thioflavine T staining of secondary amyloidosis of kidney. Amyloid deposits (in glomeruli) fluoresce bright yellow on a dark green background

Method

(1) Immerse the section in Lugol's iodine for 2–3 minutes.
(2) Wash in tap-water.
(3) Mount in glycerin jelly.

Results

Amyloid	Mahogany brown
Background	Yellow

Thioflavine T Staining (*see* page 611): **Congo Red Staining Techniques**

Alkaline Congo Red Technique (Puchtler, Sweat and Levine, 1962)

This method has the advantage of not requiring differentiation. This is probably because the Congo red is in alkaline solution; since aqueous

alkaline solution causes sections to become detached from slides it is employed in an alcoholic solution. The addition of sodium chloride gives more intense staining. Examine slide also for dichroism (*see* page 309).

Fixation

The best results are obtained after alcohol or Carnoy fixed tissues, however formalin or Zenker fixed tissues were found to stain better than with other techniques.

Special Reagents

(1) *Alkaline salt solution.* To 50 ml of 80 per cent alcohol saturated with sodium chloride add 0.5 ml of 1 per cent aqueous sodium hydroxide. Filter and use within 15 minutes.

(2) *Stock stain solution.* 80 per cent alcohol saturated with Congo red and sodium chloride.

(3) *Staining solution.* Add 0.5 ml of 1 per cent aqueous sodium hydroxide to 50 ml of stock stain, filter and use within 15 minutes.

Method

(1) Bring sections to water.
(2) Stain in haematoxylin for 5 minutes.
(3) Rinse well in distilled water.
(4) Pretreat in alkaline alcohol-salt solution for 20 minutes.
(5) Stain in alkaline Congo red solution for 20 minutes.
(6) Dehydrate rapidly in three changes of absolute alcohol.
(7) Clear in xylol and mount in D.P.X. or H.S.R.

Results

Amyloid	Deep pink to red
Nuclei	Blue
Elastic tissue	Pale pink

Congo Red Staining Techniques

Bennhold's Technique

Method

(1) Bring sections to water.

(2) Stain with Ehrlich's haematoxylin for 20 minutes.
(3) Differentiate with 1 per cent acid alcohol.
(4) Wash in running water for 1 minute to remove the acid (sections will blue in the lithium carbonate used in Stage 6).
(5) Stain with 1 per cent aqueous Congo red for 20–30 minutes.
(6) Pour off stain and flood slide with a saturated aqueous solution of lithium carbonate; leave for 15 seconds.
(7) Differentiate in 80 per cent alcohol until excess Congo red is removed.
(8) Wash in running water for 10 minutes.
(9) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Amyloid	Pink to red
Nuclei	Blue

Metachromatic Staining Technique

Staining with 1 per cent aqueous methyl violet for 5 minutes, followed by differentiation with 1 per cent acetic acid, gives quite good results, but the following method gives preparations which remain stable for a number of years, and is therefore recommended.

Lendrum's Technique

Method

(1) Bring sections to water.
(2) Stain in 1 per cent aqueous methyl violet (or 1 per cent aqueous dahlia) for 3 minutes.
(3) Differentiate in 70 per cent formalin (controlling microscopically).
(4) Wash in running water for 1 minute.
(5) Flood with saturated aqueous sodium chloride for 5 minutes.
(6) Rinse in water, and mount in corn syrup.

Results

Amyloid	Pink to red
Other elements	Violet

Wolman's Toluidine Blue Technique (1971)

The stained section is examined with polarized light (crossed polaroid discs).

Method

(1) Bring sections to 95 per cent alcohol.
(2) Stain in 1 per cent toluidine blue in 50 per cent isopropanol for 30 minutes at 37°C.
(3) Blot carefully with filter paper.
(4) Immerse in absolute isopropanol for 1 minute, and blot again.
(5) Clear in xylene (two changes).
(6) Mount in Canada balsam or resinous mountant.

Result

Amyloid	Red (under polarizing microscope)
Collagen	Silvery blue

Silver Impregnation Method

King's Silver Technique

Solution Required

Stock silver carbonate solution. – To 5 ml of 10 per cent silver nitrate add concentrated ammonia (0.880) drop by drop until the precipitate that first forms is just dissolved. Add 6.8 ml of 3.5 per cent sodium carbonate (anhydrous) and dilute to 75 ml with distilled water.

Method

(1) Bring thin frozen sections to distilled water, and leave for 5 minutes.
(2) To 10 ml of the stock silver solution, in a small beaker, add a few drops of pyridine. Using a hockey stick, place the sections in the silver solution and warm gently to 45°C. Leave the sections until they become light brown.
(3) Transfer directly to 5 per cent sodium thiosulphate for 3 minutes.
(4) Rinse well in water.

(5) Counterstain with safranine if desired.

(6) Mount on slides, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Amyloid, nuclei and some lipids . . . . Brown to black

Chapter 15

Enzymes

The importance of enzymes has perhaps best been expressed by defining life as an orderly or disorderly function of enzymes, their state of function determining health or disease. Thus, enzymes control, regulate and maintain an orderly balance of physiological processes necessary for the preservation of life.

The number of enzymes recorded by Dixon and Webb in 1958 was over 800; of these only relatively few can be demonstrated histochemically. At this moment, there are techniques for the visualization of some 80 enzymes, and in view of the fact that only about a dozen reliable enzyme techniques were available in 1950, it will be seen that this is a rapidly expanding field.

An enzyme is a catalyst of vital origin, that is, a substance that initiates or accelerates a chemical reaction. Although the enzyme takes part in the reaction its role is that of a 'bystander' who initiates a quarrel (or reaction) but takes no direct part in it and is not degraded by it. Enzymes are generally proteins with a very specific action, and they usually derive their name from this action; for example, the enzyme that hydrolyses sucrose to monosaccharides is a sucrase. The ending 'ase' indicates an enzyme, but some enzymes were named before this system was adopted and these usually end in 'in', such as trypsin, pepsin, ptyalin, and so on. The substance (or kind of chemical bond) acted upon by an enzyme is known as its substrate and since enzymes are highly specific they tend to be limited to one type of substrate or

group of related substances. The demonstration of enzymes, therefore, is dependent upon the effect they have upon a given substrate, either natural or artificial. In this way their visualization differs from that of other, inactive, tissue components, since enzymes must be active to be demonstrated. One cannot see the enzyme itself, only the effect it has had upon a substrate. This effect, to be seen, must result in the formation of an insoluble substance at the site of activity by the action of the enzyme on the specific substrate. This insoluble substance must be subsequently rendered coloured or opaque, if not already visible.

Preservation

Because of the nature of enzymes, their preservation is much more difficult than that of other tissue constituents. For a good demonstration it is important to preserve the maximum amount of enzyme activity, together with accurate localization by the prevention of diffusion.

Enzymes are sometimes classified as *lyo-enzymes* (for example, glucuronidase) which are dissolved in the cytoplasm and thus are likely to diffuse; and *desmo-enzymes* (for example, leucine amino-peptidase) which are attached to cytoplasmic constituents (mitochondria) and are much less likely to diffuse.

It will be obvious that tissue must be as fresh as possible and, although there are exceptions, autopsy tissue is generally unsuitable for the demonstration of enzymes. Although there have been papers published which record only a slight loss of alkaline phosphatase activity 5 hours after death, this should not be relied upon for an estimate of total enzyme activity. Tissue which cannot be treated immediately should be kept in a refrigerator. For most enzymes fresh preparations (cell cultures on coverslips), or frozen, cryostat or freeze dried tissue sections are necessary. There are, however, some enzymes such as acid and alkaline phosphates, esterase, and so on, which will sufficiently resist fixation to allow their demonstration.

Seligman (1951), Nachlas and colleagues (1956), Holt (1959) and Pearse (1965) have shown that fixatives, if used, should be refrigerator cold (4°C) to preserve maximum enzyme activity. Following fixation they should be transferred to, and frozen for cutting in, 0.88 M sucrose (30 per cent) in 1 per cent gum acacia. We have found that Holt's ice-cold (0–4°C) gum sucrose treatment improves cryostat section cutting, tissue morphology and enzyme localization: tissues should be left for 24 hours but may be left for longer periods (*Table 15.1*)

TABLE 15.1

Effects of Fixation Upon Enzyme Activity

Enzyme	*Fixative*	*Time*	*Temperature °C*	*Enzyme activity (percentage)*	*Reference*
Alkaline phosphatase	Buffered formalin	60 min.	4	80	Nachlas (1956)
	10% Formalin (pH 7·0)	2 hrs.	4	73	Seligman (1951)
	Acetone	30 min.	4	95	Nachlas (1956)
	Ethanol	30 min.	4	62	Nachlas (1956)
Acid phosphatase	Buffered formalin	60 min.	4	67	Nachlas (1956)
	Formol calcium	24 hrs. *	2	60	Holt (1959)
	Acetone	60 min.	4	81	Nachlas (1956)
	Ethanol	60 min.	4	50	Nachlas (1956)
β–Glucuronidase	Buffered formalin	15 mins.	4	38	Nachlas (1956)
	Buffered formalin	60 mins.	4	16	Nachlas (1956)
	Acetone	15 min.	4	76	Nachlas (1956)
	Acetone	30 min.	4	73	Nachlas (1956)
	Ethanol	15 min.	4	33	Nachlas (1956)
Esterase	Formol calcium	24 hrs. *	2	50	Holt (1959)
	Acetone	30 min.	4	68	Nachlas (1956)
Leucine amino peptidase	Buffered formalin	60 min.	4	85	Nachlas (1956)
	Acetone	60 min.	4	84	Nachlas (1956)

*Post-fixation treatment in gum-sucrose at 2°C.

Methods of Demonstration

These may be discussed under the four following headings.

(1) Simultaneous capture.
(2) Post-coupling (post-incubating coupling).
(3) Self-coloured substrate.
(4) Intra-molecular rearrangement.

Two terms used in enzyme techniques are:

Primary reaction product (P.R.P.), the product of the reaction of an enzyme on a substrate;

Final reaction product (F.R.P.), the product of an insoluble uncoloured P.R.P. which has been rendered coloured or opaque.

(1) *Simultaneous capture*

In this case the P.R.P. is coloured or opaque. This is seen when a diazonium salt, present in the incubation medium (substrate), combines with the coupler which is released from the substrate by the enzyme. The diazo technique for alkaline phosphatase is an example of this type of azo-coupling technique.

(2) *Post-coupling reaction*

This type of reaction is based on the production of a colourless, insoluble P.R.P. which is then coupled with a coloured or opaque substance. The absence of diazonium salts in the substrate has two advantages: (1) they have been thought by some workers to interfere with, or inactivate enzymes (Pearse, 1968); and (2) long exposure of diazonium salts in an acid solution may result in non-specific staining (Nachlas and colleagues, 1957). The technique of Rutenberg and Siligman (1955) for acid phosphatase is an example of such a technique.

(3) *Self-coloured substrate*

This type of reaction employs a water-soluble dye. This dye is then made insoluble, due to the removal of a hydrophilic group, by the enzyme. This gives a coloured precipitate at the site of enzyme activity. The fluorescent technique of Burstone's for alkaline phosphatase (*see* page 619) is an example of such a technique.

(4) *Intramolecular rearrangement*

This type of reaction is based on the rearrangement of the molecular structure of a colourless substrate to give a coloured insoluble precipi-

tate at sites of enzyme activity. Such a technique has been described by Nachlas (1957) for carboxylic acid esterase but the F.R.P. is not sufficiently insoluble to give good localization.

It will be seen that the vast majority of methods in current use are either of the simultaneous capture or post-coupling type of reaction. The other types of reaction, while at present confined to only a few methods, may become of importance in the future.

The Use of Controls

Nowhere in the field of histochemistry are controls more important than in the demonstration of enzymes. Many substrates or their components break down on keeping which may lead to false negatives and on occasion to false positives. Therefore, control sections should always be carried through in parallel with the test sections. A positive control, if available, will demonstrate that the reagents are all working. A negative control is prepared by destroying the enzyme in the test section (or a positive control) by immersing in boiling water for 15 minutes, or by a specific chemical method for the enzyme to be demonstrated. A useful control is to incubate one of the test sections in the reaction mixture, without the substrate, for the same time that the test section proper is in the complete reaction mixture; both sections are then taken through the remainder of the technique together. A positive result in a given area in both sections indicates that this is not due to enzymic action. Appropriate control methods are given for each of the enzyme techniques.

ENZYME TECHNIQUES

ALKALINE PHOSPHATASE

The Gomori method for the identification of this enzyme is based on the action of the enzyme (at pH 9) on a substrate containing organic phosphate, in the presence of calcium ions, to form calcium phosphate *in situ*. The calcium phosphate so formed may then be directly demonstrated by von Kossa's silver technique (page 471), or by Gomori's method. Alternatively the azo-coupling (simultaneous capture) technique may be used.

Controls

Among the disadvantages of this method is the danger of false positives, due to pre-formed calcium or other black pigment present in the

section. For this reason two control sections should be carried through which are incubated in (*a*) distilled water, and (*b*) in substrate from which the glycerophosphate is omitted. Sites which are blackened in the uncontrolled section only and not in the two control sections are alkaline phosphatase.

Location of Enzyme

Alkaline phosphatase is normally found in the bladder, suprarenal, kidney (convoluted tubules and Bowman's capsule), endothelial cells of liver and spleen, and in breast and ovary.

Gomori's Technique

The technique depends on treatment, of the calcium phosphate formed, first with cobalt nitrate (when cobalt phosphate is formed) and then with ammonium sulphide to form cobalt sulphide, which is black.

Special Reagents Required

Substrate

2 per cent sodium glycerophosphate	25 ml
2 per cent sodium barbitone	25 ml
Distilled water	50 ml
2 per cent calcium chloride	5 ml
2 per cent magnesium sulphate	2 ml
Chloroform	a few drops

This solution does not keep well and is best made up fresh for each batch of sections.

Method

(1) Fix *fresh* tissue in 80 per cent alcohol for 24 hours, followed by normal paraffin wax processing, or better, fix in cold acetone at −20°C for 24 hours, followed by 2 changes of acetone (at room temperature) of 2 hours; clear in benzene, 2 changes of 45 minutes; embed in paraffin wax as quickly as possible. The best results are usually obtained by the latter method.

(2) Cut thin paraffin sections.

(3) Bring sections to water.

(4) Leave in substrate for 1–3 hours at 37°C.

(5) Rinse rapidly in distilled water.

(6) Treat with 2 per cent cobalt nitrate for 2 minutes.
(7) Wash out the excess cobalt nitrate for 1 minute (this stage is critical).
(8) Treat with 1 per cent yellow ammonium sulphide for 1 minute.
(9) Wash in water for 2–3 minutes.
(10) Counterstain in neutral red or safranine for 1 minute.
(11) Wash in tap-water.
(12) Dehydrate, clear, and mount in D.P.X. or Canada balsam.

Results

Structures possessing alkaline phosphatase activity, and pre-formed calcium	Brown to black
Other structures	Red

Azo-coupling Technique

The sections are incubated in a substrate containing a diazonium salt and sodium alpha-naphthol phosphate. The enzyme liberates alpha-naphthol which couples with the diazonium salt to form an insoluble coloured precipitate.

Controls. Treat control slides as above, omitting the alpha-naphthyl phosphate.

Special Reagents Required

Substrate

Sodium alpha-naphthol phosphate	10–20 mg
Michaelis's veronal – HCl buffer pH 9.2 (page 172)	20 ml
Fast blue RR or Fast Black B	20 mg

This substrate is prepared immediately before use.

Method

Frozen, cryostat or freeze-dried sections of fresh or cold formalin fixed tissue may be used. Paraffin sections of cold acetone fixed tissue also give good results.

(1) Bring paraffin sections to water.

(2) Filter enough substrate on to each slide to cover section adequately, and leave at room temperature for 30–60 minutes. Paraffin sections may require longer periods up to 4 hours, and should be put into a covered dish on wet blotting paper to control evaporation.

(3) Wash in running water for 2–3 minutes.

(4) Counterstain lightly with haematoxylin, and blue.

(5) Wash in running tap water for 15 minutes.

(6) Mount in glycerin jelly or Apathy's medium.

Results

Sites of enzyme activity	Black if Fast Blue RR or Fast Black B is used
Nuclei	Pale blue

5-NUCLEOTIDASE

Nucleotidase is an alkaline phosphatase having an optimum pH of about 7.8 in human tissue. Since by the following technique alkaline phosphatase is also demonstrated, an extra control section must be stained by the method for alkaline phosphatase at pH 7.5 (page 319) and an estimation of the 5-nucleotidase activity made by allowing for the amount of colour due to the alkaline phosphatase present.

Controls. Replacement of adenosine-5-phosphate with sodium-β-glycerophospahte in substrate, or 0.1 M sodium fluoride.

Pearse and Reis' Technique

Special Reagent Required

Substrate

Barbiturate buffer (pH 7.5)*	30 ml
12 per cent calcium nitrate ($Ca(NO_3)_2$)	6 ml
2 per cent magnesium chloride ($MgCl_3$)	6 ml
0.04 M adenylic acid (adenosine-5-phosphate)	6 ml

*This buffer is made as follows:

0.1 M sodium diethyl barbiturate	15 ml
N/10 hydrochloric acid	10 ml
Distilled water	5 ml

Method

(1) Cut thin sections of tissue fixed as for alkaline phosphatase (page 320).

(2) Incubate sections in the substrate at 37°C for 3–18 hours (*see* note on control, *see* above).

(3) Wash all the sections with 2 per cent calcium nitrate solution (pH 8) and then in distilled water.

(4) Expose to daylight for 1 hour in 1 per cent silver nitrate.

(5) Rinse in distilled water.

(6) Transfer to 5 per cent sodium thiosulphate for 10 minutes.

(7) Wash in running tap-water, dehydrate, clear, and mount in Canada balsam or D.P.X.

Result

5-nucleotidase activity . . . . . Black

Unless the reaction is strongly positive it tends to be diffuse.

Lead Method (Wachstein and Meisel, 1952; Chayen and colleagues, 1969)

This is the better of the two methods for the demonstration of 5-nucleotidase in all tissues.

Control. Same as for the calcium method above.

Special Reagent Required

Substrate

Adenosine-5′-monophosphate	31 mg
0.1 M acetate buffer pH 6.5	40 ml
0.1 M lead nitrate	1 ml
0.1 M magnesium sulphate	5 ml

This must be freshly prepared.

Method

(1) Incubate frozen or cryostat sections of fresh unfixed tissue in substrate at 37°C for ½–1 hour.

(2) Wash in running tap-water.

(3) Rinse in distilled water.

(4) Immerse in 0.5 per cent ammonium sulphide for 1 minute.

(5) Rinse in distilled water.
(6) Float sections on to slides and mount in aqueous mountant.

Result

Site of enzyme activity	Brown (precipitate of lead sulphide)

ADENOSINE TRIPHOSPHATASES

It now seems likely that there are three types of ATPase (one activated by calcium and two by magnesium at different pH optima). This has led to a great deal of confusion in the literature. In relation to the Wachstein and Meisel (1957) lead method, Moses and colleagues (1966) showed that 3.6 mM lead nitrate inhibits ATPase in both unfixed sections (80 per cent inhibition) and fixed sections (50 per cent of remaining activity), and they showed that fixation itself inhibited 88 per cent activity.

Rosenthal and colleagues (1966) showed that lead (in the concentration used in the method) hydrolysed ATP at pH 7.2 at 37°C. Moses and colleagues (1966) also showed that increasing or decreasing the concentrations of either the lead or ATP resulted in a complete alteration in the reaction product. This method has therefore been the subject of considerable discussion in the literature since 1966.

There is today considerable doubt as to whether ATPase is actually demonstrated. In fact, Tormey (1966) obtained results which seemed to indicate the impossibility of the histochemical localization of Na/K-ATPase. Nevertheless, positive results for ATPase are obtained, at predictable sites, with the techniques described below. This suggests that the activity being demonstrated, if it is not in fact ATPase, may be used as a marker for the enzyme.

Calcium Activated ATPase Technique (Niles and colleagues, 1964)

The method involved is essentially the same calcium trapping procedure discussed above for alkaline phosphatase but with adenosine triphosphate as the substrate.

Control. Substitute sodium-β-glycerophosphate for ATP in substrate, or use 2.5×10^{-3} M/p-chloromercuribenzoate as inactivator.

Special Reagent Required

Substrate

0.1M (2.06 per cent) Sodium barbiturate	10 ml
0.18M Calcium chloride solution	5 ml
Adenosine triphosphate	76 mg
2,4-dinitrophenol	30 mg
Distilled water	35 ml

This solution must be freshly prepared.

Method

(1) Cut frozen or cryostat sections of unfixed tissue and incubate in substrate at 37°C for 10–30 minutes.

(2) Wash sections in three changes of 1 per cent calcium chloride.

(3) Transfer to three changes of 1 per cent cobalt nitrate solution for 2 minutes each.

(4) Wash well in distilled water.

(5) Treat sections with 1 per cent ammonium sulphide for 1 minute.

(6) Wash sections in distilled water, float on to sides and mount in aqueous mountant.

Lead Method for Mg–Activated ATPase (Wachstein and Meisel, 1956; Chagen and colleagues, 1969)

The calcium-trapping method *(above)* cannot be used in this technique since the magnesium (used as an activator) would compete for the enzyme released phosphate. The resultant magnesium phosphate being relatively soluble would then be lost. For this reason, in spite of the fact that lead may inhibit ATPase *(above)* Wachstein and Meisel use the lead-trapping procedure.

Control. As for Ca-activated ATPase.

Special Reagent Required

Substrate

Adenosine triphosphate (ATP)	25 mg
Distilled water	22 ml
2 per cent Lead nitrate	3 ml
0.25 per cent Magnesium sulphate	5 ml

Dissolve the ATP in distilled water, and add the other reagents; 40 mg of 2, 4-dinitrophenol may be added to the substrate to activate mitochondrial ATPase (Lehninger, 1965).

Method

(1) Cut frozen or cryostat sections of fresh tissue (formalin-fixed tissue may be used but is not as good).
(2) Incubate sections (free floating or mounted) in substrate at 37°C for 2 hours.
(3) Wash in running water, then rinse in distilled water.
(4) Treat sections with 1 per cent ammonium sulphide for 1 minute.
(5) Wash in distilled water.
(6) Mount in an aqueous mountant.

Results

Sites of enzyme activity . . . . Brown-black precipitate

ACID PHOSPHATASE

The Gomori acid phosphatase method is based on the action (at pH 5.0) of the enzyme on a substrate containing organic phosphate in the presence of lead ions to form lead phosphate. This in turn is treated with ammonium sulphide to form lead sulphide *in situ.* The post-coupling technique will be found to give more reliable results, and is therefore recommended for fresh, fixed frozen or cryostat sections.

Control. Add 0.01 M sodium fluoride to control substrate.

Location of Enzyme

The enzyme is found in bone, the prostate and in certain tumours.

Gomori's Technique

Special Reagent Required

Substrate

M/1 acetate buffer, pH 5	3 parts
5 per cent lead nitrate	1 part
Distilled water	6 parts
2 per cent sodium glycerophosphate	3 parts

Shake well and stand for a few hours. Filter and dilute 1 : 3 with distilled water before use.

Method

(1) Fix *fresh* tissue in chilled acetone and leave in refrigerator for 24 hours. Dehydrate in 2 changes of acetone (at room temperature) each of 2 hours, clear in benzene (2 changes each of 45 minutes) embed in paraffin wax as quickly as possible.

(2) Cut thin sections float on warm water (not above 35°C if possible).

(3) Bring sections to water (including controls, page 319).

(4) Incubate in substrate at 37°C for 1–24 hours (a moderately good result is usually obtained in 1 hour).

(5) Rinse rapidly in distilled water.

(6) Treat with 2 per cent acetic acid for 1 minute.

(7) Rinse in distilled water.

(8) Treat with 1 per cent ammonium sulphide for 1 minute.

(9) Wash in water for 2–3 minutes.

(10) Counterstain with neutral red or safranine for 1 minute.

(11) Wash in tap-water.

(12) Dehydrate, clear, and mount in D.P.X. or Canada balsam.

Results

Structures possessing acid phosphatase activity	Brown to black
Other structures	Red

Non-specific lead impregnation occasionally occurs. Such areas would be black in all 3 sections (*see* Control Sections, page 319). Structures which are black *only* in the test section show acid phosphatase activity.

Post-Coupling Techniques for Acid Phosphatase (Rutenberg and Seligman, 1955)

Special Reagents Required

Substrate

Sodium 6-benzoyl-2-naphthyl phosphate	25 mg
Distilled water	80 ml
Walpole's acetate buffer pH 5.0	20 ml
Sodium chloride	2 g

Diazonium Salt Solution

Fast blue B or Garnet GBC	50 mg
Distilled water	50 ml

This solution is made alkaline with sodium bicarbonate.

Method

Fresh or cold formalin fixed frozen or cryostat cut sections are used. Fresh tissues are placed sequentially in 0.85 per cent, 1 per cent and 2 per cent sodium chloride solutions for 2–3 minutes in each.

(1) Place sections into substrate at room temperature for 10–60 minutes (fresh tissue) and 1–2 hours (fixed tissue).

(2) Wash fresh sections in three changes of cold saline, and fixed tissues in three changes of water.

(3) Place in freshly prepared cold diazonium salt solution and agitate for 3–5 minutes.

(4) Wash in cold saline or water (fixed tissue), three changes of five minutes each.

(5) Mount in glycerin jelly.

Result

Sites of enzyme activity	Blue to red

GLUCOSE-6-PHOSPHATASE

This enzyme, which is found chiefly in the liver, is absent in Von Gierke's disease.

The method is based on the action of the enzyme on a substrate containing glucose-6-phosphate and lead nitrate to form lead phosphate, which is then converted to lead sulphide. Since this enzyme is easily destroyed, particularly by formalin, the reactions should be carried out on unfixed tissue.

Control. Substitute sodium β-glycerophosphate (25 mg/20 ml distilled water) for glucose-6-phosphate in substrate.

Wachstein and Meisel's Method (1956)

Special Reagent Required

Substrate

Potassium glucose-6-phosphate (0.125 per cent solution)	20 ml
0.2 M 'Tris' buffer pH 6.7 (page 174)	20 ml
2 per cent lead nitrate	3 ml
Distilled water	7 ml

Method

(1) Incubate cryostat or frozen sections of *fresh tissue* in the substrate at 32°C for 5–15 minutes.
(2) Wash sections in distilled water.
(3) Treat sections with dilute ammonium sulphide for one minute.
(4) Wash in water.
(5) Post fix in 10 per cent formalin for 2 minutes.
(6) Mount in glycerin jelly.

Result

Sites of enzyme activity	Brownish-black deposit

PHOSPHAMIDASE (PHOSPHOAMIDASE)

This enzyme acts upon phosphoamide bonds. The substrate used is p-chloroanilidophosphonic acid, the released phosphate is trapped by lead, which is visualized as lead sulphide.

Control. Two controls should be used; (1) omission of solution B from substrate to show reaction due to lead adsorption; and (2) the addition of 21 mg of sodium fluoride to 50 ml of substrate. This does not inhibit true phosphamidase activity.

Lead Nitrate Method (Gomori, 1948; Chayen and colleagues, 1969)

Special Reagent Required

Substrate

Solution A

0.05 M Acetate buffer pH 5.4	50 ml
3.3 per cent (0.1 M) Lead Nitrate	1.9 ml
Sodium chloride	0.11 g
Polyvinyl alcohol (M05/140)	5 g

Add lead nitrate and sodium chloride to the buffer. Then dissolve the PVA by heating and stirring continuously; cool to 37°C.

Solution B

p-Chloroanilidophosphonic acid	.0.15 g
1 M Sodium hydroxide	0.5 ml

This must be prepared immediately before use. Add to solution A.

Method

(1) Place mounted cryostat sections of unfixed tissue in substrate at 37°C for up to 20 minutes.
(2) Wash well in running water and rinse in distilled water.
(3) Treat with 1 per cent ammonium sulphide for 1 minute.
(4) Wash in distilled water and mount in an aqueous mountant.

Result

Sites of enzyme activity . . . . Brown-black precipitate

ESTERASE

The esterases are found in the liver, pancreas, stomach, kidney, pituitary, and nerve cells. They may be demonstrated by the Gomori azo coupling technique, or by the bromoindoxyl acetate method of Pierson and Defendi. These enzymes are partially resistant to cold acetone fixation and paraffin embedding, but for maximum enzyme activity cryostat sections of unfixed tissue should be used.

Burstone's Azo-Coupling Technique

This method depends upon the liberation of naphthol from the naphthol acetate. The naphthol then couples with fast blue RR to form an insoluble azo-dye.

Control. Using 10^{-6} M disopropyl fluorophosphate (DFP) as an inhibitor, esterases have been classified as (A) resistant and (B) sensitive, Gomori (1955) found A-esterases in stomach (chief cells), kidney and other organs, whereas in the liver and pancreas he found sensitive B-esterases. Eserine ($1 - 5 \times 10^{-5}$ M) inhibits the cholinesterases but not lipase, A- or B-esterases.

Special Reagent Required

Substrate

Naphthol AS–LC acetate	3 mg
Acetone	0.3 ml
Distilled water	15 ml
0.1 M Phosphate buffer pH 7.2	15 ml
Fast blue RR (or fast garnet G.B.C.)	15 mg

Dissolve the naphthol acetate in the acetone, then add in the order given. When the fast blue RR is added, stir to dissolve, *filter and use immediately.*

Method

(1) Cut frozen or cryostat sections of unfixed tissue (acetone-fixed, paraffin sections may be used).

(2) Place free-floating or mounted sections in substrate at room temperature for 10–30 minutes, until sufficient red or blue colour develops. Leave for 2 hours to report a negative reaction (Lillie, 1965).

(3) Wash well in water and mount in aqueous mountant.

Result

Sites of enzyme activity	Red (or blue)

Holt's Indoxyl Acetate Technique for Esterases

This method will demonstrate non-specific esterase, lipase and cholinesterase (Lillie, 1965) and may be controlled by use of inhibitors *(see above).* It is based upon the liberation of the indoxyl group which is then oxidized by the ferri-ferro-cyanide oxidant to indigo.

Control. See above.

Special Reagents Required

Oxidant

Potassium ferricyanide	210 mg
Potassium ferrocyanide	155 mg
Distilled water	to 100 ml

Substrate

Tris buffer (0.2 M) at pH 8.3	2 ml
Oxidant	1 ml
1 M calcium chloride	0.1 ml
2 M sodium chloride	5 ml
Distilled water	2 ml

Dissolve 1.3 mg of 5-bromoindoxyl acetate (or 1.5 mg of 5-bromo-4-indoxyl acetate) in 1 ml of absolute ethanol in a small beaker, then add the above solution (separately mixed) agitating to mix.

Method

(1) Place frozen or cryostat sections of unfixed tissue in substrate at 37°C, leave for 30–120 minutes (usually 30 minutes).
(2) Wash in distilled water.
(3) Float sections on to slides and mount in aqueous mountant.

Result

Enzyme activity	Blue

LIPASE

The Tween compounds are esters of long-chain fatty acids and either Sorbitan or Mannitan. The Tween numbers indicate the type of fatty acid – 20 (lauric), 40 (palmitic), 60 (stearic) and 80 (oleic). An appropriate Tween compound is used as the substrate, usually 60 or 80.

The method is based upon the fact that the enzyme splits the fatty acid off which is trapped *in situ* as an insoluble calcium soap. These are converted into lead soaps, which are then blackened by ammonium sulphide.

Control. Leave Tween compound out of the substrate.

Gomori's Technique

Special Reagent Required

Substrate

2 per cent Tween 60 or 80 in water	5 ml
0.2 M Tris buffer pH 7.2	20 ml
4 per cent Calcium chloride (anhyd.)	5 ml
Distilled water	20 ml

Method

(1) Cryostat sections on slides, of unfixed tissue (taken directly to step 4) will show the maximum activity Gomori's original technique may be used as follows. Fix *fresh* tissue in chilled acetone and leave in refrigerator for 24 hours. Dehydrate in 2 changes of acetone (at room temperature) each of 2 hours, clear in benzene (2 changes each of 45 minutes); embed in paraffin wax as quickly as possible.

(2) Cut thin sections; float on warm water (not above 35°C if possible).

(3) Bring sections to water.

(4) Incubate in substrate at 37°C for 6–24 hours.

(5) Rinse in distilled water.

(6) Treat with 2 per cent lead nitrate for 10–15 minutes.

(7) Treat with 1 per cent ammonium sulphide for 1 minute.

(8) Stain nuclei lightly with haematoxylin.

(9) Mount in glycerin jelly, or dehydrate, clear in dichloroethylene, and mount in Gurr's Xam. Xylol causes fading of the brown colour.

Results

Sites of lipase activity	Brown
Nuclei	Blue

CHOLINESTERASES

These may be classified as acetyl-cholinesterase (true cholinesterase) and cholinesterase (pseudo-). Although they may be demonstrated by the methods above, they are more specifically visualized by the following method.

Acetylthiocholine Method (After Karnovsky and Roots, 1964)

The basis for the method is that the thiocholine ester is hydrolysed by the enzyme, and the liberated thiocholine is believed to reduce ferricyanide to ferrocyanide which combines with the copper ions to form the insoluble copper ferrocyanide (Hatchett's Brown).

Controls. (*a*) ISO-OMPA (tetraisopropylpyrophosphoramide; Burstone, 1962) in a concentration of 3×10^{-6} M should inhibit (pseudo-) cholinesterase. It is added to a portion of the substrate lacking acetylthiocholine, sections are incubated for 30 minutes at 37°C, then the acetylthiocholine is added and the sections incubated for a further 30 minutes. A positive reaction after this treatment should be considered due to acetylcholinesterase.

(*b*) 3×10^{-5} M Eserine sulphate should inhibit both these enzymes, without affecting the A and B type esterases (*see* above). The eserine is added to the normal substrate mixture. Dr. H. McLennan in the Physiology Department, UBC, uses 30 minute pre-incubation in 10^{-6} M Disopropylfluorophosphate (DFP) as an inhibitor of (pseudo-) cholinesterase in brain tissue.

Special Reagents Required

Substrate

Acetylthiocholine iodide	5 mg
0.1 M Acetate buffer pH 5.5	6.5 ml

Dissolve the acetylthiocholine in the buffer then add the following in order, with stirring between each addition.

0.1 M Sodium citrate	0.5 ml
30 mM Copper sulphate	1 ml
Distilled water	1 ml
5 mM Potassium ferricyanide	1 ml

If eserine sulphate is used as an inhibitor it is added instead of the distilled water.

The stock solutions keep in the refrigerator for several weeks. The prepared substrate, which is clear and greenish in colour, is only stable for a few hours.

Method

(1) Tissue is fixed overnight in cold formol calcium, and then transferred to sucrose-gum solution (*see* page 316). The best results are obtained if tissues are left in the latter for 7–30 days, but reasonable results are possible after 24 hours. Blocks are washed in distilled water and frozen or cryostat sections are cut. The sections may be free-floating or affixed to slides or coverslips.

(2) Incubate sections in substrate at room temperature for 2–6 hours (brain sections take about 3 hours).

(3) Wash in water, float on to slides (if necessary) and mount in an aqueous mountant.

Result

Enzyme activity . . . . . Fine brown precipitate

LEUCINE AMINOPEPTIDASE

This enzyme is found in highest concentration in kidneys or small intestine. It is also present in muscle, skin, C.N.S., spleen and lymph nodes.

Simultaneous Coupling Method (Burstone and Folk, 1956)

The aminopeptidase is demonstrated by its action on l-leucyl-β-naphthylamide, it releases β-naphthylamide which couples with the garnet G.B.C. to produce an insoluble red precipitate at the sites of reaction.

Control. Omit l-leucyl-β-naphthylamide from substrate.

Special Reagents Required

Substrate

1 per cent l-leucyl-β-naphthylamide	1 ml
Distilled water	40 ml
pH 7.1 'Tris' buffer (page 173)	10 ml
Garnet G.B.C. (fast garnet G.B.C.)	30 mg

This solution should be freshly prepared and filtered before use.

Method

(1) Bring frozen, cryostat or freeze dried sections of *fresh* tissue to distilled water.

(2) Place sections into substrate at room temperature for 15 minutes to 3 hours.

(3) Wash well in water.

(4) Mount in glycerin jelly or Apathy's medium.

Result

Sites of enzyme activity	Red

Chelation Method (Nachlas, Crawford and Seligman, 1957)

In this method copper is chelated to the azo dye formed in the simultaneous coupling method. This allows sections to be dehydrated, cleared and mounted in a synthetic resin (D.P.X. or H.S.R.) for better definition and more permanent preparation.

Special Reagents Required

Stock substrate solution

(A) 0.8 per cent L-leucyl-β-naphthylamide

(B) 0.8 per cent L-leucyl-4-methoxy-β-naphthylamide

Either of these solution may be used. They can be prepared and stored in the refrigerator for 2-3 months.

Substrate

Stock solution (A) or (B)	1 ml
0.1 M acetate buffer pH 6.5	10 ml

0.85 per cent sodium chloride	8 ml
Potassium cyanide (2×10^{-2} M)	1 ml
Fast Blue B salt	10 mg

Method

(1) Bring frozen, cryostat or freeze dried sections of *fresh* tissue to distilled water.

(2) Incubate sections in substrate for 15 minutes to 4 hours at 37°C (until sections are red).

(3) Rinse in 0.85 per cent saline.

(4) Place in 0.1 M cupric sulphate.

(5) Rinse in 0.85 per cent saline.

(6) Mount in glycerin jelly, or dehydrate, clear, and mount in D.P.X. or H.S.R.

Results

Sites of enzyme activity	Red with (A) Purple with (B)

β-GLUCURONIDASE

This enzyme occurs in most tissues, with higher concentrations being reported in liver, kidney, spleen, lung, adrenal, thyroid and uterus. Within cells its location seems to be mainly in the mitochondrial or lysosomal fractions. The histochemical demonstration of this enzyme is used by some pathologists (Zugibe, 1970)to distinguish carcinomas (by its presence) from sarcomas in which it is minimal or absent.

Post-Coupling Technique (Chayen and colleagues, 1969)

The simultaneous coupling technique is not recommended because of the possible inhibitor effect of the diazonium compounds, and because with post-coupling the substrate can be used at the optimal pH of the enzyme (4.5) at which the coupler is not efficient.

The method is based upon the liberation of the naphthol AS-B1 which is deposited at the site of enzyme activity, and subsequently coupled with the fast dark blue R to visualize it.

Controls. Potassium hydrogen saccharate *(B.D.H.)*, 0.05 g, is added to 22 ml of substrate. This gives complete inhibition of β-glucuronidase activity.

Special Reagents Required

Stock Substrate Solution

Naphthol AS–B1 glucuronide	11.4 mg
0.05 M Sodium bicarbonate (0.42 per cent)	1 ml
0.2 M Acetate buffer (pH 4.5)	49 ml

Dissolve the naphthol AS–B1 glucuronide in the bicarbonate and add the buffer. This will keep indefinitely in the refrigerator.

P.V.A. Solution

Calcium chloride ($CaCl_2 \cdot 6H_2O$)	0.5 g
0.1 M Acetate buffer (pH 4.5)	100 ml
Polyvinyl alcohol (MO5/140)	10 g

Dissolve the calcium chloride in the buffer. Add the P.V.A. and heat (but not boil) with frequent mixing to dissolve. Cool before use.

Substrate

P.V.A. solution	20 ml
Stock substrate	2 ml

Coupling Solution

Fast dark blue R	8 mg
Ice-cold, 0.01 M phosphate buffer (pH 7.4)	20 ml

Dissolve fast blue in buffer at 4°C, filter while still cold (4°C) and use immediately.

Activator Medium

Calcium chloride, 0.5 per cent, in 0.1 M acetate buffer (pH 4.5) Sections may be immersed in this medium for 2 minutes prior to transfer to substrate.

Method

(1) Cut cryostat or frozen sections of unfixed tissue and attach to slides or coverslips.

(2) Immerse in substrate at 37°C for 30–180 minutes. This time may be reduced by pre-treatment with activator.

(3) Wash in activator medium.

(4) Immerse sections in ice-cold coupling solution for 5 minutes.

(5) Rinse in distilled water.

(6) Mount in glycerin jelly.

Results

Enzyme activity . . . . . Dark blue granules

PHOSPHORYLASES

Phosphorylase occurs in tissue in two forms, an enzymatically active form, *phosphorylase a* (α-glucan phosphorylase), and an enzymatically inactive form *phosphorylase b.* The 'inactive' (b) enzyme can be converted to the 'active' (a) by the enzymatic addition of two phosphate groups to each molecule of (b). This conversion is utilized in the technique below to give *total potential phosphorylase activity,* in addition to the *manifest existing activity.* Phosphorylases appear to play a major role in controlling glycogen metabolism in animal cells; the highest concentrations being found in the liver, and in voluntary and heart muscle. Godlewski (1962, 1963) has shown its decreased activity in tumours.

Takeuchi's Method (Chen and colleagues, 1969)

The substrate contains glycogen, and glucose-1-phosphate, the phosphorylase (if present) will increase the size of the glycogen by accretion of glucose units from the glucose-1-phosphate. AMP is added as an activator. The extended molecules of glycogen, which should become attached to the section, are demonstrated by their reaction with the iodine. The iodine is thought to stain linear amylase blue, and branched polysaccharide red-brown; the latter is interpreted by some workers as demonstrating the presence of the *branching enzyme.*

Control. Leave glucose-1-phosphate out of substrate. Pre-treatment of sections with diastase ensures that glycogen demonstrated has been formed during the reaction.

Special Reagents Required

Substrate for manifest activity

Glucose-1-phosphate	50 mg
Sodium fluoride	15 mg
AMP (adenosine-5-phosphate)	10 mg
EDTA	10 mg
Glycogen	5 mg
Insulin	0.5 mg
Distilled water	15 ml
0.5 M acetate buffer (pH 5.8)	10 ml

Substrate for Total Potential Activity

As above, *plus* 10 mg of ATP and 10 mg of magnesium sulphate but *minus* the EDTA.

Glycerin–iodine solution

Equal parts of glycerin and Lugol's iodine.

Method

(1) Cut cryostat sections of unfixed tissue. Leave section at room temperature for 10 minutes to enhance binding of glycogen (Chayen and colleagues, 1969).

(2) Incubate in substrate for 20 minutes at 37°C.

(3) Wash briefly in distilled water.

(4) Immerse in Gram's iodine, diluted 1:2 with distilled water for 30 seconds.

(5) Wash in water.

(6) Mount in glycerin-iodine solution.

Results

Blue or red-brown deposits of synthesized polysaccharide indicate sites of enzyme activity.

HAEMOGLOBIN PEROXIDASE

Haemoglobin peroxidase is a relatively stable enzyme, resistant to short fixation in formalin. The peroxidase of the granular leucocytes and their precursors is almost as stable but not so resistant to the action of formalin.

Lison-Dunn Technique

Special Reagent Required

Leuco patent blue V. – To 100 ml of a 1 per cent aqueous solution of patent blue V, add 10 g of powdered zinc and 2 ml of glacial acetic acid. Boil this mixture until it is a pale straw colour (approximately 10 minutes). Cool, filter and store in a stoppered bottle. The stock solution is stable.

Just before use, add 2 ml of glacial acetic acid and 1 ml of 3 per cent (10 vol.) hydrogen peroxide to 10 ml of the stock leuco patent blue.

Method

(1) Fix tissues in buffered formalin (page 45) for not more than 48 hours.
(2) Embed in paraffin wax and cut sections 5–7 μ thick.
(3) Bring sections to water.
(4) Stain for 5 minutes in leuco patent blue reagent.
(5) Rinse in water.
(6) Counterstain nuclei in safranine for 1 minute.
(7) Rinse in water.
(8) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Haemoglobin	Dark blue
Oxidase granules	Dark blue
Nuclei	Red

DOPA-OXIDASE (TYROSINASE)

Dopa-oxidase is thought to be responsible for the conversion of tyrosine into melanin. The initials DOPA stand for dihydroxyphenylalanine,

and this was originally thought to be the precursor of melanin, and it is for this reason the enzyme concerned in this conversion was called Dopa-oxidase.

Controls. Pre-incubate sections in 10^{-3} potassium cyanide (3.25 mg /50 ml), also add 10^{-3} of potassium cyanide to control substrate (step 3).

Dopa-oxidase Reaction (Becker, Praver and Thatcher, 1935)

Method

(1) Fix pieces of tissue 5 mm thick in 10 per cent formalin for 1 hour at room temperature.
(2) Wash in running water for 3–4 minutes.
(3) Place in 0.1 per cent DOPA in 0.1 M phosphate buffer (pH 7.4) at 37°C and leave for 1 hour.
(4) Change into fresh DOPA reagent, and leave for 12 hours at 37°C.
(5) Wash in running water.
(6) Fix in Bouin's fluid (*see* page 49) for 24 hours.
(7) Dehydrate, clear in benzene, and embed in paraffin wax.
(8) Cut thin sections, attach to slides.
(9) Bring sections to water, counterstain with haematoxylin and eosin (or tartrazine in Cellosolve).
(10) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Dopa-oxidase (tyrosinase) . .	Dark brown granules
Other structures	Depending on the counterstain

CYTOCHROME OXIDASE

The demonstration of this enzyme is based on the fact that cytochrome oxidase will act as a catalyst for the oxidation reaction between α-naphthol and a dimethyl-p-phenylaminediamine hydrochloride to form indophenol blue ('Nadi' reaction).

Controls. Add 10^{-3} M of potassium cyanide (3.25 mg/50 ml) to control Nadi reagent.

Gräff's G-Nadi Reaction

Special Reagents Required

(1) *α-Naphthol solution.* – α-Naphthol, 0.1 g, is dissolved in 1 ml of alcohol, and then made up to 100 ml with distilled water.

(2) *Oxidase reagent.* – Dissolve dimethyl-p-phenylaminediamine hydrochloride, 0.12 g, in 100 ml of distilled water. This solution should be colourless, or just tinted, otherwise it should be discarded. If this reaction is performed infrequently it is better to prepare small amounts fresh for each batch of sections. It should be stored in a dark brown bottle.

(3) *Nadi reagent.* – Mix 20 ml each of α-naphthol solution and oxidase reagent, and to the mixture add 8 ml of 0.1 M phosphate buffer pH 7.5.

Method

(1) Cut frozen sections of fresh, unfixed tissue.
(2) Incubate in Nadi reagent at 37°C for 1 hour.
(3) Transfer sections to normal saline solution.
(4) Counterstain nuclei (optional).
(5) Float on to slides, drain, and mount in 20 per cent potassium acetate solution.
(6) Ring coverslip with paraffin wax, and examine. These preparations are not permanent.

Results

Cytochrome oxidase . . . . . Blue-violet

DIAPHORASES AND DEHYDROGENASES

These enzymes are among the most important in cellular metabolism. They are concerned with the transfer of hydrogen from a substrate to a hydrogen acceptor and as such are oxidative enzymes. To reduce diffusion to a minimum when demonstrating DPN and TPN dehydrogenase, Braunstein and colleagues (1962) added 3.5 per cent PVP to the substrate. In the newer nomenclature the co-enzyme DPN is known as NAD (nicotinamide-adenine-dinucleotide, and TPN as NADP (nicotinamide-adenine-dinucleotide phosphate).

Methods are described using the monotetrazolium salt, 3- (4,5 -dimethylthiazolyl-2) 5-diphenyl tetrazolium bromide (MTT), which at

the time of writing is difficult to obtain. For this reason methods are also described using (2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3′-(3,3′-dimethoxy-4,4′-biphenylene) ditetrazolium chloride (Nitro BT).

MTT Method for DPN and TPN Diaphorase (Hess, Scarpelli and Pearse, 1958)

In addition to its use for enzyme localization, this method is recommended by Hess and colleagues for the demonstration of mitochondria.

Special Reagents Required

Stock solution

MTT 1 mg/ml	2.5 ml
Cobaltous chloride 0.5 M	0.9 ml
0.2 M Tris buffer pH 8.0 (page 174)	2.5 ml
Distilled water	4.1 ml
Polyvinylpyrrolidine	0.77 g

The cobaltous chloride and buffer are first mixed and the resultant precipitate is removed by filtration. The remaining constituents are then added. The solution is now adjusted to pH 7.2 with pH 10.4 Tris buffer, using a glass electrode. This solution keeps for 3–4 weeks in the refrigerator.

Substrate

Stock solution	1 ml
DPNH or TPNH	6 mg

Adjust pH to 7.0 with one or two drops of Tris (pH 8.5) buffer. Increased activity can be demonstrated in most tissues by the addition of sufficient amytal to give a final concentration 1×10^{-2}M (22.6 mg per 10 ml substrate). If amytal is added to the substrate the pH should again be checked and adjusted if necessary.

Method

(1) Cut frozen or cryostat sections of *fresh* tissue and mount on slides or coverslips.

(2) Place 0.1 to 0.2 ml of substrate on each section, and incubate uncovered at 37°C for 5–30 minutes.

(3) Fix in formol calcium or formol saline for 10 minutes.

(4) Rinse in distilled water.
(5) Mount in glycerin jelly.

Nuclei may be counterstained with dilute carmalum if desired.

Results

Sites of enzyme activity (DPN (NAD) or TPN (NADP) diaphorase)	Black

Since the black cobalt formazan deposits are confined to mitochondria, this may be used as a method of demonstrating them.

Nitro BT Method for DPN (NAD) Diaphorase (Nachlas, Walker and Seligman, 1958)

Special Reagents Required

Substrate

*0.5 M sodium lactate	0.6 ml
†1.5 per cent aqueous lactic dehydrogenase	0.2 ml
†DPN (NAD) (5 mg/ml)	0.3 ml
†Nitro BT (5 mg/ml)	0.3 ml
0.2 M phosphate buffer pH 7.4	1.0 ml
Distilled water	0.6 ml

Method

(1) Frozen or cryostat sections of *fresh* tissue are mounted on slides or cover slips.
(2) Place 0.1 to 0.2 ml of substrate on each section, and incubate uncovered at room temperature for 5–30 minutes.
(3) Rinse in saline.
(4) Fix in formol saline for 10 minutes.
(5) Wash in 15 per cent alcohol for 5–30 minutes.
(6) Rinse in distilled water.
(7) Mount in glycerin jelly or Apathy's media.

Result

Sites of enzyme activity	Blue deposit

*Must be stored in a deep freeze.
†Adjust to pH 7.4 and store in refrigerator.

Nitro BT Method for TPN (NADP) Diaphorase (Nachlas, Walker and Seligman, 1958)

Special Reagents Required

Substrate

Sodium DL-isocitrate (1.1 M)	0.6 ml
Sodium L-malate (2.5 M)	0.5 ml
Manganese chloride (0.005 M)	0.3 ml
TPN (NADP) (5 mg/ml)	0.2 ml
Nitro BT (5 mg/ml)	0.3 ml
Veronal acetate buffer pH 7.4 (0.05 M)	1.1 ml

The TPN (NADP) solution is kept in the deep freeze, the remaining stock solutions are adjusted to pH 7.4 and stored in the refrigerator.

The method and results are as given in Nitro BT method for DPN (NAD) diaphorase (page 345).

Nitro BT Method for Succinic Dehydrogenase (Nachlas, Crawford and Seligman, 1957)

Special Reagents Required

Substrate

Phosphate buffer pH 7.6 (0.2 M)	5 ml
Sodium succinate (0.2 M)	5 ml
Nitro BT (1 mg/ml)	10 ml

Method

(1) Mount frozen or cryostat sections of *fresh* tissue on slides or coverslips.
(2) Incubate in substrate at 37°C for 5–20 minutes.
(3) Wash in saline.
(4) Fix in 10 per cent formol saline for 10 minutes.
(5) Wash in 15 per cent alcohol for 5–10 minutes.
(6) Rinse in distilled water.
(7) Mount in glycerin jelly or Apathy's media.

Result

Sites of enzyme activity	Blue deposit

Methods for Demonstrating DPN (NAD) and TPN (NADP)-linked Dehydrogenases (Hess, Scarpelli and Pearse, 1958)

These methods all employ a standard medium in which the substrate respiratory inhibitor, buffer and DPN (NAD) or TPN (NADP) are varied. The standard medium is given below and the variations are given with each method.

Standard Medium

Substrate	0.1 ml
DPN (NAD) or TPN (NADP)	0.1 ml
Respiratory inhibitor	0.1 ml
Buffer pH 7.4	0.25 ml
Magnesium chloride (0.05 M)	0.1 ml
Tetrazolium salt (MTT or Nitro BT) (1 mg/ml)	0.25 ml
Distilled water	to make 1 ml
Polyvinylpyrrolidone (M.W. 11,000)	75 mg

Note. – With the exception of DPN (NAD) and TPN (NADP) which are stored at – 20°C, all the constituents can be buffered to pH 7.0 and stored in a refrigerator. Sodium cyanide is adjusted to pH 7.2 with normal hydrochloric acid.

(1) *Glucose-6-phosphate dehydrogenase*

Substrate – 1.0 M glycopyranose-6-phosphate disodium or calcium salt.
TPN (NADP) (1.0 or 1 M)

Respiratory inhibitor	0.1 M sodium azide or amytal
Buffer	0.2 M Tris buffer
Tetrazolium salt	MTT (1 mg/ml)
Cobaltous chloride 0.5 M	0.05 ml
Sodium fluoride 0.01 M	0.05 ml

(2) *Isocitrate-dehydrogenase*

Substrate	1.0 M DL-isocitric acid (adjusted to pH 7.0 with Tris buffer)
Co-enzyme	DPN (NAD) or TPN (NADP)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.06 M phosphate buffer
Tetrazolium salt	Nitro BT

(3) *Malate dehydrogenase*

Substrate	1.0 M Sodium-L-malate or L-malic acid (adjusted to pH 7.0 with Tris buffer)
Co-enzyme	DPN (NADP) or TPN (NADP)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.06 M phosphate buffer
Tetrazolium salt	Nitro BT

(4) *Glutamate dehydrogenase*

Substrate	1.0 sodium-L-glutamate monohydrate
Co-enzyme	DPN (NAD) or TPN (NADP)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.06 M phosphate buffer
Tetrazolium salt	Nitro BT

(5) *α-Glycerophosphate dehydrogenase*

Substrate	1.0 sodium-L-glutamate (neutralized with 0.1 M hydrochloric acid)
Co-enzyme	DPN (NAD)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.2 M Tris buffer
Tetrazolium salt	Nitro BT

(6) *Alcohol dehydrogenase*

Substrate	Ethyl alcohol
Co-enzyme	DPN (NAD)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.06 M phosphate buffer
Tetrazolium salt	Nitro BT

(7) *Lactate dehydrogenase*

Substrate	Sodium DL-lactate
Co-enzyme	DPN (NAD)
Respiratory inhibitor	0.1 M sodium cyanide
Buffer	0.06 M phosphate buffer
Tetrazolium salt	Nitro BT

Method

The methods for all the enzyme techniques listed above are identical. The incubating medium is made from the standard

medium with the appropriate substrate, co-enzyme, respiratory inhibitor and buffer included.

(1) Frozen or cryostat cut sections of *fresh* tissue are mounted on slides or coverslips.

(2) Cover sections with incubating medium, leave uncovered at 37°C for 5–30 minutes.

(3) Rinse sections quickly in saline.

(4) Fix in 10 per cent formol saline for 10 minutes.

(5) Wash sections in 10 per cent alcohol for 10 minutes for all methods using Nitro BT. This removes or reduces a red reaction product which may be present.

(6) Mount in glycerin jelly.

Results

Sites of enzyme activity . . . Purple diformazan deposit.

Nuclei may be counterstained if desired.

Chapter 16

Lipids (Fats, Lipoids, Lipins)

As early as 1823 it was established that common animal and vegetable fats were a combination of glycerol with fatty acids, but little further research occurred during the next 100 years.

The rather loose and indefinite terminology that has been applied to fats and fat-like substances is a reflection of the lack of systematic nomenclature in the early days. The use of the words lipoid, lipin, lipide and lipid has varied from author to author, the same name occasionally being used to mean all fats and fat-like substances, or fat-like substances alone (waxes, and so on). In recent years attempts have been made to classify lipids and standardize terms, Bloor (1925–6), Lison (1936) and Cain (1950) having made the most notable contributions. The terminology used in this work is adopted from Deuel (1951) which is based on that of Bloor *(above)*.

DEFINITIONS

The term 'lipid' will be used to describe all naturally occurring fats and fat-like substances.

Lipids are usually defined as those naturally occurring substances which are insoluble in water but soluble in the so-called 'fat solvents' (chloroform, benzene, petroleum, ether, acetone, and so on), which are related either actually or potentially to fatty acid esters, and which are capable of being utilized by the animal organism. For practical purposes this definition may be adopted but it cannot be accepted too rigidly since a typical lipid 'lecithin' is slightly soluble in water and insoluble in acetone; another lipid 'lysolecithin' is freely soluble in warm water and insoluble in ether, and sphingomyelin and the cerebrosides when purified are insoluble in a range of fat solvents *(see Table 16.1)*.

Method		Page	Hydrocarbons	Derived lipids		
				Higher alcohols	Fatty acids	
			Carotenoids (chromolipids)	Cholesterol	Unsaturated fatty acids	Saturated fatty acids
Keilig's extraction	Cold acetone . . .	357		+		
	Hot acetone . . .	357				
	Hot ether	357				
	Hot chloroform and methanol	357	+	+	+	+
Fat-soluble stains (Sudan, oil red O)		359	+ †	+ †	+	+
Fluorescent with phosphine red		613				
Sudan black (McManus)		370				
Lillie's Nile-blue sulphate		363			+	+
Controlled chromation procedure		367				
Performic acid–Schiff .		374	+			
OTAN reaction . . .		368				
NaOH–OTAN reaction .		369				
Fischler's method . .		371				+ *
Schultz technique . .		373		+		
Bismouth trichloride method		373		+		
Plasmal reaction		376				
Periodic acid–Schiff		267				

* A negative result is not diagnostic.

Compound Lipids							*Simple lipids*	
Cerebrosides			*Phosphatides*				*Waxes*	*Neutral lipids*
Nervone	*Phrenosine*	*Kerasine*	*Acetal phosphatides*	*Sphingomyelin*	*Kephalin*	*Lecithin*	*Cholesterol esters*	*Neutral fats and oils*
			+				+	+
+	+	+						
					+	+		
+	+	+	(±)	+	+	+	+	+
+	+	+		+	+	+	+ †	+ + +
+	+	+	(±)	+	+	+	+	+
+ *	+ *	+ *		+ *	+ *	+ *		
			(±)	(±)	(±)	+		
+ (pale)	+ (pale)	+ (pale)		+	+	+		
+	+	+	+	+	+	+		
			(+)	+	+	+		
				+				
							+	
			+					
(±)	+	+		+	+	+		

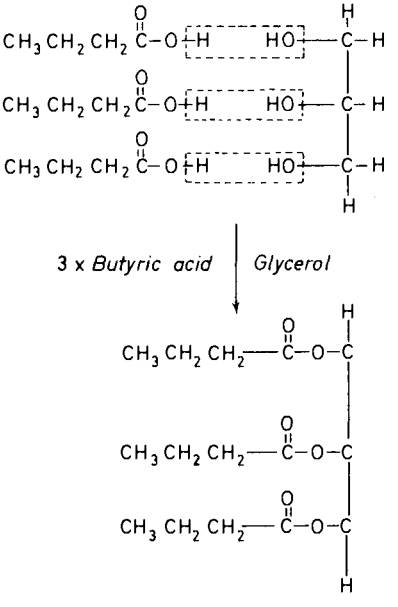
† Only when lipid is melted, not at room temperature.

Whereas certain pure lipids are insoluble in a particular fat solvent, the same lipids when contaminated with other lipids are rendered soluble in that solvent. An excellent solvent in general for crude lipids is a mixture of chloroform and methanol.

Fat and oil are terms used to describe the physical state of a lipid, a *fat* being a lipid which is solid at room temperature, an *oil* being liquid. A substance classified as a fat in a temperate climate may be an oil in the tropics.

GENERAL CHEMISTRY OF LIPIDS

The true fats (or oils) are the most abundant of the lipids. Chemically, they are neutral esters of fatty acids and glycerol. In nature, they are



Tributyrin (a butter fat)

Figure 16.1

generally three fatty acid molecules (saturated or unsaturated) combined with one molecule of glycerol, with the splitting out of three molecules of water *(Figure 16.1)*.

Most of the triglycerides which occur in nature are mixed triglycerides; that is, they contain two or three different fatty acids. Since most natural lipids contain glycerol their chemical and physical characteristics are determined by the nature of their fatty acid components. Some of the fatty acids are *saturated* with hydrogen, some are partially *unsaturated.* All have one property in common, in addition to the terminal carboxyl group (COOH), they contain an even number of carbon atoms if derived from naturally occurring fat. The latter is probably because of the mode of synthesis of fatty acids from 2-carbon fragment.

Physical Properties

Triglycerides have a specific gravity of less than one (approximately 0.86) and are therefore lighter than water. The fact that they are solid *fat* or liquid *oil* will depend upon the room temperature and their melting point (MP). The MP will be dependent upon two factors: (1) the chain length of the component fatty acids, and (2) their degree of unsaturation. In general, saturated fatty acids containing more than 8 carbon atoms are solid (with 4–8 they are liquid) and their MPs increase with the length of the chain. They confer these properties on the lipids containing them. The introduction of one carbon-carbon double bond into the fatty acid molecule will markedly lower the MP, so much so that even a fatty acid with 18 carbon atoms becomes an oil at room temperature (for example, oleic acid).

The commonly occurring fatty acids are as follows. *Saturated* – stearic (18 carbons) and palmitic (16 carbons); *unsaturated* – oleic acid $(CH_3CH_2)_7\ CH = CH(CH_2)_7COOH$.

Arichidonic acid, said to be found in animal fat and adrenal phosphatides, is a *polyunsaturated fatty acid* having 20 carbon atoms with 4 carbon–carbon double bonds.

Compound Lipids

As will be seen below, compound lipids are those containing other components in addition to alcohols and fatty acids. They may contain sphingosinein place of glycerol (for example, sphingomyelin and glycolipids).

CLASSIFICATION

Lipids may be classified according to their chemical structure and divided into three main groups, each of which may be further subdivided.

Group I – Simple Lipids

Simple lipids are neutral esters of fatty acids (palmitic, stearic or linoleic) with alcohols. They may be divided into the following.

(*a*) *Neutral lipids* comprising fats and oils in which the alcohol is glycerol.

(*b*) *Waxes,* which contain alcohols higher than glycerol (for example, cholesterol esters).

Group II – Compound Lipids

These are lipids in which products other than fatty acids and alcohol are present. They comprise:

(*a*) *Phosphatides* (phospholipids, phospholipins) are lipids containing a phosphoric acid molecule.

(i) *Lecithin* – glycerol, saturated and unsaturated fatty acid residues, phosphoric acid and choline.
(ii) *Kephalin* (cephalin) – similar to lecithin, the choline being replaced by ethanolamine.
(iii) *Sphingomyelin* – nitrogenous base, sphingosine, a single fatty acid, phosphoric acid and choline, but no glycerol.
(iv) *Acetal phosphatides* (plasmals) – similar to lecithin and kephalin but containing acetals of fatty aldehydes in place of fatty acids, combined with cholamine glycerophosphate.

(*b*) *Cerebrosides* (glycolipids, galactolipids) are lipids containing sphingosine, a molecule of sugar (glucose or galactose) and one fatty acid.

(i) *Kerasine* (cerasine) – containing lignoceric acid.
(ii) *Phrenosine* – containing cerebronic acid.
(iii) *Nervone and oxynervone* – containing nervonic and oxynervonic acid respectively.

Group III – Derived Lipids

These are derivatives of Groups I and II, obtained by hydrolysis, and can be subdivided as follows.

(*a*) *Fatty acids* derived from natural products.

(i) *Saturated fatty acids* – such as palmitic or stearic acid which contain no double bond in the molecule.
(ii) *Unsaturated fatty acids* – such as oleic acid, which contain double bonds in the molecule.

(*b*) *Alcohols* of higher molecular weight which are obtained by hydrolysis of waxes. Some authors include glycerol although it is water soluble.

(i) *Sterols* – containing the steroid nucleus, the commonest being cholesterol.
(ii) *Straight chain alcohols* – these are not of importance histologically.

Hydrocarbons

These are non-saponifiable lipids containing only carbon and hydrogen.

Carotenoids (lipochrome, chromolipid) – coloured unsaturated hydrocarbons, of which the most important histologically are carotene and lycopene. These lipid pigments do not fall strictly into any of the above groups, but are included in this classification for the sake of completeness. Those of importance histologically are dealt with in Chapter 17 on Pigments (page 387).

IDENTIFICATION OF LIPIDS

The histological identification of lipids is usually based on the following.

(1) Solubility.
(2) Examination by polarized light.
(3) Reduction of osmium tetroxide.
(4) Demonstration by fat-soluble dyes.
(5) Other staining and histochemical methods.

Of these methods, only 1, 4 and 5 may be used with any certainty of identification, and for routine purposes 4 and 5 are the most commonly employed.

Solubility

Keilig (1944), using a continuous extraction apparatus, demonstrated that lipids may be differentiated by their solubility in various 'fat solvents'. Using a Soxhlet extraction apparatus and blocks of fresh human brain not more than 3 mm in thickness, tissues were extracted for 24 hours, with at least 3 changes of solvent. The results of such extractions are shown in the *Table* for extracting with cold acetone. The continuous extraction with equal parts of hot chloroform and methyl alcohol may be used to demonstrate that a given substance is a

lipid. Following extraction, blocks are hydrated through descending strengths of alcohol to water, and frozen sections cut and stained by Sudan black B.

It is not anticipated that this method would often be employed in the routine laboratory, although occasionally it might assist in the identification of a tissue element in conjunction with other techniques.

Since the last edition of this work, several papers have been published on the subject of lipid extraction but none of the techniques has proven to be definitive. Baker's hot pyridine method (page 366) is still one of the few methods routinely employed. Zugibe (1970), using cryostat sections of unfixed tissue, notes that glycolipids and phospholipids are the only members of the major groups that are insoluble in cold acetone, and only phospholipids in hot acetone, which is a useful method to supplement the existing ones.

Examination by Polarized Light

Most lipids are highly refractile, and some are bi-refringent (anisotropic). By examining sections with polarized light (Chapter 33) it can be determined whether material is isotropic or anisotropic. In the past this method was considered to be of assistance in the identification of lipids, but in view of the variability of results (dependent on the state of the lipid at the time of examination) it is now accepted that the information gained by such examination is ambiguous.

Three types of refractility may be found (Lison, 1936).

Isotropic (mono-refringent). – This occurs in neutral fats, fatty acids, cholesterol esters, and lipins in any state which prevents the formation of liquid crystals.

Anisotropic (bi-refringent). – An appearance which may be found in any lipid in a crystalline state.

Maltese cross (bi-refringent). – A type of bi-refringence found in cholesterol esters of lipins (not neutral fats or fatty acids). If at first absent, this Maltese cross bi-refringence may sometimes be produced by heating and then cooling before examination.

Reduction of Osmium Tetroxide (Osmic Acid)

The reduction of osmium tetroxide from colourless OsO_4 to OsO_2, which is black, is of limited value histochemically for the identification

of lipids, although it may be usefully employed for their demonstration (for example, by Marchi's method). It does not react with all lipids, but does react with many non-lipid substances (for example, tannic acid and eleidin.

It remains, however, a useful method of demonstrating lipids in paraffin sections since the osmicated lipids are insoluble in the processing reagents.

Primary blackening with osmium tetroxide is said to be due to unsaturated fatty acids (oleic acid), but the secondary staining of fat (blackening which takes place only after alcohol treatment of certain lipids) has not been fully explained, although it is said to be due to the presence of saturated fatty acids (palmitic and stearic).

The use of osmium tetroxide histochemically should be limited to frozen sections of formol calcium fixed tissue, when a positive reaction after 6 hours is regarded as specific for reducing lipids, provided that the only structures considered are those known to contain lipid.

Demonstration with Fat-soluble Dyes

The staining of neutral fat with a fat-soluble dye is still the most common routine method employed in histological laboratories. It is based on the fact that the dyes used are more soluble in fat than in the solvent employed.

The first of these dyes to be used was Sudan III (1896), and it is probably still the most commonly used fat stain today, in spite of the introduction of new and better dyes. The first solvent employed was 70 per cent alcohol, for it was thought that this dilution of alcohol was not a lipid solvent; it has since been shown that small amounts of lipid may be dissolved out by this method. Herxheimer's method employs equal parts of acetone and 70 per cent alcohol which gives a greater concentration of stain but again dissolves small amounts of lipid.

Of the more recent methods, that of Lillie and Ashburn, using oil red O in isopropyl alcohol, gives a more intense stain with minimal removal of lipid particles. Chiffelle and Putt's Sudan IV or Fettrot in propylene glycol, which does not remove even minute lipid particles, gives the best results and is recommended for both routine and research work.

Colloidal suspensions of Sudan dyes in gelatin give first-class results, and there is no danger of dissolving any lipids, but they have the disadvantage of being messy and time-consuming to prepare and are consequently not likely to become adopted as routine stains.

Notes on Technique

, (1) To avoid precipitation on the sections due to evaporation of the solvent, these stains should always be employed in *closed vessels* (screw-capped wide-mouthed jars, or Petri dishes).

(2) Loose frozen sections stain more rapidly than paraffin sections attached to slides; 50 per cent haematoxylin for 10 minutes should, therefore, be used to stain nuclei; 1 per cent hydrochloric acid in water should be used to differentiate fat sections as acid-alcohol will remove lipids.

(3) Sections stained for the demonstration of lipids should never be blotted, or pressed with a needle to remove air bubbles under the coverslip, because such pressure will displace droplets of lipid. If air bubbles are present, wash off the coverslip in warm water and remount. The most common fault in mounting frozen sections is allowing them to get too dry before applying mountant. Sections should be just moist, with the excess water wiped from the slide.

Chiffelle and Putt's Propylene Glycol Method (Modified)

Staining Solution

Prepare a saturated solution of the dye by dissolving 1 g of Sudan IV, Sudan black or Fettrot in 100 ml of propylene glycol and heating to 100°C for a few minutes. Filter while hot through Whatman No.2 filter paper. Cool and refilter through a coarse sintered glass filter, or through glass wool using a vacuum pump.

Method

(1) Cut frozen sections, and wash in several changes of water to remove formalin.

(2) Dehydrate for 5 minutes in 2 changes of pure propylene glycol.

(3), Transfer to staining solution for 5–10 minutes.

(4) Differentiate in warm 85 per cent propylene glycol for 2–3 minutes, occasionally agitating the sections.

(5) Rinse in 50 per cent propylene glycol.

(6) Wash in distilled water.

(7) Counterstain nuclei lightly with haematoxylin (5–10 minutes in 50 per cent Ehrlich's haematoxylin in distilled water).

(8) Wash in tap-water to blue sections, and mount in glycerin jelly.

Results

Lipids	Red or black (depending on the stained used)
Nuclei	Blue

Myelin and mitochondria may be stained by this method.

Lillie and Ashburn's Isopropanol Oil Red O Method (Modified)

Staining Solution

A saturated solution of oil red O (0.25–0.5 per cent) in isopropyl alcohol is kept in stock. For use dilute 6 ml of stock solution with 4 ml of distilled water, allow to stand for 5–10 minutes, then filter. This solution does not keep for more than 1–2 hours.

Method

(1) Cut frozen sections.
(2) Wash well in water.
(3) Place in fat stain in a closed container for 10–15 minutes.
(4) Differentiate in 60 per cent alcohol to clear background.
(5) Wash in water.
(6) Counterstain nuclei lightly with haematoxylin.
(7) Blue in 1 per cent disodium phosphate or tap-water.
(8) Float on to slides, and mount in glycerin jelly.

Results

Lipids	Bright red
Nuclei	Blue

Alcoholic Sudan III or IV Staining

This method is inferior, in the author's opinion, to the other methods given.

Staining Solution

A saturated solution of Sudan III or IV or Black B in 70 per cent alcohol was originally employed. Herxheimer used equal parts of acetone and 70 per cent alcohol as a solvent. Rinehart uses an absolute alcohol-saturated solution, which is diluted 6:4 with distilled water in the same manner as the isopropanol method.

Method

(1) Cut frozen sections.
(2) Wash in water, and rinse in 70 per cent alcohol.
(3) Transfer to staining solution for 10–35 minutes.
(4) Differentiate in 70 per cent alcohol ro remove excess stain.
(5) Rinse in water.
(6) Stain nuclei lightly in haematoxylin and differentiate rapidly in 1 per cent aqueous hydrochloric acid.
(7) Blue in tap-water.
(8) Mount in glycerin jelly.

Results

Lipids	Orange to red
Nuclei	Blue

Govan's Gelatin Method

Solution Required

Gelatin dye suspension. – Add drop by drop, a saturated solution of Sudan III or IV in acetone to 1 per cent gelatin in distilled water containing 1 per cent acetic acid. The stain solution is added, with constant stirring, until the gelatin becomes a bright red, milky fluid. The acetone is evaporated from the suspension in the 37°C incubator for 2–3 hours. Filter through coarse filter paper.

Method

(1) Cut formol-fixed frozen sections 8–10 μ in thickness.
(2) Wash in water.
(3) Transfer to 1 per cent gelatin solution for 2–3 minutes.
(4) Stain in gelatin dye suspension for 30 minutes.
(5) Transfer to 1 per cent gelatin solution for 2–3 minutes.
(6) Wash in water.
(7) Stain nuclei lightly with haematoxylin, differentiate as above.
(8) Blue in tap-water.
(9) Mount in glycerin jelly.

Results

Lipids	Orange-red
Nuclei	Blue

Other Staining or Histochemical Methods

Cain's Nile Blue Sulphate Method

The Nile blue sulphate method, which is usually described as a technique for differentiating neutral fat and fatty acids, is based on the fact that Nile blue sulphate normally contains a red oxazone (Nile red). The Nile blue is said to combine with fatty acids, and the oxazone (Nile red) to be more soluble in neutral fat than in the staining solution.

It is generally agreed that this method is not specific for fatty acids, but may be used to demonstrate acidic lipids if employed at a temperature above their melting points. Palmitic acid melts at 63°C, stearic acid at 70°C, and oleic acid at 14°C; the temperature suggested for this method by Cain (1947) is 60°C, although 70°C may be necessary to demonstrate stearic acid. While oleic acid (if liquid) will stain with concentrated solution, other fatty acids (if liquid) stain better with dilute solutions *(see Note*).*

Staining Solution

The staining solution – 1 per cent aqueous Nile blue sulphate – should be tested for the presence of oxazone (nile red), by shaking a little of it with a small amount of xylol. The xylol, after 20–30 seconds, should be a definite red. A poor oxazone content may be improved by boiling 1 per cent Nile blue sulphate in 5 per sulphuric acid in a reflux condenser for 1–2 hours.

Method

(1) Fix tissue in Baker's formol calcium fixative.

(2) Cut 3 frozen sections of 8–10 μ thickness.

(3) Stain one section (*a*) in saturated Sudan black B in 70 per cent alcohol (or in isopropyl alcohol) as a control.

(4) Stain the other 2 sections (*b* and *c*) in Nile blue solution at 60–70°C for 5–10 minutes.

(5) Wash all sections in warm water (60°C).

(6) Differentiate in 1 per cent acetic acid at 60°C for 30 seconds.

(7) Re-stain section (*c*) in 0.02 per cent Nile blue at 60°C for 10–15 minutes.

(8) Wash in water.

(9), Mount in glycerin jelly.

Results

Neutral lipids	Red
Lecithin (and possibly kephalin and sphingomyelin) oleic acid	Blue
Nuclei	Blue
Cytoplasm and other tissue elements	Pale blue

Note. – By comparison with the Sudan-black-stained section, differentiation of lipids and acidic lipids is possible (*see also* extraction methods on page 357). *Compare sections (*b*) and (*c*) and if they are similar, discard (*b*). It is most likely that if a substance is equally well stained in (*b*) and (*c*) it is oleic acid. If sections (*b*) and (*c*) are dissimilar the deeper stained elements in section (*c*) are probably palmatic or stearic acid compounds.

Lillie's Sulphuric Nile Blue Technique for Fatty Acids

This method is based upon the use of Nile blue sulphate at a very low pH (about 0.9). At such a pH carboxylic and phosphoric acid radicles should not be ionized, they should therefore be unable to bind the dye; the fatty acids, however, do bind the dye (Lillie, 1965).

Staining Solution

Acid Nile blue sulphate

Nile blue sulphate	0.05 g
Distilled water	99 ml
Conc. sulphuric acid	1 ml

Method

(1) Bring frozen, cryostat or paraffin sections to water.

(2) Stain in acid Nile blue sulphate for 20 minutes (for the reasons given above it is best to stain at 70°C).

(3) Wash in running water for 10 minutes.
(4) Mount in Apathy's or glycerin jelly.

Results

Fatty acids	Dark blue
Neutral fats	Pink to red

Note. – Dunnigan (1968) adds 10 ml of 1 per cent sulphuric acid to 200 ml of 1 per cent Nile blue sulphate, and boils it for 4 hours in a reflux condenser to generate more oxazone. He also extracts parallel sections with acetone at room temperature for 1 hour to remove hydrophobic lipids. He further notes that phospholipids give a more intense blue stain than free fatty acids.

Luxol Fast Blue Methods for Phospholipids

Luxol fast blue G has been recommended for phospholipids (Salthouse, 1965) as well as for myelin, collagen and elastin. Salthouse notes that the dye solvent employed is of prime importance since the tissue component-dye complex may be soluble. He showed that ethanol or isopropanol solutions of luxol fast blue G will stain phospholipids, but that metholic solutions stain collagen and elastin but not myelin or phospholipids (*see* page 418).

Baker's Acid Haematein Method for Phospholipids

Baker's acid haematein method (used in conjunction with the pyridine extraction test) is superior to, and is given in place of, the Smith-Dietrich technique.

Special Reagents Required

(1) *Formol calcium fixative*
(2) *Dichromate calcium*

Potassium dichromate	5 g
Calcium chloride	1 g
Distilled water	100 ml

(3) *Acid haematein.* – To 0.05 g of haematoxylin (*B.D.H.*) in

49 ml of distilled water, add exactly 1 ml of 1 per cent sodium iodate. Heat in a flask until boiling. Cool and add 1 ml of glacial acetic acid. This solution does not keep and can be used only on the day it is prepared.

(4) *Borax ferricyanide differentiator*

Potassium ferricyanide	0.25 g
Borax (sodium tetraborate 10 H_2O)	0.25 g
Distilled water	to 100 ml

This solution should be kept in the dark.

Method

(1) Fix small pieces of tissue in formol calcium for 6–8 hours.
(2) Transfer to dichromate calcium solution for 18 hours at room temperature.
(3) Transfer to dichromate calcium solution for 24 hours at 60°C.
(4) Wash well in distilled water, and cut frozen sections at 8–10 μ.
(5) Mordant sections in dichromate calcium solution for 1 hour at 60°C.
(6) Wash in distilled water.
(7) Stain in acid haematein for 5 hours at 37°C.
(8) Rinse in distilled water.
(9) Differentiate in borax ferricyanide for 18 hours at 37°C.
(10) Wash in water.
(11) Mount in glycerin jelly, or for routine work, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Phospholipids: lecithin, kephalin and sphingomyelin (myelin sheaths) and nucleoprotein	Dark blue to black
Cerebrosides (in brain)	Pale blue to blue-black
Mucin, fibrinogen	Dark blue

Baker's Pyridine Extraction Test for Phospholipids

Method

(1) Fix frozen sections of fresh, unfixed tissue for 20 hours in a dilute Bouin's fluid (saturated aqueous picric acid 50 ml, formalin 10 ml, glacial acetic acid 5 ml, and distilled water 35 ml).

(2) Wash in 70 per cent alcohol for 30 minutes.
(3) Wash in 50 per cent alcohol for 30 minutes.
(4) Wash in running water for 1 hour.
(5) Dehydrate in 2 changes of pyridine at room temperature for 1 hour each.
(6) Extract in fresh, pure pyridine for 24 hours at 60°C.
(7) Wash in running water for 2 hours.
(8) Transfer to Stage 2 of the acid-haematein method above.

Results

Phospholipids: lecithin, kephalin and sphingomyelin, and cerebrosides . . . . Colourless

Mucin, fibrinogen and other non-lipid elements are stained.

Controlled Chromatin Procedure for Phospholipids (Elftman, 1954)

This is a simplified form of Baker's acid haematein method. Elftman controlled the pH of the dichromate fixative and shortened the time of exposure of the tissue; he also omitted the iodate and added dichromate to the haematoxylin solution to prevent background staining of the tissue thus obviating the use of the borax-ferricyanide differentiation. The embedded tissues tend to be brittle and thicker sections may need to be used; *alternatively*, thin (2–3 μ) cryostat sections of fresh tissue may be mounted directly on to slides, and fixed and stained by this method. It is thought that the haematoxylin binds to the lipid chromous hydroxides.

Solutions required

Buffered dichromate fixative

2.5 per cent Potassium dichromate adjusted to pH 3.5 with 0.2 M acetate buffer.

Buffered haematoxylin solution

0.M Acetate buffer pH 3.0 . . . . . 50 ml
Potassium ferricyanide . . . . . 0.25 g
Haematoxylin . . . . . 50 mg

Method

(1) Fix fresh tissue (or cryostat sections of fresh tissue) in buffered dichromate at 56°C for 18 hours.

(2) Wash tissues in running water (or syphon washer) for 4–6 hours (cryostat sections proceed directly to step 5).

(3) Dehydrate, clear and embed in Paraplast (or paraffin wax).

(4) Section, and bring sections to water.

(5) Stain in buffered haematoxylin solution (pre-heated to 56°C) at 56°C for 2 hours.

(6) Rinse in distilled water, dehydrate, clear and mount in synthetic resin.

Results

Phospholipids (myelin, and so on) in dark blue. Staining a parallel section in Oil Rid O (*see* page 361)will indicate that the structure stained is lipid in nature, or Baker's pyridine extraction test may be used.

Osmium Tetroxide –α Naphthylamine (OTAN) Reaction for Phospholipids (Adams, 1959)

This method is useful for differentiating hydrophilic lipids (sphingomyelins, cerebrosides, gangliosides, and so on) from hydrophobic triglyceride esters, cholesterol esters and fatty acids. It is based on the reaction of osmium tetroxide with the non-polar ethylene bonds of phospholipids and the subsequent chelation with α–naphthyamine to give an orange-red colour.

Solutions required

Osmium tetroxide solution

1 per cent Osmium tetroxide . . . . . 1 part
1 per cent Potassium chlorate . . ($KClO_3$) . . . 3 parts

Saturated α–naphthylamine solution

Heat distilled water to 40°C, and add α–naphthylamine to make a saturated solution, and filter. Since carcinogenic β–naphthylamine may be a contaminant it should be handled with care. The solution is used at 37°C.

Method

(1) Cut frozen or cryostat sections of formol calcium fixed tissue at 5–15 μ (depending upon the type of investigation or demonstration).

(2) Place free floating sections in osmium tetroxide solution for 18 hours. The container should be filled and stoppered tightly to prevent volatilization of osmium tetroxide.

(3) Wash sections in distilled water for 10 minutes and mount on slides.

(4) Treat with saturated α–naphthylamine solution at 37°C for 15–20 minutes.

(5) Wash in distilled water for 5 minutes.

(6) Counterstain the sections wtih 2 per cent Alcian blue in 5 per cent acetic acid for 15–60 seconds (optional).

(7) Mount in an aqueous mountant such as glycerin jelly.

Results

Phospholipids	Orange red
Cholesterol and triglyceride esters	Black

Note 1. Normal myelin will stain orange-red with degenerate myelin being black.

Note 2. Black staining hydrophobic lipids which may mask the orange-red phospholipid staining may be avoided by preliminary extraction of control sections with chilled acetones.

NaOH–OTAN Technique for Sphingomyelin (Adams, 1965)

If the OTAN method (above) is preceded by treatment with NaOH only alkali-resistant lipids are stained. The most important of these is sphingomyelin and this method may be used with some degree of specificity for its demonstration.

Method

As for OTAN method above except that sections are pre-treated in 2N sodium hydroxide at 37°C for 1 hour. They are then washed gently in water, rinsed in 1 per cent acetic acid for 1 minute, and again rinsed in water. Then transfer to osmium tetroxide solution (steps 2–7 above).

Result

The black staining of hydrophobic lipids can be avoided by acetone treatment (as above).

McManus's Sudan Black B Method for Compound Lipids in Paraffin Sections

A positive result with this method indicates that the substance demonstrated is a compound lipid. A negative result, however, has little significance since some compound lipids are not blackened. Myelin and mitochondria may be demonstrated using this technique. The method is based on the ability of calcium and formalin to render these lipids insoluble in acetone and the reagents used in paraffin processing. They may then be stained with Sudan black B whereas neither osmium tetroxide nor Sudan III gives a positive result.

Method

(1) Fix tissues for 2–4 weeks in Baker's formol calcium fixative to which 1 per cent cobalt nitrate or sulphate has been added. The precipitate which forms when the sulphate is used is covered with cotton-wool, and the specimen laid upon the latter.

(2) Post-chrome for 24–48 hours in 3 per cent potassium dichromate. Wash in running water overnight. (This stage is optional, but gives an improved result.)

(3) Process through paraffin keeping the length of time in alcohol, xylol and molten wax to a minimum. If fixation has been for less than 2 weeks, McManus recommends dehydration in 3 changes of acetone, each of 30 minutes; place directly into molten paraffin wax, change 2 or 3 times within 1 hour, and embed. Cut sections 4–6 μ in thickness and attach to slides.

(4) Bring sections to 70 per cent alcohol.

(5) Stain for 30 minutes in saturated Sudan black B in 70 per cent alcohol (sections of routine formol saline-fixed tissues should be stained for 30 minutes to 3 hours at 60°C).

(6) Differentiate in 70 per cent alcohol.

(7) Counterstain in carmalum (page 479) for 3 minutes.

(8) Wash in water, and mount in glycerin jelly.

Results

Compound lipids (phosphatides and cerebrosides)	Blue-black *(see above)*
Nuclei	Red

Fischler's Method for Fatty Acids

It is generally agreed that there is no specific histochemical method for the demonstration of fatty acids. However, Fischler's method is known to demonstrate fatty acids when present in large amounts.

The method is based on the fact that fatty acids form calcium soaps if fixed in formol calcium. After mordanting with copper acetate, a lake is formed between the soaps and the haematoxylin, which is very resistant to borax ferricyanide differentiator.

Special Reagents Required

(1) *Weigert's lithium haematoxylin*

Solution A; 10 per cent haematoxylin in absolute alcohol.

Solution B; Saturated lithium carbonate . . . . 10 ml
Distilled water . . . . . 90 ml

Mix equal parts of A and B just before use.

(2) *Weigert's borax ferricyanide differentiator*

Borax . . . . . 20 g
Potassium ferricyanide . . . . . 25 g
Distilled water . . . . . to 1,000 ml

Method

(1) Fix tissue in 10 per cent formalin, which has been saturated with calcium salicylate (1.3–1.5 per cent).

(2) Cut frozen sections 8–10 μ in thickness.

(3) Mordant sections in a saturated aqueous solution of copper acetate for 12–24 hours at 37°C.

(4) Wash in distilled water.

(5) Stain in lithium haematoxylin for 20 minutes.

(6) Differentiate in borax ferricyanide (this may be diluted to give greater control) intil red blood cells are very pale blue or colourless

(7) Wash in distilled water. Counterstain, if desired, to show neutral lipid red.

(8) Mount in glycerin jelly.

The method may be controlled by extracting sections in alcohol-ether mixture for 24 hours before mordanting (Stage 3); this will dissolve fatty acids, but not pre-formed calcium soaps.

Results

Fatty acids	Dark blue
Neutral lipid (if counterstained)	Red

Perchloric Acid-naphthoquinone (PAN) Method for Cholesterol and Cholesterol Esters (Adams, 1961)

This method is based on the formation of cholesta–3, 5–diene by the action of perchloric acid which subsequently reacts with naphthoquinone to give a dark blue-grey product. Although the mechanism of the latter is not known, its specificity has been shown to be high for cholesterol and related steroids. Tryptophan may give a pink colour as may some mucosubstances. It is recommended as a method of choice.

Solution Required

Pan reagent

0.1 per cent 1,2-naphthoquinone-4 sulphonic acid in ethanol	4 ml
Perchloric acid (60 per cent)	2 ml
Formalin (40 per cent formaldehyde)	0.2 ml
Water	1.8 ml

Method

(1) Cut frozen or cryostat sections, leave in formol calcium fixative for 1 week or more to allow oxidation of cholesterol to occur.
(2) Mount sections on slides and allow to dry.
(3) Cover sections with minimum amount of PAN reagent.
(4) Heat slides on hot plate at 60–70°C for 5–10 minutes (until original red colour changes to blue).
(5) Place a drop of 60 per cent perchloric acid on section and apply a coverslip (water or glycerin jelly cannot be used).

Results

Cholesterol, demosterol and cholesterol esters	Dark blue

Schultz Method (Romieu's Modification, 1927) for Cholesterol and Cholesterol Esters

Method

(1) Mount frozen sections (10–15 μ in thickness) on slides.

(2) Put 1 or 2 drops of concentrated sulphuric acid on the sections, leave for 5–15 seconds.

(3) Put 3 or 4 drops of acetic anhydride on the sections to develop the colour.

(4) Drain sections, and wash with a few drops of acetic anhydride.

(5) Mount in acetic anhydride, and examine immediately. (Stages 3, 4 and 5 should be performed rapidly.)

Result

Cholesterol, and cholesterol esters . .	.Red-violet, changing to green

Bismuth Trichloride Method of Differentiating Between Cholesterol and Cholesterol Esters (Grundland, Bulliard and Maillet, 1949)

Special Reagent Required

Bismuth trichloride	0.2 g
Acetyl chloride	1.0 ml
Nitrobenzene (anhydrous)	to 100 ml

Method

(1) Fix the tissue (not more than 2–3 mm in thickness) in a saturated solution of digitonin in 70 per cent alcohol for 36 hours.

(2) Press tissue gently between layers of filter paper to remove excess alcohol, and allow the remainder to evaporate in a 56°C oven for 5–10 minutes.

(3) Infiltrate with paraffin wax, with 5 per cent glycerol monostearate added, for 12–16 hours.

(4) Embed in paraffin wax.

(5) Cut sections 6–10 μ in thickness, and mount on slides.

(6) Without removing the wax, treat the sections with bismuth trichloride reagent for 15–45 minutes.

(7) Rinse rapidly in 10 per cent acetyl chloride in nitrobenzene (anhydrous).
(8) Wash in 75 per cent nitric acid (concentrated) in absolute alcohol.
(9) Rinse rapidly in absolute alcohol.
(10) Treat with 20 per cent ammonium sulphide (yellow) for a few seconds.
(11) Wash in absolute alcohol.
(12) Clear in xylol, and mount in Canada balsam.

Results

Cholesterol	Dark brown
Cholesterol esters	Uncoloured

Pearse's Peracetic or performic acid–Schiff Method for Phospholipids and Cerebrosides (Lipids Containing Unsaturated Bonds)

Fixation

Formol saline or Zenker–formol should be employed.

Special Reagents Required

(1) *Performic acid.* – To 40 ml of 98 per cent formic acid, add 4 ml of 100 vol. (30 per cent) hydrogen peroxide. Allow to stand for 1½ hours before use. Fresh reagent must be made daily.
(2) *Peracetic acid.* – Peracetic acid (40 per cent) is available commercially.
(3) *Schiff reagent* (*see* page 249).

Method

(1) Bring paraffin or frozen sections to water. Remove mercury precipitate if present.
(2) Blot dry.
(3) Oxidize with performic or peracetic acid for 2–5 minutes.
(4) Immerse in Schiff reagent for 30 minutes.
(5) Wash in warm running water for 10 minutes.
(6) Mount in glycerin jelly.

Result

Lipids with unsaturated bonds (phospholipid or cerebroside) . . . . . Red

Bromine-Silver Method for Unsaturated Lipids (Norton and Colleagues, 1962)

This method is based on the bromination of unsaturated lipids, which is the combination of bromine at the site of double bonds. This is then reacted with silver to give a silver bromide and finally metallic silver. According to Adams (1965) this method has the advantage that it does not stain proteins; however, it also fails to react with double bonds of hydrophobic lipids (phospholipids and glycosphingosides) so that a negative result does not exclude them.

Method

(1) Cut frozen or cryostat sections of formol calcium fixed tissue, mount on slides, dry in air.
(2) Treat with bromine-potassium bromide solution (1 ml of bromine in 390 ml of 2 per cent potassium bromide) for 1 minute.
(3) Wash in water.
(4) Treat with 1 per cent sodium bisulphite for 5 minutes.
(5) Rinse in several changes of distilled water.
(6) Treat with 1 per cent silver nitrate in 1N nitric acid for 18 hours.
(7) Rinse in several changes of distilled water.
(8) Reduce for 10 minutes in Kodak Dektol developer (diluted equal parts with water).
(9) Wash well and mount in an aqueous mountant such as glycerin jelly.

Results

Unsaturated lipids (*see* above) . . . . . Brown to black

Bromine/P.A.S. method

Steps (2) and (3) above followed by the P.A.S. method (page 268) with obviate P.A.S. staining of unsaturated lipids.

The Use of Fluorescent Dyes to Demonstrate Lipids

Fluorescent dyes, such as phosphine 3 R, and 3:4 benzpyrene, have been described for the demonstration of lipids. Techniques are given under fluorescent microscopy (page 613).

Plasmal Reaction

Method

(1) Frozen sections, or smears, are washed in distilled water.
(2) Transfer sections to 1 per cent aqueous mercuric chloride for 5–10 minutes.
(4) Transfer to Schiff reagent for 5–15 minutes.
(5) Bring sections through 3 sulphite rinses (*see* page 252).
(6) Wash in water, and mount in glycerin jelly, or float on slide, dehydrate, clear, and mount in D.P.X.

Results

Acetal lipids	Red-purple

Notes. – (*i*) A glass hockey stick should be used to transfer the sections throughout. (*ii*) A control which has not been through stage 2 should be compared with the test slide to ensure that the colour present in a specific element is due to a plasmal reaction.

Chapter 17

Pigments

The pigments encountered in normal and pathological conditions may conveniently be classified and discussed under the following headings: (1) artefact; (2) haematogenous; (3) autogenous; (4) exogenous.

ARTEFACT PIGMENTS

The principal artefact pigments are formalin pigment (acid formaldehyde haematin (page 44) and mercury pigment (page 40), which occur as a result of fixation. Both these pigments should be easily identified by their distribution over the whole of the section, by their presence outside the cells, and also by the ease of their removal.

Water droplets in a section may occasionally simulate a light brown pigment.

HAEMATOGENOUS PIGMENTS

Haematogenous pigments are derived from the colouring matter of the blood.

Haemoglobin

Haemoglobin is stained in red blood cells by the acid dyes such as eosin. It is best demonstrated specifically in sections by one of the peroxidase techniques, or the patent blue method (page 341), the latter generally giving the best results.

Haemoglobin occurs pathologically (for example, in renal casts) as droplets or granules of a yellow or yellow-brown colour. Distinction between the various types of haemoglobin (for example, methaemoglobin and sulphmethaemoglobin) is only possible spectroscopically.

Haemosiderin

Haemosiderin is a breakdown product of haemoglobin and is thought to be composed of ferric iron and protein. It occurs in pathological conditions as yellow-brown granules.

The iron-containing pigments are soluble in acids, and insoluble in alkalis and fat solvents; they are demonstrated by Perls' Prussian blue reaction. Tissues which are to be examined for iron-containing pigments must be fixed in non-metallic containers, and iron-free distilled water used for the reaction to avoid contamination.

Fixation

Although alcohol fixation was specified originally, buffered formol saline gives equally good results, with improved preservation of tissue elements.

Perls' Prussian Blue Reaction (for Ferric Salts)

Method

(1) Bring frozen, paraffin or celloidin sections to distilled water.

(2) Transfer to a fresh solution of equal parts of 2 per cent aqueous potassium ferrocyanide and 2 per cent hydrochoric acid, for 30 minutes. With a doubtful result, the reaction may be carried out at 60°C, but this is not usually necessary.

(3) Wash thoroughly in several changes of distilled water.

(4) Counterstain lightly with 1 per cent neutral red or safranine for 10–15 seconds.

(5) Wash in water, dehydrate, clear, and mount in Canada balsam, D.P.X., or Euparal (the last-named makes a more permanent mount).

Results

Ferric-iron-containing pigments (haemosiderin) . . .	Blue
Nuclei	Red

Tirmann-Schmelzer's Turnbull Blue Technique for Ferrous Salts

Method

(1) Bring sections to water.

(2) Treat with a dilute solution of yellow ammonium sulphide for 1–3 hours.

(3) Rinse in distilled water.

(4) Treat with a freshly prepared solution of equal parts of 20 per cent potassium ferricyanide and 1 per cent hydrochloric acid for 15 minutes.

(5) Wash thoroughly in several changes of distilled water.

(6) Counterstain nuclei lightly with 1 per cent neutral red or safranine for 10–15 seconds.

(7) Wash in water, dehydrate, clear and mount.

Results

Ferrous salts and ferric salts converted by treatment with ammonium sulphide . . . . Deep blue
Nuclei . . . . . Red

Note. – If Stage 2 is carried out ferric salts are converted to ferrous salts and are therefore demonstrated; to differentiate between the two, parallel sections are taken and Stage 2 omitted in the treatment of one of them.

Haemozoin (Malaria Pigment)

This pigment is found in the parasites, and in brain capillaries, liver, spleen, bone marrow and lymph nodes in malaria. It is similar to formalin pigment in every respect except that it does not occur throughout the whole of the sections and is found intracellularly in phagocytes (*see* Chart).

Haematoidin (Bile Pigment)

Haematoidin occurs as yellowish granules or masses. It is found in old haemorrhages, particularly in infarcts of the spleen or brain.

Of the methods of demonstration available, Gmelin's and Glenner's probably give the most consistent results, although Fouchet's reagent gives a much longer lasting reaction.

Fouchet's Reagent for Bile Pigment

Solutions Required

Fouchet's Reagent

Dissolve 25 g of trichloracetic acid in 100 ml of distilled water. Dissolve 1 g ferric chloride in 10 ml of distilled water and add to the trichloracetic acid solution. Mix and store in a dark bottle.

Method

(1) Bring frozen or paraffin sections to water.
(2) Add 3–4 drops of Fouchet's reagent to section, lower coverslip on to section and examine.

Result

Bile pigments are coloured green by reagent.

Glenner's Method for Bilirubin (1957)

This method is based on the oxidation of the pale yellow bilirubin pigment, by the potassium dichromate, to its oxidized form which is bright emerald green. The pH of the dichromate is critical since it must be low enough to ensure complete oxidation without being sufficiently acid to remove the pigment.

Special reagent required

Buffered dichromate

3 per cent Potassium dichromate	25 ml
N/10 Hydrochloric acid	8 ml
N/10 Potassium dihydrogen phosphate (*see* page 168)	17 ml

Mix and check to ensure that pH is 2.2.

Method

(1) Cut frozen or cryostat sections of fresh tissue, attach to slides.
(2) Treat with buffered dichromate in Coplin jar at room temperature for 15 minutes.*
(3) Wash in running water for 5 minutes.
(4) Fix in formol calcium (*see* page 45) for 15 minutes.
(5) Wash in water and counterstain if desired.
(6) Mount in Apathy's or glycerin jelly.

*An unoxidized control section (omitting step 2) should be carried through to ensure that any green colour present has been produced by oxidation.

Result

Bilirubin . . . . . Bright emerald green

Glenner's Method for Bilirubin, Haemosiderin and Lipofuscin (1957)

This method is a modification of the one above, with the Prussian blue and oil red 0 methods also being performed upon the section. It may prove useful in the identification of a yellow pigment.

Method

(1) Cut frozen or cryostat sections of fresh tissue and attach them to clean slides.
(2) Treat sections with 2 per cent potassium dichromate for 5 minutes.
(3) Place sections in equal parts of 5 per cent acetic acid and freshly prepared 2 per cent potassium ferrocyanide for 20 minutes.
(4) Rinse in running water and treat with buffered dichromate solution (*see* above method) for 15 minutes.
(5) Rinse in water.
(6) Place in oil red O solution (*see* page 360) for 20 minutes.
(7) Rinse in 70 per cent alcohol to remove excess stain.
(8) Washing in running water.
(9) Mount in Apathy's medium or glycerin jelly.

Results

Bilirubin . . . . . Green
Haemosiderin . . . . . Blue
Lipofuscin . . . . . Red

It should be remembered that (*a*) neutral lipids will stain red, and (*b*) lipofuscin may stain faintly or not at all.

Gmelin's Reaction for Bilirubin and Haematoidin (Bile Pigments)

Method

(1) Bring frozen or paraffin sections to water.
(2) Wipe excess water from around sections and mount (in water) with coverslip avoiding air-bubbles.

(3) Place section on microscope stage, and focus with a 16 mm objective on the pigment to be identified.

(4) Using a pipette, place a few drops of 50 per cent nitric acid at one end of the coverslip. Apply a small piece of filter paper to the other end of the coverslip to draw the nitric acid over the section.

(5) Watch the unidentified pigment under the microscope for a change of colour.

(6) Discard section as the reaction lasts only for a few seconds.

Results

Bilirubin and haematoidin change colour from yellow to green, then through blue to purple and red.

Stein's Technique for Bilirubin

Method

(1) Bring sections to water.

(2) Treat with a mixture of 3 parts Lugol's iodine and 1 part tincture of iodine for 6–12 hours.

(3) Decolorize with a 5 per cent aqueous solution of sodium sulphite for 15–30 seconds.

(4) Counterstain nuclei in alum carmine for 1–3 hours (or in 1 per cent neutral red for 5 minutes).

(5) Wash in distilled water.

(6) Dehydrate in acetone, clear in xylol and mount in Canada balsam.

Results

Bilirubin	Green
Nuclei	Red

AUTOGENOUS PIGMENTS

Melanin (*see* Chart)

Melanin occurs normally as yellow-brown to black granules in hair, skin and the eye. It may be found pathologically throughout the body.

This pigment may be demonstrated by the Masson-Fontana silver technique (page 468), or by Schmorl's reaction (page 386), and is differentiated from similarly coloured pigments by the ease with which

it is bleached by 20 vol. hydrogen peroxide or Mayer's technique, which is as follows.

Method

(1) Place a thin layer of potassium chlorate at the bottom of a Coplin, jar, and fill it with 70 per cent alcohol.

(2) Bring section to water, and place it in the jar.

(3) With a pipette add 1 ml of concentrated hydrochloric acid to the bottom of the jar.

The nascent chlorine produced will bleach melanin within a few hours; if necessary, add more acid.

Rost and Polak (1969) recommend examining unstained formalin-fixed, paraffin sections (dewaxed and mounted in liquid paraffin or DPX or HSR) *by fluorescence microscopy* (using a BG 12 exciter filter and a Zeiss 51/44 yellow barrier filter (*see* page 605). They feel that the presence of yellow fluorescence demonstrates a specific property of malignant melanomas and naevi, particularly in amelanotic melanomas. It is thought that DOPA (page 341) is partly, but not completely, responsible for the fluorescence.

Lipofuscin

Lipofuscin, known also as 'brown atrophy pigment' and 'wear and tear' pigment, occurs as yellowish-brown granules in heart muscle, liver, adrenals, and ganglion cells. It is probably a heterogenous group, and Pearse traces it from a lipid precursor which on progressive oxidation gives varying reactions. It is often positive with performic acid–Schiff (page 374) and occasionally with P.A.S. (page 268).

The best method of demonstration is that of Schmorl, although Sudan black B staining of paraffin sections often gives good results (*see* Chart), as also does carbol-fuchsin (a Ziehl-Neelsen with prolonged exposure to carbol-fuchsin).

Dubin-Johnson pigment (1954), one of the family of lipofuscins, is found centrolobularly in otherwise normal livers in a type of chronic idiopathic icterus (Barone and colleagues, 1969); its identification is important in the differential diagnosis of these conditions. It is positive with the Masson-Fontana (page 468), methen-amine silver (page 469) and Schmorl's reaction and Barnett and Seligman's method for sulphydryl groups (page 235). There is complete disagreement in the literature on its reactivity with Sudan black B and the P.A.S. reaction with the majority agreeing that it is positive to faintly positive by both methods.

	Pigment	Appearance in sections	Sites		Birefringence
			Normal	Pathological	
ARTEFACT	Formalin pigment (acid formaldehyde haematin)	Dark brown-black granules	Blood-containing tissues		+
	'Mercury pigment'	Brown-black	All tissues		–
HAEMATOGENOUS	Haemoglobin	Yellow-brown droplets	Red blood cells	Renal casts	–
	Haemosiderin (iron pigment)	Yellow-brown granules or clumps	–	Liver, etc.	–
	Haematoidin (bile pigment)	Yellow-brown, red granules, or needles	–	Old haemorrhages	–
	Haemazoin (malarial pigment)	Dark brown to black granules	–	Vascular and reticulo-endothelial system	+
AUTOGENOUS	Argentaffin	Pale yellow	Stomach, intestine, appendix	Carcinoid tumours	–
	Melanin	Yellow-brown, black granules	Eyes, skin, hair	Tumours, freckles, Addison's disease	–
	Chromaffin	Brown if mordanted	Adrenal medulla	Phaeochromocytoma	–
	Lipofuscin	Yellow and brown droplets	Adrenals	Heart, liver, ganglion cells, testis	–
EXOGENOUS	Carbon	Black particles or jagged masses	–	Lungs and associated glands	–
	Silica	Greyish crystals	–	Lungs and associated glands	+
	Asbestos	Beaded particles, asbestos bodies	–	Lungs and associated glands	–
	Silver	Brown or black granular deposit	–	Subcutis, intestines, nasopharynx	+
	Tattoo pigment	Vari-coloured granular deposit	–	Skin, subcutis, lymph glands	–

Pigment soluble in	*Prussian blue reaction*	*Blackening by ammoniacal silver*	*Bleaching*	*Schmorl's reaction*	*Other tests*
Alcoholic picric	–	–	–	–	–
Iodine-alcohol	–	–	–	–	–
Slightly in alcohols and water	–	–	–	–	Patent blue method
Mineral acids	+	–	–	–	Blackened by ammonium sulphide
Slightly in chloroform	–	–	–	–	Gmelin's nitric acid technique or Stein's iodine test
Alcoholic picric	–	–	–	–	–
Alcohol	–	+	–	+	–
Strong alkali	–	+	+	+	–
–	–	+	+	+	Giemsa Toluidine blue
Chloroform, benzene (slightly)	–	+	+	+	Performic acid—Schiff Sudan black Ziehl-Neelsen
–	–	–	–	–	–
–	–	–	–	–	Micro-incineration
–	+	–	–	–	–
Iodine	–	–	+	–	Blackened by ammonium sulphide
–	–	–	–	–	–

Schmorl's Reaction for Lipofuscin (Lillie, 1965)

Method

(1) Bring sections to water.

(2) Treat with a freshly prepared solution of 30 ml of 1 per cent ferric chloride, 4 ml of freshly prepared 1 per cent potassium ferricyanide and 6 ml of distilled water for 10 minutes.

(3) Wash in running water.

(4) Counterstain nuclei with 1 per cent neutral red or safranine for 15–30 seconds.

(5) Wash in water, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Lipofuscin, melanin and argentaffin	Dark blue
Chromaffin cells (after dichromate fixation) ,	Greenish blue
Nuclei	Red

Note. – Sections may be differentiated after Stage 3 with 1 per cent potassium hydroxide in 50 per cent alcohol. Differentiation is stopped by rinsing in 70 per cent alcohol and water. Stages 4 and 5 are then proceeded with. Sudan black B may be used to demonstrate this pigment (*see* page 361).

Argentaffin Granules

Argentaffin granules are sometimes listed among the autogenous pigments (*see* Chart and page 467).

Chromaffin

The dark brown, granular material normally found in the adrenal medulla is included by some authors as among this group of pigments. Since it is confined to recognized areas its demonstration is unlikely to present difficulty (*see* Chart and page 464).

EXOGENOUS PIGMENTS AND MINERALS

These are substances occurring as pigments which have gained entrance to the body: the most common are chromolipids, carbon, silica, silver and tattoo pigment.

Chromolipids (Lipochromes)

These are lipids in which are dissolved hydrocarbons of the *carotenoid* type; they are recognized in the fresh state by their obvious red or yellow colour, for example, in corpora lutea. Being soluble in fat solvents, they are usually absent from paraffin sections. Histologically, this group is of little importance.

Carbon

Carbon occurs as black particles, or jagged masses, and is most commonly found in the lungs and associated glands. It may be found rarely in the liver, the spleen and the skin (*see* Chart).

Carbon is distinguished from melanin, malaria pigment, formalin pigment, and so on, by its insolubility in concentrated sulphuric acid (which dissolves all other pigments), and the inability of Mayer's chlorine method to bleach it (page 383).

Silica

Silica (*see* Chart) is found most commonly in the lungs and associated glands of stone-grinders (silicosis); in coal miners it occurs together with carbon (anthracosis). It occurs as greyish crystals, which are birefringent (page 639). Silica may be demonstrated by its resistance to micro-incineration, and the fact that it is birefringent.

Asbestos

Asbestos is a special form of silica and is found in the lungs and associated glands of asbestos workers. It occurs as beaded bodies known as 'asbestos bodies' (*see* Chart and Frontispiece) having a characteristic shape and giving a positive Prussian blue reaction.

Silver

Silver may be found in skin, kidney or other parts of the body as a result of medical investigation or treatment, and as an occupational hazard among silver nitrate workers in the condition known as argyrea.

It occurs as a brown or black granular deposit, and is blackened by ammonium sulphide. It may be removed by borax ferricyanide differentiator (*see* page 371).

Tattoo Pigment

Under this heading is found a great variety of coloured pigments. They are, fortunately, usually confined to the skin which has been tattooed, but may be found in associated glands.

Iron-ore Pigment

Iron-ore pigment may be encountered in the lungs in certain occupations (for example, miners). The pigment may be black, blue, green, yellow or red, according to the composition. It is soluble in 5 per cent oxalic or dilute acid (1 hour or more), and reacts in acid solutions with potassium ferricyanide or ferrocyanide, or both. It is, however, sometimes necessary to carry out the Prussian blue reaction at 60°C, using stronger (even concentrated) hydrochloric acid to get a reaction.

Lead and Copper

Lead and copper in tissues may be demonstrated with fresh alkaline haematoxylin. The colours described are for fresh unripened haematoxylin, and since the ripening may take place even in minutes, it is essential that the solution used is prepared immediately before use.

Mallory and Parker's Haematoxylin Method for Lead and Copper

Solution Required

Dissolve 10 mg of haematoxylin in a few drops of 95 per cent alcohol. Add 10 ml of a filtered 2 per cent potassium dihydrogen phosphate.

Method

(1) Bring alcohol or formalin-fixed tissues to water.
(2) Stain in fresh haematoxylin solution for 2–3 hours at 50–60°C.
(3) Wash in running water for 10 minutes.
(4) Dehydrate in 95 per cent alcohol.
(5) Clear in terpineol and mount in terpineol balsam.

Results

Lead	Dark grey-blue
Copper	Blue

Nuclei	Blue
Haemosiderin alcohol fixed	Black
Haemosiderin formalin fixed	Brown

Bismuth

Bismuth in tissues is well demonstrated by the brucine-iodide method.

Wachstein and Zak's Method for Bismuth

Special Reagents Required

(1) *Modified Castel's reagent.* – Dissolve 0.25 g brucine sulphate in 100 ml of distilled water, add 3 drops of concentrated sulphuric acid and then 2 g of potassium iodide. This should be stored in a dark bottle and filtered before use.

(2) *Castel's light green solution.* – Add 0.1 ml of 1 per cent aqueous light green to 10 ml of Castel's reagent.

Method

(1) Bring sections to water and blot dry.
(2) Treat with 100 vol. (30 per cent) hydrogen peroxide for 1–15 seconds.
(3) Wash in running water for 1 minute.
(4) Treat with modified Castel's reagent for 1 hour.
(5) Transfer to 25 per cent modified Castel's reagent in distilled water, and agitate gently to remove precipitate.
(6) Counterstain in Castel light green for 4 minutes.
(7) Mount in laevulose syrup (*see* page 181).

Results

Bismuth	Orange red
Tissue	Shades of green

Demonstration of Uric Acid

Uric acid which occurs in tissue (gouty tophi), or in synovial fluids from acute gouty joints, as acid sodium urate crystals may be demonstrated as argentaffin with methenamine silver (at 37°C for 1–2 hours, *see* page 469) or with the polarizing microscope (McCarthy and Hollander, 1961; Currey, 1968). With either tissue or fluid the more

specific method is examination with the polarizing microscope (*see* page 640). In joint fluids, particularly, it is important to differentiate between calcium pyrophosphate dihydrate (CPPD), which may occur in synovial fluid from acutely inflamed joints, and true sodium urate; this differentiation is relatively simple using a polarizing microscope.

Chapter 18

Micro-Organisms

BACTERIA

Bacteria in sections are usually stained by slight modifications of those stains employed for smears, such modifications usually being designed to demonstrate the tissue constituents; for example, Gram-Weigert.

Gram-Positive Bacteria

Gram-positive bacteria may be stained by the simple acetone-Gram technique which will suffice for routine purposes, or by the Gram-Weigert method in which the Gram's stain is superimposed on an haematoxylin and eosin stain.

Gram's Method (Modified)

Special Reagent Required

Lugol's iodine

Iodine	1 g
Potassium iodide	2 g
Distilled water	to 100 ml

Dissolve the potassium iodide in 4–5 ml of water; dissolve the iodine in this. Dilute to 100 ml to make Lugol's iodine, or to 300 ml to make Gram's iodine.

Method

(1) Bring sections to water.

(2) Stain with 0.5 per cent aqueous methyl violet 6 B. for 1–3 minutes.

(3) Rinse with water.

(4) Pour on Lugol's iodine for 1–3 minutes (or Gram's iodine for 2 minutes).

(5) Differentiate rapidly with acetone (1–2 seconds) and wash immediately in running water.

(6) Counterstain with 1 per cent neutral red or safranine for 1 minute.

(7) Wash in water.

(8) Dehydrate, clear and mount.

Results

Gram-positive organisms	Blue-black
Other tissue structures	Shades of red

The Claudius modification of Gram's stain, which employs a half-saturated aqueous solution of picric acid as a mordant in place of the iodine solution, with a 0.1 per cent picric acid-aniline differentiator, is more complicated and less efficient that the acetone-Gram method, and is therefore not recommended.

Gram-Weigert Stain

Special Reagent Required

Aniline crystal violet

*Crystal violet	5 g
Absolute alcohol	10 ml
Aniline	2 ml
Distilled water	88 ml

This solution keeps well.

**Note.* – An explanation of the relationship of methyl, crystal and gentian violet to each other is thought worthwhile. These violet dyes consist of a *para*-rosanilin structure with a varying number of methyl groups attached, the number of which decide the actual shade of violet ranging from reddish to bluish violet. Methyl violet may be obtained as 2R, R, B, 2B, 6B, and in that range the number indicates the depth of colour, and the letter the shade, therefore 6B is a bluer shade than B, or 2B. Gentian violet is a mixture of ill-defined rosanilins and is in no way standardized. Crystal violet is an

hexamethyl *para*-rosanilin, and is sometimes known as methyl violet 10B. It is a definite bluish violet in colour, with a specific formula.

For these reasons, methyl violet of a specified shade or crystal violet should always be used to obtain standardized results.

Method

(1) Bring sections to water.
(2) Stain nuclei lightly with alum haematoxylin.
(3) Blue in tap-water.
(4) Stain in 2.5 per cent aqueous phloxine or eosin for 10 minutes at 56°C.
(5) Wash in water.
(6) Stain in aniline crystal violet for ½–1 hour.
(7) Rinse in water.
(8) Treat with Lugol's iodine *(see above)* for 1 minute.
(9) Blot with fine filter paper.
(10) Differentiate with equal parts of xylol and aniline until only bacteria and fibrin are blue-black (it is almost impossible to over-differentiate).
(11) Rinse in xylol to remove aniline, and mount in Canada balsam or D.P.X.

Results

Gram-positive organisms	Blue-black
Nuclei	Blue
Other tissue constituents	Shades of red

Gram-negative Bacteria

Gram-negative bacteria may usually be demonstrated by staining sections with Leishman or Giemsa stain in addition to 'control' sections stained by Gram's stain. Organisms present in the Leishman slide which are not Gram-positive are assumed to be Gram-negative. In practice this method works well.

There are a number of other methods described for the differentiation of Gram-positive and Gram-negative bacteria, but of these only the following has given consistent results.

Ollett's Modification of Twort's Stain

This method (Ollett, 1951) is simple and gives reasonable contrast

between Gram-positive bacteria, Gram-negative bacteria and tissues. The light green in Twort's stain is replaced by fast green F.C.F. to avoid fading.

Special Reagent Required

Modified Twort's stain

0.2 per cent alcoholic neutral red (C.1. No. 825) . .	90 ml
0.2 per cent alcoholic fast green F.C.F.	10 ml

For use, dilute one volume of the above stock solution with three volumes of distilled water.

The pH of this stain, like that of Twort's stain, is 4.9; it may depend on the dye samples used, but uniformity can be secured by diluting the stock solution in M/5 acetate buffer instead of distilled water.

The proportions of the dyes given are only approximate, and the optimum formula will depend on the dye content of the samples used. The stock alcoholic solution should be of reddish-magenta tint; too much green will weaken the red bacterial staining; the total dye concentration is less critical.

Method

(1) Fix material in 5 per cent formol saline, pass through the alcohols and embed in paraffin.
(2) Cut sections 3 μ in thickness.
(3) Bring sections to distilled water.
(4) Stain in aniline crystal violet for 3–5 minutes.
(5) Pour off stain and wash quickly in distilled water.
(6) Treat with Gram's iodine for 3 minutes.
(7) Pour off the iodine, wash quickly in distilled water and blot dry.
(8) Decolorize with 2 per cent acetic acid in absolute alcohol until no more colour comes away—the section should be a dirty straw colour at this stage.
(9) Wash quickly in distilled water.
(10) Counterstain in the modified Twort's neutral red-fast green stain, diluted 1 part with 3 parts of distilled water, or pH 4.9 buffer, for 5 minutes.
(11) Wash quickly in distilled water.

(12) Differentiate with 2 per cent acetic acid alcohol until no more red stain (neutral red) comes away (15–30 seconds).

(13) Clear in xylol and mount in D.P.X. or H.S.R.

Results

Nuclei	Red
Cytoplasm	Light green
Red blood corpuscles	Green
Gram-positive bacteria	Dark blue
Gram-negative bacteria	Pink

Actinomyces

Actinomyces, sometimes referred to as the 'ray fungus,' has a characteristic appearance, both to the naked eye and microscopically. The yellow pus present in actinomycosis contains numerous small granules (so-called 'sulphur granules'), which on section are seen to consist of a matted mass of branching mycelia; the mass may be surrounded by radiating club-shaped bodies. The filaments are sometimes segmented and give the appearance of cocci or short bacilli.

Staining Reactions

The mycelium is Gram-positive, P.A.S. negative, and non-acid-fast; the clubs are Gram-negative, P.A.S. positive and acid-fast (using Ziehl-Neelsen's stain).

Gridley's method (page 401) may be used to demonstrate these organisms, as may the Giesma or Leishman stains.

Acid-fast Bacilli

In spite of the multiplicity of methods now available, the author prefers the traditional Ziehl-Neelsen technique for the demonstration of *Mycobacterium tuberculosis* as a conventional method. If the equipment is available the fluorescent technique (*see* page 618) should be used. A satisfactory alternative is the use of night blue or Victoria blue R in place of basic fuchsin, with a safranine or tartrazine in Cellosolve counterstain; this gives blue bacilli.

Fixation

Formol saline gives excellent results, although any of the routine fixatives may be used with the possible exception of Carnoy's fluid,

which would tend to remove lipid material from the bacilli, and render them non-acid-fast.

Processing

Although the use of water-soluble wax has been recommended to avoid clearing reagents, for routine purposes this is unnecessary.

Ziehl-Neelsen Method

Special Reagent Required

Carbol-fuchsin

Basic fuchsin	1 g
Absolute alcohol	10 ml
5 per cent phenol (aqueous)	100 ml

Dissolve the basic fuchsin in the alcohol, then add the 5 per cent phenol.

Method

(1) Bring section to water.

(2) Stain in hot carbol-fuchsin, *either* in a Coplin jar in a 56°C oven for 30 minutes, *or* by covering section with a small square of filter paper (to avoid precipitate on sections), flooding the slide with stain, heating until the stain steams and leaving for 10 minutes.

(3) Wash in water to remove excess stain.

(4) Differentiate in 3 per cent hydrochloric acid in 70 per cent alcohol until tissue is a very pale pink colour when washed in water (approximately 5–10 minutes).

(5) Wash in water.

(6) Counterstain lightly in 0.1 per cent methylene blue for 10–15 seconds (if too heavy a counterstain is used, bacilli may be difficult to find).

(7) Wash in water.

(8) Dehydrate, clear, and mount in H.S.R. or D.P.X.

Results

Acid-fast bacilli	Red
Nuclei	Blue
Other tissue constituents	Pale blue

Note. – In a well-stained section tubercle bacilli should be clearly visible when using a 4 mm objective.

Myco. leprae

Since this organism is not so acid-fast as *Myco. tuberculosiis,* either a 1 per cent acid alcohol should be used to differentiate in the above technique, or the Wade-Fite method should be employed.

Wade-Fite Technique for Myco. leprae in Paraffin Sections (Modified)

According to the originators of this method, the bacilli are protected from extraction of their lipids during the deparaffinization, but Azulay and Andrade (1954) suggest that this new technique is not exclusively one of protection of the acid-fastness of *Myco. leprae* in the lesions, but that it also has the property of restoring acid-fastness which has been lost in the processing.

Method

(1) Deparaffinize sections with a mixture of equal parts of liquid petrolatum and rectified turpentine.
(2) Blot with filter paper until of semi-dry appearance.
(3) Wash in water for 5 minutes.
(4) Stain in carbol-fuchsin for 25–30 minutes at room temperature.
(5) Wash in water, and blot with filter paper.
(6) Decolorize with 10 per cent sulphuric acid.
(7) Wash in water
(8) Stain with alum haematoxylin for 5–10 minutes.
(9) Wash in water for 10 minutes.
(10) Blot with filter paper and place in an incubator at 56°C to dry.
(11) Clear in xylol and mount in Canada balsam or D.P.X.

Results

Myco. leprae	Red
Nuclei	Blue

SPIROCHAETES

In Fluids

In fresh fluid material, such as exudates, spirochaetes are best demonstrated by dark-ground illumination or phase contrast. Smears may be stained with dilute Giemsa (1 drop to 1 ml of distilled water) if

heat is applied, and 5–10 changes of stain made over a period of 10–15 minutes. The addition of 1 or 2 drops of 0.1 per cent sodium carbonate to 100 ml of the staining solution sometimes increases the intensity of staining.

The traditional and better method of demonstrating spirochaetes in smears is that of Fontana, modified by Hage.

Hage-Fontana Method

Special Reagents Required

(1) *Fixative (Ruge's fluid)*

Acetic acid	1 ml
Formalin	20 ml
Distilled water	to 100 ml

(2) *Mordant*

Phenol	1 g
Tannic acid	5 g
Distilled water	to 100 ml

Method

(1) Make film and allow to dry in the air.
(2) Pour on fixative and leave for 1 minute.
(3) Wash in running water for 10 seconds.
(4) Pour on mordant, heat gently until it steams, and leave for 30 seconds.
(5) Wash in running water for 20 seconds.
(6) Rinse in distilled water.
(7) Flood slide with 0.5 per cent silver nitrate; add 1 drop of concentrated ammonia; heat until steam rises and leave for 20 seconds.
(8) Rinse in distilled water.
(9) Blot dry and examine.

Results

Spirochaetes	Black
Cells and general background	Shades of yellow

In Tissue

Spirochaetes in blocks of tissue are best demonstrated by Levaditi's method.

Levaditi's Method

Special Reagent Required

Reducing fluid

Pyrogallic acid	4 g
Formalin	5 ml
Distilled water	to 100 ml

Method

(1) Fix small thin pieces (1 mm in thickness) of tissue in 10 per cent formol saline for 24 hours.

(2) Wash for 1 hour in water, and then place in 96 per cent alcohol for 24 hours.

(3) Transfer to 1.5 per cent silver nitrate in a dark bottle for 3 days at 37°C.

(4) Wash in distilled water for 30 minutes.

(5) Place in reducing fluid for 48 hours at room temperature in the dark.

(6) Wash well in water, dehydrate, clear and embed. Cut thin sections and mount in the usual way.

(7) Remove paraffin from sections and mount in Canada balsam or D.P.X.

Results

Spirochaetes	Black
Tissue	Shades of yellow

Paraffin Sections

Warthin-Faulkner Method

The Warthin-Faulkner method is the only one for use on sections of routinely fixed paraffin-embedded tissue which gives reasonably reliable results.

Fixation

Formol saline should be employed for fixation.

Special Reagent Required

Stock gelatin (for use with developer solution)

Gelatin	5 g
Walpole's sodium acetate–acetic acid buffer (pH 3.6) (page 170)	to 100 ml

Add 1 ml of 1:10,000 merthiolate to prevent the growth of moulds.

Method

(1) Bring paraffin sections to water.

(2) Wash in 1:25 dilution of stock pH 3.6 acetate–acetic acid buffer (page 170) for 5 minutes.

(3) Impregnate with 1 per cent silver nitrate in dilute pH 3.6 buffer (as in (2)) for 45 minutes at 60°C.

(4) While sections are in silver nitrate prepare the following developer solution. Mix 15 ml of the stock gelatin solution and 3 ml of 2 per cent silver nitrate in pH 3.6 acetate–acetic acid buffer, both previously heated to 60°C. Add 1 ml of fresh 3 per cent hydroquinone in pH 3.6 acetate–acetic acid buffer. Mix and use immediately.

(5) Place slides on staining rack and flood with freshly prepared developer. When sections become brown to greyish-yellow, and the developer turns brownish-black, rinse with warm water (55–60°C), and then with distilled water.

(6) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Spirochaetes	Black
Tissue elements	Shades of yellow

Note. – Over-development will give precipitation and thickened black spirochaetes, and should be overcome by carrying through 2 or 3 sections with varying times of developments.

Sections may be toned in gold chloride, and counterstained if desired.

FUNGI

Most fungi in sections may be demonstrated, at least in part, by Gram's method, the branching mycelium (hyphae) being Gram-positive and the spores (conidia) Gram-negative. Gridley's method is the best for their selective demonstration. Since practically all fungi are P.A.S. positive, these techniques may be used for their general demonstration. The standard technique gives good results, the fluorescent method of Culling and Vassar (1961) gives bright yellow fungi on a dark background, especially if tissue sections are prestained in Weigert's haematoxylin for 2 minutes before treating with periodic acid *(see Figure 32.6).* Gomori's silver methenamine technique gives excellent results for photography.

Skin scrapings may be smeared on to slides, fixed in alcohol, and stained by the above techniques.

Gridley's Method

Method

(1) Bring sections to water.
(2) Oxidize in 4 per cent chromic acid for 1 hour.
(3) Wash in running water for 5 minutes.
(4) Transfer to Schiff reagent (page 251) for 15–20 minutes.
(5) Take through 2 sulphite rinses (as in Feulgen technique, page 252).
(6) Wash in running water for 15 minutes.
(7) Stain for 15–20 minutes in Gomori's aldehyde fuchsin (*see* page 420).
(8) Rinse in 95 per cent alcohol, and wash in running water for 5–10 minutes.
(9) Counterstain in 0.25 per cent metanil yellow in 0.25 per cent acetic acid for 2–5 minutes.
(10) Wash in water.
(11) Dehydrate, clear, and mount in D.P.X.

Results

Hyphae	Deep blue
Conidia	Rose to purple
Elastin and mucin	Deep blue
Yeast capsules	Deep purple
General background	Yellow

RICKETTSIAE AND INCLUSION BODIES

Rickettsiae and inclusion bodies may be stained by Giesma, using a pH 7.2 buffer as a diluent, or alternatively by Macchiavello's method. Most of the inclusion bodies may be demonstrated with Lendrum's phloxine-tartrazine method which is given on page 484. This will not stain Negri bodies, which may be stained with Giemsa, Macchiavello or Mann's methyl blue-eosin. D.N.A. inclusions may be demonstrated by the Feulgen techniques.

Macchiavello's Technique (Modified)

Method

(1) Bring paraffin sections to water.
(2) Stain in 0.25 per cent aqueous basic fuchsin for 30 minutes.
(3) Differentiate rapidly (about 3 seconds) in 0.5 per cent citric acid.
(4) Wash in tap-water.
(5) Counterstain for 15–30 seconds in 1 per cent methylene blue.
(6) Wash in water.
(7) Dehydrate, clear, and mount in Canada blasam or D.P.X.

Results

Rickettsiae, inclusion bodies	Red
Tissue elements	Blue

Mann's Methyl Blue-eosin Method

Solution Required

Staining solution

1 per cent aqueous methyl blue	35 ml
1 per cent aqueous eosin	45 ml
Distilled water	100 ml

This solution keeps well.

Method

(1) Tissues are fixed in Zenker or formol saline.
(2) Bring paraffin sections to water.

(3) Stain for 8–24 hours (dependent on the age of the stain) in a Coplin jar.

(4) Wash in water.

(5) Differentiate in absolute alcohol to which 2–3 drops of N/1 sodium hydroxide per 100 ml have been added.

(6) Wash rapidly in several changes of absolute alcohol.

(7) Clear in xylol and mount in neutral mounting medium or D.P.X.

Results

Negri bodies, red blood cells, oxyphil cytoplasm and granules	Red
Nuclei and other tissue elements	Blue

Chapter 19

Tissues Requiring Special Treatment or Techniques

BONE MARROW AND BLOOD-FORMING ORGANS

Specimens of these may be received in four forms, as follows.

(1) *Films* of loose masses of bone marrow, which are made and stained by the usual methods for blood films.

(2) *Impression smears* of bone marrow or spleen, made at operation or autopsy by pressing a small piece of unfixed tissue against a slide, are fixed wet in Schaudinn's fluid (page 53) and stained in the same way as blood films.

(3) *Needle biopsies* are fixed in Susa which causes the particles to aggregate or clot, or better and more rapidly by Cappell, Hutchison and Harvey-Smith's method. These biopsies rarely need to be decalcified.

(4) *Bone marrow, spleen, and so on.* After fixation in Helly's fluid, and washing to remove dichromate, bone marrow may, if small bone spicules are present, be decalcified in Gooding and Stewart's fluid (page 66); 6–12 hours will usually be sufficient.

Cappell, Hutchison and Harvey-Smith Method

This method gives excellent results with needle biopsies.

Method

(1) Fix fragments of bone marrow in the following freshly prepared fixative in a 500 ml Erlenmeyer flask.

Zenker's fluid	45 ml
Formalin	5 ml
Formol saline	50 ml

Leave for 20–30 minutes; more than 30 minutes will cause shrinkage and impair subsequent staining.

(2) When fixation is adequate add 400 ml of distilled water to flask, mix, and allow the fragments to settle. (When the fixative is diluted the blood proteins precipitated remain as a flocculent suspension and are removed with the supernatant.)

(3) Remove the supernatant fluid, using a Pasteur pipette with an upturned end, and a water pump.

(4) Wash the fragments in 2 changes of distilled water; leave in fresh distilled water for 10 minutes.

(5) Syphon off the water, and add 100 ml of 60 per cent alcohol; leave for 15 minutes. The remains of the protein precipitant are removed at this stage leaving only the marrow fragments.

(6) Syphon off the 60 per cent alcohol and replace with 100 ml of 70 per cent alcohol for 15 minutes. Marrow particles may be left in this solution for 12–24 hours without damage.

(7) Replace with 80 per cent alcohol for 15 minutes.

(8) Replace with 96 per cent alcohol for 15 minutes.

(9) Replace with 2 changes of absolute alcohol for 15 minutes each.

(10) Replace with toluol for 15 minutes.

(11) Using a pipette, transfer marrow particles to a flat-bottomed test-tube and add molten paraffin wax.

(12) Impregnate in paraffin wax for 2 hours, with 2 changes.

(13) Cool wax. To free the block, use a hot wire or break the tube. Do not heat the tube as this will cause shrinkage of the cells.

(14) Cut sections without preliminary trimming since the fragments will be at the surface of the block.

Staining Methods

Difficulties arising in staining sections with the Romanowsky techniques are usually due to the pH of the section being incorrect, and for this reason it is preferable, as a routine, to incubate such sections in a phosphate buffer of pH 6.8 for 30 minutes at 56 or 37°C, after removing the mercury pigment with iodine-sodium thiosulphate treatment.

Leishman's Stain

Solution Required

Leishman's stain (powder)	0.15 g
Pure absolute methyl alcohol	100 ml

Dissolve the stain in the alcohol by grinding it up in a pestle and mortar with some of the alcohol, then pour the alcohol into a stock bottle. Add more alcohol and repeat the procedure until all the powder is dissolved. Leave overnight in a 37°C incubator. Dilute a few ml of the prepared stain with distilled water and pour on to a clean slide; unless a metallic scum forms on the surface the stain is not fit for use.

Method

(1) Bring sections to water.
(2) Remove mercury pigment (if necessary).
(3) Incubate in pH 6.8 buffer at 56°C for 30 minutes.
(4) Stain section in Leishman's stain, diluted 1:3 with pH 6.8 buffer for 30–60 minutes (a more dilute stain for a longer period will give an even better result).
(5) Rinse in pH buffer.
(6) Differentiate in 1:10,000 acetic acid to remove excess blue stain. Stop differentiation by rinsing in pH 6.8 buffer, and control staining under the microscope.
(7) When differentiation is complete, blot the section almost dry with fine filter paper.
(8) Flood with xylol. If section does does not clear completely, blot and flood with fresh xylol. Examine in immersion oil with 2 nim objective to ensure that differentiation is complete.
(9) Flood with xylol to remove immersion oil.
(10) Mount in D.P.X. or neutral mountant.

Results

Nuclei	Bluish-red
Acidophil granules	Pink to red
Basophil granules	Blue
Red blood cells	Salmon-pink

Maximow's Stain

Maximow's stain gives better nuclear staining than Leishman's method.

Special Reagents Required

(1) *Stock stains*

Solution A	1:1,000 aqueous solution of eosin
Solution B	1:1,000 aqueous solution of azure II

(2) *Staining solution.* – To 100 ml of pH 6.8 buffer, add 10 ml of solution A, then add 10 ml of solution B. This stain should be used in a Coplin jar since it tends to precipitate.

Method

(1) Bring sections to water (removing mercury pigment if necessary).
(2) Stain nuclei with Ehrlich's haematoxylin for 5–10 minutes; dip into acid alcohol, and blue.
(3) Incubate in pH 6.8 buffer *(see above).*
(4) Stain for 30 minutes–24 hours in eosin-azure mixture.
(5) Differentiate, clear and mount as described above for Leishman's stain (Stages 5–10).

Results

The results of the Maximow staining technique are the same as those of Leishman's stain, but with more definite blue nuclear staining.

May-Grünwald-Giesma Technique

The May-Grünwald-Giesma technique is excellent for the demonstration of bone-marrow smears.

Method

(1) Bring sections to water (removing mercury pigment if necessary).
(2) Treat with pH 6.8 buffer for 30 minutes at 37 or 56°C.
(3) Stain with May-Grünwald stain diluted 1:5 with pH 6.8 buffer for 10–15 minutes.
(4) Rinse in buffer solution.
(5) Stain in Giesma's stain, diluted 1:10 with pH 6.8 buffer for 1–12 hours.
(6) Wash in buffer.

(7) Differentiate in glycerin–ether until excess blue is removed, controlling under the microscope.

(8) Rinse in buffer and dehydrate. Clear in xylol and mount in D.P.X. or neutral mounting medium.

Results

The results of the above method are similar to those of the Leishman technique.

Other Methods

Acridine orange (page 612) or *Unna-Pappenheim's stain* (page 254). – These stains may also be used, especially for plasma cells.

Oxidase and peroxidase. – These are discussed in the chapter on Enzymes, pages 315–349.

Lipids. – (*See* pages 351–376).

Collagen. – (*See* pages 414–418).

Reticulin. – (*See* pages 425–428).

BONE

Decalcification

Excellent sections of bone can be prepared after the removal of calcium salts (*see* the chapter on Decalcification, page 63). Material should be embedded in celloidin for the best results, but paraffin embedding can be used with reasonable results.

Un-decalcified Sections

In cases of osteomalacia, or rickets, the demonstration of the osteoid seam may be impaired by over-decalcification. In such cases it is best to try to cut an un-decalcified frozen section. Bone (preferably rib) is fixed in formol saline for 1–3 hours or longer before freezing and cutting. The section is then stained by von Kossa's silver method with van Gieson's counterstain.

Ground-bone Sections

Ground-bone sections of un-decalcified bone may be prepared as follows:

(1) Cut thin slice of bone with a fine saw.

(2) Rub both sides of the bone on a glass plate with moistened carborundum powder (or on a carborundum stone).

(3) When the slice of bone is as thin and as even as possible (without breaking), wash well in water, dehydrate in alcohol and allow to dry.

(4) Mount, without staining, in Canada balsam under a coverslip.

Staining Methods

Schmorl's Picro-thionin Method

This method gives more reliable results than the thionin-phosphotungstic acid method next described. It is dependent on the precipitation of stain in the bone canaliculi, the picric acid combining with thionin to form precipitated picrates.

Fixation

Müller's fixative or formol saline should be used, but not a mercury-containing fixative.

Method

(1) Wash sections (celloidin or frozen) in distilled water for 10 minutes.

(2) Stain in ½ saturated aqueous thionin* for 5–15 minutes to which 1 drop of 0.880 ammonia per 100 ml of stain has been added. This solution is unstable and slowly deteriorates after the ammonia has been added.

(3) Rinse in water.

(4) Treat with saturated aqueous picric acid for ½–1 minute.

(5) Rinse in water.

(6) Differentiate in 70 per cent alcohol until clouds of stain cease to pour out; this usually takes 5–10 minutes, but may require up to 30 minutes.

(7) Dehydrate rapidly, clear, and mount in Canada balsam.

*The original method specifies Nicolle's carbol-thionin (10 ml of 0.25 per cent thionin in 50 per cent alcohol; 100 ml of 1 per cent phenol), but the aqueous solution gives more consistent results. The pH of the staining solution is critical and poor results can usually be traced to the amount of, or lack of, ammonia in it.

Results

Ground substance	Yellow to brown
Lacunae and canaliculi	Dark brown to black
Cells	Red
Ground substance of cartilage	Purple

Schmorl's Thionin-phosphotungstic Acid Method

This method is said to give better results with specimens of bone from children.

Fixation

Fixation should be in Müller's fixative or formol saline. Mercury-containing fixatives should not be used.

Method

(1) Bring sections to distilled water; leave for 10 minutes.

(2) Stain in alkaline thionin, as described above, for 10–30 minutes.

(3) Rinse rapidly in water.

(4) Transfer to saturated aqueous phosphotungstic or phosphomolybdic acid (with glass needle) for a few seconds (sections become blue, green or grey).

(5) Wash in water until sections are sky blue in colour, usually 5–10 minutes.

(6) Treat with 10 per cent ammonia in distilled water for 3–5 minutes to fix the colour.

(7) Transfer to 90 per cent alcohol (several changes) to remove ammonia.

(8) Examine under microscope; if the ground substance is too deeply stained, treat with 1 per cent acid-alcohol, and wash well.

(9) Dehydrate, clear and mount.

Results

Ground substance	Pale green to blue
Lacunae canaliculi and bone fibrillae	Blue to blue-black

CARTILAGE

Cartilage may be stained by routine stains, the picro-thionin method described above, or metachromatically with safranine (which stains it yellow), methyl violet or toluidine blue. Calcium in cartilage is demonstrated by von Kossa's method (*see* page 471).

CONNECTIVE TISSUE

A large number of the methods used for the demonstration and identification of connective-tissue elements require selective differentiation; a short explanation of its composition is therefore given.

Classification

Connective tissue may be of various kinds, usually consisting of fibres and cells divided into the following types:

(1) *Areolar tissue* is the loose connective tissue connecting the skin to underlying tissues, filling spaces between organs, and sometimes forming membranes. It is variously composed of collagen fibres, elastic fibres and connective-tissue cells.

(2) *White fibrous tissue* is found where great strength is required, such as tendons and ligaments; *white fibrous membranes* occur as periosteum, dura and cornea. These membranes usually consist almost entirely of bundles of collagen fibres.

(3) *Elastic tissue* is connective tissue in which elastic fibres predominate, and is found where strength and ability to stretch are required, for example, in blood-vessel walls and lung.

(4) *Reticular tissue* is found as the framework of lymph glands, spleen, and elsewhere. It is composed of reticulin fibres.

(5) *Adipose tissue* is peculiar in that it is composed mainly of cells instead of fibres. The cells, which are packed with lipid, have a nucleus which is usually flattened against the cell membrane. Such tissue, when treated with fat solvents (for example, during paraffin processing), appears as a series of empty rings.

(6) *Neuroglia* is the special connective tissue of the nervous system, and is dealt with on page 434.

Connective-tissue Elements

Connective tissue is composed of (1) ground substance, (2) fibres and (3) cells.

Ground Substance

Stretched preparations of fresh tissues which have been treated with silver nitrate show a dark brown background with light brown spaces indicating the presence of cells. This ground substance is thought to be mainly composed of mucopolysaccharide.

Fibres

There are three types of fibres: (1) white or collagen, (2) yellow or elastic, and (3) reticulin fibres.

White or collagen fibres. – These appear as bundles of fibres, which may be straight or wavy, and run in every direction. Although individual fibres do not branch, bundles of fibres may do so. Collagen, on boiling, yields gelatin. It swells in acetic acid, and is digested by pepsin. Collagen shows both positive form and positive intrinsic birefringence (*see* page 639) and these characteristics have been used as diagnostic criteria in fibro-osseous lesions of the jaw (Giansanti, 1970 *a* and *b*).

Yellow or elastic fibres. – Occurring singly and not in bundles, these fibres branch to join other fibres and are highly refractile. They are unaffected by boiling, acetic acid or pepsin, but may be digested with trypsin.

Reticulin fibres. – Easily distinguished from collagen fibres histologically, reticulin fibres are thought by some to be immature collagen fibres, but by others to be chemically and physically different. Their important points of difference are that collagen is anisotropic, slightly Gram-positive, and coloured brown to mauve by silver techniques, whereas reticulin is Gram-negative, isotropic when stained (but showing form birefringence when unstained, *see* page 639), and is coloured black by silver techniques.

Cells

The most important of the cells found in connective tissue are as follows.

Fibroblasts. – These are found in all connective tissue proper, and are thought to be concerned in the formation of collagen fibres.

Histiocytes. – Also known as macrophages, histiocytes are most numerous in areolar tissue. They act as scavengers, and take up effete cells and foreign bodies such as dye particles.

Mast cells. – These cells have coarse basophilic and metachromatic granules in their cytoplasm, and an indented nucleus. Although their function is still not completely clear, they are known to produce heparin (which being a sulphated mucopolysaccharide accounts for their metachromasy) and also the bulk of the histamine in the body. They also contain small quantities of 5 HT (5-hydroxytryptamine) or serotonin, which after formalin fixation (*see* page 467) will give a positive argentaffin reaction. They are best demonstrated by Czaba's Alcian blue-safranin method (page 419) or the aldehyde fuchsin technique (page 283).

Plasma cells. – These have a highly characteristic appearance. They are larger than a lymphocyte with a more abundant cytoplasm and an eccentric nucleus. The chromatin in the nucleus is aggregated into small masses around the periphery and is sometimes likened to a cart wheel. The cytoplasm stains a brilliant crimson with acridine orange or Unna-Pappenheim's stain, due to the high concentration of RNA. Under the electron microscope the cytoplasm is seen to contain a well-developed endoplasmic reticulum with a high concentration of ribosomes (*see Figure 1.3d).* They are now believed to be primarily responsible for the production of antibodies.

Staining Methods for Connective Tissue

The most commonly employed method for demonstrating connective tissue is that of van Gieson (page 219), either as a counterstain with iron haematoxylin, or following on the elastic fibre stains. Of the many methods available, Heidenhain's azan stain, or Lillie's allochrome method probably give the best and most consistent results, but this is largely a matter of personal reference, and both the Masson and Mallory trichrome techniques are very popular. Lendrum's acid-picro Mallory (page 475), Lison's Alcian-blue–chlorantine fast red (page 305) and Mallory's P.T.A. haematoxylin (for the central nervous system) may also be used. Techniques for the demonstration of elastic fibres and reticulin will be discussed separately.

Masson's Trichrome Technique

The tissues should be fixed in Bouin's or Zenker's fluid, formol-fixed sections should be mordanted in Zenker's fluid overnight.

Method

(1) Bring sections to water.

(2) Remove mercury pigment with iodine-sodium thiosulphate sequence if necessary.

(3) Stain in Weigert's iron haematoxylin (page 215) for 20–30 minutes, wash in water.

(4) Differentiate in 1 per cent acid-alcohol until only nuclei are stained.

(5) Wash in water until sections are blue.

(6) Stain for 5 minutes in 1 per cent ponceau 2 R in 1 per cent acetic acid.

(7) Rinse rapidly in distilled water.

(8) Mordant in 1 per cent aqueous phosphomolybdic acid for 5 minutes.

(9) Drain slide, and pour on 2.5 per cent aniline blue in 2.5 per cent acetic acid, or 2 per cent light green in 2 per cent acetic acid, for 5 minutes.*

(10) Differentiate in 1 per cent acetic acid for 1–2 minutes to remove excess blue or green.

(11) Dehydrate, clear and mount in Canada balsam or D.P.X.

Results

Nuclei	Blue black
Cytoplasm, muscle and acidophil granules	Red
*Collagen, cartilage, mucin and basophil granules	Blue or green *(see above)*

Mallory's Method (Crooke-Russell Modification)

Fix in Zenker's fluid, or mordant formol-fixed tissues in 5 per cent acetic acid in 2.5 per cent potassium dichromate for 12–18 hours.

Special Reagent Required

Aniline blue–orange G mixture

Aniline blue	0.5 g
Orange G	2.0 g
Phosphotungstic acid	1.0 g
Distilled water	to 100 ml

Method

(1) Bring sections to water and wash for 10 minutes.

(2) Treat with iodine-sodium thiosulphate sequence (this mordants and removes mercury precipitate).

(3) Wash well in water.

(4) Stain nuclei in Ehrlich's haematoxylin. Wash, differentiate in acid-alcohol, and blue in tap-water.

(5) Stain for 5 minutes in 1 per cent aqueous acid fuchsin.

(6) Wash in tap-water for 30 seconds or longer, until collagen is almost colourless, then rinse in distilled water.

(7) Transfer to aniline blue–orange G mixture for 20 minutes.

(8) Wash in running tap-water for 2–5 minutes.

(9) Dehydrate and differentiate in 95 per cent alcohol, followed by absolute alcohol.

(10) Clear in xylol, mount in Canada balsam or D.P.X.

Results

Collagen, reticulin fibres and basophil granules	Deep blue
Cartilage, mucin, amyloid	Lighter blue
Fibrin, glia fibres, and acidophil granules	Red
Red blood cells and myelin	Orange vermilion
Nuclei	Blue-purple

Heidenhain's Azan Stain

Tissues may be fixed in Zenker's, Bouin's or Carnoy's fluid, or formol saline.

Special Reagents Required

(1) *Azocarmine B*

Azocarmine B	0.5 g
Glacial acetic acid	1.0 ml
Distilled water to	100 ml

Filter before use

(2) *Aniline blue–orange G–acetic*

Aniline blue	0.5 g
Orange G	2.0 g

Glacial acetic acid	8.0 ml
Distilled water to	100 ml

Warm to dissolve and filter when cold. Dilute 1:3 with distilled water before use.

Method

(1) Bring sections to water.

(2) Post-mordant in 2.5 per cent potassium dichromate in 5 per cent acetic acid for 15 minutes, unless fixed in Zenker's fluid.

(3) Wash in running water for 5 minutes.

(4) Stain in azocarmine B for 45–60 minutes at 56–60°C (10 minutes*).

(5) Wash in distilled water.

(6) Differentiate in 0.1 per cent aniline in 95 per cent alcohol for 50–60 minutes (0.5 per cent aniline for 15 minutes*).

(7) Rinse in 1 per cent acetic acid in 95 per cent alcohol to stop differentiation, checking with microxcope to ensure that only nuclei and red blood cells are stained red.

(8) Mordant in 5 per cent phosphotungstic acid for 1–3 hours (45 minutes*).

(9) Stain in diluted aniline blue–orange G–acetic for 1–3 hours (20–40 minutes*). Use stain undiluted for pituitary or pancreas.

(10) Wash quickly in water.

(11) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Nuclei and red blood cells	Red
Collagen, reticulin and basophil granules	Deep blue
Mucin	Blue
Muscle and acidophil granules	Orange to red
Neuroglia	Pale red

*Times given in brackets may be used for a shortened version of the technique. The colours may not be quite as intense as those in a section given the full staining times.

Lillie's Allochrome Method

Method

(1) Bring sections to water.
(2) Remove mercury precipitate if necessary.
(3) Oxidize with 1 per cent aqueous periodic acid for 10 minutes.
(4) Wash in running water for 5 minutes.
(5) Treat with Schiff reagent for 10 minutes.
(6) Treat with 3 changes of 0.5 per cent sodium metabisulphite.
(7) Wash in running water for 5 minutes.
(8) Stain for 2 minutes in Weigert's iron haematoxylin (page 215).
(9) Wash in running water for 4 minutes.
(10) Stain in 0.04 per cent methyl blue (or aniline blue) in saturated aqueous picric acid for 6 minutes.
(11) Dehydrate, and differentiate in 2 changes each of 95 per cent and absolute alcohol.
(12) Clear in xylol and mount in D.P.X.

Results

Nuclei	Grey to black
Cytoplasm and muscle cells	Greyish-green to greenish-yellow
Collagen and most reticulin	Blue
Muscle reticulin and epithelial basement membranes	Reddish-purple
P.A.S. positive material	Red
Fibrin	Red to orange
Amyloid	Purplish-red to pink

Luxol Fast Blue G for Selective Staining of Collagen and Elastin (Salthouse, 1965)

Salthouse comments that it is important to use Luxol fast blue G (solvent blue 34 – Matheson, Coleman and Bell) and *not Luxol fast blue MBSN* (solvent blue 38).

Method

(1) Bring formalin-fixed paraffin sections to water.*

(2) Leave sections for 1 minute in each of two changes of methanol.

(3) Stain in a saturated solution (0.9–1 per cent) of Luxol fast blue G in methanol for 3 minutes.

(4) Rinse rapidly in two changes of methanol.

(5) Counterstain in 0.1 per cent nuclear fast red in 5 per cent aqueous aluminium sulphate, rinse in water (this step is optional).

(6) Dehydrate, clear and mount in synthetic resin.

Results

Elastin	Deep blue
Collagen	Blue
Myelin and phospholipids	Unstained (these are stained if dye is dissolved in ethanol, isopropanol or isobutanol)

Csaba's Mast Cell Stain (1969)

Special Reagents Required

Alcian blue–safranin

Alcian blue	0.36 g
Safranin	0.18 g
Ferric ammonium sulphate	0.48 g

Walpole's M/2 acetate-Hcl buffer pH 1.42 (page 168) 100 ml

Dissolve the dyes and iron alum in the buffer solution.

Method

(1) Bring sections to water.

(2) Stain in Alcian blue-safranin for 10–20 minutes.

(3) Rinse in water.

(4) Dehydrate in tertiary butyl alcohol, clear in xylol, and mount in synthetic resin.

*Methylation (page 290) at 25°C for 12 hours gives increased depth of staining.

Results

Young mast cell granules (with preponderance of biogenic amines)	Blue
Mature mast cell granules (with preponderance of heparin)	Red

Elastic Fibres

Of the many methods available to demonstrate elastic fibres, Gomori's aldehyde fuchsin method is the most reliable, but Verhoeff's is probably the easiest to prepare and the most permanent (*see* Frontispiece). Wergert's resorcin-fuchsin (or Hart's modification) gives excellent results provided a good batch of basic fuchsin is available.

Gomori's Aldehyde Fuchsin Method for Elastic Fibres

Fixation in formol saline gives the best results in Gomori's aldehyde fuchsin method, although most fixatives are suitable.

Solution Required

Staining solution

Basic fuchsin	1 g
70 per cent alcohol	200 ml
Concentrated hydrochloric acid	2 ml
Paraldehyde (fresh)	2 ml

The fuchsin is dissolved in the alcohol, and the hydrochloric acid and paraldehyde are then added. The mixture should be shaken well and allowed to stand at room temperature until it is a deep purple (24–48 hours), it is then ready to use. The solution should be stored in the refrigerator, where it will maintain its selective staining properties for 2–3 months. The time of staining needs to be increased as the stain ages.

Method

(1) Bring sections to water.
(2) Treat in Lugol's iodine (page 391) for 20–30 minutes.
(3) Transfer to 5 per cent sodium thiosulphate for 3–5 minutes.
(4) Wash in running tap-water for 3 minutes.
(5) Rinse in 90 per cent alcohol.

(6) Stain for 5–10 minutes. This is a progressive stain and various tissue elements may be stained by increasing the period of time at this stage *(see below)*.

(7) Rinse in 90 per cent alcohol.

(8) Counterstain with light green, orange G, or Masson's trichrome stain (page 414).

(9) Dehydrate, clear and mount.

Result

Elastic tissue	Deep purple

The following tissue elements are stained deep purple if they are immersed in the stain for the periods of time as stated: mast-cell granules (5–10 minutes); mucin and gastric chief cells (10–30 minutes); β cells of the pancreas (15–30 minutes); β cells of the pituitary (30–120 minutes).

Verhoeff's Method

Solution required

Staining solution

Stock 5 per cent alcoholic haematoxylin	20 ml
10 per cent ferric chloride ;	8 ml
Verhoeff's iodine (iodine 2 g, pot. iodide 4 g, water 100 ml)	8 ml

This should be freshly prepared, and the solutions added to a flask in the order given.

Method

(1)) Bring sections to water (mercury precipitates are removed during the staining process, but the iodine-sodium thiosulphate treatment acts as a mordant and should be employed).

(2) Stain for 15–30 minutes until sections are jet-black.

(3) Differentiate in 2 per cent ferric chloride until elastic fibres are clearly seen; rinse in water and examine under the low power of a microscope. If over-differentiated, sections may be restained.

(4) Wash in water, then in 95 per cent alcohol to remove iodine staining.

(5) Wash in water for 5 minutes.

(6) Counterstain with van Gieson's stain (page 219) for 3 minutes.

(7) Dehydrate, clear and mount.

Results

Elastic fibres and nuclei	Black to blue-black
Cytoplasm and muscle	Yellow
Collagen	Red

Weigert's Resorcin-fuchsin for Elastic Fibres

The Weigert resorcin-fuchsin method works well after alcohol or formalin fixation. Tissue fixed in Zenker's fluid should be stained by Hart's modification.

Solution required

Staining solution

Basic fuchsin	2 g
Resorcin	4 g
Distilled water	to 200 ml

Place in a porcelain bowl, bring to the boil, add slowly 25 ml of 30 per cent ferric chloride, stir, and boil for a further 2–5 minutes. Cool and filter. Discard the filtrate and place the paper (with precipitate) into the original (unwashed) flask which should have been dried. Add 200 ml of 95 per cent alcohol, and heat gently in a water bath or on a hot plate until precipitate has dissolved. Remove filter paper, add 4 ml of concentrated hydrochloric acid and filter. Make up volume to 200 ml by pouring fresh 95 per cent alcohol through the used filter paper. This stain deteriorates on keeping.

Note. – In place of 2 g of basic fuchsin, 1 g each of basic fuchsin and crystal violet may be used and give deep blue-green elastic fibres, *or*, 2 g of crystal violet plus 2 g dextrin (known as Sheridan's stain, page 423), which will give green elastic fibres.

Method

(1) Bring sections to water.

(2) Stain for 20–60 mintes.

(3) Wash in 95 per cent alcohol.

(4) Examine under microscope; if staining is diffuse, differentiate with 1 per cent acid-alcohol.

(5) Wash in tap-water.

(6) Counterstain with haematoxylin and van Gieson if desired or with 1 per cent neutral red.

(7) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Result

Elastic fibres . . . . . Dark blue-black

Hart's Modification of Weigert's Elastic Stain

Solution Required

Staining solution. – Prepared Weigert's stain *(see above)* is diluted 5–20 per cent (batches of stain vary in strength) with 1 per cent acid-alcohol. This is more stable that Weigert's stain.

Method

(1) Bring sections to alcohol.

(2) Stain overnight at room temperature.

(3) Differentiate in acid-alcohol and wash in water.

(4) Counterstain with haematoxylin and van Gieson or 1 per cent neutral red.

(5) Dehydrate, clear and mount.

Result

Elastic tissue . . . . . Blue-black

Sheridan's Resorcin-crystal Violet Method for Elastic Fibres

This modification of Weigert's elastic stain (using crystal violet instead of basic fuchsin) is popular in many laboratories.

Solution Required

Staining solution. – The solution is prepared as for Weigert's stain using 2 g of crystal violet and 2 g of dextrin instead of 2 g of basic fuchsin. Alternatively, the prepared stain may be

purchased, in which case it should be tested on receipt to ensure that it is of the required standard.

Method

(1) Bring sections to water.
(2) Oxidize with 0.5 per cent potassium permanganate for 5 minutes.
(3) Wash in water.
(4) Rinse in distilled water.
(5) Bleach with 5 per cent oxalic acid for 5 minutes.
(6) Rinse in distilled water.
(7) Wash in tap-water for 2–3 minutes, followed by several changes of distilled water.
(8) Stain in Sheridan's stain for 1 hour at 37° or 56°C or, preferably, overnight at room temperature.
(9) Differentiate in absolute alcohol for 5–10 minutes until the background is clear.
(10) Wash in tap-water for 10 minutes.
(11) Counterstain nuclei in 1 per cent neutral red for 2 minutes.
(12) Rinse rapidly in tap-water, and blot.
(13) Rinse for a second or two in absolute alcohol to differentiate the neutral red, and then blot: too long in alcohol will result in pale red nuclei, and too short a time in a red background.
(14) Flood with 0.5 per cent aqueous picric acid for a few seconds.
(15) Dip into tap-water and blot.
(16) Flood with absolute alcohol and blot immediately.
(17) Clear in xylol and mount in Canada balsam or D.P.X.

Result

Elastic fibres	Blue-green
Nuclei	Clear red
Other tissue elements	Yellow

Taenzer-Unna Orcein Method

The Taenzer-Unna orcein method, which is reputed to be specific for elastic fibres, occasionally gives indifferent results. Synthetic orcein will be found to give better results than the natural dye. The staining solution is 1 per cent orcein in 1 per cent hydrochloric acid in 70 per cent alcohol, and should be kept in a refrigerator. Sections are stained

for 30–60 minutes at 56°C. (or overnight at room temperature), washed, and may be counterstained with haematoxylin and van Gieson stain.

Reticulin Fibres

Reticulin fibres are best demonstrated by silver impregnation. Of the methods described below, that of Gomori is recommended for its simplicity and reliability. It rarely, if ever, removes the section from the slide. Sections to be treated with silver impregnation should always be fixed to the slide with starch paste or glycerin albumin.

Gomori's Silver Impregnation Method for Reticulin Fibres

Solution Required

Silver solution (modified). – To 4 parts of 10 per cent aqueous silver nitrate, in a test-tube or flask, add 1 part of 10 per cent potassium hydroxide, which causes the silver to deposit. Having marked the volume of the fluid with a grease pencil, remove the supernatant fluid and wash the deposit several times with distilled water, finally making it up to the original volume, thus giving a cleaner background. Add strong ammonia (.880) drop by drop until the deposit is just dissolved. Add again 10 per cent silver nitrate drop by drop until the solution takes on a faint sheen. Make up this solution to twice its volume with distilled water. Although this solution is best freshly prepared, it can be used for 24–36 hours.

Method

(1) Bring sections to water.
(2) Oxidize with 1 per cent potassium permanganate for 1–2 minutes and rinse in tap-water.
(3) Decolorize with 3 per cent potassium metabisulphite for 1 minute; rinse in tap-water.
(4) Sensitize in 2 per cent iron alum for 1 minute.
(5) Wash in tap-water for 2–3 minutes, and then rinse in 2–3 changes of distilled water.
(6) Impregnate in silver solution for 3 minutes.
(7) Rinse quickly in distilled water.
(8) Reduce in 10 per cent formalin in tap-water for 3 minutes.
(9) Wash in running water for 2–3 minutes, and rinse in distilled water.

(10) Tone in 1:500 gold chloride (yellow) for 5–15 minutes.
(11) Rinse in distilled water.
(12) Reduce toning in 3 per cent potassium metabisulphite for 1 minute.
(13) Rinse in distilled water.
(14) Fix in 3 per cent sodium thiosulphate for 1 minute.
(15) Wash in water.
(16) Dehydrate, clear, and mount in H.S.R. or D.P.X.

Results

Reticulin fibres	Black
Collagen fibres	Purple
Nuclei and cytoplasm , ...	Shades of grey

Foot's Silver Impregnation Method for Reticulin

Solution Required

Silver solution. – To 10 ml of 10 per cent aqueous silver nitrate, add 40 ml of 5 per cent anhydrous sodium carbonate. When the precipitate has settled, remove the supernatant and wash the deposit several times with distilled water. Add strong ammonia (.880) drop by drop until the precipitate is almost dissolved and the solution is opalescent. Make up to 100 ml with distilled water. This solution keeps for a few weeks, but once used it must be discarded and not poured back into the stock bottle.

Method

(1) Bring sections to water.
(2) Oxidize with 0.25 per cent potassium permanganate for 3–5 minutes.
(3) Rinse in tap-water.
(4) Rinse in distilled water.
(5) Bleach in 5 per cent oxalic acid for 5–10 minutes.
(6) Wash sections thoroughly with distilled water (3–4 changes).
(7) Put sections into a Coplin jar of previously heated silver solution at 56°C for 15 minutes.
(8) Rinse rapidly with distilled water.
(9) Flood slide with 1 per cent formalin in distilled water.
(10) Wash in tap-water.

(11) Tone in 0.2 per cent gold chloride for 10–15 minutes.
(12) Rinse in distilled water.
(13) Fix in 5 per cent sodium thiosulphate for 1–2 minutes.
(14) Wash in tap-water for 5–10 minutes.
(15) Counterstain in 1 per cent safranine or neutral red if desired.
(16) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Reticulin fibres	Black
Nuclei (if counterstained)	Red

Robb-Smith's Modification of Foot's Silver Impregnation Method

Since the Foot method occasionally causes sections to float off the slide during impregnation, Robb-Smith recommends floating unmounted sections (from which the paraffin wax has not been removed) on the reagents. The method is as described above, with the following exceptions:

The silver solution is prepared as follows:

10 per cent silver nitrate	15 ml
10 per cent sodium hydroxide	10 drops

Add strong ammonia, drop by drop, until the precipitate which forms is just dissolved. Make up the volume to 50 ml with distilled water. The final solution should not smell of ammonia.

Following Stage 14 (or 15) sections are floated on to slides, the water drained off in a 37°C oven, after which they are dewaxed in xylol and mounted as described above.

Gordon and Sweet's Silver Impregnation Method for Reticulin Fibres

The Gordon and Sweet method, like that of Foot, shows only the network of reticulin and does not demonstrate cells.

Special Reagents Required

(1) *Silver solution.* – To 5 ml of 10.2 per cent aqueous silver nitrate add strong ammonia (.880) drop by drop until the precipitate, which is first formed, is just dissolved. Add 5 ml of 3.1 per cent sodium hydroxide. Add strong ammonia (.880) drop by drop until

the resulting precipitate is just dissolved (the solution should not be completely clear). Make up the solution to 50 ml with fresh distilled water.

(2) *Acidified potassium permanganate*

0.5 per cent potassium permanganate	95 ml
3.0 per cent sulphuric acid	5 ml

Method

Frozen, celloidin or paraffin sections of formalin-fixed tissue may be impregnated. The method given is for paraffin sections.

(1) Thin sections are fixed to slides using starch paste or albumin.
(2) Bring sections to water.
(3) Oxidize in acidified potassium permanganate for 1–5 mintes.
(4) Wash in water.
(5) Bleach in 1 per cent oxalic acid for 3–5 minutes.
(6) Rinse in distilled water.
(7) Wash in tap-water, and then in 2–3 changes of distilled water.
(8) Mordant in 2.5 per cent iron alum for 10 minutes to 2 hours (usually 10 minutes will suffice).
(9) Wash in 2–3 changes of distilled water.
(10) Treat with silver solution until section is transparent (30 seconds).
(11) Wash well in several changes of distilled water.
(12) Reduce in 10 per cent aqueous formalin.
(13) Wash in tap-water, and then in distilled water.
(14) Tone in 0.2 per cent gold chloride for 10–15 minutes.
(15) Rinse in distilled water.
(16) Fix in 5 per cent sodium thiosulphate for 5 minutes.
(17) Wash in water for 1–2 minutes.
(18) Dehydrate, clear and mount.

Results

Reticulin fibres only	Black

Nuclei may be counterstained (after Stage 17) with 1 per cent safranine.

THE NERVOUS SYSTEM

Neuro-histology has always been regarded as a series of very highly specialized methods which are difficult even for the practised routine technologist. Little specialized work of this nature enters the ordinary routine histology laboratory, and many of the techniques employed are lengthy and require the use of solutions with an involved preparation. These points are probably largely responsible for the apprehension with which many technologists approach neuro-histology, either in their own laboratory or in preparation for their Institute of Medical Laboratory Technology examinations. In spite of this, any competent technologist should be able to perform most of the techniques involved with a small amount of practice, and given an understanding of what he is demonstrating. It is for the latter reason that the beginning of this section has been devoted to the anatomy and composition of the nervous system, since the cells and fibres, and the nomenclature, are peculiar to it.

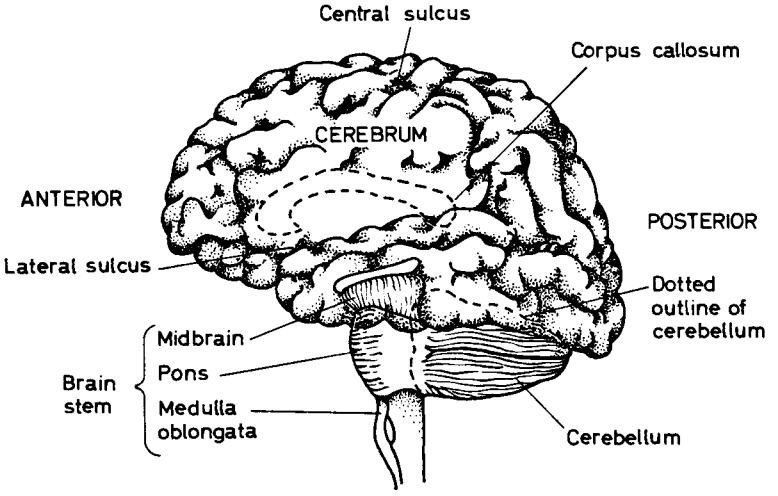


Figure 19.1 – Diagram of the brain

Anatomy

The central nervous system (C.N.S.) is made up of the following six parts *(Figure 19.1)*.

(1) *The cerebrum* is composed of right- and left-paired halves or hemispheres which are separated above, in front and behind to a depth

of approximately 1½ inches by the great longitudinal fissure; deep to this is the corpus callosum, composed of nerve fibres, which connects the two hemispheres. Each hemisphere is composed of a mass of white matter covered with a superficial layer of grey matter.

(2) *The cerebellum* is composed of two hemispheres, the surfaces of which are divided by a series of deep fissures known as sulci, which are close together. As with the cerebrum, there is a core of white matter with a superficial layer of grey matter.

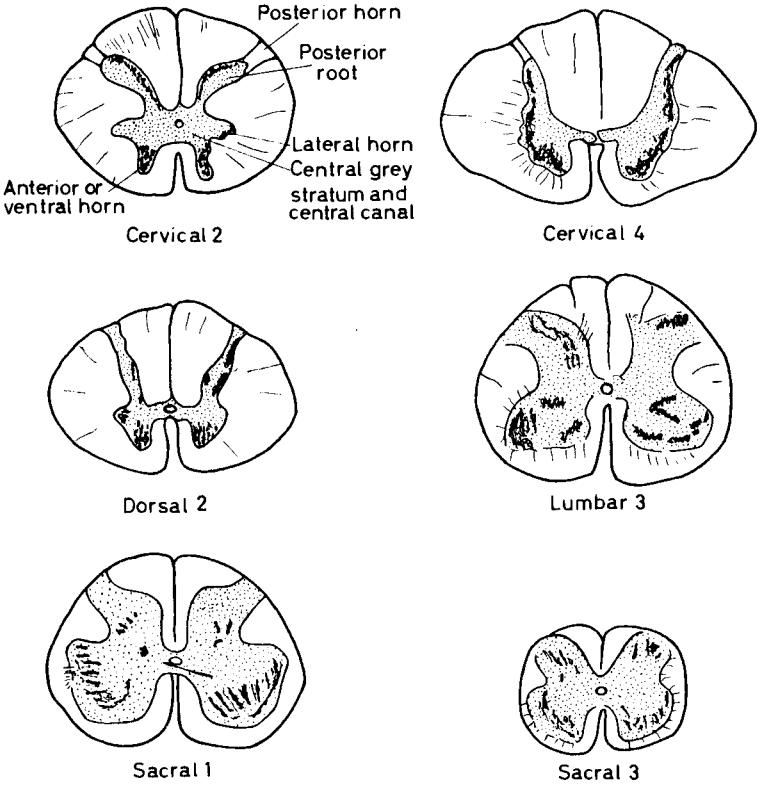


Figure 19.2 – Drawings illustrating the characteristic shapes of the spinal cord at different levels. Note the increase in grey matter (shaded) as compared with the white matter at the lower end of the cord

(3) *The midbrain and pons* connect with the medulla and spinal cord; it is from here that the optic nerves emanate.

(4) *The medulla* connects the spinal cord to the midbrain and cerebellum.

(5) *The spinal cord* connects the nervous structures to the peripheral (skeletal) nerves. The cord is composed of a core of grey matter–which increases in proportion as the nerves leave it *(Figure 19.2)*–surrounded by white matter. The cervical enlargement (where the nerves of the upper body and arms branch off), and the lumbar enlargement (where the nerves of the lower limbs leave) give characteristic shapes on cross-section *(Figure 19.2).*

(6) *The meninges* surround and protect the central nervous system. They are composed of connective tissue proper (page 412) and comprise 3 membranes: (1) the dura mater; (2) the arachnoid; and (3) the pia mater. These membranes are composed of collagen fibres, a small number of elastic fibres, and endothelial cells.

Composition

The nervous system is composed of three types of tissue:

(1) *Nervous tissue proper:* nerve cells and their processes.

(2) *Neuroglia:* the special connective tissue of the nervous system.

(3) *Connective tissue proper:* the meninges (the dura mater, the arachnoid, and pia mater).

Nervous Tissue Proper

Nervous tissue proper consists of neurones (nerve cells) and their processes. These processes (nerve fibres) may be very long and are known as axons (those conducting impulses from the cell) and dendrites (those conducting impulses to the cell). A nerve axon ends by branching into a series of smaller, finer fibres which make contact with the next neurone; where these meet the dendrites of another cell (without their actually touching) the result is known as a *synapse.* Although there is a natural tendency to think of nerve cells and nerve fibres as separate entities, because of the near impossibility of preparing sections to show the whole, one should remember that the nerve fibres are processes of the cell and that they are a single unit, although for convenience they will be dealt with separately.

Nerve Cells

These may vary in size from 4 to 5 μ in diameter (the pigment cells the cerebellum) up to 150 μ in diameter (motor cells of the anterior horn of the spinal cord). They are usually classified by the number of

their processes: unipolar (rare), bipolar (retina and cochlea) and multipolar (most common and found throughout most of the nervous system) *(Figure 19.3a* and *,b).*

The nucleus is pale-staining, large and usually circular, containing a nucleolus.

Cytoplasm

Within the cytoplasm of nerve cells are found the following.

Neurofibrils. – These are fine fibres which are arranged throughout the cytoplasm, extending into the processes *(Figure 19.3b).*

Nissl substance. – This material in fixed tissue is granular in nature and stains intensely with basic aniline dyes. The Nissl substance is usually evenly distributed throughout the cytoplasm except for the area immediately surrounding the origin of the axon; it may, however, spread into the dendrites (*see* Frontispiece). Damage to the nerve fibre usually results in the loss of Nissl substance.

Mitochondria and Golgi apparatus. – These may be well demonstrated in nerve cells.

Granules. – Certain cells, such as those in the substantia nigra, contain melanin (page 382), and all aging nerve cells may contain yellow lipofuscin granules.

Nerve Fibres

These may consist of the following.

Naked axons (axis cylinders). – These are without any form of covering and are found in the grey matter in the brain and spinal cord *(Figure 19.3a* and *b).*

Axons with a neurilemmal sheath. – These are surrounded only by flattened cells of Schwann, the nuclei of which stain intensely (nucleated sheath of Schwann). They are usually found in the sympathetic nervous system.

Myelinated nerve fibres. – These are axons surrounded by a lipid sheath (myelin sheath) *(Figure 19.3g),* which is in turn surrounded by a neurilemmal sheath.

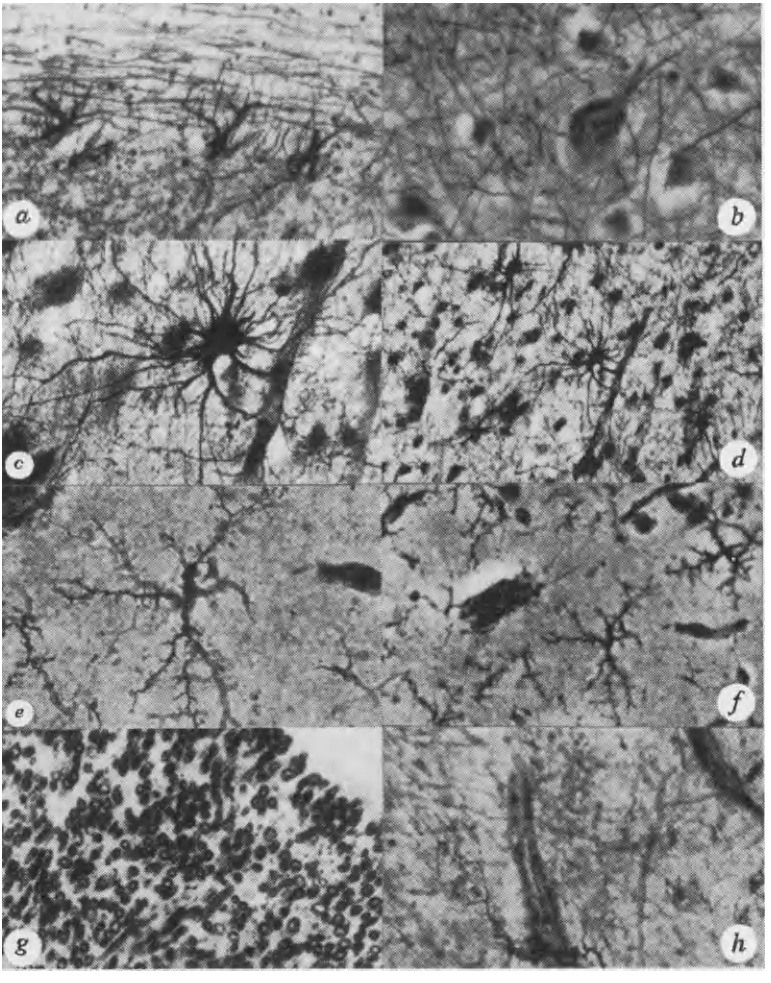


Figure 19.3 – (a) Neurones (basket cells of Purkinje) in a frozen section of cerebellum – Bielschowsky method (page 437); (b) neurofibrils in cytoplasm of a nerve cell in a paraffin section of cerebrum – Glees and Marsland's method (page 439); (c) fibrous astrocyte attached to a blood vessel by vascular feet in a frozen section of midbrain – Cajal's gold sublimate method (page 453); (d) low-power view of (c); (e) microglia in a frozen section of medulla – Weil-Davenport method; (f) low-power view of (e); (g) myelin sheaths in a celloidin section of the spinal cord – Weigert-Pal method; (h) oligodendroglia, note the few non-branching processes in a frozen section of cerebrum – Penfield's silver carbonate method

In peripheral nerve fibres the myelin sheath is constricted at regular intervals, these constrictions being known as the nodes of Ranvier. Myelinated fibres are also found in the white matter of the brain and spinal cord.

Nerve Endings

Nerve fibres end in a peripheral organ and may be either of the following:

Sensory or receptor. – These transmit nerve impulses from the peripheral organ to the nervous system. They are dendrites of a nerve cell and may end in a simple branching structure or in a complex organ.

Motor or effector. – These transmit nerve impulses from the nervous system to the peripheral organ. They are terminations of a nerve axon (myelinated in voluntary muscle and non-myelinated in cardiac and in striped muscle and glands). The nerve endings are either special motor end-plates (voluntary muscle), or simple branching of the axon terminating in thickened ends. (For details of the more complex organs a textbook of histology should be consulted.)

Neuroglia

The name neuroglia (meaning nerve glue) is given to the special type of connective tissue which is found in the nervous system. It is composed of neuroglia fibres and cells.

The astrocytes and their processes form a complicated meshwork which supports the neurones and insulates them from each other; the oligodendroglia are thought to be concerned in the formation and maintenance of myelin; and the microglia, which may become amoeboid and phagocytic, act as a defence mechanism for the nervous system.

The neuroglia cells are divided into two types:

Macroglia. – Ectodermal in origin, macroglia may be protoplasmic astrocytes, fibrous astrocytes or oligodendroglia.

Microglia. – These are mesodermal in origin.

Macroglia

These are the neuroglia proper, as classified below:

Protoplasmic astrocytes. – These cells have a large nucleus and a

granular cytoplasm with long branching processes which are protoplasmic in nature. Some of the processes are attached to blood vessels by 'sucker feet'. The cells contain no fibres. They are found most abundantly in the grey matter.

Fibrous astrocytes. – These cells (as the name implies) contain thick fibres which branch outside the cell. They are also found attached to capillaries by vascular feet, but the processes tend to be longer and finer than those of the protoplasmic astrocyte *(Figure 19.3c* and *d).* They are most abundant in the white matter.

Oligodendroglia. – These cells are small and stain intensely. They have no fibres or vascular feet, but the processes are fine and rarely branch *(Figure 19.3h).*

They are abundant in the white matter where they are found in rows along the myelinated fibres. In the grey matter they are found close to nerve cells or blood vessels.

Microglia

Microglia are small oval-shaped cells which contain a deeply staining nucleus. From each end of the cell a thick process arises which branches freely to give numerous small processes ending in terminal spines *(Figure 19.3e* and *f).*

Meninges (Connective Tissue Proper)

The brain and spinal cord are covered by 3 membranes: the dura mater, the arachnoid, and the pia mater.

Dura mater. – The strong outermost coat, the dura mater is composed of white fibrous tissue (collagen) and a few elastic fibres.

Arachnoid. – This is a very thin membrane of fine connective-tissue fibres clothed on both sides with endothelial cells. Between the pia mater is the sub-arachnoid space which contains the cerebrospinal fluid. The space has innumerable fine trabeculae crossing it.

Pia mater. – The pia mater consists of white fibrous tissue and elastic fibres, and is highly vascular.

The pia mater and the arachnoid are sometimes known collectively as the pia-arachnoid membrane because of their close attachment to each other.

FIXATION, PROCESSING AND SECTION-CUTTING OF THE CENTRAL NERVOUS SYSTEM

Fixation

For some techniques special fixation fluids are givn, but as a routine the best fixative is formol saline. This will enable special fixatives and techniques to be used subsequently if desired. The brain should, if possible, be fixed by injection (page 532).

Processing

Sections may be prepared from either paraffin or celloidin-embedded tissue, or frozen tissue, depending on the technique to be employed. While routine methods for celloidin embedding or freezing may be used, processing by paraffin embedding requires extra care.

Paraffin Embedding

Vacuum bath embedding is not recommended for tissue of the central nervous system, since this method tends to render the sections brittle, and often causes them to float off the slides during staining or silver impregnation techniques. Such tissue should be impregnated with paraffin wax for 12–18 hours, giving at least 4 changes of wax during this period.

Section Cutting

Sections are cut routinely at a thickness of 7 μ, but sections for myelin techniques should be at least 12 μ in thickness. Those sections in which complete neurones are to be demonstrated (dendrites and axons) should be even thicker (15–20 μ).

METHODS OF DEMONSTRATION

Routine Staining

As routine methods, haematoxylin and van Gieson, and Mallory's P.T.A. haematoxylin are employed as with other tissues.

Other methods, such as those for elastic tissue, reticulin, mucin and fat, are often employed for differential diagnosis.

The special methods employed for the demonstration of the various elements in nervous tissue are dealt with under the following headings.

Neurones (nerve cells and their processes).
Normal and degenerate myelin.
Neuroglia cells and fibres.
Nerve endings.

Neurones (Nerve Cells and their Processes)

The most popular technique in Great Britain for the demonstration of neurones is that of Bielschowsky, or one of its variants. Four such methods are given: (1) the long standard method; (2) the shorter Gros-Schultze method; (3) Glees and Marsland's modification for paraffin sections and (4) Holmes' tehcnique, a very reliable and simple technique, used either with or without Luxol fast blue.

All of these methods are reliable and give good results with human tissue and most animal tissue. As with all silver impregnation procedures, dishes and flasks should be thoroughly cleaned and rinsed several times in distilled water before use.

Bielschowsky's Method

Special Reagent Required

Ammoniacal silver. – To 5 ml of 20 per cent silver nitrate add 6 drops of 40 per cent sodium hydroxide. Add strong ammonia (0.880) drop by drop until the resultant precipitate is just dissolved. Add distilled water to a total volume of 25 ml and filter.

Technique

(1) Tissue should be fixed in formol saline (once adequately fixed, the length of time in formol saline is not important).

(2) Rinse tissue in water before cutting.

(3) Frozen sections are cut at 10 μ for neurofibrils, or 15–20 μ to show cells and processes.

(4) Wash sections in distilled water for 1 hour, giving several changes.

(5) Transfer to a dish of 4 per cent silver nitrate; place in the dark for 24–48 hours (sections must be flat and without creases).

(6) Rinse rapidly in distilled water.

(7) Treat with fresh ammoniacal silver nitrate for 2–10 minutes until sections, which are a very light brown, darken in shade to a deep tobacco brown.

(8) Rinse rapidly in 2 changes of distilled water.

(9) Transfer to 10 per cent formalin in tap-water for 5–10 minutes to reduce the silver.

(10) Wash in distilled water, 2–3 changes, for 5–10 minutes.

(11) Tone in 0.2 per cent gold chloride for 10–15 minutes.

(12) Rinse in distilled water.

(13) Transfer to 5 per cent sodium thiosulphate for 5 minutes.

(14) Wash in tap-water for 5 minutes.

(15) Float on to clean slides, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Nerve cells (and neurofibrils), axons and dendrites . . . . . Brown to black

(See Figure 19.3)

Note. – Toning (Stage 11) is optional and need not be employed: as it tends to clear the background and make the neurones appear more deeply impregnated it is generally included.

Gros-Schultze Method for Fibres and Nerve Endings

The Gros-Schultze method does not demonstrate neurofibrils so well as the standard technique.

The fixation and cutting of frozen sections are carried out as for the standard technique *(see above).* Paraffin sections will occasionally give good results.

Special Reagents Required

Ammoniacal silver solution. – To 30 ml of 20 per cent silver nitrate add strong ammonia (.880) drop by drop until the precipitate, which is first formed, is just dissolved. Add a further 18 drops of strong ammonia.

Technique

(1) Wash sections in distilled water (2–3 changes) for 10 minutes.

(2) Transfer to a dish of 20 per cent silver nitrate and place in the dark for 5 minutes (sections must be flat and free from creases).

(3) Transfer direct to the first of 4 previously prepared dishes of 20 per cent formalin. Agitate in this dish for a few seconds.

Transfer to the second dish of 20 per cent formalin, and repeat until all 4 dishes have been used, the overall time taking 5–10 minutes. The final dish should remain free from turbidity.

(4) Impregnate in ammoniacal silver for a few seconds.
(5) Transfer to 5 per cent ammonia for 1 minute.
(6) Rinse rapidly in 1 per cent acetic acid.
(7) Rinse rapidly in distilled water.
(8) Tone in 0.02 per cent gold chloride for 10 minutes.
(9) Wash in distilled water.
(10) Treat with 5 per cent sodium thiosulphate for 5 minutes.
(11) Wash in tap-water.
(12) Float on to clean slides, dehydrate, clear, and mount in Canada balsam or D.P.X.

Note. – Stage 9 is optional, but is preferred.

Results

Axons and dendrites	Black
Nerve cells and neurofibrils	Brown to black

Glees and Marsland's Modification of Bielschowsky's Method for Paraffin Sections

This method gives excellent results with paraffin sections of formol-fixed material and is sufficiently reliable to be used as a routine method.

Special Reagent Required

Ammoniacal silver. – To 30 ml of 20 per cent silver nitrate add 20 ml of absolute alcohol and mix; add strong ammonia (.880) drop by drop until the precipitate first formed is just dissolved, then add a further 5 drops of strong ammonia.

Method

(1) Treat sections with xylol for ½–1 minute to remove wax.
(2) Flood with alcohol for 30 seconds.
(3) Flood slide with 1 per cent celloidin for 20 seconds, or put into a glass-stoppered jar containing 1 per cent celloidin for 20–30 seconds.

(4) Wipe off excess celloidin from back of slide, and flood with 70 per cent alcohol to harden the remainder.

(5) Rinse in distilled water (2 changes).

(6) Treat with 20 per cent silver nitrate at 37°C for 25–30 minutes.

(7) Rinse in distilled water.

(8) Flood twice, 10 seconds each time, with 10 per cent formalin in tap-water.

(9) Impregnate with ammoniacal silver for 30 seconds.

(10) Drain off silver solution and flood slide with 2 changes of 10 per cent formalin for 1 minute each.

(11) Rinse in distilled water.

Note. – Sections may be toned in 0.2 per cent gold chloride for 10 minutes, followed by a rinse in distilled water. Stages 12–15 should then be continued.

(12) Fix in 5 per cent sodium thiosulphate for 5 minutes.

(13) Wash in tap-water.

(14) Blot and flood with absolute alcohol to remove celloidin film. The metallic precipitate which often occurs is usually confined to the celloidin film and is removed with it.

(15) Clear in xylol and mount in Canada balsam or D.P.X.

Results

Neurones (nerve cells, axons and dendrites) . . . . Black

(See Figure 19.3)

Holmes' Silver Technique

This is now a routine method for paraffin sections at the Vancouver General Hospital. It also give good results when used in conjunction with Luxol fast blue to show the myelin sheaths.

Special Reagents Required

Impregnating solution. – Dilute 100 ml of Holmes' boric acid–borax buffer pH 8.4 (page 175) to 494 ml with distilled water. Add 1 ml of 1 per cent aqueous silver nitrate, then 5 ml of 1 per cent aqueous solution of pure pyridine. Mix well. This solution should be freshly prepared.

Reducing solution

Hydroquinone	1 g
Sodium sulphite (cryst)	10 g
Distilled water	to 100 ml

Method

(1) Bring formalin fixed paraffin sections to water.

(2) Place in 20 per cent aqueous silver nitrate, in the dark, at room temperature for 1 hour.

(3) Wash in distilled water (three changes) for 10 minutes.

(4) Place in impregnating solution, cover container and leave overnight at 37°C. There should not be less than 20 ml of solution for each slide.

(5) Remove slides from impregnating solution, shake off excess fluid and place in reducing solution for 2–3 minutes.

(6) Wash in running water for 3 minutes.

(7) Rinse in distilled water.

(8) Tone in 0.2 per cent gold chloride for 3 minutes.

(9) Rinse in distilled water.

(10) Place in 2 per cent oxalic acid for 3–10 minures. The impregnation of the neurones is controlled at this stage, they become progressively pale red, deep red, then black. If Luxol fast blue is being used to counterstain myelin the impregnation should be stopped while axons are reddish-black.

(11) Rinse in distilled water

(12) Place in 5 per cent sodium thiosulphate for 5 minutes.

(13) Wash in tap-water.

(14) *Dehydrate, clear and mount in D.P.X. or H.S.R.*

Or

(14) Rinse in 95 per cent alcohol.

(15)
(16)
(17)
(18)
(19)
(20)

As steps 2, 3, 4, 5, 6 and 7 in Luxol fast blue–Cresyl violet stain on page 446.

(21) Dehydrate, clear and mount in D.P.X. or H.S.R.

Results

Neurones	Red–black
Myelin (if stained)	Blue

Nissl Substance (Nissl Granules)

This granular material for which the standard method of demonstration has been thionin or toluidine blue followed by Gothard's differentiator, is, in fact, almost pure ribonucleic acid. It is well demonstrated by the Luxol fast blue–Cresyl violet technique, or specifically by the acridine–orange or Unna-Pappenheim methods which stain it an intense red (*see* Frontispiece). After treatment with the enzyme ribonuclease (page 257), the substance cannot be demonstrated either with Unna-Pappenheim stain or the basic aniline dyes.

Unna-Pappenheim Stain

The Unna-Pappenheim stain as modified by Trevan and Sharrock (described on page 254), selectively stains Nissl substance red.

Thionin or Toluidine Blue Methods

Fixation

Although alcohol fixation was originally specified, formol saline fixation gives good results.

Special Reagent Required

Gothard's differentiator

Creosote	50 ml
Cajuput oil	40 ml
Xylol	50 ml
Absolute alcohol	150 ml

This original formula is really too powerful and it is advisable to dilute it with an equal amount of absolute alcohol before use.

Method

(1) Bring paraffin, celloidin or frozen sections to water.

(2) Stain for 30 minutes at 56°C (or overnight at room temperature) in 1 per cent toluidine blue in 1 per cent borax, or in 0.1 per cent thionin.

(3) Rinse in water.
(4) Treat with 90 per cent alcohol for 10–30 seconds.
(5) Differentiate in Gothard's differentiator until the Nissl substance stands out against a clear background. This stage should be controlled with a microscope.
(6) Rinse in absolute alcohol.
(7) Clear in xylol and mount in Canada balsam or D.P.X.

Results

Nissl substance	Deep blue to mauve
Nucleoli and chromatin	Blue
Other tissue elements	Colourless

Einarson's Gallocyanin Method

Einarson's method, like that of Unna-Pappenheim, has the advantage that it is progressive and does not require differentiation.

Special Reagent Required

Gallocyanin solution

Gallocyanin	0.3 g
Chromalum	10 g
Distilled water	to 100 ml

Dissolve the chromalum in the water by heating. Add the gallocyanin, bring to the boil and allow to simmer for 20–30 minutes. Allow to cool, add distilled water to the total original volume and filter.

Method

(1) Fix tissues in Carnoy's fixative, although formol saline will give reasonable results.
(2) Cut paraffin sections 6–8 μ in thickness.
(3) Bring sections to water.
(4) Stain overnight in gallocyanin at room temperature.
(5) Rinse in 2–3 changes of distilled water.
(6) Dehydrate, clear and mount.

Results

Nissl substance	Deep blue to mauve
Nuclei	Deep blue

Demonstration of Normal Myelin

The classical method of demonstrating normal myelin is the Weigert-Pal technique which is performed on paraffin or, preferably, celloidin sections. Loyez has been the routine method for its demonstration in paraffin sections, well demonstrated by the Luxol fat blue–Cresyl violet technique, or specifically by the acridine-orange or Unna-Pappenheim methods which stain. McManus's Sudan black method (page 370) demonstrates myelin well, but it is not specific. Normal myelin in frozen sections may be demonstrated by the fat-soluble stains (pages 359–363), by Baker's acid haematein method (page 365), and by the peracetic acid–Schiff technique (page 374).

Weigert-Pal Technique (Kultschitzky's Modification)

The Kultschitzky's modification of the Weigert-Pal method is based on the formation of chromium dioxide by the interaction of chrome salts with the phosphatides and cerebrosides which are constituents of normal myelin. It is this chromium dioxide which subsequently acts as a mordant and forms a lake with the haematoxylinin.

Special Reagents Required

(1) *Weigert's primary mordant*

Potassium dichromate	5 g
Fluorochrome (chromium fluoride)	2.5 g
Distilled water	to 100 ml

Add the dichromate to the water, bring to the boil and add the fluorchrome. Stir the solution well; cool and filter.

(2) *Gliabeize* *

Fluorchrome (chromium fluoride)	2.5 g
Copper acetate	5 g
Distilled water	to 100 ml

Add the fluorchrome and copper acetate to the water and bring to the boil. While the solution is boiling, add 3 ml of acetic acid. Cool, filter and keep in a well-stoppered bottle.

* *Note.* – The use of gliabeize is not essential, Stage 6 being optional, but since it gives improved staining in many cases, it is advisable to use it as a routine.

(3) *Kultschitzky's haematoxylin*

10 per cent Haematoxylin in absolute alcohol (ripened)	10 ml
Glacial acetic acid	2 ml
Distilled water	90 ml

This solution should be freshly prepared.

(4) *Pal's solution*

Oxalic acid	1 g
Sodium sulphite	1 g
Distilled water	to 100 ml

Method

(1) Fix tissue in formol saline.
(2) Transfer to Weigert's primary mordant for 5–10 days.
(3) Wash in running water overnight.
(4) Dehydrate and embed in celloidin.
(5) Cut sections 12–20 μ in thickness (cross-section of the spinal cord need not be thicker than 12 μ.
(6) Treat with gliabeize for 30 minutes (this Stage is optional, but is preferred, *see Note above*).
(7) Wash in water.
(8) Stain in Kultschitzky's haematoxylin for 12–24 hours.
(9) Rinse in distilled water.
(10) Treat with 0.25 per cent potassium permanganate for ½–1 minute.
(11) Rinse rapidly in distilled water.
(12) Treat with Pal's solution for 1–2 minutes.
(13) Wash sections in several changes of tap-water for 10–20 minutes*.
(14) Dehydrate, clear, and mount in Canada balsam.

Note. – Stages 10, 11 and 12 are the Stages of differentiation. From Stage 12 the section is returned via Stage 11 to Stage 10 and the sequence repeated until only the myelin sheaths are blue on a nearly colourless background. Sections may be left in Pal's solution, or in fresh distilled water, but never for longer than 1 minute in the 0.25 per cent potassium permanganate.

*The use of a counterstain at this point is optional but gives a pretty picture.

Results (see Figure 19.3)

Myelin sheaths and red blood cells	Deep blue-black
Other tissue elements	Colourless to creamy yellow

Luxol Fast Blue–Cresyl Violet for Myelin (Modified Kluver and Barrera, 1953)

This method is excellent for staining myelin fibres with good cellular definition.

Special Reagents Required

Luxol fast blue solution

Luxol fast blue	1 g
95 per cent alcohol	1,000 ml
10 per cent acetic acid	5 ml

Alternative Luxol fast blue solution (Salthouse, 1964)

Isopropanol	100 ml
Luxol fast blue	0.1 g

Cresyl violet solution

Cresyl violet	0.1 g
Distilled water	100 ml

In the original method 17 drops of 10 per cent acetic acid are added to the above, but the author has not found this an advantage.

Lithium carbonate solution

0.005 per cent aq. solution lithium carbonate.

In the original method 0.05 per cent solution is recommended. This can be diluted to slow down differentiation.

Cresyl violet differentiator

95 per cent alcohol	90 ml
Chloroform	10 ml
Glacial acetic acid	3 drops

Method

(1) Bring sections to 95 per cent alcohol.
(2) Stain in Luxol fast blue overnight at 37°C.
(3) Wash in 95 per cent alcohol, then in distilled water.
(4) Commence differentiation by immersing sections in lithium carbonate solution for not more than 20 seconds.
(5) Differentiate in 70 per cent alcohol until grey and white matter are clearly distinguished (30 seconds–1 minute).
(6) Rinse in distilled water and examine under microscope. If differentiation is not complete repeat steps 4, 5 and 6 with reduced times (lithium carbonate, 2–3 seconds).
(7) Wash well in distilled water.
(8) Stain in cresyl violet for 10 minutes at room temperature.
(9) Wash in distilled water.
(10) Wash in 70 per cent alcohol.
(11) Differentiate in cresyl violet differentiator for 1–2 seconds.
(12) Rinse in 95 per cent alcohol to remove differentiator.
(13) Rinse in absolute alcohol and clear in xylol.
(14) Check differentiation under microscope to *ensure* only nuclei (and Nissl substance) are stained, repeat 11, 12, 13 and 14 if necessary.
(15), Mount in H.S.R. or D.P.X.

Results

Myelin	Blue
Nuclei	Purple

Luxol Fast Blue–P.A.S.

Sections are taken through the Luxol fast blue technique *(above)* from Stages 1–7, following which the standard P.A.S. technique is performed as detailed on page 268.

Luxol Fast Blue–Phosphotungstic Acid Haematoxylin

Sections are brought to water, left in 0.25 per cent potassium permanganate for 5 minutes, rinsed in distilled water, decolorized in 5 per cent oxalic acid for 3–5 minutes and washed in running water for 5 minutes. Then Stages 1–7 of the Luxol fast blue technique *(above)* are carried out, followed by Stages 8–10 of the phosphotungstic acid haematoxylin technique on page 217.

Loyez's Technique for Myelin in Paraffin-embedded Material (Modified)

This technique, which for many years has been a standard method for paraffin sections, may be applied with equal success on frozen or celloidin sections.

Special Reagents Required

(1) *La Manna's fluid*

Potassium dichromate	9.5 g
Zinc chloride	4.5 g
Distilled water	to 100 ml

(2) *Lithium carbonate haematoxylin*

10 per cent haematoxylin in absolute alcohol (ripened solution)	10 ml
Saturated aqueous solution of lithium carbonate	2 ml
Distilled water	to 90 ml

Lithium carbonate haematoxylin should be freshly prepared from stock solution.

(3) *Loyez's differentiator*

Borax	2 g
Potassium ferricyanide	2.5 g
Distilled water	to 200 ml

Method

(1) Fix tissue in formol saline. By post-mordanting in La Manna's fluid for 24 hours at 56°C (to render the myelin insoluble in the fat solvents) before paraffin processing a much better result may be obtained.

(2) Cut paraffin, celloidin, or frozen sections to a thickness of 15–20 μ.

(3) Bring to distilled water.

(4) Treat paraffin or frozen section (on slides) with alcohol, then coat with celloidin.

(5) Mordant in 4 per cent iron alum for 12–24 hours.

(6) Rinse in distilled water.

(7) Stain in haematoxylin for 2–4 hours at 56°C, or 6–8 hours at 37°C.

(8) Wash well in tap-water.

(9) Partially differentiate in 4 per cent iron alum until myelin sheaths stand out bluish-black on a pale grey background.

(10) Wash in tap-water for 10 minutes.

(11) Complete differentiation in Loyez's differentiator (borax ferricyanide) for 2 minutes. This renders the myelin an intense deep blue against a creamy background.

(12) Wash well in tap-water.

(13) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Demonstration of Degenerate Myelin

The demonstration of degenerate myelin depends on the period of time that has elapsed since the original damage responsible for the degeneration; this period may be divided approximately into the following 3 stages.

Up to 10 days. – Myelin tract will stain more heavily with normal myelin techniques.

10–60 days. – Positive demonstration with osmium tetroxide techniques.

Over 60 days. – Degenerate myelin will have been removed by phagocytic cells, and evidence of degeneration will be given by unstained areas using the normal myelin techniques (negative demonstration method).

Positive demonstration of degenerate myelin is carried out by the classical method of Marchi, by the Swank-Davenport method, or by one of the fat-soluble stains (pages 359–362) which may be used in conjunction with a normal myelin method, such as the Luxol fast blue–oil red O technique.

Marchi's Technique

Marchi's technique is based on the fact that after normal myelin has been oxidized by chrome salts it will not react with osmium tetroxide. Degenerate myelin contains oleic acid which is not oxidized by chrome salts, it will therefore reduce osmium tetroxide, and is blackened.

Special Reagent Required

Marchi's fluid

3 per cent potassium dichromate	40 ml
1 per cent osmium tetroxide	20 ml

Fixation

Tissue should be fixed in formalin and post-chromed for 4–8 days in 3 per cent potassium dichromate: it is said that Marchi's fluid may be used directly as a fixative. Prolonged fixation in formalin (after 8–10 days) may reduce the intensity of the reaction and should be avoided if possible.

Method

(1) Fix small pieces of tissue (not more than 2 mm in thickness) in formalin, and treat with potassium dichromate as described above.

(2) Transfer to Marchi's fluid for 8–12 days for spinal-cord sections, or 12–15 days for brain tissue. Tissues should be supported on glass wool to avoid the precipitate which forms, and to aid penetration. The fluid should be changed every 4–5 days.

(3) Wash in running water for 24 hours.

(4) Dehydrate rapidly and embed in celloidin, or clear and embed in paraffin wax.

(5) Cut sections 20–30 μ in thickness and mount them in Canada balsam or D.P.X.

Results

Degenerate myelin (10–60 days after injury)	Black
Neutral fat (for example, adipose tissue)	Black

Swank-Davenport Method

The Swank-Davenport method gives better demonstration of degenerate myelin than the classical Marchi technique.

Method

(1) Fix tissue in 4 per cent formol saline.

(2) Transfer without washing to the following solution for 7–10 days.

1 per cent potassium chlorate	60 ml
1 per cent osmium tetroxide	20 ml
Commercial formalin	12 ml
Glacial acetic acid	1 ml

Approximately 15 volumes of fluid to 1 tissue should be used, the solution shaken, and the tissues turned over daily to improve penetration.

(3) Wash in running tap-water overnight.

(4) Embed in celloidin.

(5) Cut sections 20–30 μ in thickness, and mount in Gurr's neutral mountant.

Results

Degenerate myelin	Black
Neutral fats	Black

Luxol Fast Blue–Oil Red O Technique

This technique quite often gives a very pretty result, but the myelin staining is not as deep as when counterstained with cresyl violet.

Method

(1) Cut frozen sections of formalin-fixed tissue at 15–20 μ and wash in tap-water.

(2) Rinse briefly in 50 per cent and then 70 per cent alcohol.

(3) Place in Luxol fast blue solution (*see* page 446), and leave at room temperature for 3 hours.

(4) Rinse briefly in 70 per cent and then 50 per cent alcohol.

(5) Place in distilled water for 30 seconds.

(6) Commence differentiation by immersing sections in lithium carbonate solution for not more than 20 seconds.

(7) Differentiate in 70 per cent alcohol until grey and white matter are clearly distinguished (30 seconds–1 minute).

(8) Rinse in distilled water and examine under microscope. If differentiation is not complete repeat Steps 6, 7 and 8 with reduced times (lithium carbonate 2–3 seonds).

(9) Wash well in distilled water.

(10) Stain by Lillie and Ashburn's oil red O method (page 361) from Stages 3 to 10.

Results

Normal myelin	Blue-green
Degenerate myelin	Red

Demonstration of Neuroglia Fibres and Astrocytes

The demonstration of all the various elements of neuroglia (page 434) can rarely be achieved by one technique.

The methods described below are the most reliable of a great variety of techniques and their modifications, and are recommended.

Neuroglia Fibres and Astrocytes

Mallory's P.T.A. haematoxylin (page 217).
Anderson's Victoria blue technique.
Cajal's gold sublimate technique.
Scharenberg's triple impregnation.

Oligodendroglia and Microglia

Penfield's silver carbonate technique.
Weil-Davenport's technique.

Anderson's Victoria Blue Method

Anderson's method gives good results with both paraffin and frozen sections; the latter are preferable.

Special Reagent Required

Neuroglia mordant

Distilled water	to 100 ml
Sodium sulphite	5 g
Oxalic acid	2.5 g
Potassium iodide	5 g
Iodine	2.5 g

Dissolve in the above order, then add 5 ml of acetic acid and keep in a well-stoppered bottle. If the solution turns brown, add 1 or 2 crystals of sodium sulphite.

Method

(1) Frozen or paraffin sections of formalin-fixed tissue are cut to a thickness of 15–20 μ.

(2) Frozen sections are washed in, or paraffin sections brought to, distilled water for 3–4 minutes.

(3) Transfer to equal parts of neuroglia mordant and 5 per cent ferric chloride for 10–15 minutes.

(4) Wash in water, and transfer to 0.25 per cent potassium permanganate for 5 minutes.

(5) Transfer directly to Pal's solution (page 445) until sections are white (sections may remain in this solution for up to 24 hours).

(6) Rinse in distilled water.

(7) Float frozen sections on to clean albuminized slides, and drain off excess water. Blot with fine filter paper.

(8)) Boil a quantity of 1.5 per cent Victoria blue (G. Gurr's, for Anderson's method) in a test tube and pour immediately on to sections. Allow the stain to remain for 1–5 minutes.

(9) Drain slide and flood directly with Lugol's iodine for 1 minute.

(10) Blot section with fine filter paper and flood with xylol; if the section is not clear, repeat the blotting and xylol flooding process.

(11) Differentiate section with a mixture of equal parts of aniline oil and xylol. This must be controlled with a microscope until neuroglia fibres and astrocytes show up as blue on a pale blue or colourless background. All nuclei are stained by this method.

(12) Wash with xylol to remove aniline oil.

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

Results

Neuroglia fibres and astrocytes	Blue
Cell nuclei	Blue

Both fibrous and protoplasmic astrocytes are demonstrated by this technique.

Cajal's Gold Sublimate Method for Protoplasmic and Fibrous Astrocytes

This technique is the classical method for the demonstration of astrocytes *(Figure 19.3c* and *d).*

Special Reagent Required

(1) *Formol ammonium bromide (F.A.B.)*

Formalin	15 ml
Ammonium bromide	2 g
Distilled water	85 ml

(2) *Gold sublimate solution*

Mercuric chloride	0.4 g
1 per cent gold chloride (brown)	10 ml
Distilled water	60 ml

Dissolve the mercuric chloride in the distilled water with the aid of gentle heat; cool and then add the gold chloride. This solution should be prepared immediately before use.

Method

(1) Fix pieces of tissue 3–5 mm in thickness in F.A.B. for 3–8 days.
(2) Cut frozen sections to a thickness of 15–20 μ.
(3) Store sections in F.A.B. until ready for staining.
(4) Rinse well in distilled water (2–3 changes).
5) Treat in gold sublimate solution, in a flat covered dish in the dark at 22°C for 4–8 hours or longer until sections are deep purple in colour. Sections must lie flat without creasing or overlapping.
(6) Rinse rapidly in distilled water.
(7) Fix in 5 per cent sodium thiosulphate.
(8) Wash in distilled water.
(9) Float on to clean slides.
(10) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Result

Astrocytes (protoplasmic and fibrous)	Red-black

Note. – Frozen sections of formol saline-fixed tissue may be impregnated, but they should be treated overnight in F.A.B. (above) or for 1 hour in 5 per cent hydrobromic acid (supplied commercially as a 40 per cent solution) at 37°C; then continue from Stage 4 above.

Scharenberg's Triple Impregnation Method (Modified) for Glial Cells and Fibres

Scharenberg's method is a very trustworthy one and usually gives excellent results on both normal and pathological material, particularly gliomatous tissue. Occasionally, with refractory material, superior results may be obtained by omitting Stage 7 *(see below)* and using a double rather than a triple impregnation.

As with the majority of silver methods, sections may be mounted either toned in gold chloride or untoned, but in either case tissues should always be fixed in sodium thiosulphate.

Special Reagents Required

(1) *Stock silver carbonate solution*

10 per cent silver nitrate AR	100 ml
5 per cent sodium carbonate AR (anhydrous) . . .	300 ml

Add strong ammonia, drop by drop, until the creamy yellow precipitate is just dissolved; scrupulous care must be taken not to add an excess of ammonia. Dilute to a total volume of 700 ml. The solution may be used for up to 3 months if kept in a dark bottle.

(2) *Ammoniacal silver solution*

Add strong ammonia to a 2 per cent solution of silver nitrate until the precipitate, formed instantaneously, is just dissolved. Again, there must be no excess ammonia. The solution keeps indefinitely if stored in the dark.

Method

(1) Fix tissue in 10 per cent formol saline. (In the original technique it was stated that tissue must be fixed in formol–ammonium–bromide, page 454), but the technique given here gives equal if not better results).

(2) Cut frozen sections to a thickness of 15–20 μ and receive in distilled water.

(3) Wash sections overnight in 1 per cent ammonia water.

(4) Transfer direct to 5 per cent hydrobromic acid for 2–3 hours at 37°C. Rinse in two changes of 1:5000 ammonia and then in distilled water.

(5) Sensitize sections for 15 minutes at 60°C in 50 ml of 2 per cent silver nitrate, to which 20 drops of pyridine have been added.

(6) Without rinsing, transfer to 50 ml of silver carbonate solution for 15 minutes at 60°C, to which 20 drops of pyridine have been added.

(7) Transfer direct to 2 per cent ammoniacal silver nitrate at room temperature for 5 minutes.

(8) Reduce in 1 per cent formaldehyde for 2–3 minutes.

(9) Wash in distilled water.

(10) Tone in 0.2 per cent gold chloride for 10–15 minutes.

(11) Wash in distilled water.

(12) Fix in 5 per cent sodium thiosulphate for 5 minutes.

(13) Wash in distilled water and mount on an albuminized slide.

(14) Carefully blot and dehydrate in absolute alcohol.

(15) Clear in xylol and mount in Canada balsam or D.P.X.

Note. – Although this technique does not demonstrate oligodendroglia or microglia, the stock silver carbonate solution may be used with Penfield's technique (*below*). For oligodendroglia it is used undiluted, and for microglia diluted 1:4 with distilled water.

Results

Protoplasmic and fibrous astrocytes and neuroglia fibres	Jet black

Demonstration of Oligodendroglia and Microglia

Penfield's Modification of Hortega's Technique

Oligodendroglia are best demonstrated by Penfield's technique (with increased strength of silver solution), or, when this fails, by the Weil-Davenport method which occasionally gives a good result *(Figure 19.3e* and *f).*

Special Reagents Required

Silver carbonate solution

10 per cent silver nitrate	5 ml
5 per cent sodium carbonate	20 ml

Mix and add strong ammonia (.880) drop by drop until the precipitate, which is first formed, is dissolved. Make up the total volume to 75 ml with distilled water. For *oligodendroglia specifically,* better results may be obtained by using 10 ml of 20 per cent silver nitrate when preparing this silver carbonate solution.

Method

(1) Cut frozen sections of formalin-fixed tissue to a thickness of 10–15 μ.

(2) Remove traces of formalin by washing sections overnight in 1 per cent ammonia in distilled water.

(3) Transfer directly to 5 per cent hydrobromic acid* in distilled water for 1 hour at 37°C.

(4) Wash in 3 changes of distilled water.

(5) Transfer to 5 per cent sodium carbonate for 1 hour (up to 6 hours).

(6) Transfer to silver carbonate solution for 3–5 minutes, until sections turn light brown in colour.

(7) Transfer to 1 per cent formalin and agitate sections by blowing on the surface of the fluid for 2–3 minutes.

(8) Wash in distilled water.

(9) Tone in 0.2 per cent gold chloride for 10–15 minutes.

(10) Rinse in distilled water.

(11) Fix in 5 per cent sodium thiosulphate.

(12) Wash in water.

(13) Mount on clean slides, dehydrate, clear, and mount in D.P.X. or H.S.R.

Result

Oligodendroglia and microglia	Black *(see Figure 19.3e. f. h)*

Weil-Davenport's Technique

Demonstration of microglia cells is best achieved by the Weil-Davenport method, but Penfield's silver carbonate technique gives less brilliant but more reliable results.

The Weil-Davenport method was devised for frozen sections, but sometimes gives good results with loose paraffin sections which have been de-waxed and treated as frozen sections.

Special Reagent Required

Silver solution. – To 2–3 ml of strong ammonia (.880) in a flask, add 10 per cent silver nitrate, drop by drop, until about 18 ml have

*Hydrobromic acid is supplied commercially as a 40 per cent solution.

been added and the solution is still slightly opalescent. This solution gives the best results if it is prepared in a silvered flask (with a deposit of silver on the inside) which should be kept for the purpose.

Method

(1) Cut frozen or paraffin sections to thickness of 12–15 μ.
(2) Bring sections (frozen or loose de-waxed paraffin) to 3 changes of distilled water.
(3) Transfer to silver solution for 15–20 seconds.
(4) Transfer to 15 per cent formalin; gently agitate section until it is coffee-brown in colour.
(5) Rinse in 3 changes of distilled water.
(6) Tone in 0.2 per cent gold chloride for 10–15 minutes.
(7) Rinse in distilled water.
(8) Fix in 5 per cent sodium thiosulphate for 5 minutes.
(9) Wash in water.
(10) Float on to clean slides, dehydrate, clear, and mount in Canada balsam or D.P.X.

Result

Oligodendroglia and microglia . . . . . Black

Note. – Toning (Stages 6–7) is optional and sections may be mounted untoned.

Methods for Rapid Diagnosis

Toluidine blue staining, as used for frozen sections (page 206), is sometimes employed for the rapid diagnosis of brain smears following alcohol fixation. The following method gives a differential picture and is, therefore, of more diagnostic value.

Morris's Smear Technique (1947) for the Rapid Histological Diagnosis of Tumours of the Central Nervous System

This technique, which is rapid and gives a good nuclear and cytoplasmic picture, need not be restricted to brain tumours. It may be used for a variety of tissues, notably smears of fresh pituitary, when a useful differential picture is obtained.

Special Reagents Required

(1) *Dichromate–eosin*

Eosin (water soluble)	1 g
Potassium dichromate	1 g
Distilled water	to 100 ml

This stain keeps indefinitely.

(2) *Acetone–alcohol*

Absolute alcohol	30 ml
Acetone	25 ml

(3) *Polychrome blue*

Methylene blue	1 g
Potassium carbonate	1 g
Distilled water	to 300 ml

Place the constituents in a 500 ml flask and boil for 10–15 minutes. Add 3 ml of glacial acetic acid drop by drop, shaking vigorously until the precipitate is dissolved. Reduce the volume to 100 ml by boiling; then allow to cool. The stain remains stable for at least 1 year.

Method

(1) Fix smears by drying or, better, in absolute alcohol for 3–5 minutes.
(2) Rinse in distilled water.
(3) Stain for 5–10 seconds in dichromate–eosin.
(4) Wash in tap-water.
(5) Differentiate in acetone–alcohol.
(6) Wash in tap-water.
(7) Stain 10–30 seconds in polychrome blue.
(8) Wash in tap-water.
(9) Dehydrate in acetone–alcohol *(as above)*.
(10) Clear first in chloroform, then in toluol.
(11) Mount in Canada balsam.

Results

Nuclei	Blue
Other tissue elements	Shades of pink

PANCREAS

Most routine fixatives are suitable for sections of the pancreas, except that acetic acid should be avoided since it dissolves the characteristic granules. Formalin or Zenker–formol give good results with most staining methods. Since autolysis of the pancreas is rapid, tissue should be fixed as soon as possible.

For routine examination, haematoxylin and eosin staining is adequate, but special methods are required to demonstrate specifically the *alpha* (α), *beta* (β), and D cells of the islets of Langerhans and the zymogen granules which are found in the cells lining the alveoli. The β-cell granules are almost certainly concerned with the formation of insulin, and the zymogen granules with the pancreatic enzymes.

The filamentous mitochondria which are found in the alveoli may be demonstrated by the methods given on page 480. Techniques for the demonstration of other constituents, such as mucin, fat and amyloid, will be found under their appropriate headings.

Alpha (α), Beta (β) and D Cells

These may be demonstrated by most of the selective connective-tissue stains. The α-cell granules are stained red by the trichrome stains. The β-cells are pale orange brown by the trichrome methods; they are P.A.S. negative and are stained purple by Gomori's aldehyde fuchsin stain (page 420). The cytoplasm of the D cells has an affinity for aniline blue and light green. The zymogen granules are acidophil in nature and are P.A.S. positive.

PITUITARY GLAND

The pituitary gland, which is found at the base of the brain in the sella turcica, is separated into two main parts (pars anterior and pars nervosa) by the small pars intermedia.

The *pars nervosa* is composed of nervous tissue which may be demonstrated by appropriate methods.

The *pars anterior* is composed of two types of cell: (1) chromophobe cells, which contain no stainable granules and constitute about 50 per cent of the cells; and (2) chromophil cells, which contain granules. The chromophil cells are sub-divided into two further groups by their staining reactions: (*i*) *alpha* (α) or acidophil cells, which constitute about 40 per cent of the cells; and (*ii*) *beta* (β) or basophil cells, which constitute the remaining 10 per cent of the total cells.

It is the cells of the pars anterior that the technologist is often called

upon to demonstrate, and this may be achieved effectively by the trichrome methods (page 414).

Alpha-cells are stained red by the trichrome methods, and are P.A.S. negative.

Beta-cells are stained blue (or green) by the trichrome methods; are P.A.S. positive and Gram-positive after formalin or Zenker fixation.

Using a P.A.S. technique with an orange G counterstain a good differential picture of the cells may be obtained.

Slidder's Orange–Fuchsin–Green (1961)

This is a reliable method for the differential staining of the anterior lobe of the pituitary. *Fixation* is not critical.

Special Reagent Required

Celestine blue solution

Celestine blue B	0.5 g
Ferric ammonium sulphate	5 g
Glycerin	14 ml
Distilled water	100 ml

Dissolve the iron alum in the water, add the celestine blue and boil for 3 minutes. Cool, filter and add the glycerin.

Orange G solution

95 per cent alcohol	100 ml
Orange G	0.5–0.7 g
Phosphotungstic acid	2 g

Dissolve phosphotungstic acid in alcohol and saturate solution with orange G.

Method

(1) Bring sections to warer.
(2) Stain in celestine blue for 5 minutes.
(3) Rinse in water.
(4) Stain in Mayer's haemalum (page 214) for 5 minutes.
(5) Wash in water and differentiate in acid alcohol (page 199).
(6) Wash in running water.
(7) Rinse in 95 per cent alcohol and stain with orange G for 2 minutes.

(8) Rinse in distilled water.

(9) Stain in 0.5 per cent acid fuchsin (in 0.5 per cent acetic acid) for 2–5 minutes. Check slides periodically and leave until basophils are well stained.

(10) Rinse in distilled water.

(11) Place in 1 per cent aqueous phosphotungstic acid for 5 minutes.

(12) Rinse in distilled water.

(13) Stain in 1.5 per cent light green (in 1.5 per cent acetic acid) for 1–2 minutes.

(14) Rinse in distilled water.

(15) Dehydrate, clear and mount in synthetic resin.

Results

Nuclei	Blue-black
Acidophils	Orange-yellow
Basophils	Reddish-purple
Chromophobe cells	Grey
Erythrocytes	Yellow
Connective tissue	Green

SKIN

The skin is composed of two main layers: (1) the epidermis (outer covering); and (2) the dermis (underlying layer).

The Epidermis

The epidermis consists of two layers: (*a*) the malpighian layer which connects with the dermis; and (*b*) the outer horny layer (stratum corneum) which consists of dead flattened cells (keratin).

The malpighian layer is composed of polyhedral cells; the surface of these cells (prickle cells) is covered with fibres which connect with the fibres of adjacent cells (intercellular bridges). The fibres are seen in the cytoplasm of the cells, and may pass through several cells in succession. The upper 4–5 layers contain kerato-hyalin granules which stain deeply. Between these cells and the keratin of the horny layer there are clear flat cells (stratum lucidum) containing eleidin, which is thought to be an intermediate stage between kerato-hyalin and keratin.

The Dermis

The dermis consists of connective tissue and contains the hair follicles, sweat glands and sebaceous glands.

Apart from the demonstration of the various elements present in skin and nails, this tissue presents certain special difficulties in the processing and cutting of sections.

Fixation

Routine fixatives, such as formalin, Zenker's or Bouin's fluid, may be used. Pieces of skin will curl unless they have been pinned out on a small cork board (or a large cork) during fixation; either stainless steel pins, quills, or glass pins should be used to avoid rust marks.

Processing

Skin tends to become very hard during processing, and for this reason, celloidin or double embedding techniques are often employed. Skin from the hands or feet can be particularly difficult to section; this may be partly overcome by immersion in Perenyi's fluid (page 72), or 4 per cent aqueous phenol (page 72). Nails are best treated with Perenyi's fluid until softened (1–3 days), and then double embedded or celloidin embedded if thin sections are not required, and there is no urgency.

Since skin is a dense tissue it should be left in the embedding medium for a longer period than normal.

Staining

Haematoxylin and eosin may be used for routine demonstration of most structures, including the kerato-hyalin granules (blue and black), and the keratin which stains bright pink with eosin. Mallory's P.T.A. haematoxylin (page 217) demonstrates the intercellular bridges (or fibres). The Alcian blue–P.A.S. technique (page 276) is recommended for the demonstration of connective tissue polysaccharides.

For the demonstration of eleidin the picro-nigrosin technique should be employed.

Picro-nigrosin Technique for Eleidin

Technique

(1) Cut thin frozen sections of formalin-fixed tissue.
(2) Wash in water.
(3) Treat with saturated aqueous picric acid for 5 minutes.
(4) Rinse in distilled water.
(5) Stain in 1 per cent aqueous nigrosin for 1 minute.
(6) Rinse in water.

(7) Rinse in 96 per cent alcohol.
(8) Clear in origanum oil and mount in Canada balsam.

Results

Eleidin	Black
Other elements	Yellow

For the demonstration of mitochondria, connective tissue elements, melanin, and so on, *see* the appropriate chapters.

SUPRARENAL GLANDS

The suprarenal glands are situated one at the upper pole of each kidney. They are the most susceptible of all tissues to autolysis and must be fixed within 1–2 hours of death (or removal of the gland) for detailed histological examination.

The cut surface of the gland shows a yellowish cortex, which surrounds a reddish medulla. The medulla contains chromaffin tissue which, after treatment with chrome salts, turns brown owing to the reduction of the chrome salts by the adrenaline present in the cells.

The cut surface of the cortex shows 3 zones, the centre and largest one composed of cells which are rich in lipid material.

Fixation

To demonstrate the chromaffin reaction, tissue must be fresh and fixed in a non-acid dichromate-containing fixative, although formalin fixation followed by post-chroming in dichromate may rarely give positive results.

For routine histology any of the standard fixatives may be used; for example, Susa, formalin, Zenker–formol.

Staining

Suprarenal glands may be stained with routine stains; for example, haematoxylin and eosin, or Weigert's and van Gieson's, for most purposes. The connective-tissue methods on pages 414–428 also give good results.

Chromaffin Tissue

To demonstrate chromaffin, fresh material should be fixed in Régaud's fluid (page 52); post-chroming (page 55) may improve the

final picture. Frozen sections of such tissue will show the typical brown staining of the cells of the medulla. Staining of such sections (either frozen or paraffin) with azure-eosin stains (Leishman, Giemsa) show a characteristic yellowish-green colouring of the chromaffin cells.

The Vulpian reaction, which is the treatment of fresh suprarenal tissue in dilute ferric chloride, gives a greenish colour to the medullary cells.

In addition to the above methods, chromaffin tissue becomes a greenish-blue colour with Schmorl's ferric chloride-ferricyanide test (page 386) if chromate fixed, and greyish-red colour with the periodic acid-Schiff reaction (page 268).

Adrenaline

Adrenaline is demonstrated in cells by the above methods, or by Cramer's osmium tetroxide method, whereby thin slices of fresh suprarenal, which have been freed of surrounding fat, are fixed in the vapour of 2 per cent osmium tetroxide at 37°C for 2–3 hours.

The tissue is then rapidly dehydrated and embedded in paraffin wax. Alternatively, frozen sections may be cut, which give deeper staining by avoiding processing through fat solvents.

To avoid confusion, globules of fat which are blackened by osmium tetroxide are removed from sections by treatment with crude turpentine. The black granules, which remain after removal of the fat, are presumed to be adrenaline.

TEETH

Sections of teeth are prepared by the methods already described for bone on page 409. Ground bone sections are similarly prepared, but since the cutting of thin slices before grinding with a saw may be difficult, a metal wheel, the edges of which are impregnated with diamond dust, should be used if possible. Very thin slices, cut on such a wheel, may be mounted directly in H.S.R. or D.P.X.

Fixation

Most routine fixations may be employed, formalin fixation being generally preferred.

Staining Methods

The staining methods for sections of teeth are as already described for bone sections.

THE EYE

Composed as it is of very hard and very soft tissue, sections of the eye are difficult to prepare unless the correct technique is used.

Fixation

The complete eye should be fixed in formol saline or Zenker's fluid for 24–48 hours, after which it is wrapped in gutta percha and frozen in an ice-salt compound, or in frozen carbon dioxide in alcohol. When completely frozen, it is bisected with a sharp knife. The direction of the cut will normally be from the lens to the optic nerve, but occasionally a cross-section may be required. An alternative method which is preferred by some pathologicst is to make two cuts that take off the top and bottom of the eye. This leaves the whole of the cornea, lens and optic nerve in one flat circular block. Care must be taken not to detach the retina during any stage of the technique.

Embedding

It is best to embed the specimen in celloidin (page 102), although double embedding or paraffin embedding may sometimes give a reasonable result. A method of embedding in polyester resin has been described by Culling, Martin and Smith (1960) which, while it makes sectioning more difficult, results in reasonably good H. and E. sections and a plastic embedded specimen which may be used as a museum specimen.

Staining

Standard methods may be used to demonstrate connective tissue, myelin, neuroglia, and so on.

Various components of the rods and cones of the retina may be demonstrated by iron haematoxylin (page 215), fat-soluble stains (page 359), and the P.A.S. technique (page 268). The last named may also demonstrate glycogen in the retina and a web-work in the vitreous humour.

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Chapter 20

Certain Cytoplasmic Constituents and Cell Products

In this chapter most of the normal cytoplasmic constituents, and certain cell products, are discussed. Some, such as fats and enzymes, are thought to be of sufficient importance to merit a separate chapter, while others, such as pigments and neutral red vacuoles, it seemed more logical to discuss in relation to the tissues or other factors involved. The structures included in this chapter are dealt with in alphabetical order, and the appropriate page for others will be found in the index.

ARGENTAFFIN OR ENTEROCHROMAFFIN CELLS

These cells are also known as Kultschitzky cells. They are found normally in the pyloric glands of the stomach, at the base of intestinal glands (the crypts of Leiberkuhn), and in the mucous coat of the appendix. They were considered by Masson (1928) to be the type cells of carcinoid tumours, which are often referred to as argentaffin cell tumours. It now appears that the presence or absence of positive reactions for argentaffin cells in carcinoid tumours will depend on the functional state of the tumours (Lillie and Glenner, 1960). Most carcinoid tumours of the small intestine, appendix, and caecum are positive; however, Vassar and Culling (1962) described 10 bronchial carcinoids which failed to give positive reactions.

The histochemical reactions given by the granules in argentaffin cells have been shown to be dependent on the presence of 5-hydroxytryptamine (5-HT). The 5-HT must, however, be converted to the tetrahydro-4-carboline derivative by formalin fixation before these reactions can be demonstrated (Holcenberg and Benditt, 1961). In this respect

the argentaffin (or enterochromaffin) cell differs from chromaffin tissue proper (page 464) which will give the chromaffin reaction after treatment with potassium dichromate only (without prior treatment with formalin).

Gelatin (Benditt and Wong, 1957) and plasma (Vassar and Culling, 1962) models can be prepared with varying concentrations of 5-HT which, after formalin fixation, may be used to assess the sensitivity of the various reactions. Smears (formol fixation only) and formol-fixed paraffin sections were used. Autofluorescence and Schmorl's reaction, although not specific, were the most sensitive, detecting as little as 39 μg 5-HT/ml in smears, and 312 μg 5-HT/ml in paraffin sections. Argentaffin and diazo reactions which are more specific, were less sensitive, detecting 78 and 624 μg/ml respectively.

The diazo reaction in conjunction with Schmorl's ferric ferricyanide reaction (page 386) are recommended as routine methods.

Fixation

As already mentioned, the fixative must contain formalin; formol saline is probably the best, but Zenker–formol gives good results. Alcohol (or alcohol-containing fixatives) must not be used as it dissolves argentaffin granules.

Silver Impregnation Method

Fontana's Technique

Silver solution. – To 25 ml of 10 per cent aqueous silver nitrate add strong (.880) ammonia drop by drop until the precipitate which first forms has almost disappeared; then add 25 ml of distilled water.

This solution should be left for 24 hours, stored in a dark bottle and filtered before use. It is better to renew this solution after 14 days, but it may be used for 1 month.

Method

(1) Bring paraffin, frozen or celloidin sections to water.
(2) Place in Gram's iodine for 5 minutes.*
(3) Transfer to 3 per cent sodium thiosulphate for 3 minutes.*
(4) Wash in several changes of distilled water.
(5) Transfer to the silver solution in a covered container and leave in the dark for 18–48 hours at room temperature.

*If fixed in a mercuric-chloride-containing fixative.

(6) Rinse in several changes of distilled water.
(7) Tone in 0.2 per cent gold chloride (or Cajal's toner, *see* page 477) for 10 minutes.
(8) Rinse in distilled water.
(9) Fix in 3 per cent sodium thiosulphate for 2 minutes.
(10) Wash in running tap-water for 2 minutes.
(11) Counterstain with 1 per cent safranine for 1 minute.
(12) Wash in tap-water for ½ minute.
(13) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Argentaffin cell granules	.Black
Melanin granules	.Black
Other constituents	.Shades of red and pink

Note. – The technique described above may be used substituting hexamine silver solution for Fontana's silver solution. Using the former solution the technique is more rapid. The section is placed in hexamine silver in a 60°C oven for only 3½ hours.

The Gomori-Burtner methenamine (hexamine) silver solution is prepared as follows. A stock solution is made by adding 5 ml of 5 per cent silver nitrate to 100 ml of 3 per cent methenamine (hexamine) in distilled water; a precipitate forms which redissolves almost immediately. This stock solution keeps in a cool dark place for several months. *For use,* add 8 ml of Holmes' pH 7.8 boric acid-borate buffer (page 175) to 30 ml of the stock of silver solution.

Diazo Technique

Fixation

Fix tissue in formol saline.

Special Reagent Required

To 5 ml of 1 per cent aqueous fast red salt B* add 2 ml of saturated aqueous lithium carbonate. Place in a refrigerator at 4–5°C for 10 minutes before use.

*Obtainable from G. T. Gurr, Kings Road, London, S.W.6.

Method

(1) Bring section to distilled water.
(2) Stain in the above reagent in the refrigerator for 2 minutes.
(3) Rinse in distilled water.
(4) Wash in running tap-water for 2–3 minutes.
(5) Counterstain nuclei with haematoxylin.
(6) Blue in tap-water.
(7) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Argentaffin cell granules	Orange red
Nuclei	Blue

Autofluorescence

Formalin-fixed paraffin sections are brought to water and mounted unstained in Apathy's mounting medium. They are examined by fluorescence microscopy using a Schott UG 2 (3000 Å wavelength) exciter filter and a colourless ultraviolet barrier filter. Argentaffin granules fluoresce a bright golden yellow.

Chromaffin Reaction

The chromaffin reaction is not generally used to demonstrate argentaffin cells since it is less specific than the methods given above. The technique is the same as that given for chromaffin tissue on page 464.

CALCIUM

The deposition of calcium is normally restricted to bone, although hyaline cartilage may show calcareous infiltration in old age. Calcium is not uncommonly found deposited in fibroids, tuberculous foci, mesenteric lymph glands and the thyroid gland; although calcium compounds are distributed throughout all the tissues and body fluids such compounds are in a state of solution and cannot be demonstrated histologically.

Fresh Preparations

The solubility of calcium deposits in mineral acids may be used to demonstrate their presence in tissue, but it does not give their exact

location. Treatment of smears or sections with concentrated hydrochloric acid may be used to determine whether the calcium present is carbonate or phosphate:

(1) The smear or section is mounted in water.
(2) Hydrochloric acid is run under the coverslip.
(3) Solution of the deposit *with* bubbles of CO_2 indicates the presence of calcium carbonate, whereas solution of the deposit *without* bubbles indicates calcium phosphate. Dilute sulphuric acid (15 per cent) may also be used in this manner and the formation of gypsum crystals is specific for calcium.

Sections

The methods for the demonstration of calcium in sections are based on the following.

(1) The conversion of the calcium salt into the salt of another metal which itself is opaque (for example, von Kossa's technique) or, by further treatment, into a coloured compound (as in the alkaline phosphatase method, page 320).
(2) The ability of certain dyes to form lakes with calcium (such as purpurin or anthrapurpurin [alizarin], or even haematoxylin).

In spite of the new dyes and methods which have been evolved for the demonstration of calcium in sections, the method usually employed is that of von Kossa, which was first described in 1901.

Lillie's oxalic acid technique is a more specific method for the presence of calcium, but does not show the location of the deposit. The use of the anthraquinone dyes (purpurin, anthrapurpurin) is usually confined to the demonstration of bones in embryos (page 549) on account of the technical difficulty of employing it and its low specificity.

The morin fluorescent technique for calcium may be confusing for an inexperienced observer in view of the autofluorescence of other material that may be present in some tissue sections (*see* page 610). Morin also reacts with aluminium and beryllium.

von Kossa's Technique

Method

(1) Bring two sections to water.
(2) Immerse one section in pH 4.5 citrate buffer for 20 minutes *(see Note).*

(3) Wash both slides well in distilled water.
(4) Flood slides with 5 per cent silver nitrate.
(5) Expose to bright sunlight or ultra-violet light for 10–20 minutes or to a 60-watt electric bulb at a range of 4–5 inches for 30–60 minutes.
(6) Wash in several changes of distilled water.
(7) Treat with 5 per cent sodium thiosulphate for 2–3 minutes.
(8) Counterstain with neutral red, safranine, or van Gieson's stain.
(9) Dehydrate, clear, and mount in D.P.X. or Canada balsam.

Result

Calcium deposits . . . . . Black

Note. – This technique may be made more specific by removing the calcium from parallel sections with a 0.1 M citrate buffer at pH 4.5 for 20 minutes (Everson Pearse, 1951). The buffer is prepared by mixing the following.

0.2 M disodium hydrogen phosphate . . . . . 9.09 ml
0.1 M citric acid . . . . . 10.91 ml

Lillie's Oxalic Acid Technique

Method

(1) Mount the section in water.
(2) Draw 10 per cent oxalic acid under the coverslip by placing a few drops of reagent at one end of the coverslip, and a piece of blotting paper at the other.

Results

The calcium deposits dissolve with the formation of characteristic envelope-shaped calcium oxalate crystals.

Fluorescent Technique for Calcium and Aluminium (Pearse, 1960)

(1) Bring sections to 95 per cent alcohol.
(2) Stain for 2–5 minutes in 0.2 per cent Morin in 85 per cent alcohol containing 0.5 acetic acid.
(3) Wash in 95 per cent alcohol (differentiate if necessary in acid-alcohol).

(4) Rinse and mount in water; or *preferably,* rinse rapidly in absolute alcohol, clear in xylol, and mount in Fluormount or H.S.R.

(5) Examine under fluorescent microscope.

Result

A greenish-white fluorescence indicates the presence of calcium or aluminium. By treating a control section in acid buffer *(see above)* they may be differentiated since aluminium is stable in acid solutions.

FIBRIN

Fibrin is made of fine fibres, protein in nature, and is found in acute inflammatory processes.

It may be demonstrated in sections by Mallory's phosphotungstic acid haematoxylin (page 217), Weigert's modification of Gram's stain or by Lendrum's stain *(see below).*

Fixation is not critical and the methods given work after almost any fixative.

Weigert's Stain for Fibrin

Method

(1) Bring sections to water.

(2) Stain with 2.5 per cent aqueous eosin at 56°C for 10 minutes.

(3) Wash in water.

(4) Stain in 1 per cent aqueous methyl violet for 3 minutes.

(5) Rinse in water, and treat with Lugol's iodine for 3 minutes (*see* page 391).

(6) Blot dry with fine, fluffless filter paper.

(7) Differentiate with aniline oil and xylol (equal parts), until only the fibrin network is stained violet.

(8) Rinse well with xylol to remove aniline oil.

(9) Rinse in fresh xylol and mount in D.P.X. or Canada balsam.

Results

Fibrin, Gram-positive bacteria, hyalin degeneration	Blue-black
Other tissue constituents	Red

MSB Method for Fibrin (Lendrum and Colleagues, 1962)

Special Reagent Required

Martius yellow	0.5 g
Phosphotungstic acid	2.0 g
95 per cent alcohol	100 ml

Dissolve phosphotungstic acid and Martius yellow in 95% alcohol.

Method

(1) Sections* of tissue (preferably fixed in formol-mercuric chloride for a lengthy period, up to 8 weeks; or treat sections of formol-fixed tissue in formol-mercuric chloride for 24 hours) are brought to water.

(2) Stain nuclei with celestine blue-haemalum sequence (*see* steps 2–5 page 475).

(3) Wash in running water.

(4) Rinse in 95 per cent alcohol.

(5) Stain in Martius yellow solution for 2 minutes.

(6) Rinse in distilled water.

(7) Stain in 1 per cent brilliant crystal scarlet 6R (Ponceau 6R) in 2.5 per cent acetic acid for 10 minutes.

(8) Rinse in distilled water.

(9) Treat with 1 per cent phosphotungstic acid for up to 5 minutes. This fixes and differentiates the crystal scarlet staining.

(10) Rinse in distilled water.

(11) Stain in 0.5 per cent soluble blue in 1 per cent acetic acid for up to 10 minutes.

(12) Rinse in 1 per cent acetic acid, blot, dehydrate in absolute alcohol, clear in xylene and mount in a synthetic resin.

Results

Nuclei	Blue-black
Fibrin	Red
Erythrocytes	Yellow
Connective tissue	Blue

*In the absence of formol-mercuric chloride fixation, Lendrum and his colleagues recommend treatment of sections in trichlorethylene for 24–48 hours following treatment with xylene to remove wax.

Lendrum's Acid Picro-Mallory Method

This, or the preceding, method is specific for fibrin and gives a result which justifies the extra time spent on it. The best results are given after extended mercuric chloride fixation.

Solutions Required

(1) *Celestin blue.* – Dissolve 2.5 g of iron alum in 50 ml of distilled water overnight at room temperature, and to this add 0.25 g of celestin blue. Boil for 3 minutes, and filter when cool into a staining jar; add 7 ml of glycerin. This staining solution will keep for several months.

(2) *Mayer's haemalum.* – Refer to page 214.

(3) *Picro-orange.* – Dissolve 0.2 g of orange G in 100 ml of 80 per cent alcohol which has been saturated with picric acid.

(4) *Acid fuchsin.* – One per cent acid fuchsin in 3 per cent trichloracetic acid.

Method

(1) Bring sections to water.

(2) Stain with celestin blue in a Coplin jar for 3–5 minutes.

(3) Rinse in tap-water.

(4) Stain with Mayer's haemalum for 5 minutes (longer if the solution is not fresh).

(5) Wash in tap-water for 3 minutes, then rinse in 95 per cent alcohol.

(6) Stain with picro-orange for 2 minutes.

(7) Stain with acid fuchsin for 5 minutes, then rinse in water.

(8) Dip sections into equal parts of picro-orange and 80 per cent alcohol for a few seconds.

(9) Differentiate in 1 per cent phosphotungstic acid until colours are clear (5–10 minutes), then rinse in water.

(10) Stain with 2 per cent soluble blue in 2 per cent aqueous acetic acid for 2–10 minutes, then rinse in water.

(11) Dehydrate, clear, and mount in D.P.X. or Canada balsam.

Results

Fibrin	Clear red
Red blood cells	Orange
Collagen	Blue
Nuclei	Blue-black

GOLGI APPARATUS

Golgi apparatus *(see Figure 1.1)*, also known as Golgi substance, and Golgi bodies, was first described in nerve cells by Golgi (1898). It can rarely, if ever, be demonstrated in routine material, since it requires special fixation. It may be demonstrated by one of three methods: (1) silver impregnation; (2) osmium tetroxide reduction; or (3) with fat stains such as Sudan black B. None of the methods at present available is consistent or reliable for all tissues, and consequently the technique of choice will depend on the type of tissue and the experience of the operator. On occasions it may be necessary to try all three types of technique before getting a first-class preparation. It is often advisable to experiment with the time of fixation and exposure of the tissue to the stain or reagent before deciding to change to another technique. The da Fano-Cajal technique has been found most successful for adult human tissue other than that of the central nervous system, for which Ludford's modification of Weigl's technique is the method of choice. It is best demonstrated by electron microscopy.

Da Fano-Cajal Method

Fixation

The fixative of choice is 1 per cent cobalt nitrate in distilled water, to which is added 6–15 per cent of formalin. It is advisable to keep the cobalt nitrate in stock and add the appropriate amount of formalin before use.

The amount of formalin to be added depends on the type of tissue; for example, embryonic tissues give the best results with 6 per cent formalin and adult tissues with 15 per cent formalin.

To get the best results it is advisable to fix 3 pieces of tissue for varying times. They should not be more than 2 mm in thickness and taken not more than 2–3 hours after death. In general it will be found that embryonic tissues require only 3 hours, normal adult tissue 5–8 hours, and brain and spinal cord from 10 to 16 hours.

Solutions Required

(1) *Silver nitrate.* – 1.5 per cent in distilled water.

(2) *Cajal's developer*

Hydroquinone	2 g
Formalin	15 ml
Distilled water	100 ml
Sodium sulphite (anhydrous)	0.5 g

Dissolve the hydroquinone in the distilled water, then add the formalin, and finally the sodium sulphite.

(3) *Toning solutions (optional)*

Solution A	Sodium thiosulphate	3 g
	Ammonium sulphocyanide	15 ml
	Distilled water	100 ml
Solution B	Gold chloride	1 g
	Distilled water	100 ml

For use mix equal parts of A and B, the stock solutions being kept separately.

Method

(1) Fix as described above.
(2) Rinse in distilled water.
(3) Transfer to silver nitrate bath for 36–48 hours.
(4) Rinse the tissue rapidly in distilled water.
(5) Reduce in Cajal's developer for 6–12 hours.
(6) Wash well in running tap-water.
(7) Dehydrate rapidly, clear in xylol, and embed in paraffin wax in the usual manner.
(8) Cut thin sections (3–6 μ) and attach them to slides.
(9) Bring to water.
(10) Transfer to Cajal's toning solution* and leave for 5–10 minutes.
(11) Wash in running water, dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Golgi apparatus	Black
Cytoplasm	Grey
Mitochondria and myofibrillae of muscle	Sometimes medium to dark grey or black

Note. – The time of impregnation in silver is governed by the same factors as fixation (for example, embryonic tissues, 36 hours;

*Although optional, toning the sections before mounting will give them greater permanence, and a black and grey appearance instead of a dark and light brown colour. They may then be counterstained with safranine.

brain and spinal cord, 48 hours). Under-impregnation will fail to show Golgi material, and over-impregnation will cause a heavy deposit of silver.

Weigl Technique Modified by Ludford

Solutions Required

(1) *Mann's fixative*

Mercuric chloride, saturated solution in 0.75 per cent sodium chloride	50 ml
1 per cent osmium tetroxide in distilled water	50 ml

These solutions are kept in stock and mixed immediately before use.

(2) *Osmium tetroxide.* – Two per cent osmium tetroxide in distilled water.

Method

(1) Fix thin pieces of tissue (2–3 mm) in Mann's fixative for 18–24 hours.

(2) Wash in several changes of distilled water for 30 minutes.

(3) Impregnate with 2 per cent osmium tetroxide at 37°C for 2–3 days.

(4) Rinse well in distilled water, and wash in running tap-water for several hours.

(5) Dehydrate, clear and embed in paraffin wax as rapidly as possible.

(6) Cut sections, remove the paraffin wax with xylol, and mount in Canada balsam or D.P.X.

Sections may with advantage be counterstained with safranine or neutral red before mounting.

Results

Golgi apparatus, fats and lipids	Black
Mitochondria	May be blackened

Sudan Black B Technique (Baker, 1949)

Solutions Required

(1) *Formol saline* (10 per cent formalin in 0.7 per cent sodium chloride)

(2) *Formol dichromate*

Potassium dichromate	2.2 g
Sodium chloride	0.7 g
Distilled water	95 ml
Formalin (neutral)	5 ml

(3) *Embedding medium* (25 per cent gelatin, containing 0.2 per cent sodium parahydroxybenzoate)

(4) *Formalum*

Potassium alum	4 g
Distilled water	80 ml
Formalin	20 ml

(5) *Sudan black B saturated solution.* – This is prepared by boiling 0.5 g in 100 ml of 70 per cent alcohol for 10 minutes, using a reflux condenser. Leave to cool; when cold the solution is ready for use.

(6) *Mayer's carmalum.* – Dissolve 2 g of carmine in 100 ml of 5 per cent ammonium alum by boiling for 1 hour. Make up to original volume and add a few crystals of thymol to prevent the growth of moulds.

Method

(1) Fix pieces of tissue, not more than 3 mm in thickness, in neutral formol saline for 1 hour.

(2) Transfer, without washing, to fresh formol dichromate and leave for 5 hours.

(3) Transfer, without washing, to 5 per cent potassium dichromate for 18–24 hours at room temperature.

(4) Place in 60°C paraffin oven for 24 hours.

(5) Wash in running water overnight, or for at least 6 hours.

(6) Infiltrate in embedding medium at 37°C for 18–24 hours.

(7) Embed in mould as described for paraffin 'blocking', cool in refrigerator or cold room, and harden block in formalum *(see above)* for 12–24 hours.

(8) Cut sections (8–10 μ) on freezing microtome, receiving them in distilled water.

(9) Transfer cut sections to 70 per cent alcohol for 1 minute.

(10) Transfer to Sudan black B solution for ½–4 minutes (usually 2 minutes will suffice).

(11) Wash rapidly in 70 per cent alcohol to remove surplus stain.

(12) Transfer to 50 per cent alcohol for 1 minute.

(13) Wash in water for 1 minute.

(14) Transfer to Mayer's carmalum and leave for 2–3 minutes.

(15) Rinse in distilled water for 2–3 minutes, giving at least four changes.

(16) Float on to slide, allow to drain, and mount in glycerin gum arabic, glycerin jelly or other aqueous medium.

Results

Golgi apparatus	Dark blue
Cytoplasm	Pale blue to colourless
Nuclei	Shades of red
Neutral fats	Dark blue

MITOCHONDRIA

These minute filamentous or granular structures are generally found scattered throughout the cytoplasm *(see Figure 1.3c* and Frontispiece). They are lipo-protein in nature and are quickly affected by autolysis. Early and special fixation is therefore essential.

It should be remembered that the phase contrast microscope may give the simplest and best demonstration of mitochondria.

Fixation

Thin pieces of tissue should be fixed in Zenker-formol fluid (page 48) for 24 hours, followed by immersion in 3 per cent potassium dichromate for 4 days (the solution should be changed 2–3 times during this period). Alternatively, after treatment in Zenker-formol the tissue may be immersed in Champy's fluid for 12–24 hours (page 51), followed by 3 per cent potassium dichromate as above. Of these two methods the former is preferred. Following fixation the tissue must be well washed in running water for 12–24 hours before processing and embedding in paraffin wax.

Choice of Staining Procedure

The best method of demonstrating mitochondria is the Champy-Kull technique (*see* Frontispiece), but the acid fuchsin-picric acid technique is much easier to control. The Millon reaction works well on certain tissues, and Heidenhain's iron haematoxylin (page 215), if properly differentiated, will show mitochondria.

Sections should be cut as thinly as possible (2–4 μ) to demonstrate mitochondria by any of the techniques. The best material for test sections is fresh fixed kidney or intestine.

Champy-Kull's Method

This method gives very striking results when employed successfully, but it is not recommended as a technique for beginners. It is based on overstaining the mitochondria with aniline fuchsin, staining the nuclei and partially differentiating the fuchsin with toluidine blue, completing the differentiation of the fuchsin and removing the excess toluidine blue with alcoholic aurantia. The times of exposure to the toluidine blue and the aurantia are very critical and must be corrected for each group of sections. Consequently, if only a single section of a tissue is available it should be stained by Altmann's method.

Solutions Required

(1) *Aniline acid fuchsin.* – Saturated solutions of acid fuchsin (approximately 14 per cent) in aniline water (page 166).

(2) *Toluidine blue.* – 0.5 per cent aqueous solution.

(3) *Aurantia.* – 0.5 per cent in 70 per cent alcohol.

Method

(1) Bring sections to water.

(2) Remove mercury precipitate with iodine and thiosulphate (*see* page 40).

(3) Wash well in water.

(4) Place slide on staining rack, flood with aniline acid fuchsin, heat the slide until the stain steams and leave for 5 minutes. (Avoid boiling the fuchsin).

(5) Rinse slide with distilled water.

(6) Flood with toluidine blue, leave for 3 minutes *(see Note below).*

(7) Rinse slide with distilled water.
(8) Flood with aurantia, leave for 2 minutes *(see Note below).*
(9) Rinse and flood slide with absolute alcohol for 30 seconds.
(10) Clear in xylol, mount in Canada balsam, and examine with the oil immersion objective.

Results

Mitochondria	Red
Nuclei	Blue
Cytoplasm	Yellow

Note. – If the first section is not well stained, the times of exposure to toluidine blue or aurantia (Stages 6 and 8) will need to be varied as follows. (1) If the section is too red, increase the time in toluidine blue by ½–1 minute. (2) If the section is too blue, increase the time in aurantia.

Although the alcohol removes a certain amount of blue, it is better to leave these times constant and only vary the times of the toluidine blue and aurantia. It will be found as a general rule that the toluidine blue needs to be left on longer than the aurantia in the ratio of 3:2 or even 2:1.

Altmann's Acid Fuchsin-Picric Acid Technique

Solutions Required

(1) *Aniline acid fuchsin.* – Saturated solution of acid fuchsin (approximately 14 per cent) in aniline water (page 166).

(2) *Picric acid differentiator*

Picric acid, saturated solution in absolute alcohol	20 ml
30 per cent alcohol	to 80 ml

Method

(1) Bring sections to water (Stages 1–3 of the Champy-Kull method).
(2) Transfer section to slide rack and flood with aniline acid fuchsin. Heat (*see* page 192) until steaming, but not boiling, and leave for 5 minutes.
(3) Rinse in distilled water for about 2–3 seconds.

(4) Differentiate in picric acid differentiator until the bright red colour of the sections begins to fade.

(5) Continue differentiation – controlling the section under the microscope – with picric acid differentiator diluted 50:50 with 30 per cent alcohol.* Differentiation is continued until only the mitochondria are red, and the nuclei and cytoplasm are yellow. (Red blood cells will be stained red.)

(6) Rinse in 90 per cent alcohol for 1 or 2 seconds.

(7) Rinse in absolute alcohol for 1 or 2 seconds.

(8) Clear in xylol, and mount in Canada balsam or D.P.X.

Results

Mitochondria and red blood cells	Bright red
Nuclei, cytoplasm, collagen, and so on.	Yellow

If an osmium tetroxide fixative has been used, fat will be black.

Millon Reaction

This reaction (modified by Bensley and Gersh, 1933) will demonstrate all proteins containing tyrosine and was adapted to demonstrate mitochondria in freeze-dried tissues, but works satisfactorily on paraffin-embedded tissues.

Thin sections are recommended to give the best results.

Solutions Required

(1) *Millon reagent.* – Prepare 100 ml of 40 per cent nitric acid in distilled water, and allow to stand for 48 hours. To 70 ml of the diluted nitric acid add 630 ml of distilled water, mix and saturate with mercuric nitrate crystals. Filter, and to 400 ml of the filtrate add 3 ml of the original 40 per cent nitric acid and 1.4 g of sodium nitrate.

(2) *Nitric acid* (2 per cent).

Method

(1) Remove the wax from thin paraffin or freeze-dried tissues with light petroleum.

*Cain (1948) used 0.1 per cent aqueous sodium carbonate to differentiate, followed by a dip into 1 per cent hydrochloric acid, a wash in distilled water and counterstaining in 1 per cent methylene blue or methyl green.

(2) Cover with acetone, leave for ½ minute, pour off excess and allow section to dry.

(3) Cover with Millon reagent and leave until the maximum colour develops; this may be done at room temperature, but as it requires a long period it is preferable to place the slide in a covered Petri dish in the 60°C oven; the reaction should then be complete in ½–1 hour.

(4) Rinse in cold 2 per cent nitric acid; or if the 60°C oven has been used to accelerate the reaction, in warm 2 per cent nitric acid.

(5) Dehydrate rapidly through 90 per cent and absolute alcohol.

(6) Clear in xylol, and mount in Canada balsam.

Results

Mitochondria (or any protein containing tyrosine, such as Russell bodies) . . . . . Orange to red

NEUTRAL RED VACUOLES

The reader is referred to Chapter 23 on Vital Staining.

PANETH CELL GRANULES

Paneth cells are found at the bottom of the intestinal glands (crypts of Lieberkühn) of the small intestine. These cells contain large stainable granules, which are thought by some to be the precursors of the intestinal enzymes.

The granules are strongly eosinophilic, and are well demonstrated by Lendrum's phloxine-tartrazine stain (*see* Frontispiece).

Fixation

The granules are best demonstrated after neutral formol saline fixation. They are destroyed by acetic acid fixatives.

Lendrum's Phloxine-Tartrazine Stain

This stain was designed by Lendrum (1947) as a general histological stain, and also for the demonstration of inclusion bodies.

Method

(1) Bring section to water.

(2) Stain lightly with haematoxylin. (Lendrum specifies Mayer's, but any alum haematoxylin may be used.)

(3) Blue in running tap-water.

(4) Stain with 0.5 per cent phloxine in 0.5 per cent calcium chloride for 30 minutes in a Coplin jar (this stain keeps for about a year).

(5) Rinse in water, drain almost dry.

(6) Flood with a saturated solution of tartrazine in Cellosolve (ethylene glycol, monomethyl ether), which differentiates and counterstains.

(7) Rinse with 95 per cent alcohol.

(8) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Paneth cell granules and inclusion bodies	Bright red
Nuclei	Blue
Cytoplasm and collagen	Yellow
Muscle, fibrin, keratin, red blood cells	Red

Chapter 21

Exfoliative Cytology and Chromosome Techniques

Exfoliative cytology is the study of superficial cells which have been exfoliated or shed from mucus membranes, renal tubules, and so on; and also includes the study of those cells which have been scraped or pulled off such surfaces. Such cells may also be found in body fluids; for example, sputum, peritoneal fluid, and so on. Most of the techniques employed are for the rapid and early diagnosis of malignancy, particularly in carcinoma of the cervix.

Most of the difficulties encountered in applying these techniques are due to poor preparation and fixation of the smears. It is important that smears are sufficiently thin and that they are put into fixative while still moist.

Cytodiagnosis of Cancer

While a variety of stains are available to stain the smears, the Papanicolaou method is still probably the best available when searching for malignant cells.

A high concentration of RNA in cells has been used as an indication of malignancy and for this reason the acridine-orange technique of von Bertalanffy (1956) became popular. While this technique does offer a method of rapid assessment of RNA concentration, this alone has not been found completely reliable for the cytodiagnosis of malignancy and, furthermore, the smears *cannot* be permanently mounted. Since the smears are mounted in a water mount, it is more difficult to see cytological detail on which, in the majority of cases, a diagnosis will depend (Culling and Vassar, 1961). Smears stained by the acridine-orange technique may be subsequently stained by the Papanicolaou method.

Cervical Smears

These must be sufficiently thin to enable cytological detail to be studied, and must be placed into the fixative while still moist. The most commonly used fixative for this purpose is equal parts of ether and ethyl alcohol. The slides are then transported to the laboratory while still immersed in the fixative.

Treatment of Body Fluids

Urine

This may be an early morning voided sample from male patients, but in females it is usually obtained by catheterization to avoid contamination by desquamated vaginal epithelium. Specimens less than 50 ml in volume are usually impracticable because of the small number of cells likely to be present. Early morning specimens should be examined as soon as possible; some workers add an equal quantity of 95 per cent alcohol to the fresh specimen. Three techniques have been described, each of which has certain advantages.

(1) *Deden's Method (Deden, 1954)*

Collect large volumes of urine (400–500 ml), allow to sediment in separating funnels. Collect the sediment and centrifuge. Make smears of entire deposit on several albumenized slides, fix in ether–alcohol for 15 minutes and stain by Papanicolaou technique.

(2) *Millipore Filter Method (Solomon and Colleagues, 1958)*

The urine specimen is filtered through the cellulose membrane (5 μ pore size) using a negative pressure of 25 mm Hg. Large volumes may be passed through the filter which withholds the cells. The filter is finally mounted on a glass slide and stained by the Papanicolaou technique.

(3) *Sagi and Mackenzie's Method (1958)*

A 200 ml sample of urine is distributed into centrifuge tubes after being cleared of urates, phosphates, carbonates, and so on. After centrifugation, the supernatant is discarded and the deposits from all tubes are combined into one tube, a small amount of distilled water being used as a vehicle. This tube is again centrifuged and the supernatant discarded. One drop of a mixture of equal parts of cell-free saliva and glycerin are stirred into the deposit to make it cohesive. This suspension is then transferred with a pipette to a single clean

slide and spread evenly over its surface. Fix in acetone and stain by Papanicolaou technique.

Other Body Fluids

Other fluids containing cells (such as sputum, pleural, peritoneal, gastric and spinal) may be treated by methods (1) or (2) above. Alternatively, Bouin's fixative (page 49) may be added to the centrifuged deposit; this has the effect of coagulating the proteinaceous material. After 8–12 hours the material is filtered through coarse filter paper; the filter paper folded over makes a convenient container for the material which is now dehydrated, cleared and embedded in paraffin wax. The paper is opened after the last change of molten paraffin and the material is scraped into the mould for blocking (*see* page 87). The paraffin sections may be stained by Papanicolaou stain or haematoxylin and eosin. By cutting and staining serial sections every cell in the specimen may be examined.

STAINING METHODS

Papanicolaou Method (1942, 1957)

The staining solutions employed in this technique may be purchased commercially*, or prepared, as follows.

Special Reagents Required

(1) Harris's haematoxylin (without acetic acid).

(2) Orange G 6 (OG 6)
0.5 per cent Orange G 6 in 95 per cent alcohol . 100 ml
Phosphotungstic acid . . . . . 0.015 g

(3) Eosin-azure 36 (EA 36 or EA 50)
0.5 per cent Light green SF yellowish in 95 per cent alcohol . . . . . 45 ml
0.5 per cent Bismarck brown in 95 per cent alcohol . . . . . 10 ml
0.5 per cent Eosin Y in 95 per cent alcohol . . . . . 45 ml
Phosphotungstic acid . . . . . 0.2 g
Sat. aq. lithium carbonate . . . . . 1 drop

EA[65] uses 0.25 per cent light green which is preferable if an excess of mucus is present in the smear.

*Ortho Pharmaceutical Corp., Raritan, New Jersey, U.S.A.

Technique

(1) Fix smears (while still moist) in equal parts of alcohol and ether for 15–30 minutes, although they may be left in the fixative for longer periods without damage.
(2) Rinse smears in distilled water.
(3) Stain in Harris's haematoxylin for 4 minutes.
(4) Wash in tap-water for 1–2 minutes.
(5) Differentiate in acid alcohol (*see* page 181).
(6) Blue in tap-water or 1.5 per cent sodium bicarbonate.
(7) Rinse in distilled water.
(8) Transfer to 70 per cent then 95 per cent alcohol for a few seconds.
(9) Stain in OG 6 for 1–2 minutes.
(10) Rinse in 3 changes of 95 per cent alcohol for a few seconds in each.
(11) Stain in EA 36 for 1–2 minutes*.
(12) Rinse in 3 changes of 95 per cent alcohol for a few seconds in each.
(13) Dehydrate in absolute alcohol, clear in xylol, and mount in D.P.X. or H.S.R.

Results

Nuclei	Blue
Acidophilic cells	Red to orange
Basophilic cells	Green to blue green
Cells or fragments of tissue penetrated by blood	Orange to orange green

Fluorescent Acridine Orange Technique (Bertalanffy)

This method gives good differentiation of RNA and DNA, although there is doubt as to its absolute specificity. It gives a brilliant red staining of RNA and is excellent for plasma cells and those cells actively synthesizing protein.

Solutions Required

(1) M/15 Phosphate buffer pH 6.0 (page 171).
(2) Acridine orange 0.1 per cent in phosphate buffer, pH 6.
(3) M/10 Calcium chloride differentiator (1.109 g in 100 ml distilled water).

*This time will vary with different batches.

Method

Smears are fixed in ether/alcohol for at least 30 minutes. Tissue sections fixed in an alcoholic fixative (formalin-fixed tissue cannot be used) are brought to water.

(1) Hydrate by passing them through 80 per cent, 70 per cent and 50 per cent alcohol for 10 seconds each, and rinse in distilled water.

(2) Treat with 1 per cent acetic acid for 6 seconds, followed by rinsing in two changes of distilled water.

(3) Stain in 0.1 per cent acridine orange for 3 minutes.

(4) Wash in M/15 phosphate buffer, pH 6.0, for 1 minute.

(5) Differentiate in M/10 calcium chloride for 30 seconds.

(6) Mount in a drop of pH 6.0 phosphate buffer, and examine under a fluorescence microscope.

Results

DNA fluoresces	Green
RNA fluoresces	Red

Rapid Acridine Orange Fluorescent Method

This method has been recently described by Riva and Turner (1962) as a 10-second staining method for unfixed cervical smears, which can be used in offices and clinics. The author has extended the staining time and decreased the differentiation for use with fixed smears. This method gives results comparable with the longer Bertalanffy method, but it has not been controlled histochemically. It provides an excellent rapid method for checking preparations or smears for the presence of plasma cells, or cells with an RNA rich cytoplasm.

Method (Riva and Turner, 1962 Modified)

Staining solution. – 0.025 per cent acridine orange in 2 per cent acetic acid, to which 0.01 per cent merthiolate has been added to prevent the growth of moulds and bacteria.

Technique

(1) Agitate unfixed or fixed smears for 5 seconds in acridine orange solution.

(2) Differentiate in 2 per cent ethyl alcohol in physiological saline for 2 seconds.

(3) Rinse and then mount in physiological saline.

Results

As described above for Bertalanffy's method.

SEX CHROMATIN STAINING

The sex chromatin is a distinguishing characteristic of interphase nuclei of females in humans and certain other animals. A comparable chromatin body is rarely visible in cells of males (Moore, 1962).

The first demonstration of the sex chromatin body was by Barr and Bertram in 1949 in nerve cells of cats. Moore, Graham and Barr described a simple method of chromatin testing in man, and demonstrated the practical value of the test as a guide to chromosomal sex in ambisexual patients.

The chromatin body is seen, in a well-stained smear from a female, as an intensely staining dot lying against the nuclear membrane *(Figure 21.1)*.

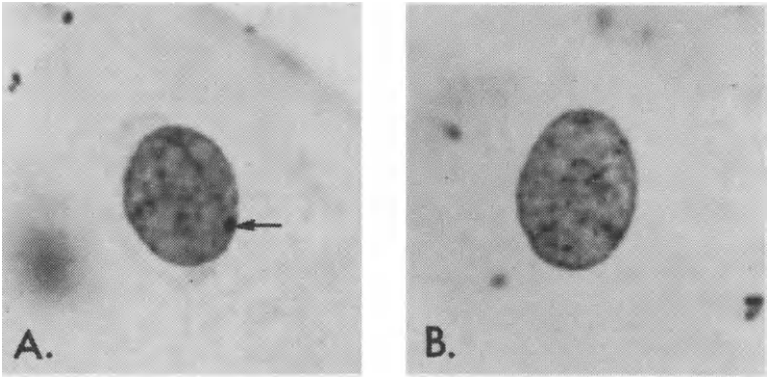


Figure 21.1 – Oral epithelial nuclei stained with cresyl echt violet x2000. A, chromatin-positive nucleus from a normal female; the arrow indicates a typical mass of sex chromatin. B, chromatin-negative nucleus from a normal male; no sex chromatin is visible. (Reproduced from Moore, K. L., and Barr, M. L. (1955). by courtesy of the Editor of The Lancet **2**, *57)*

The first methods described were for skin biopsies and then, because of ready availability of specimens, for neutrophils in the circulating blood. Most examinations are now done on cells from the buccal

mucosa which are obtained by scraping the inside of the cheek with a spatula. The cells from the vaginal mucous membrane do, however, give better staining since the sex chromatin appears to be slightly larger and the cytological detail sharper.

The haematoxylin and eosin technique (page 195) is excellent for the sex chromatin, especially in tissue sections. For a much more complete and authoritarian discussion of the demonstration and significance of the sex chromatin, readers are referred to the excellent textbook *The Sex Chromatin* with 22 contributors, edited by Professor Keith L. Moore.

Making the Smear

This is the most important single step. The scraping of the mucosa *(see above)* is smeared on to a clean slide which is *immediately* (while still moist) plunged into equal parts of ether and ethyl alcohol. Leave for at least 15 minutes, although they may be left for longer periods without great damage.

Staining Techniques

The cresyl echt violet method of Moore (1962) and the Feulgen technique (page 248) are without doubt the best and most reliable. The method of Guard is described because, when it works, the appearance of the sex chromatin is very striking and easy to read; this is due to differential staining. Both of these methods have worked well but the author uses the cresyl echt violet technique as a routine. Smears which contain numerous bacteria are sometimes easier to read when stained by Papanicolaou's technique.

Cresyl Echt Violet Method (Moore, 1962)

(1) Remove the slides from the fixative and pass through 70 per cent alcohol, 50 per cent alcohol and distilled water, 5 minutes in each, with two changes of distilled water.

(2) Immerse the slides in a 1 per cent solution of cresyl echt violet for 5–8 minutes. Coleman and Bell dye gives the best results (Moore).

(3) Differentiate in 95 per cent alcohol, 5–8 quick dips.

(4) Continue differentiation in absolute alcohol, checking with the microscope at intervals until the details of cell structure are defined clearly. Usually this takes about 1 minute.

(5) Clear in two changes of xylol, 3 minutes in each and mount in H.S.R. or Depex.

Results

Sex chromatin is seen as a deeply stained dot in the nucleus, usually lying against the nuclear membrane.

Note. – The smear is studied with the oil immersion objective of a good binocular microscope under optimal condition of illumination.

The smear will contain some shrunken and pyknotic nuclei. These must be passed over. Bacteria are inevitably present and are a source of annoyance at first, but this diminishes with experience. In satisfactory smears there are many well formed nuclei with discrete chromatin particles. If the chromatin detail is not sharp, repeat smears should be taken. The usual cause of this is that the smears were allowed to dry before fixation.

At least 100 well-formed nuclei should be carefully examined for the presence or absence of sex chromatin. If, after careful search, nuclei are not found with a definite mass of sex chromatin at the nuclear membrane, the smear may be interpreted confidently as one from a chromosomal male. The report should refer to chromatin negative nuclei rather than male nuclei.

If 40–60 per cent of nuclei in a smear contain the distinctive mass of sex chromatin, the smear may be interpreted as one from a chromosomal female. The nuclei are described as chromatin positive.

The interpretation of oral smears is relatively easy but requires experience. One should familiarize oneself with the sex difference in nuclei before attempting to sex unknown smears.

Cresyl Echt Violet Method for Nervous Tissues (Moore, 1962)

(1) Fix blocks in 10 per cent formalin.

(2) Dehydrate in graded alcohols (70 per cent, 95 per cent and absolute), with 2 minutes in each.

(3) Clear in xylol.

(4) Embed in paraffin.

(5) Section at 10 μ.

(6) Remove paraffin in xylol, 2 changes with 2 minutes in each.

(7) Hydrate to water as follows: absolute alcohol, 95 per cent alcohol, 70 per cent alcohol, with 2 minutes in each, and distilled water, 5 minutes.

(8) Stain 0.5 per cent solution of cresyl echt violet for 5 minutes.*

(9) Wash in distilled water (2 minutes).

(10) Differentiate in graded alcohols (70 per cent, 95 per cent and absolute), with 2 minutes in each.

(11) Clear in xylol.

(12) Mount with picolyte, neutral balsam, H.S.R. or D.P.X.

**Note.* – Acidification of the dye to pH 4 with acetic acid improves the preparations.

Results

As above.

Guard's Method for Sex Chromatin (1959)

Special Reagents Required

Biebrich scarlet stain

Biebrich scarlet	1 g
Phosphotungstic acid	0.3 g
Glacial acetic acid	5.0 ml
50 per cent alcohol	to 100 ml

Fast green FCF

Fast green FCF	0.5 g
Phosphomolybdic acid	0.3 g
Phosphotungstic acid	0.3 g
Glacial acetic acid	5.0 ml
50 per cent alcohol	to 100 ml

Method

(1) Fixation (as above).

(2) 70 per cent alcohol for 2 minutes.

(3) Biebrich scarlet for 2 minutes.

(4) Rinse in 50 per cent alcohol.

(5) Differentiate in Fast green FCF for 1–4 hours (usually 3 hours) until cytoplasm and nuclei are green. Pyknotic nuclei and sex chromatin are red.

(6) Rinse in 50 per cent alcohol, leave for 5 minutes.

(7) Dehydrate, clear and mount.

Results

Sex chromatin	Bright red
Pyknotic nuclei	Bright red
Cytoplasm, nuclei, and so on.	Green

Staining the Y Chromosome (Figure 21.2)

In 1969, Casperson, Zech and colleagues at the Karolinska Institute, Stockholm, were investigating DNA characteristics by means of fluorescent stains, especially the acridine (or flavine) dyes, by comparison with the Feulgen technique. They were making ultra-microspectrophotometric measurements with a scanning device to give chromosome profiles and discovered a fluorescent banding of the chromosomes, and in particular that the long arm of the Y chromosome was particularly bright. Zech then published a simple staining technique for chromosome preparations to demonstrate the Y chromosomes utilizing quinacrine mustard; this compound is the most effective, but difficult to obtain. Subsequently, Vosa, and Pearson and Vosa showed that quinacrine dihydrochloride (Atebrin) could be used effectively to demonstrate the Y chromosome in cells in metaphase (chromosome preparations) and also in cell nuclei. In the latter a characteristic fluorescent body could be seen in buccal smears (25–50 per cent of cells being positive), thus providing the ideal counterpart of the Barr body or X chromosomes method *(see above).*

Sumner, Robinson and Evans were able to distinguish between X, Y, and YY human spermatozoa and produced some remarkable figures which brought an editorial in *The Lancet* entitled 'Where have all the XYY's gone', for if their figures are correct there should be 4.7 per cent of XYY's in the population instead of the present incidence (estimated at 0.8 per cent).

Rook and his colleagues, and Shettles, are investigating the use of the technique for prenatal sex determination using amniotic cells, and it is likely to be successful although early reports recommend caution in interpretation. It is likely that the availability of quinacrine mustard from commercial sources will give much improved results.

Our experience to date with buccal smears and chromosome preparations (using quinacrine dihydrochloride) would indicate that experience in both fluorescence microscopy and cytology is necessary for interpretation; equally, it seems certain that such a useful technique will be improved and in routine use in the not too distant future.

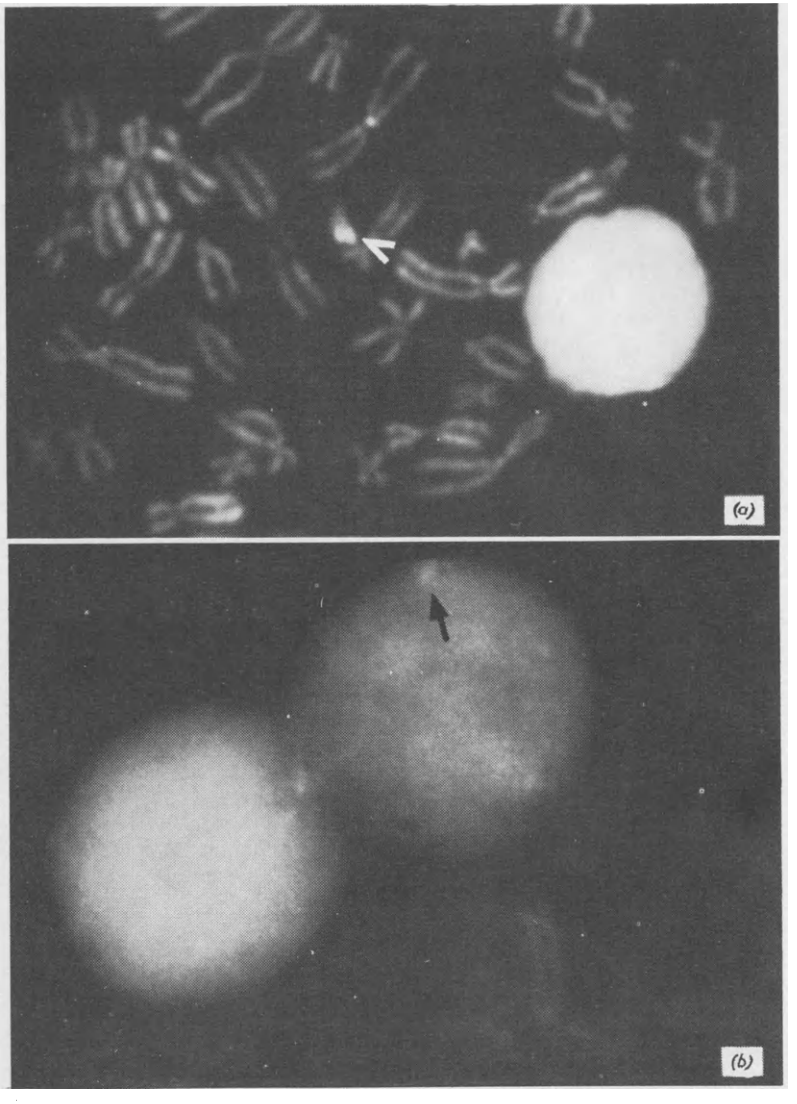


Figure 21.2 – (a) Chromosome preparation from micro-method leucocyte culture, stained with quinacrine dihydrochloride. Note the bright 'Y' chromosome (arrow). (b) Interphase nuclei of leucocytes stained with quinacrine dihydrochloride. Note the bright staining 'Y' chromosome lying against the nuclear membrane (arrow) (reproduced by courtesy of Dr. Fred Dill, Division of Medical Genetics, Vancouver General Hospital)

Fluorescent Staining Technique

(1) Fix smears or squashes in methanol or alcohol/ether in the usual manner.

(2) Stain in 1 per cent acridine dihydrochloride in distilled water for 5 minutes.

(3) Wash in water for 3 minutes.

(4) Rinse in buffer pH 5.5.

(5) Mount in buffered glycerol (1:1 glycerol and pH 5.5 buffer).

Results

Y chromosome gives a very bright yellow fluorescence, using a BG 12 exciter filter and a yellow-green (Euphos) barrier filter (page 605).

CHROMOSOME TECHNIQUES

Using currently available methods, the normal human complement of 46 chromosomes can be separated into 7 groups (A-G) but only in group A can the individual chromosome pairs be identified. The use of autoradiography resolved most of these problems in groups B, D and E, but groups C, F and G have, until recently, defied identification (Hecht, Wyandt and Erbe, 1971).

The September, 1971 Conference on Standardization in Human Cytogenetics in Paris has tentatively classified the new techniques as follows.

1.Q. – Quinacrine Fluorescent Methods
(Casperson and Colleagues, 1969)

After exposure to quinacrine derivatives *(see above)* human chromosomes fluoresce to differing degrees ranging from faint to bright with patterns characteristic for many of the individual chromosomes.

2.C – Centric or Constitutive Heterochromatin Method
(Arrighi and Hsu, 1971)

This technique embodies the denaturation of DNA by alkali, renaturation followed by staining with Giemsa's stain.

3.G – Giemsa Methods (Drets and Colleagues, 1971; Sumner and Colleagues, 1971; Schnedl, 1971)

The G-bands (Giemsa bands) are almost identical in location to the Q-bands, and the technique of Patil and colleagues (1971) is basically a conventional Giemsa stain at pH 9.0.

4.R – Reverse Giemsa Method (Dutrillaux and Lejeune, 1971)

This method demonstrates the reciprocal pattern of the G methods and vividly stains the ends of the chromosomes, which is a great advantage for the study of deletions.

The great advantage of the new techniques is that, with the possible exception of the fluorescent Q methods, they can be performed in any routine laboratory.

I have not experience with the C, G or R methods, but have found the quinacrine methods relatively simple to perform. Time and experience will determine which of these methods, or all of them, since they are in some ways complementary, are the most useful. Interested readers are referred to the individual papers for details of the methods, particularly that of Hecht, Wyandt and Erbe (1971).

Chromosome preparations may be made from squash, smear or touch preparations of tissue, or directly from tissue cultures.

Colchicine

The use of colchicine (or Colcemid) has become popular for chromosome studies and is almost essential for chromosome counts. When added to a cell culture (or injected into animals) it prevents spindle formation and allows the chromosomes to spread; they also shorten and become thicker and straighter. Colchicine is used in a final concentration of 1/10,000 to 1/100,000, being added to cultures 2–4 hours before fixation.

Use of Hypotonic Solutions

Some workers treat cells with hypotonic solutions for 10–15 minutes before fixation, to swell the cells and so aid dispersion of the chromosomes.

Leucocyte Culture Technique for Human Chromosome Studies

The culture of leucocytes from peripheral blood has become a routine method of obtaining mitotic figures for chromosome counts on

patients. Skin biopsies are also occasionally used, but peripheral blood is obviously more convenient.

The technique described is modified from that described by Moorhead and colleagues (1960).

Culture Technique

(1) Cultures are set up under strictly sterile conditions.

(2) A 10 ml syringe is moistened with a minimal amount of heparin solution, excess heparin being thoroughly expelled from the syringe. Blood is taken from any convenient vein.

(3) The blood is placed in an ice-cooled centrifuge tube containing 0.2 ml phytohemagglutinin m (Difco). This agglutinates the RBC's and facilitates their removal. Mix gently and leave in the ice bath for 30 minutes.

(4) Spin while still cool for 5 minutes at 350–500 r.p.m.

(5) Aspirate the supernatant (containing the leucocytes) and transfer to a screw cap culture tube. Add 10 ml tissue culture medium (1066 Connaught) to 3.5 ml of supernatant or until the concentration of WBC's is 1,000–2,000 per mm^3. Then add 3 drops of phytohemagglutinin p (22 gauge needle) and 1 ml penicillin G potassium 500 U/cc.

(6) Divide into 4–6 aliquots and set up in culture tubes. Blow 5 per cent CO_2 in air into culture tubes and screw caps on tightly. Slant tubes in incubator at an angle of 15 degrees. Incubate for 3 days at 37°C.

Preparation of Chromosomes from Culture

(1) Four hours before harvest, add colchicine in a final concentration of approximately 1/100,000.

(2) After a 4-hour treatment with colchicine, loosen the cells from the culture tube wall, swirl and transfer to a centrifuge tube. Centrifuge at 800 r.p.m. for 7 minutes.

(3) Remove the supernatant and suspend the cells in the remaining drop of fluid. Slowly add 1.5 ml of 0.9 per cent sodium citrate. Mix and incubate for 12 minutes at 37°C.

(4) Centrifuge at 800 r.p.m. for 7 minutes. Remove the supernatant.

(5) Without disturbing the button of cells, gently add 1 ml of cool, fresh 45 per cent acetic acid. Leave in the refrigerator for 30 minutes.

(6) Remove the supernatant and add 0.5 ml of fresh acetic acid. Mix into a fine suspension by pipetting.

Preparation of Slides

(1) Place a drop of the cell suspension on a clean slide. Add a small drop of carbol fuchsin, mix and cover with a coverslip.

(2) Squash between filter paper, using steady pressure.

(3) Temporary slides may be made by sealing the coverslip with wax.

Results

Chromosomes and nuclear chromatin . . . . . Red

Chromosome Staining Techniques

Special Reagents Required

Aceto–orcein stain. – Add 1–2 g orcein to 45 ml of hot acetic acid. When cool, add 55 ml of distilled water. Filter before use.

Aceto–carmine

Stock solution. – Boil 1 g carmine in 200 ml of 45 per cent aqueous acetic acid for 5 minutes. Filter when cold.

Working solution. – Use equal parts of stock solution and 45 per cent aqueous acetic acid. One or two drops of ferric hydrate or ferric chloride solution may be added to darken the colour of the stain.

Technique

Add a few drops of either stain to prepared slide (or coverslip), lower coverslip and apply gentle firm pressure with filter paper or glass rod (*see above* for use of colchicine and hypotonic solutions). Remove excess stain by applying filter paper to the edge of the coverslip. Seal the edges of the preparation with Vaseline or paraffin wax; alternatively a mixture of equal parts of each may be used.

Results

Aceto–orcein	Deep purple chromosomes
Aceto–carmine	Red chromosomes
Iron aceto–carmine	Deep bluish-red chromosomes

Notes

(1) Gently warming the slide may improve the staining.

(2) Pieces of tissue may be soaked in stain before being squashed as above.

Our Vancouver General Hospital genetics laboratory under the direction of Doctor J. Miller is currently using commercially available chromosome culture materials with excellent results, their micro-method is given below.

A Micro-method for Leucocyte Culture for Human Chromosomes

Cultures are set up under sterile conditions.

Materials

(1) Chromosome Medium 1A . . . . . Cat. No. 167
(2) Colcemid Solution . . . . . Cat. No. 521
(3) Fetal Calf Serum . . . . . Cat. No. 614

The above are obtained from Grand Is. Biological Co. 2323 5th Street, Berkeley, Cal. 94710

(4) Orcein Natural . . . . . Cat. No. 23309 (George T. Gurr, Ltd.)
(5) 15 ml Sterile screw cap culture tubes.

Setting up the Culture

(1) The medium-containing culture tubes should be removed from the refrigerator and warmed to room temperature before the culture is set up.
(2) Aspirate blood from a convenient vein and add 0.25 ml of whole blood to a culture tube containing 10 ml of medium. Sufficient blood can be obtained from a heel or finger prick using a sterile pipette.
(3) Divide culture into two ml cultures.
(4) Incubate at 37°C for 3 days.

Terminating the Culture

(1) Colcemid treatment to arrest mitosis

1½–2 hours before harvesting add 0.25 ml of Colcemid per 5 ml culture and incubate at 37°C.

(2) Treatment with hypotonic solution.

(a) After the Colcemid treatment, transfer contents of each culture tube to a centrifuge tube and spin at 800 r.p.m. for 8 minutes.

(*b*) Remove supernate with a pipette.

(*c*) Slowly add approximately 10 ml of hypotonic solution (1:5 fetal calf serum: distilled H_2O).

(*d*) Incubate at 37°C for 15 minutes.

(3) Fixation

(*a*) Centrifuge at 800 r.p.m. for 8 minutes and discard supernate.

(*b*) Slowly add 10 ml of fresh fixative (1:3 glacial acetic acid: methyl alcohol).

(*c*) Mix gently by pipetting after each addition.

(*d*) Leave at room temperature for approximately 1 hour (or longer).

(*e*) Centrifuge at 800 r.p.m. for 8 minutes.

(*f*) Remove supernate and wash once in fixative, centrifuging at 800 r.p.m. for 8 minutes.

(*g*) Remove supernate and add fresh fixative drop by drop to obtain proper concentration of cells.

(4) Preparation of slides: air-dried technique

(*a*) Slides should be pre-cleaned and better preparations are usually obtained if the slides are cold and wet.

(*b*) Place a drop of cell suspension on the cold wet slide and flame dry immediately.

(*c*) Check the results under a microscope without stain and add more fix if the preparation is too concentrated. If results are still not satisfactory, add more fix, wash, spin and make more slides. Fixative may have to be changed 2 or 3 times. This is the most critical step in obtaining good chromosome spreads. The air-dried preparations can be stored for future use and stained just prior to microscopic examination.

(5) Staining

(*a*) Place a generous drop of aceto-orcein stain (1.5 per cent orcein in 75 per cent acetic acid) on the slide preparation.

(*b*) Cover with a coverglass and remove excess stain by pressing between bibulous paper or several layers of paper towels.

(*c*) Seal with paraffin.

Result

Chromosomes are stained deep purple.

Chapter 22

Autoradiography

Autoradiography is a technique used to determine the presence, and to study the distribution, of radioactive isotopes in tissue.

The basis of these methods is to expose photographic emulsion to a section of tissue for a sufficient period to allow the radioactivity to reduce the silver in the emulsion. The presence of black granules (reduced silver) after development of the emulsion by the standard method will indicate the presence of radioactivity, and comparison with the section will localize such activity. For such localization it is obvious that the emulsion and section must be in permanent close contact, or be capable of accurate re-orientation after processing.

The methods of fixation and processing of the tissue must not dissolve or remove the radioactive material, and although this will depend to a degree on the vehicle used for the isotopes, absolute alcohol is the most commonly used fixative, followed by clearing in benzene and embedding in paraffin wax.

Four techniques have been used in autoradiography: (1) the film and slide technique; (2) the section on emulsion technique; (3) the stripping technique; and (4) the coating technique.

Methods (3) and (4) are the most popular. The stripping technique gives the more accurate localization, but is time consuming if large numbers of slides are to be handled. The coating technique gives reasonably good localization and enables larger numbers of slides to be handled at a time.

'Film and Slide' Technique

Method

(1) Sections, which have been floated on slides, are de-waxed and covered with 1 per cent celloidin, which is air-dried.

(2) , A piece of film is cut in the darkroom to the size of the slide on which the section is mounted (for example, 3 X 1 inches), and the section is pressed against the emulsion on the photographic film.

(3) Another slide is secured with adhesive tape behind the film to act as a stiffener, and the whole is placed in a light-proof box in the refrigerator until the film has been sufficiently exposed.

(4) Following exposure, the film is developed and the section stained by any routine method. The section is then superimposed on the appropriate part of the film and examined microscopically.

Note. – Several preparations should be set up to determine the optimum exposure, these being removed and tested at intervals.

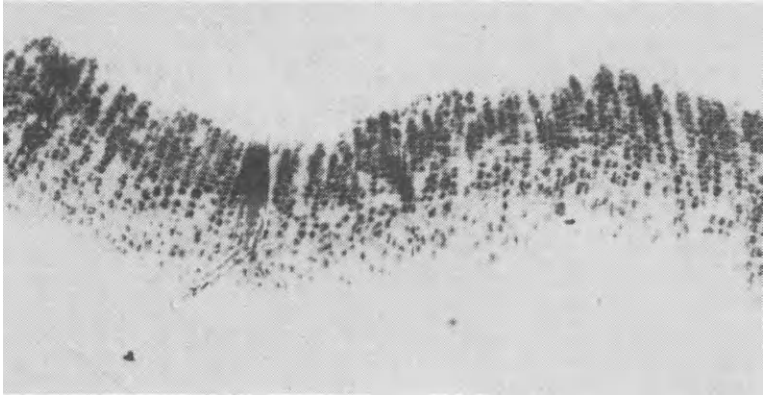


Figure 22.1 – Radioautograph of normal rat epiphyseal cartilage, two hours after injection of radioactive sulphate

'Section on Emulsion' Technique

The 'section on emulsion' technique is superior to the film and slide method described above.

Method

(1) Float a paraffin section on warm water to remove creases, pick it up on a clean slide and refloat on cold water, this avoids softening of the emulsion on the plate.

(2) Float the section on to the emulsion-covered side of a strip of photographic film or plate, and dry with a current of air. The film or plate must be handled in a darkroom, with a safe light.

(3) The film (with slides as a stiffener) or the plate is placed in a light-proof box in the refrigerator until sufficiently exposed *(see Note above).*

(4) After developing the film or plate, the section may be stained with haemalum, mounted in D.P.X., and examined.

The black granules, indicating radioactivity in the tissue, will be superimposed on the appropriate part of the section and they may be examined together. Should the section and emulsion become detached from the glass or film, they should be refloated on to clean glass slides (as frozen sections).

The Stripping Technique (Pelc, 1947)

The stripping technique is commonly used in Great Britain as the most reliable of all the methods, giving a great degree of definition and accuracy of location. A special type of film having a thick layer of gelatin is made by Kodak for this purpose.

Method

(1) Sections (prepared as above) are floated on to slides (using 0.1 per cent chrome alum in 1 per cent gelatin as an adhesive) and allowed to dry.

(2) In the darkroom, using a safe light, mark the special stripping film with a sharp scalpel (cutting through the emulsion and the gelatin backing) into a series of squares. With the edge of the scalpel, free an appropriate number of such squares into a dish of cold water.

(3) Bring mounted section to water and lower under a square of emulsion. Orientate the slide so that a corner of the emulsion overlaps each side and slowly withdraw, the overlapping corners of the emulsion will fold under the slide and help to retain it in place when it dries.

(4) Dry the slide with a current of warm air (for example, from a hair dryer).

(5) Place the slide in a light-proof box and store in the refrigerator until sufficiently exposed (*see Note* on page 508).

(6) Return the box to the darkroom and develop the photographic film, using distilled water for all solutions (the manufacturers of the film specify the method of development to be adopted).

(7) Rinse well in several changes of distilled water.

(8) Stain nuclei with Ehrlich's haematoxylin for 10 minutes.

(9) Differentiate with 1 per cent acid alcohol to clear background.

(10) Blue in tap-water.

(11) Dehydrate, clear, and mount in Canada balsam or D.P.X.

Results

Sites of radioactive isotopes show black granules on superimposed emulsion. Nuclei appear blue. The two images will be on different planes and are brought into focus separately.

Note. – Sections may be mounted in glycerin jelly at Stage 7 for examination by phase-contrast microscopy.

The Coating Technique (Kopriwa and Leblond, 1962)

This method is a modification of that described in 1958 by Messier and Leblond. The technique in 1958 incorporated the protection of sections by dipping them in 1 per cent celloidin before coating with emulsion. This step they now feel is unnecessary except when staining with celestine blue when a chemical fog appears.

Many more sections can be handled by this technique which is quicker and less tedious than the stripping technique.

Sections are stained before coating; unstained sections are carried through as a control against the loss of activity by staining. These may be examined for cellular detail by phase-contrast.

Method

(1) Fix tissues in formol saline, Carnoy's or Bouin's fixatives. Sections at 2–5 μ.

(2) Bring sections to distilled water. One section is *overstained* with haematoxylin and eosin and rinsed well in distilled water.

(3) Allow sections to dry.

(4) In a light-proof dark room (preferably maintained at 17–18°C) a Wratten safelight No. 2 is placed 3–4 feet above the bench. A waterbath at 40°C is necessary to melt the photographic emulsion (Eastman Kodak NTB 2) which is poured into a Coplin staining jar to a level such that sections are covered, but the label is not. The jar is then placed in the waterbath. It is important that the emulsion is free from air-bubbles. A clean glass slide is dipped into the jar and examined before the safelight; if bubbles are present they are scooped out with a spoon made of non-reactive material (for example, porcelain).

The section-bearing slide is held vertically and placed in the emulsion for about a second. When it is withdrawn the slide is kept vertical, the excess emulsion is drained on to tissue paper, with which the back of the slide is now wiped clean.* The slide is placed on a portion of bench covered with tissue paper, where it is allowed to dry in a vertical position. Drying in an incubator at 28°C with about 80 per cent humidity gives the best results. After drying the slides are removed from the incubator and placed on the bench face uppermost.

(5) The coated slides are stored in plastic slide boxes with slots for 25 slides. In the case of ^{3}H-labelled tissues the slides are placed in consecutive slots of the box, but this distance is increased when higher energy isotopes are employed. A tissue paper bag containing 15–25 g of Indicating Drierite is placed in the slide box to keep the atmosphere dry. The box, sealed with black adhesive tape to render it light-proof, is placed in a refrigerator (4–5°C). It is placed on edge so that slides are exposed in the horizontal position with the emulsion side up.

The duration of exposure is checked by developing test slides at different time intervals. If the exposure is prolonged for over a month, it is recommended that the Drierite bag be changed each month.

(6) After the appropriate exposure, slides are developed in freshly prepared Kodak D-170 (D-72 Dektol is not quite as good) for 6 minutes at 17–18°C. They are then taken through distilled water (as a stop bath). Fix in Eastman Kodak acid fixer (with hardener) for 3 minutes.

(7) Wash in slowly running water at 17–18°C for 15 minutes.

(8) Dehydrate in 95 per cent alcohol and two changes of absolute alcohol for 2 minutes each.

(9) Clear in xylol and transfer to a staining jar which is filled with mounting medium (Canada balsam, H.S.R. or Depex, and so on). Slides should be left in mounting media for at least 1 hour, and preferably 2–3 hours. Slides are then removed, the excess balsam wiped off and a coverslip applied.

Results

Sites of radioactivity are shown by discrete black granules superimposed on the H. and E. stained sections.

*At the Department of Pathology, University of British Columbia, the emulsion is applied to celloidin coated H. and E. stained slides by painting it on with clean gauze. This simple technique has given good results *(see Figure 22.1)*.

Chapter 23

Vital Staining

Vital staining is the staining of cells or tissue in the living state.

The earliest technique (developed by Paul Ehrlich in 1885) involved immersion of freshly removed tissue in methylene blue. This method has been largely superseded by the use of the phase-contrast and interference microscopes which enable the elements in living cells to be seen and studied without staining.

Vital staining has several limitations, some of which are mentioned below.

(1) Only a few specific elements may be demonstrated.

(2) Nuclear material cannot be stained.

(3) The strength of the dye employed is very critical; if too weak there is little or no staining, if too strong the cells are killed.

(4) Intra-vital staining *(see below)* is mostly confined to the demonstration of the reticulo-endothelial system. Dye particles from a colloidal solution are ingested by the phagocytic cells and are subsequently found loose in the cytoplasm, no specific elements being coloured.

(5) Some of the methods (for example, Ehrlich's) are not truly vital in that staining will take place after the death of the tissue.

In addition to the above limitations, certain of the dyes used (for example, methylene blue) retain their colour only if there is an excess of oxygen, and too bright a light will cause preparations to fade. When using a warm stage, therefore, the microscope lamp should be switched on only when the preparation is being studied.

Techniques of vital staining will be discussed under the following two headings.

(1) *Intra-vital staining,* in the living body *(in vivo),* usually by the injection of colloidal solutions of dyes.

(2) *Supra-vital staining,* outside the body *(in vitro),* usually applied to slide preparations of detached cells.

INTRA-VITAL STAINING

Intra-vital staining is usually confined to the demonstration of phagocytic cells of the reticulo-endothelial system. A colloidal solution of one of the following dyes is injected into a living animal and, after an interval, the animal is killed. Tissues are then fixed, processed, and sectioned. Sections are stained by a routine method which will contrast with the vital stain, and examined microscopically. The type of fixation and processing will depend on the solubility of the dye employed *(see below).*

Vital Stains Employed

Trypan Blue

Trypan blue is used as a 1 per cent aqueous solution. It withstands fixation in formalin and processing by routine paraffin wax embedding.

Vital New Red

This is a relatively non-toxic dye and is used as a 5 per cent aqueous solution. Tissues may be fixed in saturated aqueous mercuric chloride and, if the times in dilute alcohol are reduced to a minimum, processed by the paraffin wax technique.

Indian Ink

Commercial Indian ink is diluted with distilled water and, after filtration, sterilized in an autoclave. It withstands routine fixation and processing in paraffin wax.

A form of intra-vital staining is the Congo red test for amyloidosis. Following injection of this dye, all the amyloid deposits in the body are stained bright red. Amyloid deposits in various specimens from such a case still retain their bright red staining 8 years after mounting as museum specimens.

SUPRA-VITAL STAINING

Ehrlich's Methylene Blue Technique

Ehrlich's method is still used occasionally to demonstrate nerve endings in dissociated tissue (for example, muscle).

Method

(1) Tissue is teased out in warm saline solution in a watchglass.

(2) Small pieces are transferred to a dilute (0.025–0.25 per cent) solution of methylene blue in normal saline solution either on a slide or in another watchglass for 10–45 minutes. Evaporation is controlled by having the watchglass inside a dish containing a piece of wet cotton-wool. The preparation should be examined under the microscope from time to time. If staining is insufficient or absent after 45 minutes, the strength of the stain should be increased and the technique repeated.

Note. – The staining fades quite quickly if the preparation is covered (oxygen is excluded). Treatment of stained tissue with saturated aqueous ammonium molybdate for 2 hours before mounting in glycerin helps to preserve the colour.

Results

Nerve fibres and nerve endings	Blue
Other tissue constituents	Colourless to pale blue

Staining of Leucocytes or Other Living Cells

Vital staining is used in haematology laboratories to differentiate between acute leukaemias of the blastic types; for example, the mitochondria in myeloblasts are small and tend to aggregate, whereas in lymphoblasts they are large and diffuse. Saline-washed scrapings of living tissues may occasionally give good results by this technique.

The technique is based on the affinity of Janus green B for mitochondria and neutral red for the neutral red vacuoles.

Both dyes are toxic for cells and their strengths must be carefully controlled, Janus green B being by far the more toxic.

Slide preparations are used and these must be maintained at a constant temperature of 37°C either by a hot box surrounding the microscope, a warm stage, or in the incubator. (Slides may be examined for a very short time on a normal microscope stage.)

Temperatures above 40°C will kill cells, and below 36°C motility will be sluggish or absent. Since the temperature at which preparations are kept is critical, a control slide is prepared without dyes, and is examined to ensure that motility has not been impaired by factors other than the dyes.

Whitby and Hynes Method

Cleaning of slides and coverslips. – These must be scrupulously clean, and should be treated with dichromate-sulphuric acid, followed by several washings in distilled water before drying by gentle heat. They may be stored in alcohol if not immediately required, and flamed to remove the alcohol before use. In practice, it is convenient to clean, dry and coat slides with dye *(see below)* in batches since they keep almost indefinitely in a dust-proof container.

Preparation of the dyes. – Janus green B (0.4 per cent) and neutral red chloride (0.25 per cent) are dissolved separately in pure ethyl alcohol and freshly mixed before use. The actual mixture used will depend on the batch of stain and the age of the stock solution, and the optimal mixture should be tested for each batch of slides prepared. Two types of slide should be prepared: (1) for use with normal blood (labelled N.B.); and (2) for use with bone marrow (B.M.). The latter would be used for saline-washed tissue scrapings. The following concentrations of stains are made up and tried for each batch.

	Tubes			
	1	2	3	4
	No. of Drops			
Janus green B	1	1	1	1
Neutral red	14	19	24	29

A platinum loop (2 mm in diameter) is used to standardize the amount of stain per slide, and one loopful is placed on each of two slides for each concentration. The stain is spread with the edge of another clean slide (in the same way as for a blood film) and allowed to dry in a dust-proof atmosphere. The slides are tested by the technique described below and, after 20–30 minutes' incubation, the optimal concentration is decided upon. A batch of slides is then prepared for future routine use and stored in clean air-tight jars.

Method

(1) Clean the area of skin with alcohol and allow to dry.

(2) Stab with a needle and transfer a small drop of blood directly from the patient to a warm prepared slide, taking care not to touch the skin. (This is important since there is a substance normally present on the skin surface which will cause cells to die. A similar slide without stain on the surface is also prepared as a control.)

(3) Cover immediately with a specially cleaned coverslip and allow to settle; force used in flattening coverslip may kill some of the cells.

(4) Ring the coverslip with Vaseline or paraffin wax to prevent evaporation.

(5) Put the preparation on a warm stage or in an incubator.

(6) Examine after 10 minutes, but 20–30 minutes should produce optimal results.

Results

Mitochondria	Green
Neutral red vacuoles	Red
Granules	
Neutrophils	Yellow
Eosinophils	Orange
Basophils	Maroon
Red blood cells	A few cells show a few dull red granules, but red cells are otherwise unstained.

Motility of cells

Neutrophils	++++
Eosinophils, basophils, monocytes	++
Monocytes (in monocytic leukaemia)	+++
Lymphocytes	+

Upon death, cells develop vacuoles and these stain red; they must not be confused with neutral red vacuoles which are minute and numerous, whereas the vacuoles in a dying cell, although at first small, rapidly increase in size.

Chapter 24

Micro-Incineration

Micro-incineration, first described in 1833, is not likely ever to become a routine procedure because of the difficulty of interpreting results.

The technique is based on the preparation of parallel sections, one being incinerated, and the other stained by a routine method as a control. By this means mineral elements demonstrated in the incinerated section can be localized in the control section.

Micro-incineration has proved useful as a means of demonstrating silica in cases of silicosis of the lung. Examination of sections with the polarizing microscope before and after micro-incineration has, on occasions, resulted in a greater amount being revealed in the treated section.

Primary fixation and processing of the sections may present great difficulty, since the methods employed should not detract from, or add to, the mineral content of the tissue. Aqueous fixatives are likely to dissolve certain minerals, and the use of fixing agents, such as mercuric chloride or chrome salts, may add to the mineral content: freeze-dried sections are ideal for this purpose. If the apparatus required for freeze drying is not available, tissues should be fixed in 10 per cent formalin in absolute alcohol, and transferred directly to fresh absolute alcohol before clearing and embedding in paraffin wax.

Technique

(1) Fix and process tissues as described above, and cut sections 3–5 μ in thickness.

(2) Float 2 adjacent sections on warm absolute alcohol to remove creases and transfer to clean slides. Dry in a 37°C oven. Stain 1 section (the control) by the routine haematoxylin and eosin technique and treat the other section as follows.

(3) Flood with xylol for 1–2 minutes to remove wax.
(4) Transfer to absolute alcohol for ½–1 minute.
(5) Drain off alcohol and allow the section to dry.
(6) Place the glass slide carrying the test section on to a quartz slide of equal size and insert it into a special quartz oven (if a slide touches the sides of the oven it will crack).
(7) Raise the temperature of the oven slowly, reaching 100°C during the first 10 minutes. During the next 25 minutes, slowly raise the temperature to 650°C (the oven should be red-hot during the last few minutes). Switch off oven.
(8) When the slides have cooled, but before they are cold, remove them from the oven with previously heated forceps (to avoid cracking) and transfer them to a sheet of asbestos.
(9) Cover the incinerated section with a No. 1 coverslip using great care not to disturb the tissue ash. The coverslip, which is to protect the section and prevent moisture being absorbed from the air, is then ringed with paraffin wax or cement.
(10) The section is examined by the methods described below, in parallel with the control stained by haematoxylin and eosin.

Methods of Examination of Section Ash

The recognition and identification of minerals in these sections require a great deal of experience, and even then positive identification is not always possible. The following techniques may be employed:

The use of low-power objectives with *oblique light* to determine the disposition and colour of deposits may be helpful.

Some minerals have a characteristic colour and appearance under *dark ground illumination;* for example, calcium and magnesium form a dense white ash.

Various *chemical tests* can be applied; for example, the formation of gypsum crystals by calcium as a result of treatment with 3 per cent sulphuric acid.

Sections may be pre-treated chemically to assist in the identification of specific elements; for example, treatment with hydrogen sulphide will convert lead to lead sulphide, which has a characteristic colour and appearance.

Some minerals are fluorescent and these show characteristic colours when examined by *fluorescent microscopy.*

Birefringent (anisotropic) minerals rotate the plane of polarized light (for example, silica) under polarizing microscopy.

Metals or certain of their compounds emit a stream of electrons when heated *in vacuo* and the emission of these electrons varies with the temperature and type of compound. Although at present only calcium and magnesium may be so identified in incinerated sections, electron microscopy holds great promise for the future.

Chapter 25

Injection Techniques

Injection techniques may be used in two ways: (1) to investigate capillary circulation by microscopic examination of cleared and mounted sections of injected and processed tissues; and (2) to demonstrate venous, arterial and capillary circulation and the bronchial tree in gross specimens.

There is a degree of overlap between these two classes, and many methods may be employed for both microscopic examination and gross specimens. Injection is easiest immediately after death, and tissue should be treated as fresh as possible.

The injected material must be in colloidal solution, since, if it is in true solution it will diffuse through vessel walls. When it is intended to study capillaries the particles must obviously be sufficiently small to enter them.

The injection mass may be used cold (for example, latex or plastic), or hot (for example, gelatin chrome yellow). The incorporation of 0.25 per cent sodium nitrite to promote vascular dilation is useful in all injection masses, but in gelatin masses it is particularly useful since gelatin has a vasoconstricting action.

Techniques for Microscopic Examination

Although gelatin masses may be used for the demonstration of capillaries, Fischer's method is probably best for this purpose.

Fischer's Method

Technique

(1) Attach a glass cannula to the main artery of the organ and, using a two-way syringe or an aspirator bottle suspended 4–6 feet

above, inject normal saline solution until the solution issuing from the main vein is free from blood.

(2) Without detaching the cannula, inject the organ with milk or 20 per cent lard in ether. When the milk (or lard solution) flows from the main vein, tie both artery and vein.

(3) Fix the organ in 7.5 per cent aqueous formalin to which 1.5 per cent acetic acid has been added.

Cut frozen sections to a thickness of 15–20 μ and stain with oil red O or another fat-soluble stain (page 361).

Result

The path of the capillaries can easily be traced by the red colouring.

Techniques for Gross Specimens

Many of the following techniques may be used for either gross or microscopical examination. Gelatin masses were very popular, but since they must be kept hot, latex rubber and plastic, which are used cold, have in many cases supplanted them. Latex and plastic have the additional advantage that tissue may afterwards be digested, leaving a cast of the circulatory system. Plastic casts are rigid and hold their shape after digestion of the tissue; rubber casts collapse unless kept in fluid, but have the advantage that fine arterioles and capillaries may be cut off and examined microscopically.

Positive pressure for injections may be obtained by forcing air into an aspirator jar (containing the mass) fitted with a non-return cycle valve at the top, the tube carrying the mass to the cannula going from the bottom of the jar. The pressure may be obtained by using a cycle pump, or a compressed air cylinder, great care being necessary to ensure that the pressure employed will not burst the aspirator or damage the specimen. A pressure of 6–7 lb per square inch will suffice for most purposes. As a precaution the apparatus should be fitted with a wooden or metal covering.

Gelatin Masses

Gelatin masses are made of molten 5–10 per cent gelatin (the weaker strength for capillaries) containing various coloured pigments, and injected under pressure. They must be kept hot (60°C) throughout to ensure an easy flow through the smaller vessels; it is best to immerse

the organ to be injected in a bath of saline solution at the same temperature to avoid setting of the gelatin in the smaller vessels.

Gelatin masses are injected as described for Fischer's method, and the following colouring agents may be used for the masses.

Chrome yellow. – This pigment is mixed with molten gelatin until the colour is sufficiently dense when viewed in a capillary tube.

Carmine gelatin. – Mix 5 g of carmine with 10 ml of distilled water in a stoppered flask, and add 100 ml of strong ammonia (.880). Shake well and allow to stand for ½–1 hour. Shake well again and filter through glass wool. To the filtrate add 15–20 g of gelatin and a few crystals of thymol. Stopper flask, and keep at 37°C until the gelatin is dissolved. Remove stopper and keep at 37°C until the smell of ammonia has disappeared. Make up to 150 ml with warm distilled water and the mass is ready for use; it should be stored in the refrigerator.

Ranvier's Prussian blue. – This is prepared by adding a saturated aqueous solution of ferric sulphate to a saturated aqueous solution of potassium ferrocyanide. The resultant blue precipitate is well washed in a Buchner funnel. This pigment may be used in aqueous colloidal solution or a gelatin mass.

Fixation. – Following the injection of gelatin masses, the organ should be fixed in cold formol saline. The gelatin is converted to an irreversible gel by the formalin.

Neoprene Latex and Plastic Injections

These masses are injected by one of the methods described above, the cycle pump or oxygen cylinder pressure method being the most efficient. After injection the organs are fixed in formalin and may be treated with dilute hydrochloric acid to demonstrate the circulatory or the respiratory system.

By the use of two different coloured plastics, both the circulatory and respiratory systems of the lungs may be demonstrated as a skeleton. (For more complete details of the many methods available the reader is referred to the work of D. H. Thompsett (1956).)

Chapter 26

Preparation, Colour Maintenance, Fixation and Storage of Specimens

The mounting of pathological specimens in Perspex containers, for long regarded as the prerogative of the larger teaching hospitals, is now becoming commonplace in many of the smaller hospitals.

Even small museums, in addition to their teaching value, play a part in recording the history of medicine, since the common diseases of today may well be the rarities of tomorrow. To fulfil such a purpose it is essential that the original shape and colour of such specimens is retained and that accurate records of the patient's medical history and, if possible, relevant photographs and radiographs, are readily available. The presentation, labelling and cataloguing are of equal importance. Lack of interest in a museum can almost always be traced to poor presentation and documentation of specimens: nothing is more uninteresting than a large number of dirty, badly labelled specimens crowded into a series of cupboards.

PREPARATION OF THE SPECIMEN

Good museum specimens are generally only obtained and preserved by care and planning at the time of autopsy, and careful treatment after removal. Indiscriminate examination of organs in the post-mortem room, and the careless removal of sections for histological examination can easily ruin a potentially valuable specimen.

Cut surfaces should be smooth and even and this is achieved by using a continuous stroke with a long-bladed, sharp knife. The usual type of brain knife may be used, but a butcher's knife with a 14-inch blade is preferred because even large organs may be cut with one long stroke; also, such knives are usually much cheaper than brain knives.*

*Knives with 14-inch Swedish steel blades are obtained from Chimo Labs. Ltd., 1200 West 6th Avenue, Vancouver, B.C., Canada.

Tissue for histological examination should either be taken from the bakc of specimens intended for preservation, or neatly removed from the front with a scalpel so that when stained slides accompany the specimen their position in relation to the rest of the specimen can easily be seen.

Specimens should be put into a fixative almost immediately. If a fixative is not available they should be immersed in saline solution, and stored in a refrigerator. If the specimen is allowed to dry, a permanent darkening will result.

Specimens from the operating theatre will, if properly treated, provide the best museum specimens, and containers with formol saline should always be readily available to theatre staff.

METHODS OF COLOUR MAINTENANCE

The fixing fluid to be used will depend on the technique employed, but 10 per cent formol saline can always be used for primary fixation and the specimen transferred to a special fixative afterwards.

The technique most widely used, and still by far the best available, is a modification of the method described by Kaiserling (1897).

The original technique employed 3 solutions: the first for fixing, the second for restoring colour, and the third a mounting fluid in which the colour should be maintained. Pulvertaft (1936) described a method of restoring colour to tissues by the addition of a reducing agent (sodium hydrosulphite) to the mounting medium. The original specimens mounted by Pulvertaft's technique show remarkably little fading even after 35 years.

Pulvertaft–Kaiserling Technique

Solutions Required

(1) *Kaiserling's fluid I – fixing fluid*

Formalin	400 ml
Potassium nitrate	30 g
Potassium acetate	60 g
Tap-water	to 2,000 ml

Specimens may be transferred to this fluid after fixation in formol saline, or they may be directly fixed in it.

(2) *Kaiserling's fluid II*

80 per cent ethyl alcohol

This fluid may be used to restore colour in an emergency (for example, for colour photography) but it is not necessary when using a sodium hydrosulphite mounting fluid. If colour is restored with 80 per cent alcohol the time should be carefully controlled, since once the full colour has been restored (½–4 hours, depending on the size of the specimen), continued immersion in alcohol has a permanent bleaching effect and the colour so lost is not afterwards restored by the mounting fluid.

(3) *Pulvertaft-Kaiserling mounting fluid III*

Glycerin	300 ml
Sodium acetate	100 g
Formalin	5 ml
Tap-water	to 1,000 ml

0.4 per cent sodium hydrosulphite is added immediately before sealing the jar (page 541).

Dissolve the sodium acetate in warm tap-water, add the glycerin and formalin, and make up the volume with cold tap-water. If the reaction is more acid than pH 8, a few drops of N/1 sodium hydroxide should be added.

If the solution is not crystal clear it should be filtered through a paper pulp filter. Cloudiness of the solution is usually due to impurities in the sodium acetate. On rare occasions, simple filtration will fail to produce the desired clarity, in which case 50 ml of a saturated solution of camphor in alcohol should be added to 1 litre of the solution; refilter as before.

The use of camphor will always produce a fluid of sparkling clarity, but the overwhelming smell of camphor in the curator's room is a decided disadvantage.

Schultz's Carbon Monozide Technique

The Schultz method employs carbon monoxide to convert haemoglobin into the more stable compound carboxyhaemoglobin. It has two main disadvantages: (1) the colours are unrealistic; and (2) there is a danger of explosion during processing.

The technique, based on Kaiserling's method, is as follows.

(1) Bubble carbon monoxide (or coal gas) through Kaiserling's fixing fluid I containing the fixed specimen. This stage must be carried out in a fume cupboard with a good draught.

(2) When the specimen shows the characteristic colour change it is mounted in Pulvertaft-Kaiserling fluid (without the addition of

sodium hydrosulphite) which has been saturated with carbon monoxide.

FIXATION OF SPECIMENS

Certain additional rules should be observed in the fixing of museum specimens:

(1) To ensure adequate fixation, specimens should always be injected with fixative if this is possible. Whole brains *must* be fixed in this manner by injection into the basilar artery, after first washing through with saline solution to remove the blood. After injection of the fixative, the basilar artery is tied off with a long length of linen thread; the brain is then transferred to a bucket containing fixative and suspended by the thread from a glass or wooden rod laid over the top of the bucket so that the brain (which almost floats) remains suspended in the centre of the fixing fluid. Lungs, whole limbs and kidneys are more easily and speedily fixed by injection.

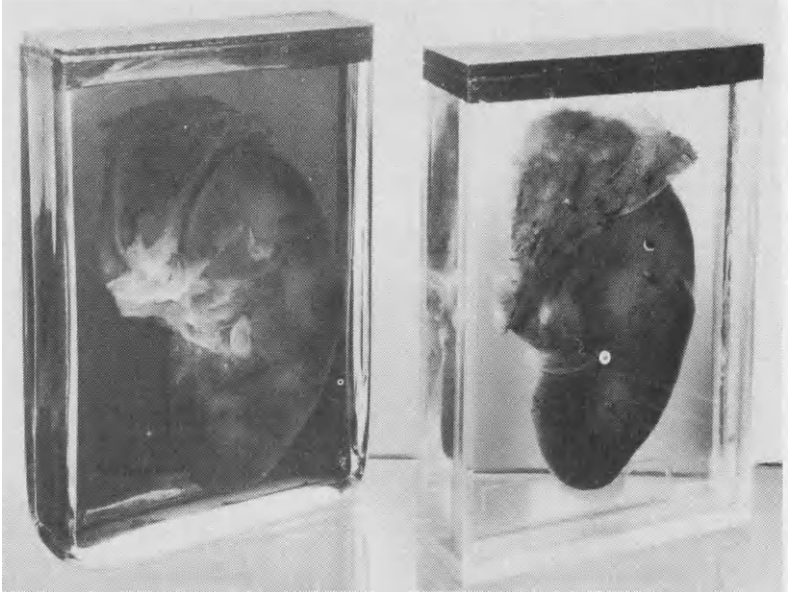


Figure 26.1 – Two halves of the same kidney. The half shown on the left was washed overnight in running water after fixation, following which both specimens were mounted in routine mounting fluid. Note the haemolysis in the specimen shown on the left (by courtesy of the Editor of J. clin. Path.)

(2) Specimens containing much blood must not be washed in water at any time, either before or after fixation. If excess blood or mucus is to be removed, the specimen should be washed in saline solution or formol saline. *Figure 26.1* shows two halves of a fixed kidney, one washed in saline solution and the other in running tap-water. The half washed in water is gelatinous and in ordinary light is almost completely obscured by haemolysis.

(3) Fresh soft specimens should be fixed individually since contact with other specimens or with the container may alter their contours.

(4) The specimen, together with its attached structures, must be fixed so that it is in the position in which it is to be finally displayed. Membranes, skin, intestine and so on may be pinned to cork boards which are then floated (specimen downwards) on the fixing fluid; the pins used must be rustless (glass or stainless steel) to avoid marking the specimen.

(5) Cystic cavities, if unopened, are inflated with fixative; or if opened are packed with cotton-wool soaked in fixative so as to maintain their natural shape.

(6) Bile-stained or bile-containing specimens must be fixed and stored separately or they will stain other specimens.

STORAGE OF SPECIMENS

The storage of potential display specimens forms an important part of museum technique since the supply of these specimens usually exceeds the number actually mounted, the latter being governed by the type of specimens currently required, the space available for display and the technical assistance to mount them.

The method of storage must permit easy and certain identification of each specimen. The following system is recommended.

The specimen, together with a duplicate label, is wrapped in gauze or muslin and the label attached with a piece of linen thread. A duplicate label wrapped in with the specimen can be of great value when linen threads break or become tangled.

Specimens are stored in large rectangular earthenware tanks measuring 34 X 22 X 19 inches. The fluid used may be Kaiserling's fixing fluid I for periods up to 6 months, after which time specimens should be treated with 80 per cent alcohol to restore the colour and then transferred to Pulvertaft–Kaiserling mounting fluid (without sodium hydrosulphite).

The labels are arranged in order in a series of saw-cuts in strips of wood on each side of the tank *(Figure 26.2).* A teak-wood cover is used to control evaporation.

The number of tanks in use will depend on the flow of specimens, but fragile specimens, particularly brain, should be stored in separate containers to avoid damage which may be caused by contact with other specimens.

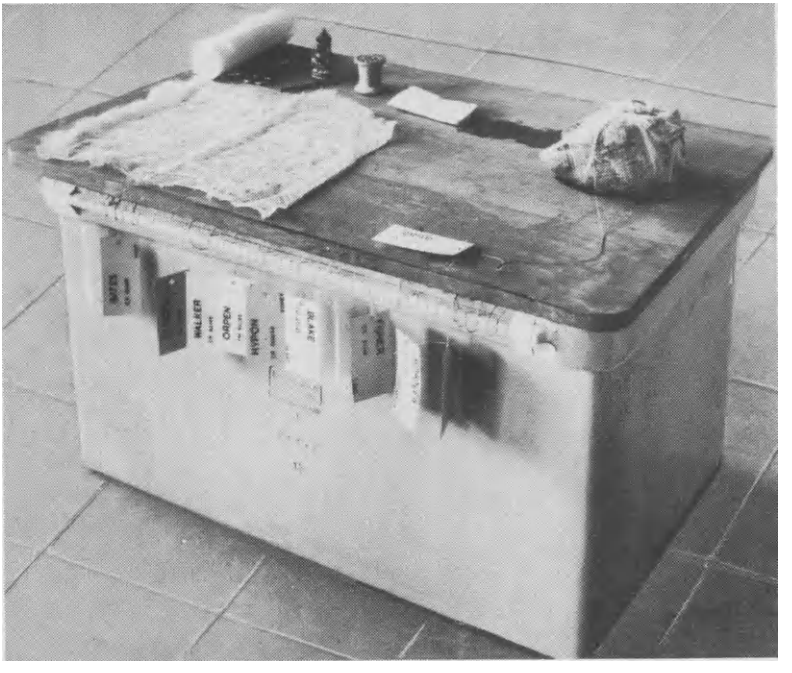


Figure 26.2 – Storage tank. Note the materials for wrapping and labelling on the top of the tank, also the method of arranging labels in order on the strip of wood (by courtesy of the Editor of J. clin. Path.)

Preparation of Storage Labels

Old radiographs, of no further clinical use, should be cleaned in hot water to remove the emulsion, and when dry cut into strips 1–2 inches in width. They are then placed in glacial acetic acid for 10 seconds to dissolve the surface layer, drained for 5 seconds and plunged into 2 per cent aqueous sodium carbonate until opalescent. They are then washed in running water for 3–10 minutes, allowed to dry and cut to the required size. A hole is then punched at one side to which the linen thread is attached. Indian ink should be used to record particulars of the specimen.

A set of numbered brass discs may be used in place of written labels. The numbers may be prefixed by letters, for example: A–clinical; B–post-mortem; or the letter may be used to designate the type of specimen: breast, brain, and so on. A special reference book should be kept to identify the specimens.

Chapter 27

Mounting of Museum Specimens

When specimens are brought from the storage tank they will usually require some final attention before being actually mounted.

Slight irregularities may have developed in the surface of the specimen during fixation and it may need to be recut. In such cases, only a minimum amount of tissue should be removed since, owing to the uneven penetration of the fixative, the removal of a thick slice will reveal a surface which recolours in a concentric pattern of differing shades.

If specimens of membranes, skin, or intestine have been pinned out on cork boards, the outer edges will require trimming.

If, after the removal of cotton-wool packing from cavities, the specimen will not remain in a natural position by normal mounting methods, such cavities should be filled with arsenious acid-gelatin.* Unopened cysts and cavities may, if thin-walled, need to be supported by the injection of gelatin, after removal of injected fixative.

Coloured Perspex arrows or rods may be used to identify anatomical details. A loop of black horse hair may be used in the same way for delicate structures.

Specimens which are particularly friable may be covered with a thin layer of arsenious acid-gelatin (Wentworth, 1947), and it may also be used locally to hold fragments such as blood clot in position.

**Arsenious acid-gelatin* – Boil 20 g of arsenious acid in 1 litre of water, using a reflux condenser, for at least 2 hours. Cool the solution, make up the volume to 1 litre, and add 120 g of gelatin. Dissolve the gelatin by steaming for 1–2 hours, and filter while hot through chardin filter paper, or sand and paper pulp. Add 100 ml of glycerin.

To render the gelatin almost colourless, add 0.5 per cent Victoria blue (or 1 per cent methylene blue) drop by drop until the bulk is faintly blue, but appears colourless in a 6 × $^5/_8$ inch test tube. Bottle and store in the dark.

Add 0.5 per cent formalin before use; this converts the gelatin to an irreversible gel.

Bile-stained specimens will colour the mounting fluid for some time after mounting. It has been suggested that the soaking of specimens in a saturated solution of calcium chloride for 24 hours will obviate discoloration. Although this reduces the degree of colouring, it will not prevent it and frequent changes of fluid are necessary to keep the discoloration of fluid at a minimum.

MUSEUM JARS OR BOXES

Perspex boxes are used almost universally today, and the technique described will be that which applies to their use. Methods of mounting in other containers are described at the end of the chapter.

Perspex boxes are available commercially, or may be made in the laboratory. The prices of commercially-made boxes may appear to be high, but it will generally be found that if the cost of Perspex sheeting and labour is estimated, laboratory-made boxes are quite often more expensive. The method employed commercially to join the sides is far superior to the cementing process described below, and gives the boxes a much longer life. Early boxes now show a whitening of the joints which eventually give way, and this has been shown to be due to a breakdown of the cement used.

For the benefit of those who prefer to make their own boxes, however, the technique described below has the advantage that such boxes may be designed to fit each specimen exactly.

Perspex Boxes

Perspex boxes may be made quite simply by cutting four sides, a top and a bottom from Perspex sheeting and cementing them together with Perspex cement. An alternative and better method, as devised by Professor Duguid, is to bend a strip of Perspex to form the top and sides of the box. This is done as follows.

There are several methods of heating a Perspex strip for bending. A simple one is to use a finely drawn Pasteur pipette as a gas jet and draw it across the Perspex strip on both sides at the desired place. An alternative is to use 2 copper rod heating elements connected with a transformer so as to pass a high current of 700 amperes at ½ volt. This heats the Perspex to a state of flexibility in about 5 minutes, when it may be bent to any angle. A framework is used to ensure that the angle is square, and the Perspex is then held or clamped in this position until cold.

Bend a rectangular strip of 1/8 inch Perspex at right-angles at 2 points to form the top and sides of the box, and trim the edges accur-

ately with a circular saw. Soak one edge in ethylene dichloride or chloroform for 10 minutes by propping the strip on 3 pieces of fine wire or nylon thread laid on a glass slab and run chloroform or ethylene dichloride around the lower edge to form a pool of the fluid. When the edge is softened it is applied to a roughly cut sheet of 1/8 inch Perspex to form one face of the box, gentle pressure being applied by weights. In 10–15 minutes the joint is firm enough to permit manipulation; the other face is cemented by the same process. Weights up to 14 pounds should then be applied and left overnight. On the following day the face edges are cut off, flush with the sides of the box, sandpapered and polished on the buffing machine. The bottom of the box is sawn, squared and sandpapered, leaving an even rim to which the base is fixed. A rectangular slab of ¼ inch Perspex is cut and polished to form a base, and a 1/8 inch hole is bored in it through which the box can finally be filled with mounting fluid.

ROUTINE MOUNTING OF SPECIMENS

The specimens should be laid on a flat, waterproof (preferably formica-covered) bench. The position in which they are to be mounted is decided upon, and this should, as far as possible, be anatomically correct to enable students to recognize the various structures. The specimen is then measured, allowing a ½ inch clearance at the top and sides and 1 inch at the bottom. The extra clearance at the bottom is to enable a label to be fitted without obscuring part of the specimen. The depth of the specimen is measured, and approximately ¼ inch added for the centre plate. A suitable box is then taken from stock, ordered or made.

Centre Plates

One of the great advantages of mounting in Perspex is its flexibility when heated, since it can be moulded or bent to satisfy the requirements of individual specimens. In spite of this fact, however, it has been found that the great majority of specimens may be simply stitched to a flat sheet of Perspex which just fits into the box with about ¼ inch clearance at the top (the centre plate). Commercial boxes may be obtained already fitted with centre plates.

Coloured opaque Perspex centre plates may be used to enhance the colour of the specimen or to enable specimens to be attached to both sides.

Special methods of mounting are dealt with later in the chapter.

Stitching Specimens to Centre Plate

The specimen is arranged in the desired position, and crosses are made on the centre plate with a scribe where stitches are to be placed. With solid specimens the number of stitches will depend on the weight and consistency of the tissue: for example, half a kidney is adequately supported with a stitch at each pole. Hollow or cystic organs, or organs with attached structures, may require stitches to hold the specimen in the correct position in addition to providing support: for example, the oesophagus and stomach may require up to 12 stitches. Attached structures may need to be stitched to the main organ or to each other to hold them in position. Stitches must not be placed through pathological lesions if this is avoidable.

When the centre plate has been marked, holes, 1/16 inch in diameter, are drilled at those points (a No. 0 dental drill is useful for this purpose). If linen thread with a glass bead is to be used, one hole is drilled at each point; if nylon thread, two holes are necessary. Nylon thread has the advantage of being almost unbreakable, but is so hard it tends, in time, to cut through specimens and for this reason linen thread should be used for all specimens except bone. Lengths of linen thread are cut and a small clear glass bead is threaded on and tied in the centre; the bead should be slightly larger than the hole in the centre plate since it acts as a retainer for the tie.

The centre plate is thoroughly washed in a detergent, and dried on a fluffless cloth. The specimen is stitched on by passing first one end of a tie and then the other through the centre plate and the specimen, pulling on both ends until the glass bead is tight against the centre plate. The threads should emerge from the specimen about 3/8 inch apart, so as to form a V of tissue on which to tie. A reef knot is tied with sufficient tension to cut slightly into the tissue, so that with the ends cut the knot is hardly discernible. With soft tissue, such as brain, 3/8 inch squares of celluloid, in which two holes have been made, are threaded on to the ties on top of the tissue; the knot is then made on the celluloid to avoid cutting the tissue, and it should be made as a simple reef knot, except that instead of passing the left thread over the right once, it is passed over twice, then pulled tight and finished by passing the right thread over the left. Such a knot gives a tight tie which will not slip, and must always be used with nylon thread which will otherwise become loose.

Fixing the Centre Plate

The centre plate, with specimen attached, is put into the box, and marks are made with a grease pencil if 'stops' are required to hold the

centre plate in position. If the box is of the correct depth there will be no movement of the specimen, but if a deeper box has been used (as for a thin membrane) two rectangles of 1/8 inch Perspex measuring 1 × ¼ inch, with polished edges, are cemented to the wall of the box to keep the centre plate in position. These 'stops' may be used to hold centre plates that have been bent in any desired position.

Filling and Sealing

When the specimen is in position, museum fluid, to which 0.4 per cent sodium hydrosulphite has been added, is run in to within ½ inch of the top. Air-bubbles trapped between the specimen and centre plate are released with a broad-bladed spatula.

A hole, 1/8 inch in diameter, is drilled in one corner of the lid, through which is introduced the remaining mounting fluid.

The top of the box is wiped dry and Perspex cement applied with a Pasteur pipette; ethylene dichloride may be used in place of Perspex cement. After 30 seconds the lid is laid lightly in position, surplus Perspex cement being carefully removed. After a further 30 seconds, a lead weight is applied and left for at least 1 hour, preferably 2–3 hours. A short length of Perspex rod, 1/8 inch in diameter, is tapped lightly into the hole in the lid and the specimen left for 24–48 hours to remove residual air-bubbles. The Perspex plug is removed, and the box filled with museum fluid by means of a Pasteur pipette. When the last bubble is removed, the Perspex plug is replaced and tapped firmly into position and, when dried, a small amount of Perspex cement is applied.

It will be found that large specimens develop air-bubbles over a period of 2–3 weeks after mounting; these may be removed by drilling a fresh hole, filling up and replugging, but it is advisable to wait 2–3 months before removing this residual air.

MOUNTING IN GLASS JARS

Specimens may be mounted in glass jars on Perspex centre plates, as described above, or on glass centre plates if the mounting medium dissolves Perspex (for example, Spalteholz fluid). The specimens are mounted as described above except that holes are drilled with an engraver's tool (a metal rod with a diamond-shaped end) in a hand drill, using camphor dissolved in turpentine as a lubricant. When drilling such holes the glass sheet must be on an absolutely flat surface. An alternative but inferior method of holding the specimen is to bend glass rod to form a frame which just fits into the jar, the specimen then being stitched to the frame.

Glass jars are sealed with an asphaltum-rubber compound (Picein) which must be applied to a perfectly dry ground-glass surface. It is best to wash the empty jar, dry it thoroughly with a cloth, and then gently apply a Bunsen burner flame to the ground-glass edges until they turn white. One edge of the Picein should then be warmed in the flame and scraped along the edge of the jar until there is an even amount all round. Flame with a Bunsen burner until the Picein runs and completely covers the ground edge, but does not run over the edge. Place the specimen in the jar and fill with mounting fluid to within $^3/_8$ inch of the top; if glass jars are completely filled they will crack with atmospheric changes. Hold the lid in a pair of forceps, heat it and the Picein on the jar gently with a Bunsen burner, then press the lid firmly into position on top of the jar with a cloth. When set remove the excess picein with a hot knife. These jars look neater if, after sealing, the edges are painted with black enamel or asphaltum varnish.

GELATIN EMBEDDING

Delicate or intricate structures (for example, the circle of Willis) which are difficult if not impossible to stitch, may be embedded in a thin layer of arsenious acid-gelatin on a centre plate, and then mounted by the routine method. A trough is formed by applying Sellotape around the edge of the centre plate to a depth of $^1/_4$ – $^3/_8$ inch and filled with gelatin. The Sellotape is removed after the gelatin has set. Although after 4–5 years this layer tends to become detached from the centre plate, it is so easily replaced that the method is worth while.

Specimens may be mounted in a solid block of gelatin by fixing the specimen in position with a thin layer on one surface of the jar. When this layer is set, the jar is filled to within ½ inch of the top with gelatin, which is allowed to set before the top is affixed. This method was popular with glass containers since they were easily broken and the gelatin protected the specimen and avoided the sudden release of a glycerin solution. It has the disadvantage that the gelatin tends to become yellow with age, and also to undergo liquefaction with the resultant formation of air-bubbles. The durability of Perspex boxes offers most of the advantages of the old method of gelatin embedding without its disadvantages.

EMBEDDING IN SOLID PLASTIC BLOCKS

Embedding in a solid block of plastic would appear to offer the ideal method of presenting museum specimens, but unfortunately there is as

yet no method available which preserves the colour of soft tissues. Such methods* while adequate for hard tissues (certain insects, plants, and so on) are useless for the normal pathological museum specimen.

*I.C.I. Ltd., Plastics Division, Imperial Chemical House, Millbank, London S.W.1, will give details of such a method using Perspex monomer.

Chapter 28

Special Methods

MACERATED SPECIMENS OF BONES

Cutting the Surface

Macerated specimens of bones, like all others, should have a clean-cut even surface. This is best achieved by cutting the specimen on a circular saw or a band-saw, by which method slices as thin as $^1/_8$ inch may be cut. The specimens are easiest to cut if they have been frozen hard in a deep freeze cabinet before cutting, since the soft tissue then has a similar consistency to the bone. Specimens of soft tissue may also be cut into thin even slices by the freezing technique.

Maceration

Maceration is used to demonstrate bony lesions, such as are produced by osteogenic sarcomas, osteomas, and the effects of chronic osteomyelitis and tuberculosis.

The technique employed will depend, to a degree, on the type of lesion present. The finer spicules of bone in an osteogenic sarcoma are easily damaged or dissolved, whereas an osteoma will withstand comparatively harsh treatment.

The method which will preserve even the finest spicules of bone is that of putrefaction, where the bone and soft tissue is put into a tank of water and left for several months, but this method is almost completely impracticable owing to the nauseating smell.

As a routine method, the specimen, after trimming off the excess soft tissues, may be boiled in tap-water or very dilute (N/100) sodium hydroxide. At intervals during the boiling, the bone is removed from

the fluid and the softened tissue removed with forceps, care being taken not to damage the specimen.

A gross method for hard compact bone is autoclaving in N/10 sodium hydroxide for 5 minutes. This will effectively remove all the soft tissue, but will also remove the fine spicules of bone.

Degreasing and Bleaching

Following removal of the soft tissue by one of the methods described above, any fat is removed by immersion of the bone in chloroform for 3–4 hours. Specimens are then dried in an incubator and bleached in hydrogen peroxide.

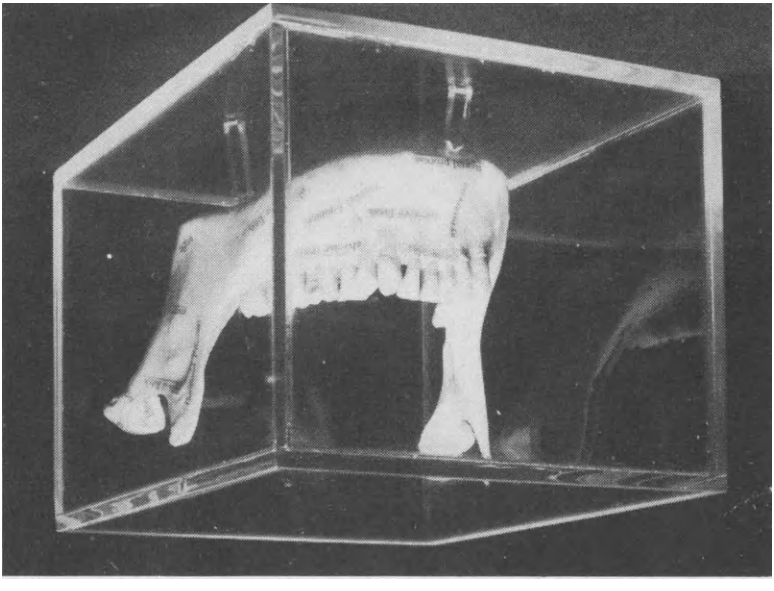


Figure 28.1 – Method of mounting dry specimens (by courtesy of the Editor of J. clin. Path.)

Mounting

Macerated bones are mounted dry, either on a centre plate as described for routine mounting, or with Perspex supports designed for individual specimens *(Figure 28.1)*. When tied to a centre plate, nylon thread should be used, employing a double knot (page 540); a drop of

Perspex cement should be applied to the knot and to places where the specimen touches the centre plate to give added support to the specimen.

CALCULI

Calculi are often presented by either (*a*) dry mounting in boxes with removable glass lids or (*b*) mounting in gelatin to which formalin has been added. The disadvantages of these older methods are that in the former the specimens became very dirty and in the latter the gelatin slowly dissolved. Several methods were tried in an endeavour to meet the following requirements.

(1) Laminations must show clearly.
(2) It should be possible to see both surfaces of the calculus.
(3) Variants of one particular type should be mounted together to allow of easy comparison, and the method of labelling should be such that the observer's knowledge can be tested.
(4) Containers should be dust-proof.
(5) Students should be able to handle the specimens.

It was obvious that the last two conditions would be the most difficult to satisfy with any one method; it was decided, therefore, to utilize both halves of calculi by polishing and mounting one half and labelling the other half with Indian ink, the latter being kept for students to handle and study more closely at lectures.

The polished specimens are cemented halfway through a sheet of Perspex which, in turn, is cemented into the box, thus ensuring a minimum of disturbance. The stencilling is done on a sandpapered rectangle with a Uno stencil, size 1½ *(Figure 28.2)*.

Technique

(1) Stones are cut in halves with a fine fretsaw, or coping saw, and the cut surfaces polished with sandpaper, using grade 1 at first and then grade 0. The polishing may be completed on a fine oil stone, but this is not usually necessary.

(2) The calculi are assembled in their appropriate groups, and the size of the museum jar decided upon, allowance being made for a label under each specimen.

(3) A centre plate of ⅛ inch clear Perspex sheeting is cut to fit exactly the inside of the museum jar, and the edges are polished.

(4) The stones are arranged on the centre plate and the outlines of the stones, and rectangles for the labels, drawn with a metal scribe.

(5) The outline of each stone is cut round with an 'Amprofile' in a hacksaw frame, and filed until the stones fit tightly when pressed halfway through.

(6) The label rectangles are sandpapered to give a ground glass appearance on both sides of the Perspex the scribed line giving a clean edge, a metal shield being used to protect the remainder of the Perspex. The object of preparing both sides of the Perspex is merely to prevent the stencilled label from being seen too easily from the front. The metal shield is easily made from a piece of tin cut to an appropriate shape.

(7) The museum number or details of the composition of the calculi are stencilled on to the rectangle *(Figure 28.2)*.

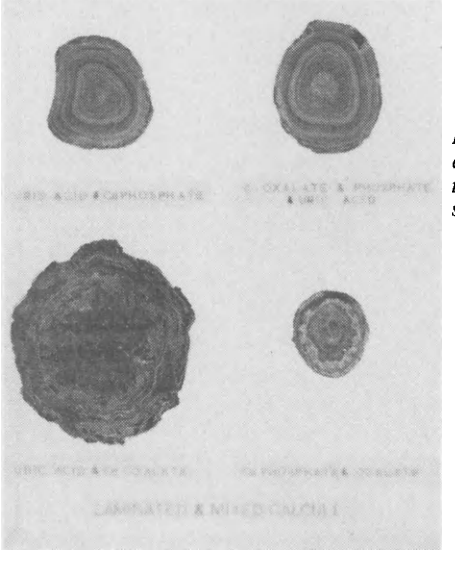


Figure 28.2 – Method of mounting cut and polished calculi. Although the wording can be seen it is sufficiently obscured to test the observer's knowledge

(8) The stones are pressed gently into position and cement applied to the edge of the centre plate touching the calculi.

(9) The cement is allowed to harden overnight, having been covered to keep the specimen free from dust.

(10) On the following day the centre plate is placed in a museum jar with the cut surface of the stones tight to the face of the

jar. Being a close fit, 1 drop of cement halfway down each side of the centre plate is sufficient to hold it firmly in place.

(11) The lid of the museum jar is cemented on in the usual manner and allowed to stand with a light weight on it for 1–2 hours.

The extra work involved in the above method is fully justified by the results *(Figure 28.2)*.

An alternative method is to mount calculi in a Perspex box, the back and sides of which should be black. The cut surface of one half, and the outer surface of the other are secured to the bottom with Perspex cement so as to show both surfaces from above.

TRANSPARENT SPECIMENS

The techniques used in the preparation of transparent specimens are dependent on the replacement of the tissue fluids by fluids of a higher refractive index.

Such techniques are usually employed to demonstrate either the bones of embryos or circulatory systems *(Figure 28.3* and *28.4)*.

Dawson's Technique

Dawson's technique is excellent for the demonstration of bone in embryos or small animals, and depends on clearing the soft tissues in potassium hydroxide, the staining of bone with alizarin, and the replacement of body fluids with glycerin.

Method

(1) Fix embryos, or small animals, in 95 per cent alcohol for 48–72 hours. Prolonged fixation in alcohol renders the tissues less liable to maceration in the caustic potash solutions.

(2) In specimens prepared by this method, any fat present is partially saponified and appears in the cleared material as opaque white masses. Therefore extract fat immediately after fixation by treatment in acetone for 2–4 days. Return the specimen to 95 per cent alcohol for 12–24 hours.

(3) Place the tissue in 1 per cent potassium hydroxide until the bones are clearly visible through the muscle. Transfer to a solution of 0.1 per cent alizarin red S in 1 per cent potassium hydroxide.

(4) Leave the specimens in the alizarin red S solution until the bones are stained the desired colour. If the dye is absorbed from the solution before the maximum intensity is obtained the specimen can

be transferred to a fresh solution of stain. If clearing in the initial caustic potash solution has progressed to the proper stage, nothing but the bone will take up the stain. If the clearing was not complete the muscles and other tissues take up the stain almost as readily as the bone itself.

(5) Following the staining, place the tissues in the following fluid:

Potassium hydroxide	1 g
Distilled water	79 ml
Glycerin	20 ml

(6) When properly cleared the tissues are passed through increasing concentrations of glycerin and finally stored and mounted in pure glycerin *(Figure 28.3)*.

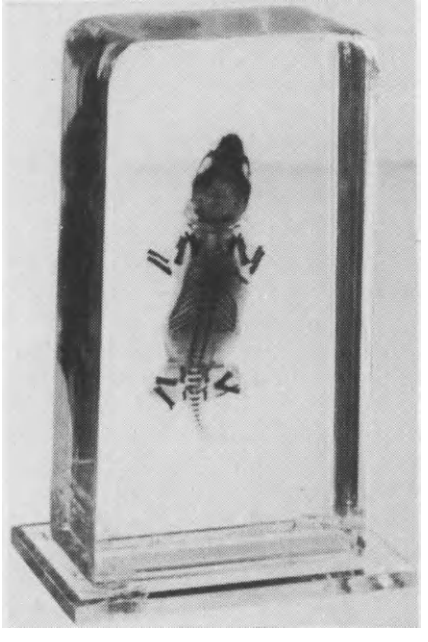


Figure 28.3 – Mouse embryo treated by Dawson's alizarin technique (by courtesy of the Editor of J. clin. Path.)

Spalteholz Technique

This technique, which completely clears the soft tissues, is excellent for the demonstration of the circulatory system after injection with

coloured pigments. Its success depends on the complete dehydration of the specimen with graded alcohols, and replacement of the tissue fluids with benzyl benzoate and oil of wintergreen.

Method

(1) Fix specimens in 10 per cent formol saline.
(2) Bleach in 10 vol. hydrogen peroxide for 1–2 days.
(3) Transfer to 50 per cent alcohol for 14 days, followed by immersion in 60, 70, 80 and 90 per cent alcohol, each for 14 days.
(4) Transfer to absolute alcohol for 14 days.
(5) Transfer to fresh absolute alcohol with a layer of anhydrous copper sulphate at the bottom of the container covered with 5 layers of filter paper, for a further 14 days.
(6) Transfer to benzene for 14 days.
(7) Transfer to fresh benzene for a further 14 days.
(8) Transfer to benzyl benzoate for 14 days.
(9) Transfer to fresh benzyl benzoate for a further 14 days.
(10) Mount in a mixture of equal parts of benzyl benzoate and oil of wintergreen. If the specimen is not completely clear at this stage it should be left in this fluid until clearing is completed.

Since the mounting fluid is a Perspex solvent, the specimens must be mounted in glass museum jars, and Spalteholz cement used to seal the lid.

Spalteholz cement

Powdered gum arabic	50 g
Sugar	50 g
Sodium silicate (waterglass)	2 g
Formalin	1 ml

All the ingredients are mixed into a paste with as little water as possible.

The specimen, fixed on a glass centre plate or frame, is placed in the jar which is filled with mounting fluid to within ½ inch of the top. Dry the top of the jar and apply the cement evenly to the ground-glass edge. Place the lid in position and apply pressure with a lead weight. Leave undisturbed for 24–48 hours. (The specimen shown in *Figure 28.4* was injected with 5 per cent gelatin containing finely ground chrome yellow.)

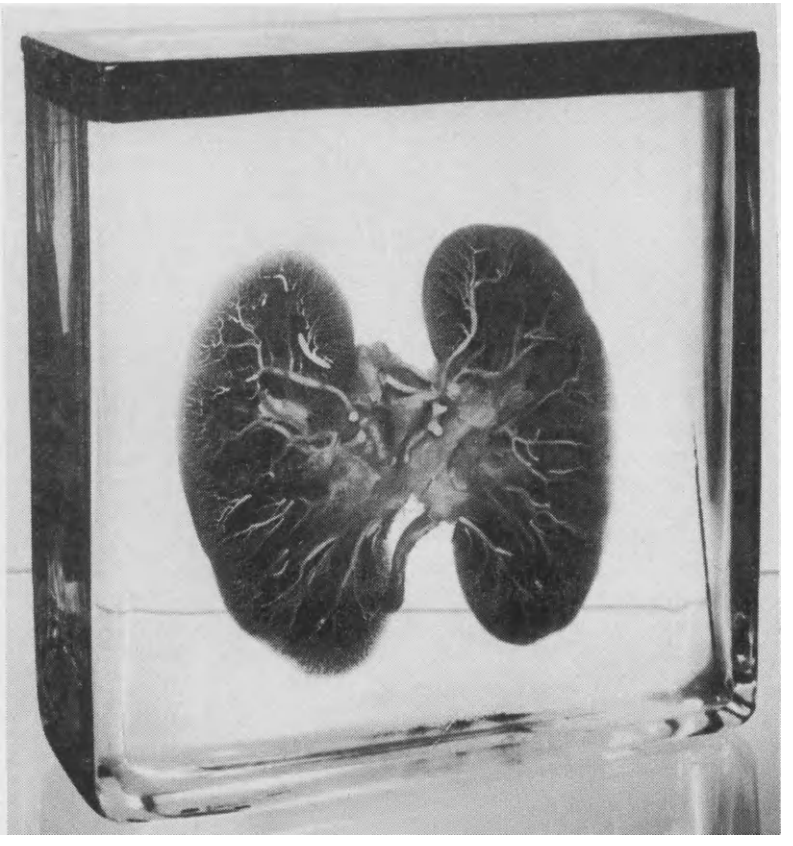


Figure 28.4 – Specimen of kidney treated by the Spalteholz technique, after injection with chrome yellow in gelatin (by courtesy of the Editor of J. clin. Path.)

AMYLOID

Amyloid degeneration may be stained deep brown with iodine, or bright red with Congo red.

Iodine Technique

Method

(1) Place slices of formol-fixed tissue in Lugol's iodine to which 1 per cent sulphuric acid has been added; leave for 1–2 hours.

(2) Wash in running water overnight.

(3) Mount in liquid paraffin. The colour will fade in ordinary mounting fluid.

Congo Red Technique

Method

(1) Place slices of formol-fixed tissue in 1 per cent Congo red for 1 hour.

(2) Transfer to a saturated aqueous solution of lithium carbonate for 2 minutes.

(3) Differentiate in 80 per cent alcohol until only the amyloid (and arteries and veins) are coloured red.

(4) Mount in normal mounting fluid (page 531).

HAEMOSIDERIN (IRON)

Haemosiderin in gross specimens may be demonstrated by the Prussian blue reaction.

Method

(1) Specimens are fixed in formol saline, taking care to avoid contamination with rust.

(2) Place in a solution of equal parts of 10 per cent hydrochloric acid and 5 per cent potassium ferrocyanide until, after a few minutes, the specimen develops a blue colour.

(3) Wash overnight in running water.

(4) Mount in 5 per cent formol saline. The colour diffuses out of the specimen in normal mounting fluid.

Note. – (1) Specimens fade after several months, but the colour is completely restored by treatment with hydrogen peroxide.

(2)) The fluid of haemochromatosis specimens becomes milky and the colour diffuses after a few months; this seems to be unavoidable.

STAINING OF SPECIMENS

Fat in gross specimens may be demonstrated by staining with Sudan III, followed by differentiation in 70 per cent alcohol, The specimen may

be counterstained with Ehrlich's haematoxylin to show infiltration with malignant tissue.

GOUGH AND WENTWORTH PAPER MOUNTED SECTIONS

By the Gough and Wentworth technique thin sections of entire organs are mounted on paper. Such a method lends itself to the storage of a large number of such sections in the form of a book, with a great saving in space; they may be examined as transparencies and compared and demonstrated side by side with the corresponding radiographs. The organ most commonly treated is the lung, but the liver, the kidney and the heart may also be used.

The most convenient machine for cutting the large sections is the MSE 'Large Section' Microtome.

Technique

(1) Remove the lungs from the body whole and without rupturing the pleura. If there are dense adhesions remove the parietal pleura with the lung (a few small tears do not matter except where there are large emphysematous bullae). One or both lungs may be used; one may be reserved for bacteriological and chemical investigation.

(2) Cut off at the hilum and fully distend by running the following solution into the major bronchi, by means of a tube and cannula from a reservoir about 4 feet above the lung.

Liq. formaldehyde (40 per cent)	500 ml
Sodium acetate	200 g
Tap-water	to 5,000 ml

There is no need to tie the bronchi after expansion. The amount necessary to distend the lung varies up to about 2 litres and the containers used by Gough and Wentworth contained a further 3 litres.

(3) Place the lung in a container of fixative large enough for it to float freely with no distortion from pressure. Cover with a cloth wetted with the fixative.

(4) Fix for 2 days or longer and then cut a slice about ¾ inch in thickness; this may be in any direction but a sagittal one is most convenient. Good results are usually obtained after a few days' fixation,

but in the absence of any urgency the slice is allowed to continue to fix for some weeks to reduce proteolytic enzyme activity.

(5) Wash the slice in running water for at least 72 hours to remove the formalin. Use a syphon system and have a drip of copper sulphate solution or nitric acid running into the washing water to give a dilution of 1:500,000 to 1:1,000,000. The copper or acid reduces enzyme action which – especially in summer – may digest the gelatin in the next stage of the technique.

(6) Place the slice in the following solution.

Gelatin	250 g
Cellosolve (ethylene glycol monoethyl ether)	40 ml
Capryl alcohol	5 ml
Tap-water	850 ml

Note. – In the United States of America gelatin of the specification 80–100 bloom should be used.

Remove the air from the slice to assist penetration by the gelatin. To do this, place the slice in a jar containing the gelatin solution heated to about 60°C, and put under a bell jar connected to a vacuum pump. A solution of agar is useful as a seal around the jar. With an efficient glass pump sufficient air can be removed within an hour, during which time the gelatin remains fluid at ordinary room temperature.

(7) Place the specimen, still in the gelatin solution, in an incubator at 35°C for 48 hours, in a container in which it can lie flat and be completely immersed.

(8) Cast the gelatin and specimen into a block by allowing the gelatin to set in a container with a loose bottom.

(9) Remove the block by pushing out the loose bottom of the container.

(10) Fix the block to the microtome holder by warming the latter and then put weights on top of the block. The undersurface of the gelatin melts and as it resets the block sticks to the holder. Put in an icebox at −15°C for several hours, preferably overnight.

(11) Cut as thawing takes place; a warm cloth rubbed on the surface hastens thawing. Do not try to cut sections until the block is thawed sufficiently to cut easily. For lungs the optimum thickness is 400 μ. For solid organs like liver somewhat thinner sections of 300 μ are usually preferable.

(12) Put the sections into 10 per cent formalin-sodium acetate solution for 24–48 hours to harden the gelatin.

(13) Wash in cold water for 1–2 hours to remove the formalin.

(14) Pour some fresh solution of the following over a sheet of Perspex.

Gelatin	75 g
Glycerin	70 ml
Cellosolve	40 ml
Water	805 ml

Note. – Perspex is an acrylic resin. Other plastics would probably work as well. Glass cannot be used for this purpose as the sections would then adhere to the glass and not to the paper.

(15) Trim the surplus gelatin from the edges of a section, place it flat on the Perspex and cover with a sheet of Whatman's No. 1 filter paper.

(16) Run a rubber roller squeegee lightly over the paper to remove surplus solution and air-bubbles.

(17) Stand the Perspex sheet on end for 15–30 seconds and then lay flat until the gelatin sets.

(18) While still wet, place in a radiograph drying cabinet, and when thoroughly dry strip the paper with the section attached from the Perspex. If no 'dryer' is available, dry as thoroughly as possible at room temperature, and when there are no wet patches left, complete the drying at 37°C.

Chapter 29

Presentation of Museum Specimens

The value of a museum specimen as an aid to teaching cannot be measured simply on its appearance or the technical excellence of its mount. It must be clearly labelled, and a system of cataloguing employed which allows easy and rapid access to a description of the specimen, a précis of the relevant history of the patient and, if possible, copies of photographs and radiographs *(Figure 29.1)*. In smaller museums it may be possible to have the relevant stained histological sections available also.

Labelling

The type of label employed and the type of information the label is to carry will depend on personal preference. It may be a rectangle of Perspex sheeting, 1/16 inch in thickness which is cemented in the centre at the bottom of the outside of the box, or at the bottom of the centre plate. The advantage of the former method is that alterations are simply and easily carried out. These labels may all be white, or a variety of colours used to indicate the different types of specimens; for example, red for undergraduate students, yellow for postgraduate teaching, and white for duplicate specimens. Perspex labels are lightly sandpapered, marked with Uno stencils, and finally covered with a thin layer of Perspex cement which is allowed to harden before fixing to the box. Alternatively, rectangles may be painted on the boxes with white or coloured cellulose paint and on these the number or diagnosis is painted; or information may be written or stencilled with Indian ink on celluloid labels which may be stitched to the specimen itself.

Where specimens are used for examinations it is best to employ a numerical system of labelling to avoid revealing the diagnoses, which should be available in a catalogue.

A good method is a modification of the decimal system whereby the first two figures of a four figure number indicate the type of conditions; for example, 01, normal organ or congenital lesion; 02, traumatic and mechanical; 03, 04 and 05, acute infection and sequelae. The remaining figures divide the subsection, and re-divide it again. Four, five or six figures may be used for this purpose. By prefixing the number with a letter the section of the museum is shown; for example, D, breast; E, nervous system; F, endocrine.

Figure 29.1 – General museum layout. Note pyramidal stand in the foreground which can be adapted for display of specimens, radiographs or photographs

Cataloguing

A loose-leaf system of cataloguing is essential in order to enable new specimens to be easily listed, but the method of attachment and the quality of the cards or catalogue pages must be of sufficient strength to withstand rough handling, and the pages or cards should be covered with a transparent material to protect them from damage. Such a material which is simple to use is Symax*, it is cut roughly to the size

*Available from A. Warne and Co., 153 Queen Victoria Street, London, E.C.4.

of the card and inserted in a photographic hot press (or pressed under brown paper with an electric iron), the heat causing it to adhere, leaving a strong shiny surface which may be washed if necessary.

Duplicate cards should be kept in the library or in the director's office as a protection against loss.

A useful type of catalogue is that made by Shannon Ltd. These open book-like, with 48 wires on each cover. The 96 cards are attached to the wires with clips and, when not in use, lie flat with the lower ½ inch of each card showing. On the visible edge is typed the number and diagnosis of each specimen.

Photographs

Photographs may be attached to the catalogue cards, or even cemented on the museum boxes (back, top or sides) as described by Martin (1952). A sheet of dense white Perspex, 1/16 inch in thickness, is cut slightly smaller (1/8 inch margin allowed) than the back (or top, top, or even both) of the specimen box and the edges polished. A further sheet of clear Perspex of the same thickness is cut slightly smaller (1/16 inch all round) than the white Perspex, and the edges chamfered and polished. The photograph is trimmed to allow a margin of 1/8 inch inside the clear Perspex.

The white Perspex is held in position with a small weight, and Perspex cement No. 2 is applied to the edges by means of a 10 ml syringe with a blunt-edged medium-bore needle; this is left for 30 minutes with a heavier weight in position. The photograph is then positioned, covered with the clear Perspex, and a weight used to maintain the position, and cement is applied sparingly but evenly to all the edges. Several weights are then applied to exert an even pressure, and the mount left to harden overnight. By this method only a minimum of air-bubbles is formed in the cement, and these are not usually visible.

Photographs may be attached to a centre plate inside the box by the following technique, but have a tendency to fade slightly over a long period.

A coloured centre plate (usually black or white) is cut to fit the box, and is then washed and dried. The photograph is trimmed to size, laid on chloroform in a developing dish for 3–4 minutes, the excess chloroform drained off and the photograph positioned on the centre plate. A rubber roller (of the photographic type) is then moved quickly backwards and forwards over the print until it is dry and has become firmly attached to the centre plate. A loose corner or edge may be moistened

with chloroform on a piece of cotton-wool and rolled flat. The centre plate is then put into position at the back of the specimen and the box sealed. The addition of sodium hydrosulphite to the mounting fluid appears to have no effect on the photograph.

ADDITIONAL DISPLAY MATERIAL

On the subject of additional material that can be displayed to advantage, Dr. Peter Hansell, of the Department of Photography and Illustration, Westminster Medical School, in a personal communication suggests: 'A museum should not only be a place for the storage and exhibition of preserved and mounted specimens, it should also provide a suitable location for the display of quite different material'.

Colour photography is playing such an increasing part in medical illustration that a note of warning should be sounded regarding the display of colour records in the medical museum.

Briefly, three types of material are involved: (1) colour prints; (2) colour transparencies; and (3) large, stained histological preparations mounted between glass.

(1) Colour prints are expensive and of doubtful permanence; they have the advantage that they are usually derived from a colour negative or transparency and can therefore be replaced as they become faded or defaced. To prolong their valuable life it is essential that they are protected in an album; this has the further property of shielding them from bright light when not in use, thus avoiding premature fading of the colours.

(2) Colour transparencies can only be displayed by trans-illumination, either in the form of a simple radiograph viewing box for large transparencies or an illuminated magnifying system for miniature slides. The latter are today becoming increasingly popular for reasons of economy and ease of storage. The dyes used in transparency processes appear to be more stable than those in present-day colour prints. Fading, accelerated by exposure to heat and light, therefore takes longer, but it is a factor to be contended with. This feature forms one objection to the replacement of specimens by collections of colour transparencies, a system which has found some favour in the United States of America. When displaying any form of colour transparency, attention must be paid to the following points.

(*a*) The illuminating source must be cool-running to avoid overheating of the transparency or its support.

(*b*) The light should be of a colour temperature best suited to the particular type of transparency: that is to say, no undue yellow or blue cast should be added by the lighting itself. These two conditions are met by 'natural' or 'daylight' fluorescent tubes.

(*c*) All extraneous light should be masked off with black card around each transparency and overhead lighting should not compete in brilliance with the transillumination.

(*d*) Some form of press-button time switch, permitting a suitable viewing interval, should be fitted to the transilluminator. The same stringencies need not be applied to black and white transparencies which may also be used with effect.

(*e*) So-called 'giant' sections of large, stained histological preparations may be mounted on lantern-slide glasses for projection or display on illuminated panels. They are apt to suffer the same ill-effects from light and heat as colour transparencies and similar counter-measures should be taken.

Static displays are a quite separate adjunct to teaching, and as they may be ranged with equal ease on walls or central stands, the museum may offer a good location. If this is possible, mounted specimens may

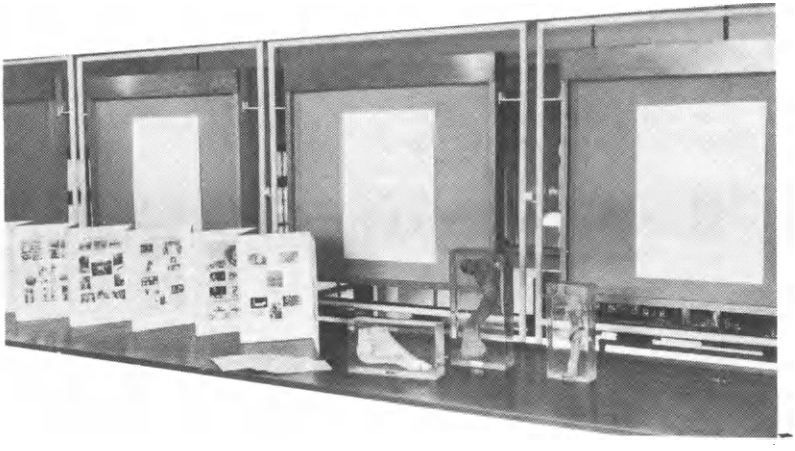


Figure 29.2 – Removable panels erected on framework above museum bench. The smaller exhibits shown may be arranged in 'concertina' form on any available flat bench or table

be worked into the display itself without depriving the museum of reference material. Teaching displays may take several forms; it is sufficient here to say that the problem involves medical illustration as a whole. Photographs, radiographs, mounted specimens, drawings, dia-

grams, models and microscopical preparations may all have a part to play in a pattern which is usually knit together with text *(Figure 29.2)*.

Taking example from public museums, in which an attempt is often made to restore a breath of life to otherwise dull inanimate objects, it seems logical that there should be a similar approach to medical museums, however modest. The minimum requirement in this direction is that the normal products of a medical illustration department can be conveniently and adequately displayed therein.

LIGHTING

A well-lit museum is more likely to be used than one where the specimens may not easily be examined. Fluorescent lighting offers great

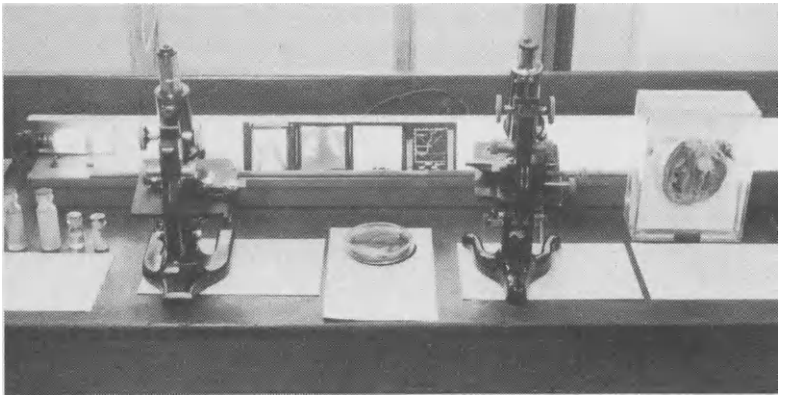


Figure 29.3 – Composite demonstration arranged on bench in museum. Note the spring clips holding the fluorescent tube to the base, and holding the aluminium shade to the tube, seen on the left of the picture. The choke for the light is incorporated in the metal base

advantages over other types in being very adaptable when used in conjunction with reflectors.

A bench, or benches, fitted with light points for microscope lamps, or a portable mounted 6 feet fluorescent tube, is an advantage for weekly displays which can then include stained histological sections. The mounted fluorescent tube can also be used to display giant sections, culture plates, transparencies, and so on *(Figure 29.3)*.

LAYOUT

The layout of a museum will depend on the space and money available, in addition to the type of material to be displayed and the audience for which it is intended. The normal medical school might base various sections of the museum relative to the organs of the body, in contrast to the Wellcome Museum of Medical Science where selected diseases each have a separate section.

Special display cabinets with semi-permanent displays on selected diseases may, however, be incorporated in a conventional medical museum *(Figure 29.1)*.

Chapter 30

The Compound Microscope

The microscope is the most commonly used piece of apparatus in the laboratory, and yet it is probably the instrument about which least is known by its users. It is generally thought that the microscope can be used effectively without any knowledge of its limitations or construction, but this is, of course, a complete misconception. An ill-adjusted, badly illuminated microscope can, when one is using high-power objectives, give completely misleading information as to the structure of an object. For this reason it is advisable to gain a knowledge of how the magnified images are produced by the microscope before attempting to assess the information obtained by its use.

The first part of this chapter is devoted to the lens and its faults, after which the component parts of the microscope, its use and maintenance are discussed.

A LENS

A lens is the name given to a piece of glass or other transparent material, usually circular, having the two surfaces ground and polished in a specific form in order that rays of light passing through it shall either converge (collect together) or diverge (separate).

A lens is called positive when it causes light rays to concentrate or converge to form a real image *(Figure 30.1a–d)*; or it is negative, in which case light rays passing through will diverge or scatter and positive or real images will not be seen *(Figure 30.1e–g)*. These two types are easily differentiated since positive lenses are thicker at the centre than at the periphery, whereas negative lenses are thinner at the centre and although the shapes may vary considerably, these characteristics remain *(Figure 30.1a–g)*.

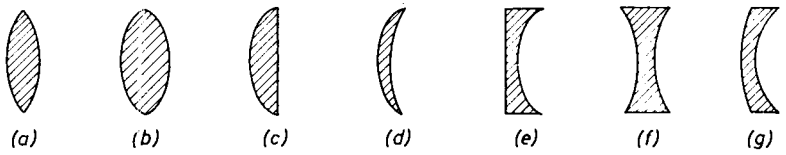


Figure 30.1 – Types of lenses; (a–d) positive lenses; (e–g) negative lenses

Refraction of Light Rays

The effect of a lens on a ray of light is due primarily to the density of the glass (or other material) which reduces the speed at which light travels through it. (Light is usually considered as a vibration in the ether – a hypothetical substance which fills the whole of space). In a dense medium (for example, glass) the light rays are retarded, or slowed down. If a beam of light containing two parallel rays (AB, $A_1 B_1$ *Figure 30.2a*) strikes a sheet of plane glass at right-angles, its speed of travel through the glass will be reduced, but its direction unchanged. If it strikes the plane glass or the curved surface of a lens at an angle (CD, $C_3 D_3$ *Figure 30.2b*) its speed will be reduced and its direction changed. The bending of light rays, known as refraction, is due to the fact that one part (D) strikes the surface of the glass first (D_1) and is retarded while the other part (C) is still travelling at normal speed, thus causing the ray to be bent and its direction altered. From C_1 the two portions travel in the same direction at a common reduced speed until C emerges from the dense medium (C_2) and travels at its original speed while D is still retarded in the dense medium, causing a further bending of the light ray. After D emerges from the dense medium (D_2), the two portions travel in the new direction at their original speed.

It will be obvious that the degree of refraction will be dependent not only on the angle of the surface of the lens to the light ray, but also on the optical density of the material from which the lens is made. The optical density of a substance is indicated by its refractive index (R.I.), which is the ratio of the velocity of light in air to the velocity of light in that substance.

The behaviour of a beam of light passing from one medium to another can be estimated from the rule that light entering a more dense medium bends towards the 'normal' (A O in *Figure 30.2c*), and when entering a less dense medium it bends away from the normal (O B in *Figure 30.2c*).

Light can always enter a lens, no matter what the angle at which it strikes, but it is not always possible for it to leave. As the angle between

the beam of light leaving and the 'normal' (the angle of incidence) increases the emerging beam is bent closer and closer to the surface of the glass, until it is parallel with the surface (C O D in *Figure 30.2c*).

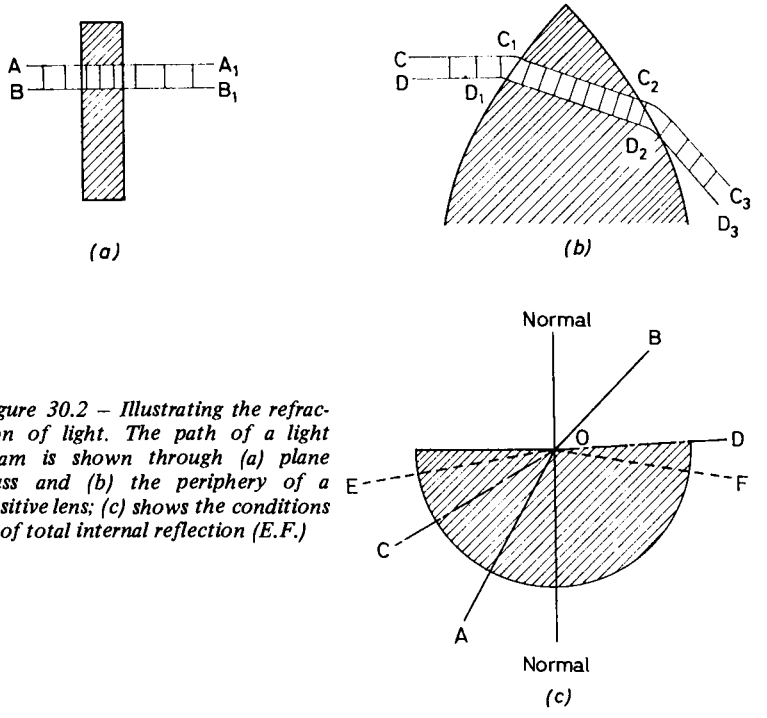


Figure 30.2 – Illustrating the refraction of light. The path of a light beam is shown through (a) plane glass and (b) the periphery of a positive lens; (c) shows the conditions of total internal reflection (E.F.)

Any further increase in the angle of incidence will result in the beam being reflected from the surface instead of emerging – a condition known as total internal reflection (E O F in *Figure 30.2c*).

Focus

If, through the centre of one side of a box, a pinhole is made, so small that only one ray of light can pass through it in each direction, then the image of an object outside the box will be formed on the back of the box *(Figure 30.3a)*. The ray of light from each point of the object entering the box is very narrow and it can only travel in a straight line. Therefore, each point of the object will have a corresponding point in the image *(Figure 30.3a)*, and since the light rays from the

bottom of the object form the top of the image, and vice versa, the image will be inverted. Similarly, variations of brightness and colour will be reproduced. Such a box may be used as a camera, though not a very efficient one; a long exposure would be needed owing to the small amount of light allowed to enter. To enlarge the hole and fit a lens would result in the production of a much brighter image, owing to the fact that instead of only one light ray entering from each point of the object, a large number will enter through the fitted lens *(Figure 30.3b).*

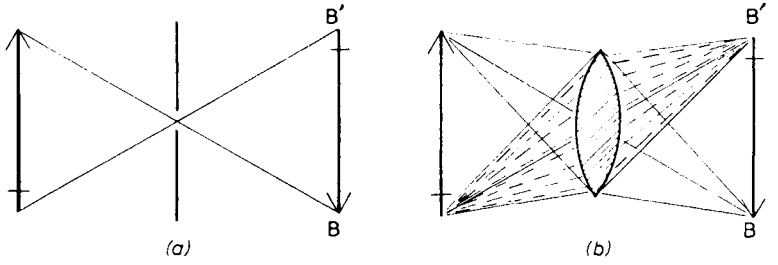


Figure 30.3 – Production of (a) image through pin-hole aperture, and (b) brighter image by using a lens instead of pin-hole

A notable difference in the production of images by a pinhole and a lens is that a pinhole will produce an image, regardless of the depth of the box or the nearness of the object, whereas in the case of a lens the screen and the objet must be in exactly the correct positions, or the image will be indistinct and hazy. The lens will cause the light rays to converge to a single point at only one position (BB′, *Figure 30.4*), and at either side of that position each point of the object will be represented by a solid circle of light; each circle being overlapped by the adjoining ones (CC′, *Figure 30.4*).

When a lens concentrates the light rays to form a clear sharp image of an object, the object is said to be in focus. The terms 'focus' or 'principal focus' are used to indicate the position in which a lens will form a sharp, clear picture of a distant object, such as the sun. (The word focus originally meant burning place, and was used to indicate the point at which a lens concentrated the sun's rays to form a sharp image having the power to burn.)

In addition to the principal focus, a lens also has conjugate foci; these are two points, one on each side of the lens, in one of which a clear image will be formed on a screen of an object placed in the other. The positions of the conjugate foci vary: as an object is moved away from the lens, so the image will be formed closer to it and vice versa

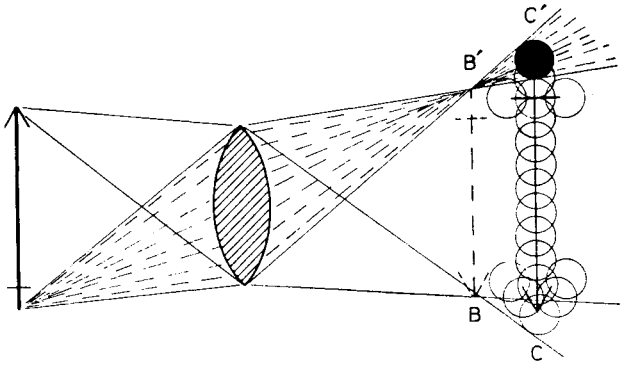


Figure 30.4 – The production of an in-focus image BB', and an out-of-focus image CC'

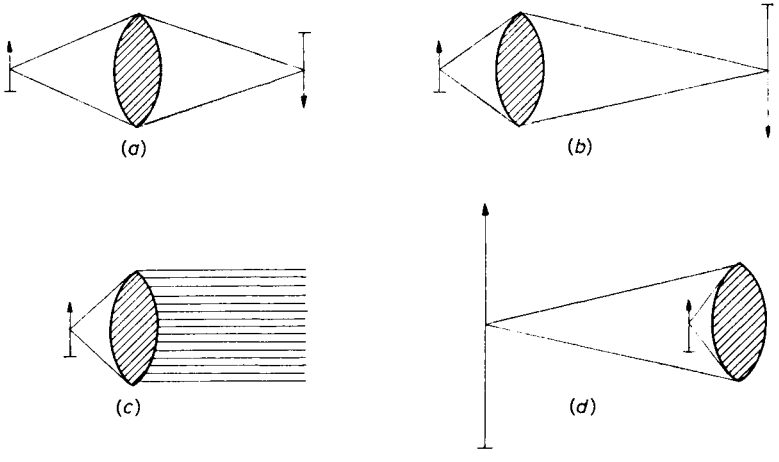


Figure 30.5 – Showing the effect of moving a lens in relation to a static object (reproduced from 'The Microscope' by courtesy of R. and J. Beck Ltd.)

(Figure 30.5a and *b)*; and any pair of such positions are called conjugate focal planes. The magnification of the lens is affected by this movement of the lens or object since the further away from the lens the image is formed, the larger it appears *(Figure 30.5a* and *b)* and, consequently, the greater the magnification.

Images, as those described, which can be seen on a screen, are known as real images. As the object is brought closer to the lens, the image will move further away until it reaches infinity and cannot be seen *(Figure 30.5c)*. If the object is brought still closer, the image will re-appear on the opposite side of the lens – that is, the same side as the object – but it will be a ghost image which can be seen only by looking through the lens, and which cannot be focused on a screen. The image has undergone a further change in that the image will appear the right way up *(Figure 30.5a)*. This is known as a virtual image.

Defects of a Lens

For a microscope to be efficient, it must not only produce a magnified image, but one which will be clear and well defined. To use a simple lens of the type described will not give such good results because: (1) white light is not a single vibration but is composed of a series of vibrations of differing wavelengths; and (2) faults are inherent in its shape.

Chromatic Aberration

When white light is split into its component parts, each part vibrates to a different degree, producing to the eye a different colour. These colours (red, orange, yellow, green, blue, indigo and violet) are known as the primary spectrum, and are seen in the rainbow, or through a spectroscope. Red has the longest wavelength, with a vibration of 0.7μ, blue 0.45μ and violet 0.35μ.

It will be seen that the vibrations of red light are twice the length of those of violet light.

Since light rays cannot vibrate as easily in a dense medium as in a rare medium, it follows that the various colours will be affected by a lens to differing degrees, the colours with shorter wavelengths, such as blue violet, being affected to a greater degree than those having a longer wavelength, such as red and orange. It is for this reason that rays of white light, having passed through a prism, emerge as a spectrum, each ray having been refracted to a different degree, and each emerging at a different point. Violet and blue are refracted to a greater degree than red and orange.

In *Figure 30.6*, W represents a point source of white light entering a lens which, on emerging, forms a different point of focus for each of the component colours, blue(B) being focused at a point nearer the lens than red(R).

A screen placed at R will show a red point surrounded by the colours of the spectrum, having a blue edge; at B a blue point with a red periphery will apear. This colour defect is called chromatic aberration, and its correction is known as achromatism.

Since different types of glass have different optical properties, chromatic aberration can be corrected to within useful limits by using a two-component lens. A positive lens (of greater magnifying power than is finally required) is combined with a negative lens made of glass producing a greater chromatic aberration, but with the same refractive index. The negative lens corrects the chromatic aberration in the positive lens, and only partially neutralizes its magnifying power. This method will correct a thin positive lens for any two colours, leaving a small error in the intermediate colours (secondary spectrum). This type of lens is known as an achromatic lens.

If fluorspar is incorporated in the glass of the achromatic lens, three colours can be brought to the one focal point, and the amount of chromatic aberration visible in the image will be negligible. Such lenses are known as apochromatic lenses.

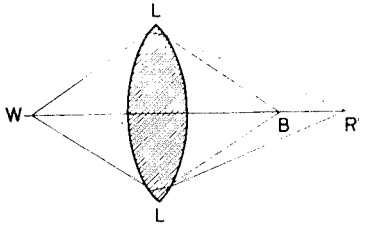


Figure 30.6 – Chromatic aberration

Since the correction of an apochromatic lens involves the use of a larger number of lenses, its other defects are corrected at the same time so that the final lens, provided that it is correctly used, will show hardly any defects.

Spherical Aberration

Spherical aberration is a further defect of a single lens, due to the fact that it has a curved surface.

Since the angle at which light rays enter (and leave) the surface of a lens varies with each part of the lens, those rays passing through the periphery (AA) will be refracted to a greater degree than those travelling through the central area (CC, *Figure 30.7*). There is no position, therefore, where the light from a point source will be in sharp focus,

and since each point is hazy the composite image is bound to be indistinct. This fault could be minimized by using only the central area of a lens, but since a microscope objective must have a short working distance and a high magnification, a large angle of light is required from each point of the object, and the correction of this aberration is most important.

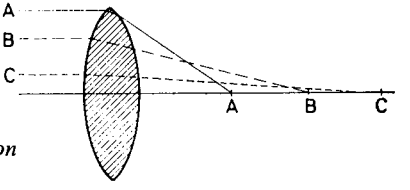


Figure 30.7 – Spherical aberration

The degree of spherical aberration will depend on the actual shape of the lens, and by varying the shape, although the focus may be the same, the spherical aberration will vary. The method of correction follows the same pattern used in correcting achromatism, namely, that of using a powerful positive lens and partially neutralizing its magnifying power with a negative lens made of glass having a greater relative aberration. Complete correction is extremely difficult and the above account presents the problem and its solution only in a very simple form.

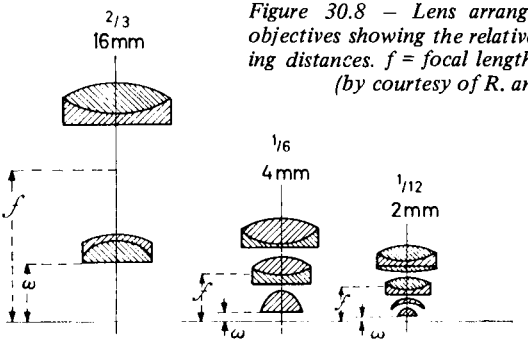


Figure 30.8 – Lens arrangement of the common objectives showing the relative focal lengths and working distances. f = focal length; w = working distances (by courtesy of R. and J. Beck Ltd.)

Chromatic and spherical aberration are the two principal faults to be found in lenses; there are others, but since correction is achieved by similar means – variation in shape and composition, and the distance apart of component lenses – they will not be discussed in detail. For a

more comprehensive description of these faults the reader is referred to *The Microscope* by Beck (1938). Some idea of the complexity of various lens systems may be gathered from *Figure 30.8.*

COMPONENT PARTS OF A COMPOUND MICROSCOPE

A simple microscope is composed of one or several lenses mounted closely together, as in the case of a hand lens, whereas a compound microscope is composed of two widely separated lenses, or sets of lenses, capable of producing greatly enlarged images.

The standard monocular microscope *(Figure 30.9)* is composed of two main parts: (1) the microscope proper, incorporating the body tube with the objective at one end and the eyepiece at the other; and (2) the stand, which includes the supporting, adjusting and illuminating apparatus.

The Microscope Proper

The Eyepieces (or Oculars)

These are designed to further magnify the primary image from the objective, they also limit the field of view as seen by the eye. They may be used to correct residual errors in the objective lenses and may then be either: *under-corrected,* when a blue ray of light will be refracted to a greater degree than the red, this can be identified by the blue fringe that is seen around the edge of the field diaphragm; or *over-corrected,* when the reverse is the case and an orange fringe may be seen at the edge of the field diaphragm. Compensated eyepieces are usually over-corrected.

There are two basic types of eyepieces, as follows.

(1) With the *negative* eyepiece the focus is within (between) the lenses of the eyepiece. It is composed of two lenses; the lower or field lens collects the image that would have been formed by the objective (virtual image plane) and cones it down to a slightly smaller image at the level of the field stop (or field diaphragm) within the eyepiece *(Figures 30.10a* and *30.16);* the upper lens then produces an enlarged virtual image which is seen by the microscopist. An engraved scale placed in the field stop will be superimposed (in focus) on the image (*see* the section on Micrometry on page 593).

(2) With the *positive* eyepiece the focus is outside the eyepiece lens system; for this reason it may be used as a simple microscope. The

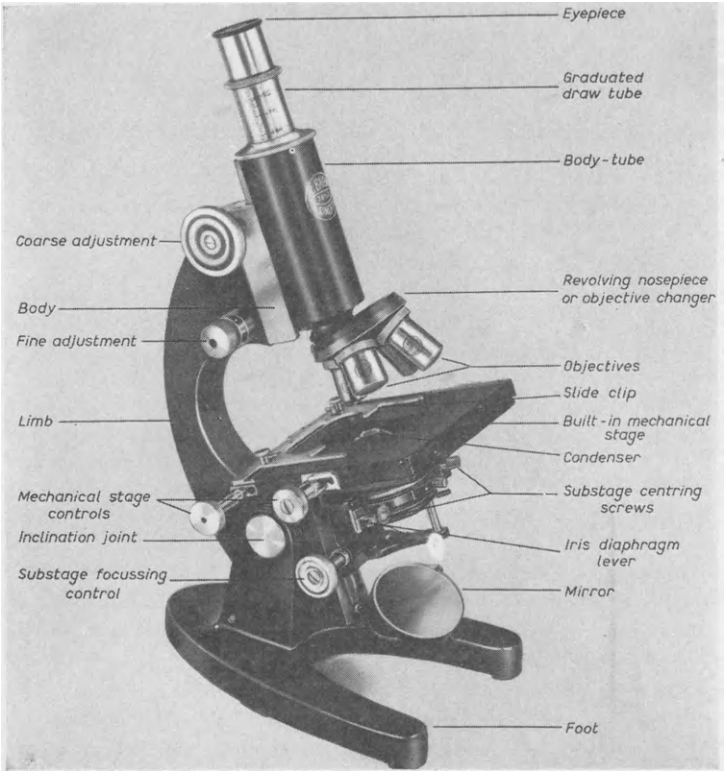


Figure 30.9 – The monocular microscope (by courtesy of C. Baker of Holborn Ltd.)

field stop (or diaphragm) is outside the eyepiece, from which the virtual image (from the objective) is focused and magnified by the entire eyepiece *(Figure 30.10b).* As with the negative type of eyepiece, a scale placed on the field stop will be superimposed (in focus) on the image formed by the objective.

Huygenian Eyepieces

These eyepieces *(Figure 30.10a,),* originally designed by Huygens for the telescope, are the type most commonly used in microscopy. They are negative, under-corrected *(see above),* and are best suited for use with achromatic objectives.

Ramsden Eyepieces

As will be seen in *(Figure 30.10b),* these are positive oculars. It will be noted that the lower lens has its plane side toward the object. Most

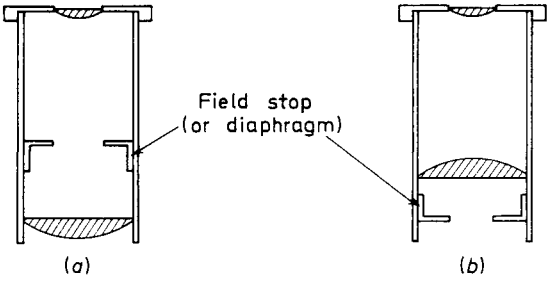


Figure 30.10 – Basic types of eyepieces (or oculars): (a) negative type (Huygenian); (b) positive type (Ramsden)

of the compensated eyepieces are of the Ramsden type, having doublet or triplet lenses instead of the single lenses shown in *Figure 30.10b.* Ramsden oculars are preferred for micrometer eyepieces as they impart less distortion to scales.

Wide Field Eyepieces

Within recent years improvements in ocular design have enabled manufacturers to produce lenses which give a large flat field of view which are particularly valuable in the biological laboratory.

High-eyepoint Oculars

These have also been introduced in recent years, primarily for microscopists who wear spectacles, and are usually engraved with a diagram of a pair of spectacles. With normal eyepieces, the distance between the top of the eyepiece and the exit pupil (eye point) is so small as to prevent the wearing of glasses, but the high eyepoint of these special oculars make this possible. It is advised that the rubber guards supplied with such eyepieces be used to prevent the scratching of the spectacle lenses. Such eyepieces may be used by all microscopists, but some practice is needed before their use (with the head being held slightly higher than usual) becomes familiar and comfortable. The author uses X12.5 high-eyepoint, wide-angle eyepieces routinely.

Compensating Eyepieces

These eyepieces were originally intended for use with apochromatic objectives only, and were not recommended for use with achromats. They are *now recommended for use with all modern objectives.* English speaking countries mark them 'Comp', while German lenses are designated by the letter 'K'.

Field of View

Some eyepieces are marked with their field of view number from which can be calculated the actual diameter of the specimen being viewed (the field of view number, divided by the magnification of the objective, equals the field of view in millimetres).

Magnification

Eyepieces always receive the 'virtual image' from the objective in the same plane and therefore magnify it to a constant degree, independent of other factors such as body tube length, and so on. They are consequently marked with their magnifying power and may vary from ×4 to ×50. As will be seen in the following pages it is generally inadvisable to employ powers in excess of ×12.5.

The Objective

The objective screws into the lower end of the body tube by means of a standard thread, thus all objectives are interchangeable. They are usually designated, not by their magnifying power but by their focal length (from 2 to 50 mm); this is because their actual magnifying power will depend on the tube length at which they are used. Some confusion has arisen in the past by the terms 'focal length' and 'working distance' in relation to objectives. Whereas with a simple lens these are identical, with compound lenses such as those in an objective they are different.

The 'working distance' is simply the distance from the object to the outer surface of the front lens, whereas the 'focal distance' is that from the object to a point roughly midway between the component lenses *(Figure 30.8).* The latter is correct only when the objective is used at the standard tube length of 160 mm. If the tube length is altered the focal distance will also be altered and the object will need to be refocused.

Most instrument manufacturers mark objectives with the appropriate magnifying power, usually because they produce a microscope which has no draw tube, the tube length being a standard 160 mm.

The aperture. – The first objective consisted of a single lens, and its defects were overcome by the use of a pin-hole aperture, but since only a small cone of light could enter from each point of the object, the image, although greatly magnified, showed very little detail. It is apparent, therefore, that the amount of detail seen is dependent not, as commonly believed, on the magnification but on the size of the cone of light that can be collected from the object.

The ability of a lens to define detail is known as its resolving power, and this is measured by the distance apart of two lines or dots, or the number of lines to the inch, that can be visually separated from each other; for example, a lens that has a resolution of 30,000 lines to the inch has a greater resolving power than one separating only 20,000 lines to the inch. This will be appreciated by viewing *Figure 30.11a* from a

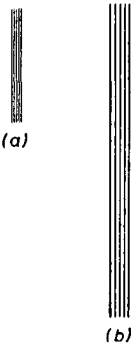


Figure 30.11 – The difference between resolution and magnification

distance of 10 feet, when only a single line will be seen. On closer examination it will be found that there are, in fact, five lines. Even when the image is magnified *(Figure 30.11b)* and viewed from 10 feet it still has the appearance of being a single line.

Resolution is restricted by two factors: (1) the numerical aperture of the lens; and (2) the wavelength of light employed. The relationship is as follows.

$$\text{'X'} = \frac{1.2\,\lambda}{2\ \text{N.A.}}$$

Where 'X' is the resolution (the smallest distance between the closest

two lines or dots that can be defined separately) and λ is the wavelength of the light employed. N.A. is the numerical aperture.

The numerical aperture. – The apertures of the early microscope lenses were at first measured by the actual angle of aperture; that is, the angle formed by the outer edges of the lens, and a point on the object *(Figure 30.12a).* The aperture of oil-immersion lenses, however, depends on the refractive index of the medium between the object and the lens and for this reason may vary. This is because the cone of light, emerging from a glass coverslip into air, is refraced away from the lens face and a much smaller cone of light enters than if there was glass, or a medium having the same refractive index as glass, between the lens and the object. In *Figure 30.13a* it will be seen that the angle of the cone of light, from a point source of the object, actually entering the lens when used dry, is only 78 degrees, compared with an angle of 120 degrees when immersion oil is between them *(Figure 30.13b).* To take account of this factor, and to be able to express a lens aperture as a simple figure, the term numerical aperture (N.A.) is used, which may be expressed as follows.

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

where 'n' is the refractive index of the medium between the lens and object, and 'sin u' is the sine of half the angle of aperture *(Figure 30.12a).* Since the sine of an angle is opposite over hypotenuse $(\frac{O}{H})$ *(Figure 30.12b),* it may also be roughly expressed as half the diameter of the lens over the distance from the periphery of the lens to the object. Since the highest N A. theoretically possible when a lens is used dry (air R.I. = 1.0) must be 1.0, and with immersion oil (R.I. 1.51) 1.51, it will be appreciated that modern high-power objectives (dry N.A. = 0.95; oil immersion N.A. = 1.32) approach very closely to the theoretical maxima.

The effects of a high numerical aperture. – Whilst a high numerical aperture increases the resolution of an objective, it has the following disadvantages: (*a*) it reduces the depth of focus, that is, the ability to focus on more than one layer of an object at the same time, and (*b*) it reduces the flatness of the field, so that the edges are out of focus.

It will be seen, therefore, that if depth of focus and flatness of field are important, then increased magnification should be obtained with high-power eyepieces, although as a general rule a change to a higher power objective, giving increased magnification and resolution, is preferred.

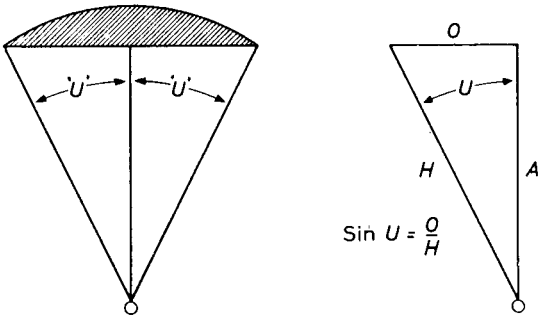


Figure 30.12 – Diagram to illustrate numerical aperture

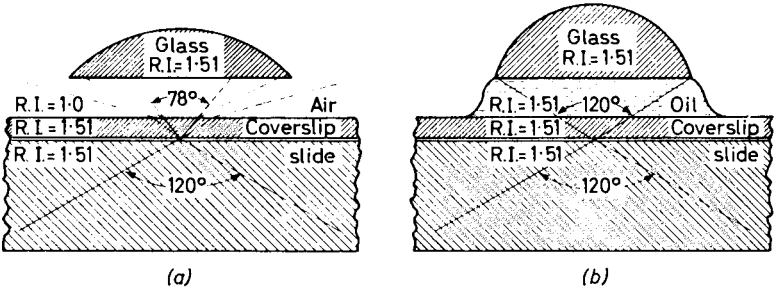


Figure 30.13 – Diagram illustrating the effect of the interposing medium on the angle of light entering the objective

Types of Objective

All objectives are engraved with the information needed to obtain their maximum performance as well as any possible limitations. Such an engraving might read:

Plan 40/0.65
160/0.17

which indicates that it is a planachromat; ×40 magnification at a tube-length of 160 mm (for its best performance), has a numerical aperture of 0.65 and should be used with a coverglass of 0.17 ± 0.01 mm in thickness (this /0.17 may instead be / = insensitive to coverglass thickness or /0 = for use with unmounted specimens).

Achromatic Objectives

These objectives are the type most commonly used and the modern well-corrected lenses are more than adequate for routine microscopy in pathology and biology laboratories.

Apochromatic Objectives

When apochromatic objectives are employed, their high degree of correction is wasted unless they are used in conjunction with a highly corrected aplanatic or achromatic condenser, and compensating eyepieces. The latter compensates for peripheral chromatic aberration due to the differing magnifications of the various coloured images. These eyepieces together with the objectives form a re-combined single image free from coloured fringes even at the periphery.

Apochromatic objectives should always be used for microphotography. To get the maximum light with high-power objectives having numerical apertures above 1.0, oil-immersion condensers should be used with an N.A. at least equal to that of the objective, and immersion oil between the condenser and the slide as well as between the objective and the slide.

These objectives are also highly corrected for the other lens aberrations (spherical, coma, and so on).

Fluorite Objectives (Neofluor)

Fluorite or semi-apochromatic objectives, have fluorite incorporated into the lens system to give better colour correction. They are corrected for three wavelengths of light in the yellow-green of the spectrum, and are free of colour fringes. They are generally more highly corrected in all other respects than the achromats and represent a quality of image midway between that of the achromat and apochromat.

Planachromat Objectives

Planachromats are principally designed to give a perfectly flat field, with the whole field in focus at the same time. They are used mainly for photomicrography.

Polarizing Objectives

Designated POL, these are strain-free objectives for use on the polarizing microscope.

Phase Objectives

These objectives contain a phase-plate for use in phase-contrast microscopy (*see* page 651).

Coverglass Thickness

It will follow that oil-immersion objectives do not have coverglass restrictions since they will have the same refractive index as the immersion oil. The coverglass thickness is only important if high-power 'dry' objectives are being used, when No. 1 coverglasses should be used, or an objective with a correction collar may be employed which allows a range of thickness of coverslip from 0.12 to 0.22 mm to be used. To check the setting for a particular specimen (where the coverslip thickness is unknown) first focus upon a high contrast area, then determine whether changing the collar setting increases or decreases the contrast. If the coverglass thickness is known it can be set directly upon the engraved scale above the collar.

The Body Tube

The body tube is attached to the limb of the microscope which, in turn, is attached to the base either directly or by a hinged joint. Since the aberrations, or faults, of a lens can only be corrected for one tube length, for critical microscopy it should always be set to the standard 160 mm if a draw tube is fitted; if there is no draw tube, the body tube will, of course, be correct.

The body tube may rarely contain a draw tube, being a telescopic tube by means of which the distance between the eyepiece and objective may be varied *(Figure 30.9)*. The draw tube usually contains a fixed diaphragm at its lower end to cut off reflections from the inside of the body tube. Such a draw tube is useful in micrometry (page 593).

A carrier or nosepiece for a number of objectives is usually fitted at the lower end of the body tube; it rotates on a central pillar, and is designated by the number of objectives it carries; for example, double, triple or quadruple nosepiece. The nosepiece should bring each objective into its correct position; that is to say, centred on the optical axis, and at the correct tube length. An increase in magnification is simply a matter of rotating the nosepiece, which is optically better than changing the eyepiece since a large aperture is being used; the oil-immersion lenses are, of course, an exception since the body tube needs to be raised to place oil on the slide.

The depth of the nosepiece will affect the tube length and this is

generally 18 mm in depth, the actual length of the body tube being only 142 mm. If, for any reason, the nosepiece is removed, it must be replaced by a compensating ring of the same depth.

For accurate centring of objectives another type of objective changer may be used, a female slide being fitted to the bottom of the body tube, and each objective screwed into a male slide which has three centring screws. Owing to the improved design of modern nosepieces such attachments are now rarely seen.

Support, Adjustment and Illumination

Supporting Structure

The body tube of the microscope is attached to a limb, which in the past was hinged to a pillar and base. The latest models have inclined eyepieces, and the main supporting structure is not hinged.

Adjustment

On old models the body tube was attached to the supporting structure by two slides which were the site of the adjustment controls *(Figure 30.9).* This was followed by placing the slides (and controls) on the base, which entailed the controls moving the whole superstructure (body tube and limb) which caused increased wear and shorter life, but it was felt that the convenience of having the controls at almost bench level outweighed this disadvantage. Models of the past few years, however, have a fixed body tube, limb and base, the adjustment slide or slides being connected to raise and lower the stage and substage; this has the dual advantage, the controls being conveniently placed with little weight bearing on them, which gives longer life and lessens the likelihood of their 'slipping'.

The mechanism of the slides is such that one of them, working by rack and pinion, enables the stage and substage to be moved rapidly up and down, and is called the coarse adjustment; the other, working by micrometer screws, and levers or cams, enables the stage and substage to be moved slowly and accurately and is called the fine adjustment. Although the designs of the latter may vary, they are based on the same general principle: the movement by a lever or cam to a steel plate fixed on the back of the coarse adjustment slide *(Figure 30.14).* The coarse adjustment therefore moves the stage and substage, but the fine adjustment moves both the stage and substage and the coarse adjustment slide. As these slides wear, a degree of play will develop and cause slackness in focusing; most manufacturers, therefore, fit screws which may be adjusted to compensate for this slackness, but they should be adjusted with care as overtightening will cause excessive wear.

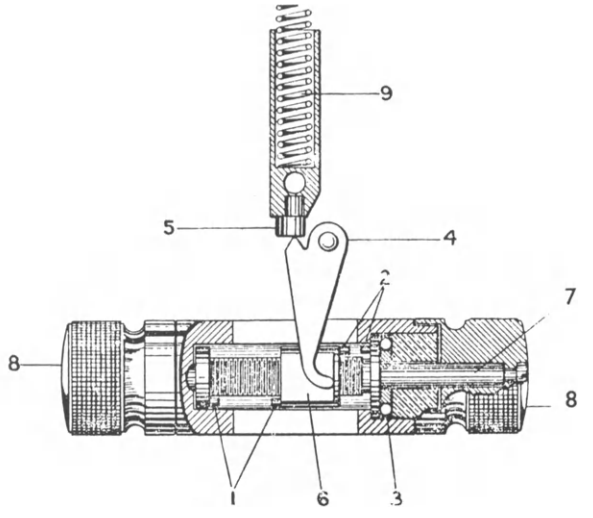


Figure 30.14 – A type of fine adjustment (by courtesy of C. Baker of Holborn Ltd.)

Stage

At the lower end of the limb supporting the body tube and adjustments is a platform, or stage, on which the objects to be examined are placed. The stage, which has an aperture of 1–1½ inches in diameter is provided with either simple metal spring clips to hold the object, which is then moved by hand to change the field, or is fitted with a mechanical stage which will give even steady movement of the object in two directions by means of two micrometer threads. The standard type of mechanical stage takes a 3 × 1 inch slide and moves over an area approximately 3½ × 1¼ inches so that a whole slide may be examined, but special stages are available to take very large slides and Petri dishes. Circular rotating stages are also available if preferred (for example, the polarizing microscope).

Most mechanical stages are fitted with a Vernier scale for recording the position of the slide in each direction, and they may be very useful if a particular field is to be found quickly at a later re-examination. By noting the reading on each scale, the slide can be replaced in much the same position almost immediately. One scale will be graduated, for example, from 0 to 80, and the other from 80 to 110 in order that the two readings will not be confused. Opposite these graduations will be the smaller Vernier scale, marked from 0 to 10. These 10 graduations,

being equal to 9 in the main scale, enable each of the latter to be subdivided by 10.

Illuminating Apparatus

Below the stage, and usually attached to it, is an adjustable substage which can be moved up and down by a helical screw or rack and pinion (as is the coarse adjustment).

The substage consists of: (*a*) the condenser to focus the light on the object when using objectives with a focal length of 16 mm of less; (*b*) an iris diaphragm to control the cone of light entering the condenser; (*c*) a filter carrier; and (*d*) a mirror, flat on one side and concave on the other, which is mounted in gimbals so that light may be directed into the condenser from almost any angle, or more commonly a built-in light source.

The Condenser

The condenser should form a perfect image of the light source, and have the same numerical aperture as the objective with which it is being used.

The two-lens Abbé condenser is in common use but is not very efficient, forming only an imperfect image of the light source. It should not be used with apochromatic or fluorite lenses (page 582). To obtain perfect results with such objectives, a condenser with a lens system equal to that of the objective being used should be employed: a three-lens aplanatic or a more highly corrected achromatic condenser will give a crisp image with good resolution. Such condensers are usually fitted with a swing-out front lens (or the front lens may be unscrewed) to illuminate the whole field for low-power lenses. By swinging out the front lens the numerical aperture of the condenser is reduced to 0.3–0.4. For critical microscopy with objectives having an N.A. exceeding 1.0, immersion oil should be applied between the condenser and the slide, as well as between the objective and the slide.

The Iris Diaphragm

Light which passes through the object but does not enter the objective is unnecessary, and may interfere with those light rays which are intended to form the image.

The iris diaphragm is employed to limit the angle of the cone of light passing through the object so that it will just fill the front lens of the objective.

The intensity of illumination should always, if possible, be reduced by using light-absorbing filters, or a variable resistance, not by closing the diaphragm and never by racking down the condenser.

The Filter Carrier

The filter carrier is usually a recessed metal ring, pivoting on a screw to facilitate the easy removal of filters.

The Mirror

The two-sided mirror is plane on one side and concave on the other, and is fitted about 4 inches below the stage. A concave mirror has a focus since it causes the light rays, which have been reflected, to converge together and form an image. The focus is approximately 4 inches (its distance from the object) and is intended to take the place of a condenser when using very low-power objectives since these require a large area of the object to be illuminated.

The plane mirror must always be used with the condenser since the latter can only be used efficiently if the whole of the back lens is filled with light.

The Binocular Microscope

The light rays emerging from the objective in the binocular microscope are equally divided between the two eyepieces. It is not sufficient simply to insert a single prism and divert one half of the rays, since this would cause eyestrain due to both the observer's eyes being focused on a single point a short distance away, and the advantage of a binocular microscope is that long periods may be spent viewing through it with the minimum amount of eye fatigue. The modern binocular microscope achieves this by the use of four prisms. It will be seen from *Figure 30.15* that the eyes are receiving two parallel beams of light. The lower central prism consists of two prisms cemented together, at the interface of which there is a semi-silvered surface: this silvering is a very special process, fine grains of silver being deposited so that alternate light rays are differentially treated, one being reflected to the right *(Figure 30.15)* and the other passing into the upper prism.

The light rays passing through the semi-silvered surface to the upper prism travel through a greater thickness of glass than those that are reflected – having the effect of retarding them – and this is compensated for by making the right-hand prism with an extra thickness of glass as will be seen by comparing the two outside prisms in *Figure 30.15.*

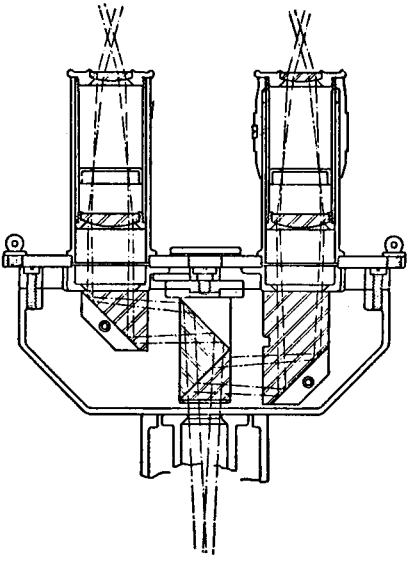


Figure 30.15 – The prism arrangement in a binocular attachment (by courtesy of Cooke Troughton and Simms Ltd.)

An additional advantage of this system is that the eyepieces, with the prisms attached, can be easily moved together or apart, and the interocular distance adjusted to suit individual requirements.

One of the eyepieces (the one on the right in *Figure 30.15* is fitted with an adjustment to compensate for the slight variation of focus occasionally required.

With a binocular body on a microscope, the optical tube length may be increased from 160 to 140 mm, and since the objectives are corrected for the shorter tube length, a compensating lens is incorporated to overcome this factor; the lens is also necessary to re-focus the virtual image for the new tube length. The increase of tube length also has the effect of increasing the magnification, and binocular attachments may have their magnifying factor engraved on them which, since the tube length is usually increased by one half, is X 1.5.

Illumination

Although daylight may be used to illuminate the field, it will generally be found inconvenient owing to its inconstancy. Artificial

illumination supplied by an electric filament lamp is therefore most commonly employed.

The lamp may be either a simple pearl bulb, or a high intensity lamp used in conjuction with a condenser and an iris diaphragm.

The source of illumination should be: (1) uniformly intense; (2) should completely flood the back lens of the condenser with light when the lamp iris diaphragm is open; and (3) make the object appear as though it were self-luminous.

(1) Uniform intensity of illumination is most difficult to obtain since the solid sources of light – a tungsten arc (where a small sphere of tungsten glows white), or a carbon arc – present great difficulties if used over long periods. The difficulty is overcome by using a closely would filament with a diffusing screen, although for routine work with a monocular microscope a 60 watt pearl bulb will suffice. Kohler illumination may be used.

(2) The source of light should be sufficient to enable its rays when directed by the plane side of the mirror to flood the back lens of the condenser uniformly. The high intensity type of lamp has an optical axis and must be correctly aligned for use, and the distance from the microscope at which it is used adjusted so that the lens magnifies the lamp image to the correct size, a built-in light source has been so adjusted.

Where separate, the lamp and the microscope should be connected so that accidental movement of one or the other will not upset the alignment. If the manufacturers do not supply such a connexion, the lamp, the microscope and the transformer (if needed) may be mounted on a wooden base.

(3) The object will behave as if self-luminous if the opal bulb or the image of the lamp condenser is focused in the object plane with the sub-stage condenser *(see below)*.

SETTING UP THE MICROSCOPE

The bench on which the microscope is mounted should be free from vibration and be in such a position that the microscopist works with his back to the window; a light screen, the back and sides of which are finished with a flat black paint to minimize back-scatter of light, is a great advantage.

Critical Illumination by Nelson or Kohler Methods

These are the two universally recognized methods for correct illumination.

Nelson Method

For this method the light source should be homogeneous and no lamp condensers used. It is normally employed with a bare light source.

Kohler Method

For this method to be used the light source does not have to be homogeneous, but a lamp condenser is essential to project an image of the lamp filament on to the substage iris diaphragm. In this system the lamp condensing lens (which is evenly illuminated) functions as the light source. This method must be used with compound lamps, and should always be used for micro-photography.

Technique

(1) The lamp should be positioned opposite the microscope (the high intensity compound light being fixed), and a blue daylight filter inserted in the filter carrier to absorb the excess yellow given by artificial light.

(2) Position the lamp so that the light strikes the centre of the mirror, and adjust the mirror so that the light is directed upwards into the condenser.

(3) With a compound lamp focus the condensing lens so that an image of the source of light is formed on the substage iris diaphragm; if necessary hold a piece of white paper at this position so that the image is visible. The daylight should be removed to get a clear image.

(4) Focus on an object on the stage with the 16 mm objective, and, with the eyepiece removed, adjust the mirror so that the field is evenly illuminated.

(5) Replace the eyepiece and, with the object in focus, rack the substage condenser up or down until a sharp image of the lamp iris appears; this renders the object self-luminous. In practice it has been found that the best position for the condenser is just below this point. This is 'Kohler illumination'.

(6) If the instructions given in (5) are followed with the exception that the condenser is racked up and down until a mark on the bulb, or the image of the lamp iris diaphragm, is focused, this will be *critical illumination* (Nelson method).

(7) Adjust the substage iris diaphragm so that only the area to be examined is illuminated; with objectives having a N.A. in excess of 1.0 the condenser diaphragm will need to be fully open.

(8) If the microscope is fitted with a centring substage, move the condenser up until a sharp image of the closed diaphragm is seen; the condenser is then adjusted until this image is central. Stages 4, 5, 6 and 7 are then carried out.

The microscope is now critically illuminated and the optical train co-axial.

For critical microscopy and microphotography, the lamp iris and the condenser may need to be re-centred each time the objective is changed.

One cardinal rule for the microscopist is always to rack the objective down near the object before looking through the eyepiece and then to focus on the object by racking the objective up and away from the object. This will avoid damaging the object, or the front lens of the objective, and is particularly important when using oil-immersion lenses, which have such short working distances.

CLEANING AND MAINTENANCE

It must be remembered that the microscope is an exceedingly complicated and delicate piece of apparatus, and a great deal of experience is required to completely service and maintain it. Component parts should be returned to the manufacturer when faulty, since amateur attempts at repair usually result in further damage: apart from cleaning the outer surface of their lenses objectives are best left alone. Prisms should never be touched, and cleaning should be confined to blowing off the dust with a rubber bulb fitted with a small-bore metal tube, since the slightest disalignment of the prisms will cause enormous eye fatigue. Lenses should be wiped only with fresh lens paper or well-washed silk, otherwise they may be scratched. Immersion oil should be removed immediately after use, although old oil can be removed with lens paper damped with xylol.

Daily Cleaning Routine

(1) The microscope should be dusted daily, and the outer surface of the lenses of objectives polished with lens paper or well-washed silk.

(2) The top lens of the eyepiece should be polished to remove dust or fingermarks, and the microscope set up for critical illumination.

(3) Rotation of the eyepiece will show if any dust is still present, in which case, the eyepiece may need to be dismantled and both lenses cleaned.

(4) The substage condenser and the mirror are cleaned in a similar manner: dust on the condenser will be apparent when this is racked up and down, since it will come in and out of focus.

A little attention to cleaning the microscope daily will, by the removal of chemically-active and sharp pieces of grit and foreign matter, prolong the life of the instrument and make the weekly cleaning task a short and simple one.

Weekly Cleaning Routine

(1) The slides of the coarse adjustment, the mechanical stage and the substage condenser should be wiped with a cloth damped with xylol to remove dust which would otherwise damage the slides. A little oil (as supplied for lubricating microscopes) is applied and the slides replaced: later models do not require this treatment.

(2) The lens system should be checked and cleaned.

(3) Clean the eyepieces as described in (2) and (3) of the daily routine, and then trace dirt in other places by a similar system.

(4) Dust is removed from the back lenses of objectives by use of the rubber bulb described above.

(5) Interocular adjustment slides will usually require cleaning only once a month, and great care should be taken not to damage or disturb the prisms during this operation.

MAGNIFICATION

The magnification of a lens will depend on its conjugate foci (page 571); that is, the distance from the object to the lens and that from the lens to the image. In the microscope the objective forms a real inverted image in the upper part of the body tube, which is then further magnified by the eyepiece. Therefore, the magnification of the microscope is the product of the magnifications of the objective and the eyepiece, and is dependent on the following three factors: (1) the focal length of the objective; (2) the distance between the focal plane of the objective and the image it produces (since the optical tube length and the mechanical tube length are approximately the same, the latter is always used *(Figure 30.16)*); (3) the magnification of the eyepiece.

Magnification therefore equals:

$$\frac{\text{Tube length}}{\text{Focal length of objective}} \times \text{Eyepiece magnification}$$

To take an example: the magnification obtained with a 16 mm objective, used with a X 10 eyepiece at the standard tube length of 160 mm would be:

$$\frac{160 \text{ mm}}{16} \times 10 = 100$$

Where the magnification is marked on an objective it is only correct when used at the standard tube length. It should be remembered that this magnification is a linear one, and in the example above the object will be magnified 100 times in all directions; the actual area magnification will be 100 X 100 = 10,000 times.

MICROMETRY

The standard unit of measurement in microscopy is a micron (μ), which is 0.001 mm.

To measure microscopic objects an eyepiece micrometer scale is used in conjunction with a stage micrometer. The eyepiece micrometer scale is usually a disc on which is engraved a scale divided into $^1/_{10}$ and $^1/_{100}$ graduations. This is placed inside the Huygenian eyepiece, resting on the field stop *(Figure 30.16)*. Eyepiece micrometers may be purchased with the scale permanently in position; these are usually Kellner eyepieces which have a focal plane below their bottom lens. They give a sharp image of the scale and have a greater eye clearance; they are an advantage (without a scale) for general work if spectacles are worn.

The stage micrometer consists of a 3 X 1 inch slide on which a millimetre scale is engraved in $^1/_{10}$ and $^1/_{100}$ graduations.

An object may be measured by the following method.

(1) Insert a micrometer eyepiece scale and place the stage micrometer on the stage.

(2) Select the objective to be used when measuring the object, and focus on the stage micrometer scale.

(3) Determine the number of divisions of the eyepiece scale equal to an exact number of divisions of the stage micrometer scale. A drawtube is useful at this stage since a slight alteration in magnification by increasing or decreasing the size of the stage micrometer scale, may greatly simplify calculations.

(4) Remove the stage micrometer, focus on the object to be measured, and determine the number of eyepiece divisions exactly covered by the object.

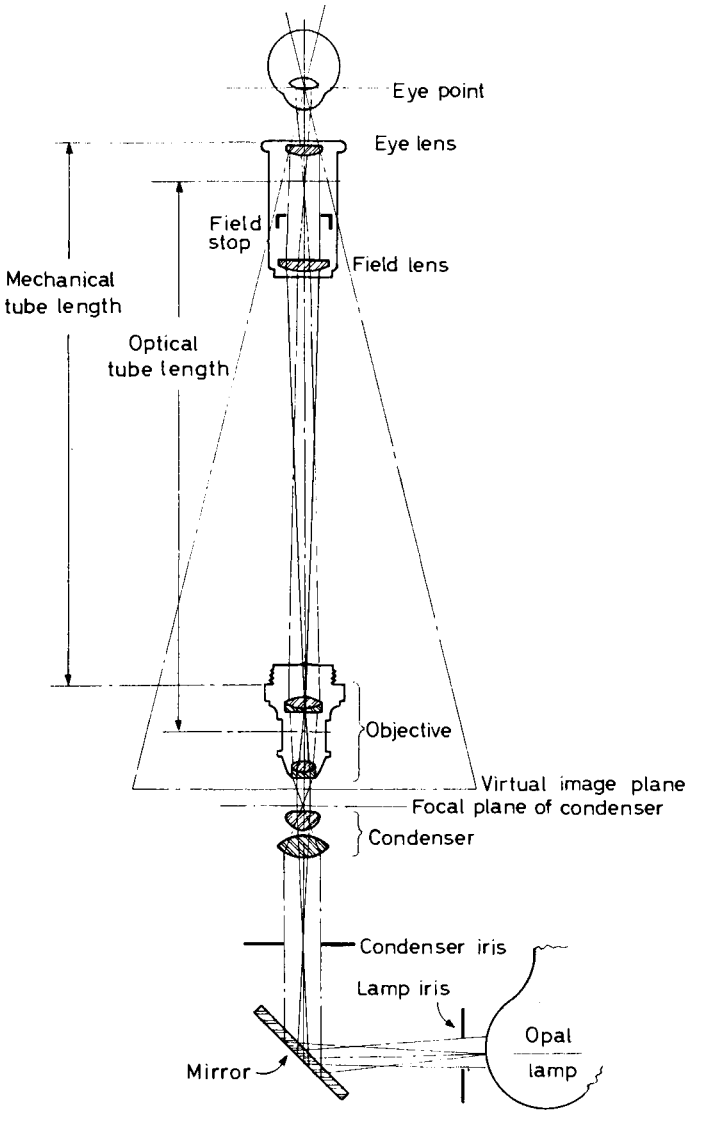


Figure 30.16 – Showing the optical path of light through a microscope

Calculate the size of the object as follows, assuming that 100 eyepiece divisions were equal to 10 stage divisions, and that the diameter of the object was exactly covered by 12 eyepiece divisions.

100 stage divisions = 1 mm = 1,000 μ
$\therefore$ 10 stage divisions = 100 eyepiece divisions = 100 μ
$\therefore$ 1 eyepiece division = 1 μ
$\therefore$ 12 eyepiece divisions = 12 μ

The diameter of the object, therefore, was 12 μ.

Chapter 31

The Dark-Ground Microscope

For an object to be examined microscopically, it must first be visible. Visibility is dependent on contrast, as is illustrated by the fact that a black cat is invisible in a coal cellar because there is no contrast between the object and the background. For the same reason, a spider's web is difficult to see against the sky, yet stands out clearly when viewed against a dark background with the sun shining on it; this is because the fibres reflect the rays of light from the sun and give the web the appearance of being self-luminous, the dark background increasing the contrast.

Most objects examined microscopically are naturally transparent, but in general they reflect or scatter light rays, and if, as in dark-ground illumination, oblique light is thrown upon them which does not enter the objective, they will appear as self-luminous objects on a dark background.

Objects examined by dark-ground illumination give a misleading impression of size; fine particles appear to be much larger than they are, owing to their light-scattering properties. This factor is of advantage when examining fine structures such as spirochaetes which are clearly visible by this method, yet when stained (by Giemsa's stain) are difficult to see. This will only apply if the object is alone or nearly alone in the field of view; therefore, preparations must be as thin as possible; if such objects are examined in a mass of light-reflecting material the contrast will be lost. Although it is impossible to completely isolate cells and organisms, extraneous refractile material such as air-bubbles, red blood cells and oil droplets must be avoided and a thin preparation used.

Objectives and Condensers

Low-power objectives work at some distance from the object and therefore dark-ground illumination is obtained simply by inserting a small circle of black paper (pasted on glass) in the filter carrier. The central rays which would normally pass through the object and into the objective are cut off and the peripheral rays from the condenser pass through the object, but do not enter the objective; the only light entering the objective will be that scattered by the object.

High-power objectives, having a much shorter working distance require a special condenser which will accurately focus a hollow cone of light at an acute angle. This angle is so acute that if oil is not used between the condenser and slide the light rays are reflected back into the condenser (total internal reflection, *see* page 569). Immersion oil

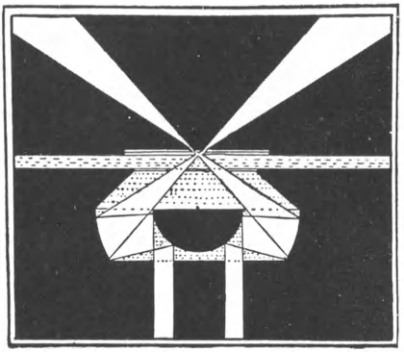


Figure 31.1 – A fixed focus dark-ground condenser

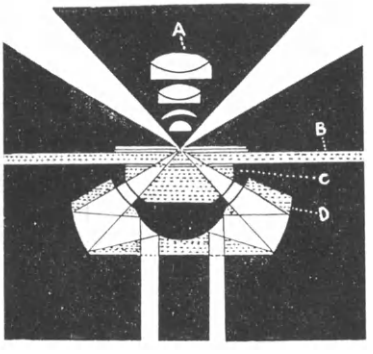


Figure 31.2 – A focusing dark-ground condenser

(Reproduced from 'The Microscope' by courtesy of R. and J. Beck, Ltd.)

must be used between object and objective to ensure that the maximum amount of reflected light from the object enters the objective. To get the best results the condenser must be accurately centred, otherwise peripheral light rays will enter one side of the objective; similarly, the condenser must be accurately focused to get the maximum amount of light on the object without its entering the objective.

Because of the very acute angle of light required, very few dark-ground condensers can be used with an objective having a numerical aperture (N.A.) in excess of 1.0. A 2 mm objective having a N.A. 1.3 can be used if a funnel stop (a small metal tube) is inserted in the back which reduces the working aperture to less than 1.0. Alternatively, a

$^1/_7$-inch oil immersion lens may be used without modification. The most convenient type of 2 mm objective is one incorporating an iris diaphragm, since this can be closed just sufficiently to stop any direct light.

The fixed-focus type of dark-ground condenser *(Figure 31.1)* is most common, but this can only be used with extra-thin glass slides and coverslips (No. 1). Focusing dark-ground condensers *(Figure 31.2)* are available which will allow a variety of slides and coverslips to be used.

Since only reflected or scattered light forms an image of the object, the source of light should be an intense one, to ensure the maximum amount of light passing through the object. A Pointolite tungsten arc lamp probably gives the best results, although the modern high intensity lamp will give almost equally good results.

SETTING UP THE DARK-GROUND MICROSCOPE

Method

(1) Make a thin preparation, using a thoroughly clean thin slide and coverslip, and taking care not to have air-bubbles in the preparation.

(2) Place the lamp in front of the microscope and focus the image of the source on the plane side of the mirror (if necessary).

(3) Direct, or adjust, light through the condenser so that it is evenly distributed.

(4) Rack the condenser down; place a drop of immersion oil on the top lens of the condenser and on the lower side of the slide. Place the slide on the microscope stage and slowly rack up the condenser until the two surfaces of the immersion oil meet without forming air-bubbles; such bubbles would reflect light in all directions.

(5) Focus on the object with a low-power objective such as the 16 mm.

Note. – If the condenser is correctly focused a small point of light will illuminate the object on a dark background. If a hollow ring of light is seen the condenser is above or below its point of focus and should be adjusted.

(6) With the centring screws, adjust the condenser until the point of light is in the centre of the field.

(7) Place a drop of immersion oil on the coverslip and focus the object with the high-power oil immersion objective. Perfect dark-ground illumination should result if a funnel-stop objective is used; if

an iris diaphragm is incorporated in the objective it is adjusted to give the maximum performance. Occasionally the objectives are not par-central and the condenser may need a slight adjustment to get a perfect result.

(8) After use the oil should be carefully cleaned off both the condenser and objective.

The following errors are the most common causes of difficulty in setting up the microscope.

(1) The slides or coverslips are too thick.
(2) The preparation has too many air-bubbles present.
(3) Condenser is not properly focused or centred.
(4) Lighting is not sufficiently intense.

Chapter 32

Fluorescence Microscopy

In 1852 Stokes first used the word 'fluorescence' to describe the reaction of fluorspar to ultraviolet light: in 1903 R. W. Wood devised a filter which would absorb visible light and transmit only ultraviolet light. These two events led to the first 'fluorescence microscope' described by Lehmann in 1911. Little use was made of this apparatus until 1935 when Max Haitinger pioneered and developed the technique of staining histological preparations and smears with fluorescent dyes. It is probably to him that most of the credit for the modern development of fluorescence techniques belong. In 1937 Hageman applied fluorescent dyes to organisms, and probably the first routine use of fluorescent microscopy was the staining of acid fast bacilli. In 1941 Coons, Creech and Jones described a technique for labelling protein with a fluorescent dye, which led to the now almost routine technique of fluorescent antibody staining.

Fluorescence

When a quantum of light is absorbed by an atom or molecule, an electron is boosted to a higher energy level. When this displaced electron returns to its original ground state it may emit a quantum of light *(Figure 32.1)*. If this light is emitted only during the time of exposure, or for a very short time afterwards (about 9–10 seconds) it is known as *fluorescence*, if the emission persists after the exciting light is cut off it is called *phosphorescence*. Since a certain amount of energy is lost as heat before the electron returns to its ground state the fluorescent (or phosphorescent) light is at a longer wavelength (lower energy) than the original exciting light. In fluorescence microscopy ultraviolet light

(which is not visible to the human eye) is used as the exciting light with the resulting fluorescence (of a longer wavelength) being in the visible range. Thus an object is illuminated with 'black' light and, when fluorescent, appears as a bright object on a dark background. It should be remembered that while an enormous number of compounds are fluorescent to some degree, only relatively few give sufficiently brilliant fluorescence that they may be detected in small quantities by their autofluorescence (*see* 5HT page 468), or used as fluorescent dyes. Certain dyes, marked in catalogues as fluorescent, are virtually useless because of their poor fluorescence. Furthermore some compounds and dyes, while brilliantly fluorescent as pure compounds, may lose their power to fluoresce when bound to other structures. This is known as *quenching* of fluorescence. This latter is sometimes a useful property, since non-specific fluorescence can be quenched to give greater contrast (*see* use of haemotoxylin to quench nuclear fluorescence, page 611).

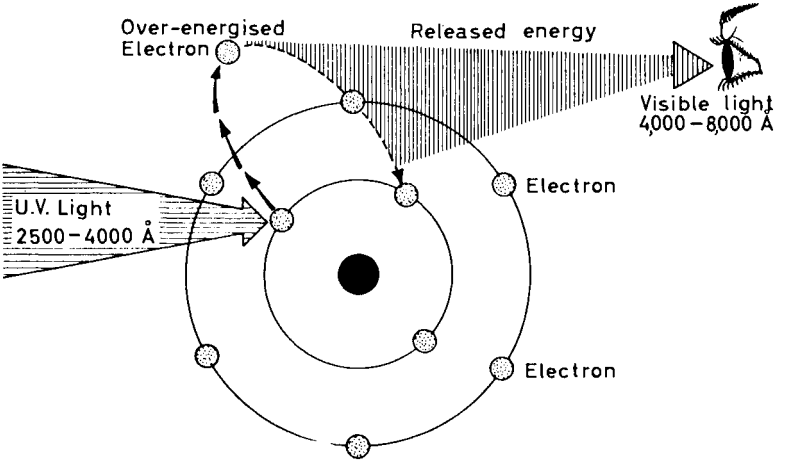


Figure 32.1 – Diagram to illustrate how ultraviolet light may excite fluorescence in a molecule

Equipment

The early workers in this field used quartz condensers and slides to bring the maximum concentration of ultraviolet light on to the object. With modern optical glass (which transmits light with a wavelength of over 300 mμ) it has been found that almost any condenser may be used for this purpose.

Slides should be checked for obvious fluorescence (those made of green glass being avoided); but most good brands of slides are suitable.

The only special equipment that needs to be purchased, provided a microscope is available, is a good ultraviolet light source, special light filters and ideally, though not necessarily, a polished metal (surface reflecting) mirror.

Illumination

Any good ultraviolet light source may be used, for example, carbon arc lamp, Mazda Mercra lamp, and so on. The ones most commonly employed are dealt with in detail below.

*Osram HBO 200 Lamp**

This is a high pressure mercury lamp which provides a steady powerful source of ultraviolet light. The mercury arc, measuring 2.5 by 1.3 mm, operates in a globe of fused quartz at a pressure of 70 atmospheres with a brightness of 25,000 stilbs (25,000 candle power per square centimetre). The starter unit provides 15,000 volts to strike the arc, which is maintained by low tension of about 60 volts. It should always be employed within the special housing provided.

This lamp is without doubt the most efficient and least troublesome of those available. With the exception of the AH6 *(see below)* it gives the most intense illumination. It gives five-sixths the light intensity of the AH6, but since it does not require water cooling it is much more convenient to use and there is no installation problem. Most microscope manufacturers (Zeiss, Leitz, Reichart) now incorporate it in their fluorescence equipment. The lamp has an average rated life of 200 hours, but the author used his first one for over 2,000 hours; his second lamp (in 4 years) is still in constant use. There is a fall in emission with wear but this was not sufficient to make any practical difference. The only rule followed was that once the lamp was switched on it was left on for the whole of that working day. The lamp gives over 30 per cent of its emission at a wavelength of 365 mμ which is the wavelength found to be most useful in fluorescence microscopy *(Figure 32.2)*.

100 Watt, Type AH4 Mercury Vapour Arc (General Electric Co.)

This lamp is a fused quartz tube, with sealed-in tungsten electrodes, giving an arc stream about 25 mm in length and 1.6 mm in width. The average rated life of this lamp is 1,000 hours.

*In the U.K. marketed under the name 'Neron'.

The AH4, available from a number of microscope manufacturers, is employed in many laboratories for routine fluorescence work. It is less expensive than the HBO 200, and has a longer life. It is not suitable for critical fluorescence, for example, fluorescent antibody technique or autofluorescence assessment, but is adequate for most fluorescent staining methods.

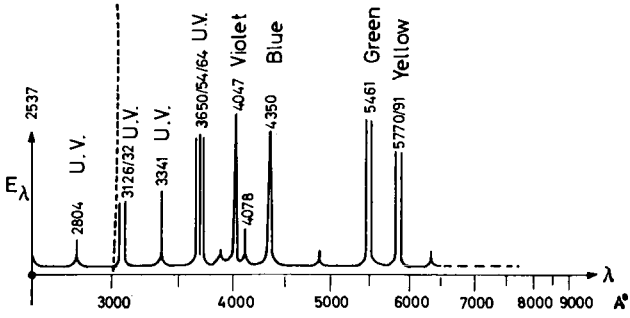


Figure 32.2 – Emission spectrum of the super pressure mercury lamp Osram HBO 200. The interrupted line at 3,000 Å indicates the transmission limit of optical glass; light of a shorter wavelength than this can only be used with a quartz condenser

1,000 Watt, Type AH6, Water Cooled Mercury Lamp (General Electric Co.)

This lamp consists of a quartz capillary tube, about 2 inches by ¼ inch. The arc stream is about 25 mm by 1.6 mm. A special transformer supplies an operating voltage of about 840 volts. It develops tremendous internal pressure which necessitates its encasement in a water-jacket to develop a counteracting pressure; this jacket also functions as a cooling system. For efficient operation of the lamp a pressure operated switch and magnetic valve must be inserted into the water line which must be attached permanently to the lamp.

This lamp is without doubt the best available for fluorescence microscopy since it has the highest intrinsic brillance of any artificial illuminant, with a continuous emission spectrum. However, the cost of installation and the loss of mobility in most instances outweigh the slight advantages.

Projection Lamps

These have been used by some workers, who utilize light in the blue–violet visible range (400–450 mμ) to excite fluorescence in the

green–yellow (550–600 mμ) range. They are not recommended for general use except where no other source is available.

Filter System

A heat filter system is essential with any intense source of illumination. The heat filter is usually located in the lamp housing between the lamp and the collecting lens.

In addition, two basic filter systems are necessary for fluorescence microscopy.

(1) *Exciter filters.* – These transmit light of a short wavelength to excite fluorescence in the specimen. They may be varied to transmit (*a*) light of a specific wavelength, (*b*) light up to 400 mμ (ultraviolet), or (*c*) light up to 500 mμ (ultraviolet-blue).

(2) *Barrier, or contrast filters.* – These are so named because they are used primarily to protect the eyes from the damaging

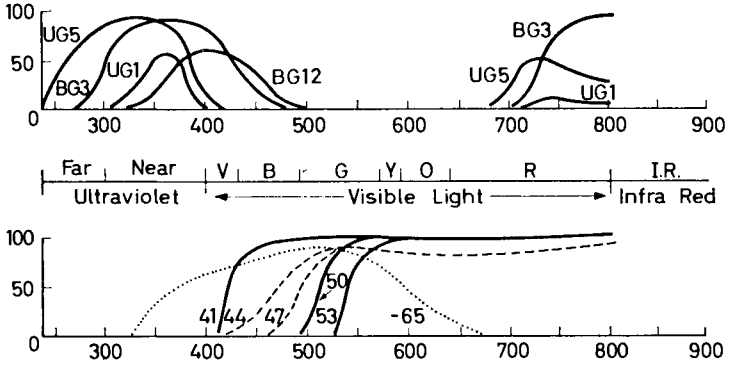


Figure 32.3 – Diagram showing transmittance curves of (top) exciter filters and (bottom) barrier filters. Figures on the ordinate scale indicate the percentage of transmission. Wavelength is shown in millimicrons (mμ)

effects of ultraviolet light. By the use of different filters, with varying absorption and transmission characteristics, non-specific background fluorescence may be extinguished, giving greater contrast. For example, when examining tissue stained by a yellow or orange fluorescent dye, Schott filters OG 4 and 5 (Zeiss 47 and 50) may be used to absorb the blue autofluorescence of the tissue. This will result in bright yellow staining against a dark background.

Exciter Filters

For routine use a 2 mm Schott BG 12 filter (325–500 mμ) should be used in conjunction with a dark-ground condenser. With a bright ground condenser a 4 mm thick BG 12 filter (or even 6 mm) should be used.

For special purposes a Schott UG 1 (275–400 mμ) may be used alone, or in combination with a 2 mm BG 12 (325–400 mμ). This latter combination is recommended for use in fluorescent antibody staining techniques, although with minimal fluorescence the 2 mm BG 12 or UG 1 alone may be preferable.

Other filters *(Figure 32.3)* alone, or in combination, may be used for specific wavelengths.

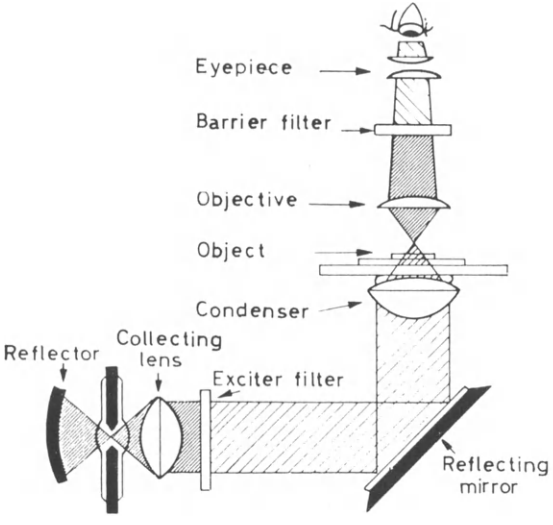


Figure 32.4 – Diagram to illustrate the component parts of the fluorescent microscope. The lamp gives out mixed ultraviolet and visible light (darkened area); the visible light is filtered out by the exciter filter. The object gives rise to visible light which is mixed with ultraviolet light (darkened area); the ultraviolet light is filtered out by the barrier filter, so that only visible light reaches the observer's eye

Barrier Filters

These may be used to specifically absorb light below a given wavelength *(Figure 32.4)*. Zeiss filters are now numbered 41, 44, 47, 50, 53, to designate the wavelength at which they transmit light, for example,

41 will transmit light with a wavelength of 410 mμ and above. The filter – 65 is used to absorb unwanted red light (above 650 mμ) which is transmitted by several of the exciter filters.

Fluorescent staining techniques. – The most useful filters are the yellow Schott OG 4 and orange OG 5 (Zeiss 47 and 50) used alone or in combination.

Fluorescent antibody techniques. – A pale green–yellow GG 9, Zeiss 44, or Euphos filter is used.

Autofluorescence. – A colourless ultraviolet stopping filter is used simply as a barrier filter (Schott GG4, Zeiss 41).

Any make of filter may be used; they are ordered by specifying the transmission wavelength required.

Microscope

Any good microscope may be used for fluorescence microscopy. It is often convenient to purchase the lamp (HBO 200) from the manufacturer of the microscope being used since there will probably be convenient points of attachment to set the lamp in the correct position (distance).

Mirror. – This should be of a front surface reflecting type (polished metal) to avoid loss of ultraviolet by double surface reflection (for example, glass face and mirror face) and to avoid the possible absorption of ultraviolet, by the glass. However, it will be found that a large number of normal microscope mirrors will give satisfactory results.

Condenser. – A *light type condenser* may be used, particularly with low-power objectives; a *dark ground condenser,* however, is almost mandatory for oil immersion objectives, since it gives a darker background and allows a thinner exciter filter to be used. The disadvantage of a dark-ground condenser is that oil must be used between condenser and slide (*see* page 599) but this is found to be far outweighed by the advantages. In practice the author uses a dark ground condenser as a routine.

Contrast-fluorescence condenser. – This combined fluorescence–phase condenser is available from Reichert, and is intended for use on their Binolux microscope. The specially designed condenser annulus

(the whole of which passes ultraviolet light) permits examination by phase contrast, fluorescence, or a mixture of the two. It is most useful as a means of identifying the source and location of fluorescence in smears and sections.

Barrier filter attachment. – Barrier filters may be as follows.

(1) Inserted into the eyepiece by removal of the top lens, or they may be screwed into the bottom of the eyepiece.

(2) Inserted in the body tube by means of specially fitted slides (carrying one, or a number of filters), or by placing a single filter in a convenient location.

(3) Incorporated in a rotary filter changer (such as that supplied by Zeiss) which is fitted below the binocular attachment.

If the microscope is used for a variety of purposes type (3) will be found the most convenient, since filters are easily and quickly changed. Since two rotating discs each carry 3 filters and one blank space, one can use a variety of filters, either alone or in combination.

Objectives. – Any non-fluorescent objective may be used. Achromats are generally preferred to apochromats as they rarely fluoresce and their colour correction is usually adequate. A high numerical aperture (N.A.) is preferred to ensure the maximum transmission of fluorescent light from the object. The oil immersion objective should be fitted with an iris diaphragm (or funnel stop) when using a dark ground condenser.

Microscopic Preparations

Microscope slides. – These should be thin and of even thickness (not of green glass). Special ultraviolet transmitting slides may be purchased, but unless a quartz condenser is used it is pointless to employ them. Optical glass (as used in condensers) will only transmit light of 300 mμ and over, and at this range thin glass slides have an adequate transmission.

Section adhesives. – Thinly applied routine section adhesives do not interfere with preparations.

Mountants. – Cleared preparations may be mounted in H.S.R. (Harleco synthetic resin) or Depex. Fluormount will probably give the best results.

Aqueous mounts. – These may be mounted in Apathy's media (*see* page 180) with the exception of acridine orange or fluorescent antibody stained preparations.

Fluorescent antibody preparations. – These are mounted in glycerin to which 10 per cent phosphate buffered saline (pH 7.1) (*see* page 629) has been added.

Acridine orange stained preparations. – These are mounted in buffer only.

AUTOFLUORESCENCE (PRIMARY FLUORESCENCE)

The ability of some naturally occurring compounds to fluoresce is on occasion a great advantage in identification. Autofluorescent material can present a great hazard to the inexperienced microscopist, because, dependent on its structure, it may fluoresce any colour and thus appear to have been stained by the technique employed. For this reason unstained smears, identically prepared in all other respects, should always be used as controls of fluorescent stains.

Preparation of Material

For the specific study of autofluorescence, unfixed smears or cryostat cut sections of unfixed tissue should be used. It may be found subsequently that fixation does not interfere with the specific fluorescence. 95 per cent alcohol (ethyl) or ether–alcohol are usually satisfactory. Formalin should generally be avoided if possible as it tends to increase the blue autofluorescence of tissue, however with 5HT *(see below)* it is essential.

Specific Autofluorescence

The number of naturally occurring autofluorescent compounds is enormous and for a more complete list of them the reader is referred to *Fluorchemistry* by De Ment (1945) (Chemical Publishing Co.), or *Fluorescent Analysis in U.V. Light* by Radley and Grant (1951) (Chapman and Hall). Those dealt with below are considered the most likely to be encountered.

Tissue. Generally fluoresces a bright blue, although this may be absorbed by use of a yellow or orange filter.

Elastic fibres. – Fluoresces an intensely brilliant blue while unstained, and may be easily seen even in an H. and E. stained section.

Ceroid and riboflavine, – These fluoresce in shades of yellow.

Lipids and lipochromes. – Many of these fluoresce in shades of yellow.

Vitamins. – Many vitamins are fluorescent in shades of yellow, green and blue.

Porphyrin. – This group (and chlorophyll) are among the very few compounds with an intense red fluorescence (Hellstrom, 1934). This characteristic has been made use of by adding a drop of concentrated H_2SO_4 to blood stains (or suspected stains), the H_2SO_4 takes the iron out of the haemoglobin forming haematoporphyrin which gives a brilliant red fluorescence. There is a small accessory lacrymal gland (Harderian) in the corner of the eye of some animals which, having a high porphyrin content, gives this characteristic fluorescence.

Nissl substance. – Fluoresces a bright yellow colour in formalin-fixed unstained sections.

5-Hydroxytryptamine. – Gives golden yellow fluorescence (in argentaffin or enterochromaffin cells) after formalin treatment (*see* page 468).

Drugs. – Certain drugs give a characteristic fluorescence. The ability of *tetracyclines* (terramycin) to form bright yellow fluorescent foci in malignant tumours has been investigated (Vassar, Saunders and Culling, 1960). The author has also used this antibiotic (since it is bound by calcium) to show areas of new bone formation in tetracycline fed animals.

Hydrocarbons. – The carcinogenic compounds, in particular, have been found to be strongly fluorescent. Vassar, Culling and Saunders (1960) utilized this method to demonstrate their presence in histiocytes in sputum from heavy smokers. 3:4 Benzpyrene has been used by Berg (1951) to demonstrate even the finest lipid granules (*see* page 614).

FLUORESCENT STAINING TECHNIQUES

The number of fluorescent staining techniques which are applicable in a routine laboratory are still moderately few. Those methods described

below have been tried in the author's laboratory and were found to be reasonably reliable. A great number of fluorescent techniques recently described have great promise for the future, particularly those in enzyme histochemistry which will enable specific demonstration of minute areas of activity. These more recent techniques have yet to be evaluated for routine work and are not therefore included.

Fluorescent antibody techniques are dealt with on page 620.

Fluorescent Stain for Amyloid

This stain, which was developed following research on twenty-six fluorescent dyes under varying conditions (Vassar and Culling, 1959), has proved to be the most specific of those stains currently available. It has the additional advantage of not requiring microscopical differentiation. It has on many occasions demonstrated amyloid when all other methods have failed; in at least three of these cases the patients have subsequently died and the diagnosis was confirmed. Because of the sensitivity of fluorescence technique even the finest deposits of amyloid are seen. The only other tissue components that stain are mast cell granules and myeloma casts in the kidney (Vassar and Culling, 1962).

In practice it will be found that a typical amyloid structure is easily recognized (*see Figure 14.2,* page 310). The technique is simple, the reagents stable and the method absolutely reliable. The stained slides may be examined using the BG 12 exciter filter with an OG 4 and/or OG 5 barrier filters which give a bright yellow on a black background; but the use of a UG 1 or UG 2 exciter filter with a colourless U.V. filter gives a brighter yellow on a blue background and will show the finest amyloid deposits (for example, heart). See discussion on specificity on page 308.

Fixation is not critical. We use formalin-fixed paraffin-embedded material. Frozen or cryostat sections may be used.

Method

(1) Bring sections to water.
(2) Stain in alum haematoxylin for 2 minutes, to quench nuclear fluorescence. The haematoxylin does not need to be differentiated, or blued.
(3) Wash in water for a few minutes.
(4) Stain in 1 per cent aqueous thioflavine T for 3 minutes.
(5) Rinse in water.
(6) Differentiate in 1 per cent acetic acid for 20 minutes.
(7) Wash in water.
(8) Mount in Apathy's medium.

Results (*see above* for details of filters employed)

Amyloid	Bright yellow
Mast cell granules	Yellow

Acridine Orange Staining Techniques

The original method for differentiation of RNA and DNA is described on page 492. In this connection it should be remembered that proof of RNA and DNA structures must be demonstrated by the use of enzymes. Acridine orange has been shown to demonstrate mucopolysaccharides although in the author's experience this is rare except with the technique given below.

Hicks and Matthaei (1958) discovered that a section previously stained by iron haematoxylin would, if subsequently stained with acridine orange, demonstrate mucins with some degree of specificity. The author made a similar observation working independently.

Method for Mucin (After Hicks and Matthaei, 1958)

(1) Fixation is not critical.
(2) Bring sections to water.
(3) Treat with 5 per cent iron alum for 10 minutes.
(4) Wash in water.
(5) Stain in 0.1 per cent aqueous acridine orange for 2–3 minutes.
(6) Wash in water.
(7) Mount in buffered glycerin (9 parts glycerin, 1 part 19/15 pG 6 phosphate buffer).

Results

Mucin fluoresces orange red, most other tissue components give dull background fluorescence.

Examine slides using BG 12 exciter filter, and OG 4 and/or OG 5 barrier filters.

Method for Fungi (Chick, 1961)

The use of iron alum as a mordant for acridine orange has been utilized in this method, which is similar to that for mucin. The fluorescent colour of the various fungi has not aided in identification; it

is recommended as a simple method for the morphological identification of fungi. A variety of fungi were demonstrated by the author: *Aspergilli, Blastomyces, Coccidioides, Cryptococcus neoformans, Histoplasma capsulatum, Rhinosporidium seeberi,* in addition to actinomycotic and maduromycotic granules. A mixture of potassium hydroxide and acridine orange is recommended for direct examination of skin scrapings and hair for fungi.

Method

(1) Fixation is not critical.
(2) Stain in Weigerts iron haematoxylin for 5 minutes.
(3) Wash in tap-water for 3 minutes.
(4) Stain in 0.1 per cent acridine orange for 2 minutes.
(5) Wash in tap-water for 30 seconds.
(6) Dehydrate, clear and mount in Gurr's Xam or Fluormount.

Results

Fungi and mucin fluoresce green–red.

Examine using a BG 12 exciter filter and a yellow (OG 4) and/or orange (OG 5) barrier filter.

Fluorescent Methods for Lipids

Fluorescent methods for lipids are much more sensitive than conventional methods, and therefore require some experience in interpretation. Popper (1944) described a method using phosphine 3R which, while not as sensitive as the 3:4 benzpyrene method, has some degree of permanency. The benzpyrene method is recommended for the demonstration of the finest lipid granules, but it must be remembered that the fluorescence fades rapidly.

Phosphine 3 R Method (Popper, 1944)

(1) Formalin fixation is preferred.
(2) Cut frozen or cryostat sections.
(3) Wash sections or smears in distilled water.
(4) Stain in 0.1 per cent aqueous phosphine 3 R for 3 minutes.
(5) Rinse quickly in water.
(6) Mount in 90 per cent glycerin.

Results

All lipids, with the exception of fatty acids, soaps and cholesterol give a silvery-white fluorescence. See benzpyrene method for recommended filter system.

3:4 Benzpyrene Method (Berg, 1951)

Staining solution. – Prepare a saturated aqueous solution of caffeine (about 1.5 per cent) at room temperature and leave overnight. Filter, and add 0.002 g 3:4 benzpyrene to 100 ml of filtrate. Incubate at 37°C for 2 days, filter and add an equal volume of distilled water.

Method

(1) Formalin fixation is preferred.
(2) Cut frozen or cryostat sections.
(3) Rinse sections or smears in distilled water.
(4) Filter staining solution on to smears or sections and leave for 20 minutes.
(5) Rinse in distilled water.
(6) Mount in distilled water and examine.

Results

Lipids, even the finest granules, give a brilliant blue–white fluorescence which fades rapidly.

Examine using a UG 1 or UG 2 (BG 12 if not available) exciter filter and a colourless U.V. barrier filter.

Fluorescent Stains for Mucin

The best fluorescent method for the demonstration of mucin is probably the fluorescent P.A.S. technique *(see below);* the acridine orange method (page 612) tends to fade on repeated examination. Vassar and Culling (1959) used 1 per cent aqueous atebrine in pH 3.95 sodium acetate–hydrochloric acid buffer for 10 minutes, followed by a brief rinse in water. This causes mucin to fluoresce a bright yellow colour, with other tissue components a pale green.

Fluorescent Feulgen Reaction (Culling and Vassar, 1961)

This technique utilizes a fluorescent Schiff reagent. It is simple in operation and, because of the intense brilliance of the fluorescence against a dark background, gives results superior to the conventional Feulgen reaction (*see Figure 13.2* page 251).

Control sections, treated with deoxyribonuclease (DNAse) to remove DNA, or bisulphite to block aldehyde groups, failed to stain by this technique which may be accepted as proof of specificity.

In addition to its use for the demonstration of DNA and nuclear patterns (including chromosomes), it has been utilized for the demonstration of the LE cell phenomena (Wignall, Culling and Vassar, 1962). The altered DNA of the inclusion body. which is thought to consist of

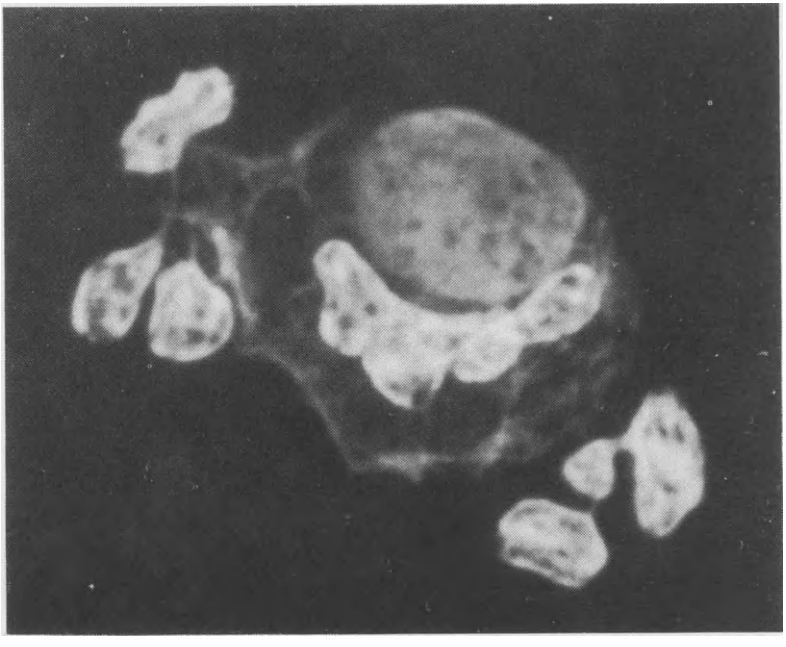


Figure 32.5 – Fluorescent Feulgen staining of L.E. cell, showing the lighter staining of the phagocytosed DNA in the cytoplasm

DNA and histone in salt linkage, fluoresces a lighter yellow than nuclear DNA and is thus easily seen even with high dry objectives *(Figure 32.5)*. Cytomegalic inclusions may also be seen.

Fixation

Carnoy or formalin fixed paraffin sections give excellent results, as do methyl alcohol fixed smears; other fixatives may require different times of hydrolysis as for conventional Feulgen reaction (*see* page 249).

Special Reagents Required

Fluorescent Schiff reagent

Acriflavine hydrochloride	1 g
Potassium metabisulphite	2 g
Distilled water	200 ml
N/1 hydrochloric acid	20 ml

Dissolve the acriflavine and metabisulphite in the distilled water, then add the hydrochloric acid. This should be kept overnight before use. This reagent is reasonably stable.

Method

(1) Bring sections to water.
(2) Treat sections (or smears) in preheated N/1 hydrochloric acid at 60°C for 10 minutes (depending on fixation, *see above*).
(3) Wash briefly in distilled water.
(4) Transfer to fluorescent Schiff reagent for 20 minutes.
(5) Wash in acid–alcohol (1 per cent HCl in 95 per cent alcohol) and leave for 5 minutes; this removes unreacted Schiff reagent and takes the place of sulphite rinses in the conventional method.
(6) Transfer to fresh acid alcohol for a further 10 minutes.
(7) Wash in absolute alcohol, a few changes to remove traces of acid.
(8) Clear in xylol and mount in H.S.R. or D.P.X.

Results

DNA	Fluoresces a bright golden yellow
Other tissue components	Green

Examine using BG 12 exciter filter and yellow (OG 4) and/or orange (OG 5) barrier filters.

Fluorescent P.A.S. (Culling and Vassar, 1961)

The fluorescent P.A.S. reaction has the advantage of demonstrating minute quantities of reactive material. It demonstrates basement membranes, mucin and fungi *(Figure 32.6)* exceptionally well; it has a high degree of specificity and may be controlled in the same manner as the

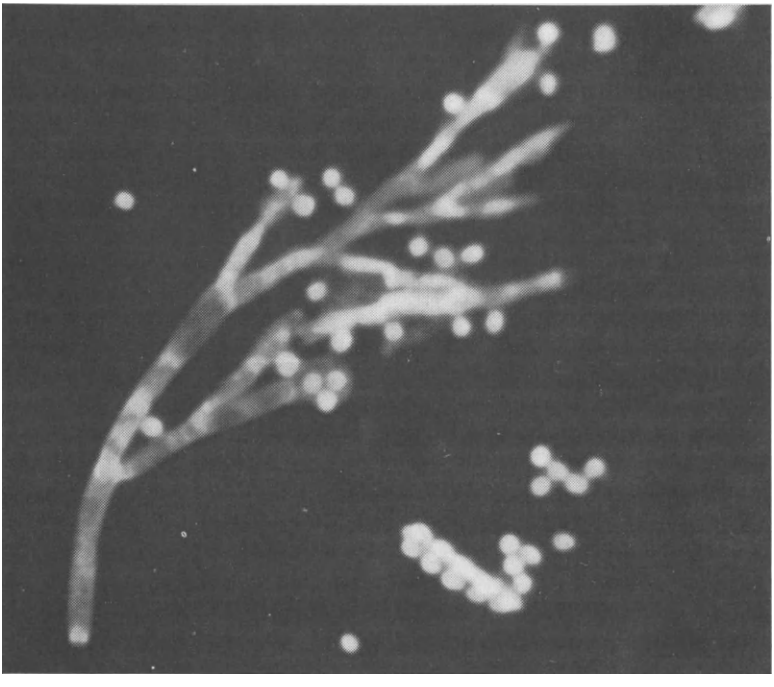


Figure 32.6 – Culture of fungi from lung, stained by the fluorescent P.A.S. technique

conventional method (*see* page 289). Because of the degree of specificity and the brilliance of the fluorescence, some experience of the method is required in interpreting results, as compared with the conventional technique.

Fixation

As for P.A.S. technique (*see* page 266).

Method

(1) Bring sections to water.

(2) Treat with 1 per cent aqueous periodic acid for 10 minutes.

Steps 3–8 and method of examination are as for the fluorescent Feulgen technique *(see above).*

Results

P.A.S. positive structures	Fluoresce bright golden yellow
Other tissue components	Green

Demonstration of Acid-fast Bacilli in Sections and Smears

Fluorescence microscopy for the detection of acid-fast bacilli has been used widely for years. It has probably not become universally used due to the lack of, or inadequacy of, fluorescence equipment. However, with the equipment now available the fluorescence method is reliable, sensitive, and permits very rapid screening of sections and smears. By using the method in duplicate with Ziehl–Neelsen technique, organisms have on several occasions been found reasonably quickly, which could only be found on repeated examinations by the conventional method. Wellman and Teng (1962) found that positive cases are three times as likely to be overlooked by the Z.N. method as they are by the fluorescence method.

Fixation

This does not appear to be critical. Formalin or Zenker fixatives give good results.

Special Reagents Required

Staining solution

Auramine O	1.5 g
Rhodamine B	0.75 g
Glycerol	75 ml
Phenol cryst, (liquified at 50°C)	10 ml
Distilled water	50 ml

Method (Kuper and May, 1960)

(1) Bring sections or smears to water* (use thin, scratch-free slices).

(2) Stain with filtered auramine-rhodamine at 60°C for 10 minutes.

(3) Wash in tap-water for 2 minutes.

(4) Differentiate in 0.5 per cent aqueous HCl in 70 per cent alcohol for 2 minutes. Use 0.5 per cent aqueous HCl for *Myco. leprae.*

(5) Wash in tap-water for 2 minutes.

(6) Differentiate in 0.5 per cent potassium permanganate for 2 minutes. This step quenches background fluorescence.

(7) Wash in tap-water for 2 minutes, blot dry.

(8) Dehydrate, clear and mount in Fluormount†.

Examine using a high dry objective, with a UG 1 or 2 exciter filter, and a colourless U.V. barrier filter.

Fluorescent Method for Calcium, Aluminium and Beryllium
(*see* page 472).

Fluorescent Technique for Alkaline Phosphatase

This method, described by Burstone (1960) as one of several new techniques for this enzyme, is one of the first applications of fluorescence to enzyme histochemistry. The enzyme releases a fluorescent naphthol compound from the substrate in a non-coupling reaction (*see* page 318).

This approach has great significance since either the use of a substrate that gives a fluorescent reaction product, or the post-coupling of the reaction product with a fluorchrome would allow, because of the increased sensitivity of fluorescence, visualization of very low levels of activity.

Special Reagents Required

Substrate. – Approximately 5 mg 5,6,7,8,-β-tetralol carboxylic acid-β-naphthylamine phosphate and 0.5 ml *N,N*-dimethylformamide (DMF) substrate are placed in a 50 ml flask.

*By using 30 per cent vegetable oil in xylol to remove paraffin wax from sections bacilli are more deeply stained. This step is essential when staining for *Myco. leprae.*

†This step is omitted when staining for *Myco. leprae.*

Twenty-five ml of distilled water is added, followed by 25 ml 'tris' buffer pH 8.7 (24.2 g tris (hydroxy methyl) aminomethane, 16.5 ml N/1 Hcl and distilled water to make 1 litre). Two drops of 10 per cent magnesium chloride are then added, the solution is shaken several times and then filtered. The solution should be clear or slightly opalescent.

Method

(1) Bring frozen dried, cryostat cut, or acetone-fixed paraffin embedded sections to distilled water.

(2) Incubate in substrate in a Coplin jar at 60°C for 15 minutes, then remove Coplin jar to bench for remainder of incubation at room temperature. Incubation period may vary from 1 to 3 hours.

(3) Wash slides in two changes of 50 per cent alcohol, then in running tap-water.

(4) Mount in 90 per cent glycerin.

Examine using BG 12 or UG 1 or 2 exciter filter and colourless U.V. barrier filter.

Results

Sites of enzyme activity . . . . . Brilliant bluish-white fluorescence

FLUORESCENT ANTIBODY TECHNIQUES

Creech and Jones (1940) showed that various proteins could be labelled with a fluorescent dye without material effect on their biological or immunological properties. The resultant fluorescent complex gave an intense blue fluorescence, but was difficult to distinguish from the blue autofluorescence of tissue.

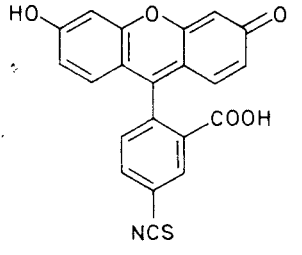
In 1941, Coons, Creech and Jones described the preparation of fluorescein isocyanate (FIC) which imparted an apple-green fluorescence to the tagged antibody which could be readily distinguished from tissue autofluorescence. Although this originally had to be prepared in the laboratory, it is now available commercially. In the past few years it has been replaced by fluorescein isothiocyanate (FITC) which is cheaper, more stable, and gives a more intense fluorescence.

Another fluorescent label for protein now available is 1-dimethylaminonaphthalene-5-sulphonic acid (DANS). This also gives an apple-green fluorescence and may be used in place of FITC. It is available

commercially in a solid form*, and is cheaper than FITC. The author has had no experience with this compound, although Nairn (1962) reports that it is possible to introduce more fluorescent groups per molecule of protein with DANS (10) than with FITC (7). Most of the reported work has been done with FITC, which gives consistent results. Lissamine Rhodamine B (RB200) has also been widely used as an orange fluorescent label. It may be used in double tracing experiments or, after conjugating with albumen, as a non-specific counterstain.

Methods of Use

Antibodies are produced by a series of injections of antigen (with or without an adjuvant) into an appropriate animal. Alternatively serum from a patient or animal with naturally occurring antibodies may be used; the gamma globulin fraction is usually then precipitated, to which the fluorescent dye is coupled (conjugated). This conjugate may then be used to detect and locate antigen (or antibody) by one of the following methods.



Fluorescein isothiocyanate

Direct Staining

Here the fluorescent antibody is used to directly locate an antigen. The section or smear is flooded with the conjugate, left to react, then washed with buffered saline to remove unreacted antibody. The presence of apple-green fluorescence will indicate the location of antigen. *Figure 32.7* illustrates this method.

Sandwich Technique (Weller and Coons, 1954)

By this technique, antibody sites may be visualized. The smear or section is first treated with unlabelled antigen which will attach itself to

*Fluka, Buchs, Switzerland.

antibody. After an appropriate time this is washed off and replaced by a fluorescent tagged antibody. The tagged antibody will react (or bind) with the antigen which has attached itself in the first stage to the antibody in the original specimen, thus making a sandwich. The presence and location of positive fluorescence now indicates antibody sites

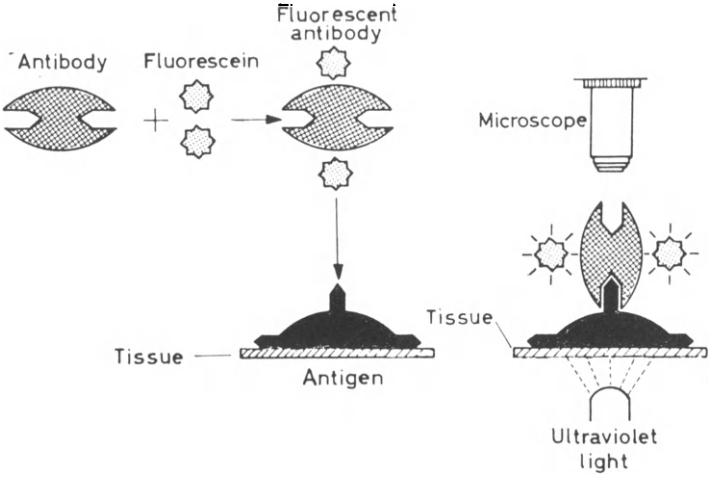


Figure 32.7 – Direct staining. Attachment of fluorescent dye to an antibody, and its subsequent attachment to an antigen in the specimen

in the original specimen *(Figure 32.8).* Sandwich staining is more sensitive than simple staining. Coons (1956) has assessed the increase in sensitivity as tenfold.

Multiple Layer Technique

This is an extension of the sandwich technique. This technique for antigen (or antibody) can be made more sensitive by successive layering of antibodies; for example, if mouse antibody (globulin) is to be detected, the specimen is:

(1) Treated with unlabelled rabbit-anti-mouse gamma globulin. This will combine with any mouse globulin that may be present. Uncombined antisera is washed off.

(2) Treated with unlabelled goat-anti-rabbit sera which will combine with the rabbit globulin used in Step 1.

(3) Theoretically one can now use alternately rabbit anti-goat, and goat anti-rabbit and build a bigger and bigger aggregate around each molecule of the original mouse antibody. It is generally assumed that there are several reactive sites on each molecule of antibody. Therefore one molecule of antibody (x) will bind x number of rabbit anti-mouse molecules (stage 1) and there will be x^2 after Stage 2, and x^3 after Stage 3, and so on. The last antibody used will, of course, be labelled.

It will be found that in practice there are a great number of technical problems involved in such a procedure, not the least of which is removing unreacted antibody.

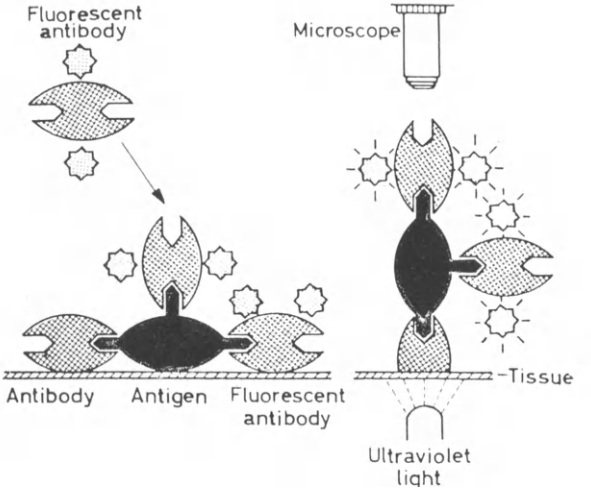


Figure 32.8 – Sandwich technique. Following attachment of an unlabelled antigen to an antibody in the specimen, a fluorescent labelled antibody is attached to the antigen

In Vivo Tracing

Pressman, Yagi and Hiramoto (1958) studied the *in vivo* localization of anti-tissue antibodies by this fluorescent antibody technique. He concluded that the *in vivo* technique was at least 4–12 times less sensitive. *Figure 32.9* shows a section of kidney from a rat injected with

rabbit (anti-rat glomerular basement membrane) sera which has been stained with labelled goat anti-rabbit gamma globulin.

Application

The fluorescent antibody technique has been used for the detection and localization of bacterial, viral, protozoal, fungal, helminthic, and animal and human tissue antigens. It has also been used, as described

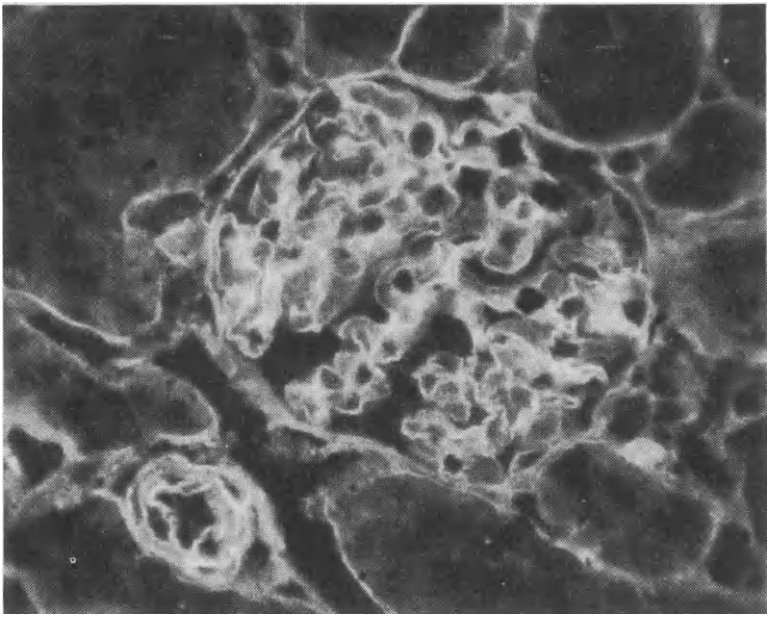


Figure 32.9 – Direct staining. Rat kidney stained with anti-basement membrane conjugate. The basement membranes fluoresce bright apple-green

above, for the detection and localization of antibody (Coons, Leduc and Connolly, 1955). By absorption and cross absorption techniques, it has the specificity of immunological techniques and has a sensitivity and precision that enables identification and localization at a microscopical level. In the diagnostic laboratory it is becoming increasingly widely used in the bacteriological field. The field of auto-immunity, in particular, has received great stimulus by this technique since fluorescent labelled patient's serum can be seen to react with various

components of the patient's own tissue. The demonstration of antinuclear antibodies in the sera of patients with various collagen diseases (Fennell, Rodnam and Vazquez, 1962); the presence of gamma globulin concentrations in the lesions of various diseases (Vazquez and Dixon, 1957); the demonstration of rheumatoid factor by Mellors and colleagues (1961), and Taylor and Shepherd (1960); and the formation of antibodies to thyroglobulin in immune thyroiditis by White (1957) are but a few examples of its use.

Non-specific Staining

Staining of a specimen which is not due to a specific reaction between a particular antigen and the labelled specific antibody is referred to as non-specific staining. In spite of many improvements in technique this still presents a serious problem, particularly when dealing with tissue antigens and antibodies.

Unreacted Fluorescent Material (UFM)

The original method for the removal of UFM (unattached FITC) from conjugated sera, was by simple dialysis; this was never completely satisfactory. This problem has now been overcome by the use of gel filtration with Sephadex, or by extraction with activated charcoal.

Conjugated Normal Serum Proteins

These are proteins, other than antibody proteins, which have been labelled during the conjugation process. Because of their non-specificity they attach themselves arbitrarily to protein in the specimen, giving the appearance of a specific reaction. Coons and Kaplan (1950) used dried tissue powders to selectively remove them. The powder is preferably prepared from the same type as the tissue to be stained. The absorbing powder should, of course, not contain the specific antigen to be detected; if the antigen is present in the powder to any significant degree it will remove the conjugated antibody. Fractionation of the serum and conjugation of only the gamma globulin is a valuable practical method of reducing this type of non-specific staining; however, this conjugate should still be absorbed with tissue powder.

Conjugated Unwanted Antibodies

These are antibodies, present in the serum to be conjugated, which have been produced either as a result of natural infection, by impurities in the immunizing antigen, or are against organisms or tissue components which share a common antigen with the material to be investigated (Coons, 1951). Such antibodies must be removed. The sera are absorbed with appropriate antigens, for example, similar tissue or strain of organism which does not contain the specific antigen being traced.

Conjugation

This is the process of coupling the fluorochrome to protein. The antisera (or protein antigen) may be as follows.

(1) Conjugated as whole sera.

(2) Fractionated after conjugation (as 1) and only the gamma globulin fraction used.

(3) Fractionated to obtain gamma globulin fraction, and only the globulin fraction conjugated.

Method (1) is not recommended for antisera since it will contain a large amount of conjugated serum proteins which will increase the amount of non-specific staining. Methods (2) and (3) are those in general use; the author uses the latter since this economizes in the amount of FITC used.

Fractionation of Sera

Antibody present in sera is concentrated by precipitating the gamma globulin present. To each ml of sera is added 1 ml of saline and 1.33 ml of cold saturated ammonium sulphate at 4–6°C. After centrifugation, the precipitate is washed once with 50 per cent saturated ammonium sulphate and dissolved in buffered saline. The globulin suspension is then dialysed overnight against buffered saline. The protein concentration is determined by the biuret reaction, and the concentration adjusted to 50–60 mg/ml. It should be not less than 25 mg/ml (Goldstein and colleagues, 1961). This solution can then be stored in a deep freeze.

Conjugation

To one volume of globulin add two volumes of cold 0.5 M carbonate–bicarbonate buffer pH 9.0*. This solution is stirred thoroughly at

**pH 9.0 buffer.* 3.7 g $NaHCO_3$ and 0.6 g Na_2CO_3 (anhyd.) are dissolved in distilled water, and made up to 100 ml.

a temperature of 0–2°C (in a cold room or ice bath) while 1 mg of FITC dissolved in the minimum amount of acetone is added for each 18–20 mg of protein. The acetone solution is slowly added over a period of 15 minutes. The stirring is continued overnight. Care should be taken to ensure that the stirring, while thorough, does not cause frothing.

Purification of Conjugates

Unreacted fluorescent material (UFM) was originally removed by dialysis against saline over a period of several days, but this method is not as effective as the charcoal or Sephadex methods.

Extraction with powdered activated charcoal, which has been washed well with saline and dried at 100°C, is much more effective. The charcoal, moistened with saline to avoid undue loss of protein, is added in the proportion of 2.5 mg/mg of protein in the sera. The mixture is shaken for one hour and the charcoal is then removed by centrifugation. The only disadvantage of this technique is the loss of protein (20–30 per cent) which may be important when conjugating small amounts of a weak antisera.

Gel filtration with a cross-linked dextran, Sephadex* depends on the diffusion of small molecules into the pores of the gel, the larger molecules being excluded because of their size. Separation takes place in a column, with the large protein molecules travelling more rapidly than the small molecules, which diffuse into the gel. Pore size G 25 or G 50 are the commonly used grades for this purpose. The manufacturers describe the method of preparation and use in detail. For volumes of conjugate up to 20 ml a column 20 cm in length and 3 cm in diameter, which has been washed with buffered saline, is adequate. The conjugate having been centrifuged, is allowed to soak into the column, and a suitable head of buffered saline applied. As the solution passes down the column, two bands separate, the faster one being the conjugate. The loss of protein by this method is very small. Dilution of the conjugate may be overcome by reprecipitation of the globulins.

Absorption by Tissue Powders

The removal of conjugated non-specific serum proteins *(vide supra)* is usually carried out by absorption with tissue powders, if possible with the same type as the tissue to be examined, provided that it does

*Pharmacia Ltd., Uppsala, Sweden.

not contain any appreciable amount of the specific antigen. The precipitation of the globulin fraction will remove these to a great degree, but more elaborate fractionation of sera by chromatography on modified cellulose (Goldstein and colleagues, 1961) reduces them to almost undetectable levels.

The tissue powders, usually liver or bone marrow (to inhibit non-specific staining of granulocytes) are prepared as follows.

Wash the organ free of blood with physiological saline, chop into small pieces with scissors or scalpels, and rewash with saline. Grind up the material in a low-speed homogenizer (or pestle and mortar) with acetone, and filter through coarse filter paper. Wash several times with acetone until completely dehydrated, and dry at 37°C. Grind to powder in a mortar, sieve through wire mesh to remove coarse material and store at room temperature. For absorption purposes approximately 100 mg of tissue powder is used for each ml of original serum. The mixture is shaken at room temperature for 1 hour, and centrifuged at about 10,000 g (preferably in a refrigerated centrifuge) for about 15 minutes. The high speed is essential to give maximum return of conjugate. The supernate is now ready for use. The conjugate should be divided in small aliquots and stored in a deep freeze, otherwise there will be a protein–dye breakdown which will necessitate re-absorption before use.

Prepared Conjugates

Commercially prepared conjugates are available which are ready for immediate use*. These give excellent results for many routine procedures.

Preparation of Material to be Stained

Cryostat Sections

These are by far the most commonly employed histological preparations. Unfixed tissue is quick frozen or quenched (*see* page 55) and sections cut in the cryostat. They may be used fresh (after air-drying) or fixed in cold 95 per cent alcohol either before or after air drying. Tissues which tend to detach during staining may need to be air-dried before fixing. The type of fixative used will depend on the antigen/antibody involved in the reaction, but cold 95 per cent alcohol has been employed successfully in a number of investigations.

*Burroughs Wellcome

Freeze-dried Sections

These have been used with success (Rey, 1960). Tissues after drying are embedded in polyester wax.

Paraffin Sections

Methods have been described using freeze-substitution techniques but they are still at an experimental stage.

Conventional paraffin sections have been used on a few occasions but are generally unsuitable.

Smears

Tissue and bacterial smears, touch preparations and tissue culture monolayers on coverslips have all been used with great success for fluorescent antibody staining techniques.

Smear preparations of tissue are best made by brushing the cut surface of the tissue with a camel hair brush which has been dipped into 7.5 per cent PVP (polyvinyl pyrollidone). Several strokes are then made on to a clean slide. One advantage of this technique is that the experimental and control smears can easily be made on the same slide.

Staining Technique

The method of mounting the stained slides recommended is that described by Culling (1967), using New Unimount. Alternate slides may, sometimes, with advantage be examined in buffered glycerin.

Reagents Required

Phosphate buffered saline (pH 7.1)

Sodium chloride	8.5 g
Disodium hydrogen phosphate (anhyd.) (Na_2HPO_4)	1.07 g
Sodium dihydrogen phosphate ($NaH_2PO_4 . 2H_2O$)	0.39 g
Distilled water	to 1 litre

Buffered glycerin mountant

Glycerin	9 ml
Phosphate buffered saline	1 ml

(1) Sections or smears may be rinsed with buffered saline. This facilitates spreading of the conjugate.

(2) Slides or coverslips are placed in a moist chamber, for example, Petri dish, with moist filter paper in the bottom. The preparation is covered with a drop (or two) of the conjugate which is applied with a platinum loop or Pasteur pipette. The chamber is kept at room temperature. The reaction time may vary (with the strength of antisera or type of antigen) from 10 minutes to 2 hours; from 15 to 30 minutes is usually adequate.

(3) The preparations are rinsed in several changes (not less than three) of buffered saline over a period of 10–15 minutes. This step is critical and should be carried out for a longer, rather than a

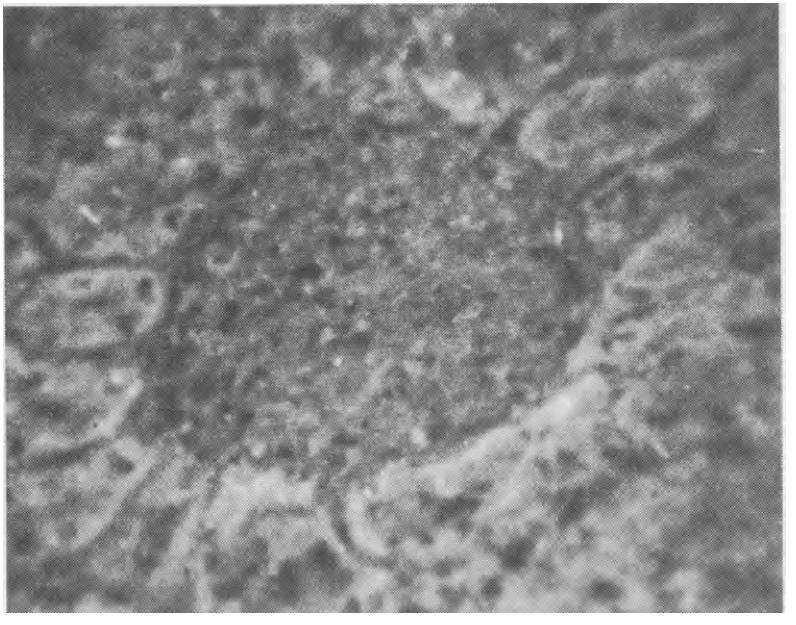


Figure 32.10 – Specific absorption. Rat kidney stained with anti-basement membrane conjugate that has been absorbed twice with antigen (basement membrane suspension, see Figure 32.9)

shorter time. Dr. Irene Batty of the Wellcome Research Laboratories uses a magnetic stirrer to agitate the buffer during washing, her stained preparations were quite the best I have seen and we now use and recommend this method. The slides to be washed are suspended

in an open-type slide holder in a beaker, over a magnetic stirrer; three changes are usually adequate.

(4) Excess buffered saline is wiped off and the specimens mounted in buffered glycerin. After examination they may be rinsed in distilled water and stored dry in the dark.

or

(4) Rinse briefly in distilled water and blot dry.

(5) Place in xylol until section is clear; it may be necessary occasionally to blot again before complete clearing is achieved.

(6) Mount in New Unimount*.

Results

Antigen–antibody reaction sites give an apple-green fluorescence (with FITC or DANS) or orange fluorescence with rhodamine B.

Background fluorescence will be blue (unless counterstained with rhodamine B) except for autofluorescent sites (*see* page 609). An unstained slide should be used to check autofluorescence.

Examine using a Schott BG 12 (alone or preferably with a Schott UG 1) exciter filter and one of the following barrier filters; Euphos, yellow green GG 9, Zeiss 44 or their equivalent.

Tests of Specificity

Tests of specificity are modelled on those used in established immunological techniques. Those most commonly employed are as follows.

Blocking

Staining should be inhibited by pre-treatment of the specimen with unconjugated antisera (at room temperature for 30 minutes) before staining. The unconjugated sera should bind all the reactive sites on the antigen, and block a reaction with the conjugated antibody. This is referred to as a *blocking test.* It will sometimes be found that only a reduction in intensity of staining can be obtained by this test.

The blocking test should also be performed with a non-specific (control) serum to prove that the blocking is due to the presence of the specific antibody.

*Obtainable from Sherwood Medical Industries, Inc. 1831 Olive St., St. Louis, Missouri, U.S.A. 63103

Absorption

Staining should be inhibited if the conjugate has been previously absorbed (usually twice) with the specific antigen (*specific absorption*), the conjugate being centrifuged after absorption to remove reacted material (if possible).

The staining should not be inhibited by absorption with a different antigen (*non-specific absorption*) using the same technique.

Absorption of the antisera with an antigen is simple when the antigen is pure, but it must be remembered that when an antigen is impure there is no guarantee that positive staining of the antibody may not be due to a non-specific antigen (impurity) blocking a non-specific antibody.

Control Conjugate

Occasionally it may be necessary to prove that staining is due to an induced antibody, and not a naturally occurring one. This is tested for by conjugation of a normal control serum from the same species of animal. There should be no staining of the antigen with this sera.

Treatment (as detailed above)	*Results if sera is specific*
(1) Conjugated antisera alone	Staining (+ + +)
(2) Unconjugated antisera, followed by conjugated antisera (blocking test)	No staining (—)
(3) Unconjugated control sera, followed by conjugated antisera (control blocking test)	Staining (+ + +)
(4) No treatment. Slide mounted unstained to control autofluorescence	No staining (—)

Routine Test and Control Technique

As a routine we set up four slides of each specimen to be examined. The treatment and expected results are shown above.

Chapter 33

The Polarizing Microscope

Theoretical Aspects

Although it is beyond the scope of this book to enter deeply into the theory of polarized light, the basic elements of the subject will be explained.

Light is assumed to be due to a wave motion, to the upward and downward vibration of ether particles. These do not move along in the direction of the light ray (*Figure 33.1,* A to B), but vibrate at right angles to it (*Figure 33.1,* C to E), and when the light ray ceases they return to their original position (*Figure 33.1,* D). Ether is supposedly an homogeneous medium and there is no reason therefore to believe that these particles will vibrate in any one direction more than another. To explain polarized light it is necessary to suppose that light normally vibrates in all planes; that is, in *Figure 33.2,* from C to E, F to G, H to I, and J to K. It is difficult to imagine a particle oscillating in all planes at one time, but it is possible to imagine that it moves – at right angles to the direction of the light ray – in all planes in such rapid succession so as to act as if it were moving in all these planes at one time.

While this theory is not strictly accurate, it is sufficiently correct to explain the behaviour of polarized light.

If a dot drawn on a sheet of white card is viewed through a block of glass laid on top, only one dot will be visible from above *(Figure 33.3a).* If the block of glass is replaced by a polished block of crystal, such as Iceland spar, two dots will be visible. Such a crystal is described as being bi-refringent or anisotropic; it has split each light ray from the dot into two rays which emerge from the crystal at different points *(Figure 33.3b).*

This splitting of light rays by certain crystals is due to their uneven optical density. It is known that light rays are retarded when travelling

through an optically dense medium such as glass, but since the molecules in glass are evenly spaced in all directions only a simple retardation or slowing takes place. The molecular structure of a crystal differs from glass in that although its molecules are regularly spaced they are closer together in one direction than in another; they are therefore unevenly dense. There are many types of crystalline structure, but all have the common property of being more dense in one direction than in another.

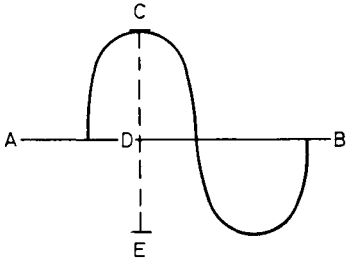


Figure 33.1 – Sine curve representing a wave of light
AB = *direction of travel*
CDE = *direction of vibration*

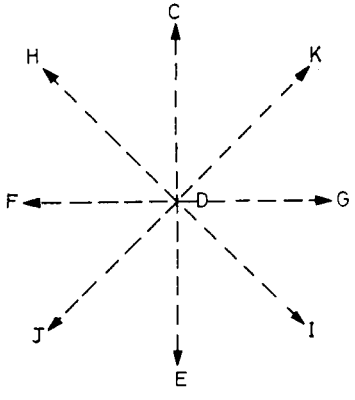


Figure 33.2 – Theoretical representation of the vibration of light in all planes

Figure 33.4 shows a downward view of a series of posts through which wind is blowing from directions W1 and W2. It will follow that from whatever angle the wind is blowing it can only leave in the direction of A or B. The intensity of the wind emerging at points A or B will depend on the angle at which it enters. If it enters from W1 then almost all the wind will emerge in direction B; if the wind enters from W2, an almost equal amount will emerge in each direction. If we now substitute the words

'crystalline structure' for 'posts driven into the ground', and 'light rays' for 'wind', an understanding may be gained of what happens when a light ray passes through a crystal: the ether particles are vibrating in all directions at right angles to the line of propagation when it enters, but two rays emerge, and each of these causes ether particles to vibrate in one plane only (A to C, or B to D in *Figure 33.4*, these two planes always being at right angles to each other).

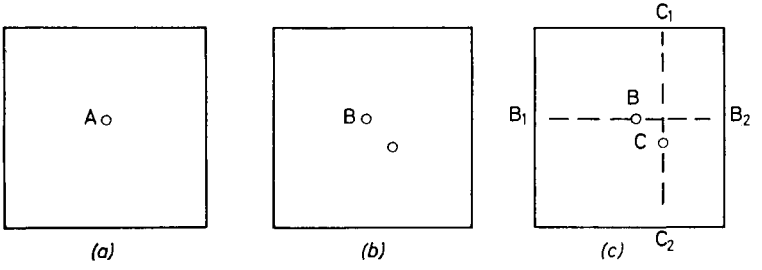


Figure 33.3 – Optical effect of (a) block of glass and (b and c) birefringent material when laid on a single dot

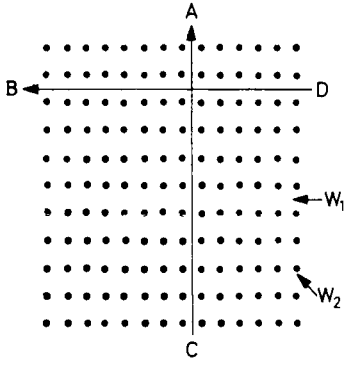


Figure 33.4 – Diagram illustrating the theoretical effect of birefringent material in splitting a beam of light (or wind) into two beams (CA, BD)

Light when entering a dense medium is retarded in speed. Further, being an unevenly dense medium, the crystal will retard the two rays to a differing degree, and since refraction is partly dependent on density, the two rays will be refracted or bent to differing degrees. This is known as double refraction or birefringence and explains the phenomena described above *(Figure 33.3)*. A ray of light entering such a crystal will be converted into two rays (*Figure 33.3*, B and C) which will emerge at different points, and the emergent light rays will be

polarized; that is, all the vibrations in one ray (B) will be in one single direction (B_1-B_2); in the other ray (C) in another single direction (C_1-C_2), and these directions will be at right angles to each other.

The Nicol Prism

Just over 100 years ago, Nicol devised a prism from which light rays, having passed through, would emerge vibrating in a single plane, that is, as polarized light. The single direction in which the light is vibrating when it emerges is known as the 'optical path' of the prism.

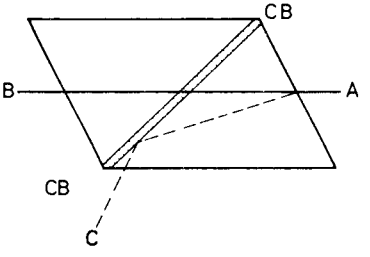


Figure 33.5 – The Nicol prism

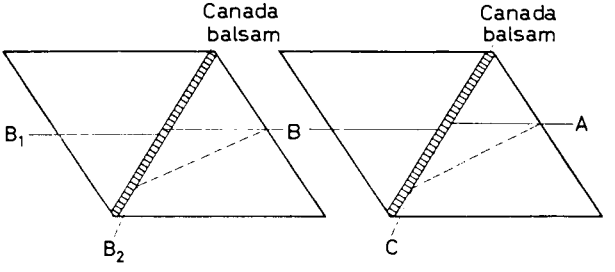


Figure 33.6 – The effects of two Nicol prisms, with optical paths aligned (ABB_1) *and optical paths crossed* (ABB_2)

The prism is composed of a crystal of Iceland spar, cut to the shape shown in *Figure 33.5,* slit in half and the halves cemented together with Canada balsam along the line CB–CB. On entering the prism, a light ray (A) is divided into two rays (B and C) which are refracted differently, ray C being refracted to one side. Owing the the difference in the refractive index between Canada balsam and the calcite spar crystal and the cement, ray C on meeting the surface CB–CB at a greater angle than ray B, is totally reflected out of the prism. Ray B passes through the

prism and emerges vibrating in the direction of the optical path of the prism only and is polarized light.

It will follow that if another Nicol prism is placed above the first one *(Figure 33.6)*, the polarized light ray B will pass through the upper prism if their optical paths are aligned (B_1) but if the upper prism is rotated through 90 degrees so that the optical paths of the prisms are crossed, then ray B will be totally reflected out of the upper prism (B_2). Such prisms are said to be crossed and it will be seen that no light will normally emerge from crossed Nicol prisms. If the upper prism is slowly rotated it will be seen that the amount of light passing through will vary with their relative positions. At a rotation of 45 degrees, from alignment of the prisms, approximately half the light will pass through the prism and so on. In practice, it will be found that with an intense light source some light will pass even through crossed Nicol prisms, but with light of moderate intensity the field will appear black.

Polaroid Discs

In 1935 'polaroids' – glass or celluloid covered discs with the ability to polarize light – were first made available for use in place of Nicol prisms. They act as a single crystal of herapathite which is not only birefringent, but has the ability to absorb the ordinary ray (which would be refracted out of Nicol prism (*Figure 33.5,* C)), only the extraordinary ray (*Figure 33.5,* B) being transmitted.

Polaroids are made by suspending ultra-microscopic crystals of herapathite in nitrocellulose. All the crystals in the suspension are orientated so that their optical paths are aligned. This suspension when mounted between two glass plates or celluloid sheets acts as a single crystal.

One glass plate is made to fit into the substage filter carrier, and the other has a metal mount to hold it in place on top of the eyepiece. The celluloid sheet may be cut with scissors and used in a similar manner. For all practical pruposes they may be used as Nicol prisms.

The Use of the Polarizing Microscope

It has been shown that certain crystals have the power to convert a single ray into two rays of light, which are vibrating in a single plane at right angles to each other *(Figure 33.3)*, and also have the power of quenching or absorbing one set of these rays.

If such a crystal is placed on the stage of a microscope having a Nicol prism (or polaroid) in the substage, the effect will be as shown in *Figure 33.7.* The light rays in the filed will be vibrating in the optical path

of the Nicol prism (AB), except those that pass through the crystal, which will be vibrating from C to D or E to F. If the set of rays CD were absorbed (as described above for herapathite), the crystal would have the effect of changing the plane of vibration from AB to EF. Therefore, a Nicol prism placed above the object, having its optical path from A to B, will absorb most of the rays which have passed the crystal, and it will appear dark on a light background. If the upper Nicol prism has its optical path in the direction EF, then the rays AB will be absorbed and

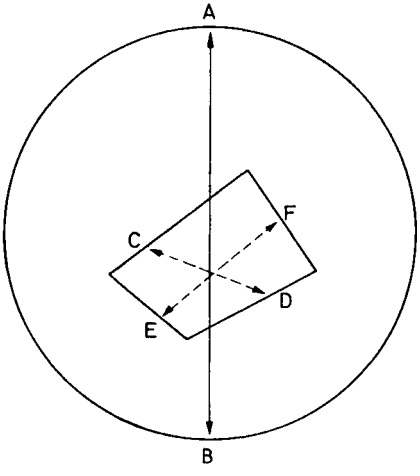


Figure 33.7 – Showing birefringent effect of a crystal

the crystal will appear light on a dark background. This latter method is the one normally used in histological laboratories, since the majority of the birefringent material to be examined consists of small particles or crystals which are easier to see as light objects on a dark background. It will be seen that such crystals have the apparent power of changing the plane of vibration (from AB to EF) and for this reason they have occasionally been referred to as 'optically active'.

The direction in which the plane of vibration is changed, and the degree to which it is turned, may be used to assist in the identification of a crystal. For this reason, petrological microscopes have the polarizing prism (in the substage) in a graduated circular mount which may be rotated through 360 degrees. In addition, the stage is usually graduated and may be rotated in the same manner, so that the object may also be orientated.

Crystals which change the plane of vibration clockwise are called 'dextra-rotary', and anti-clockwise 'laevo-rotary'.

In the histological laboratory, one is usually only concerned with simple birefringence. The microscope is set up, as described on page 589, polarized discs normally being used. When the material being investigated is in the field, the upper polaroid (analyser) is rotated. The material will appear light on a dark background if it is birefringent or anisotropic. Talc crystals, hair, myelin, silica and collagen fibres are among the many birefringent substances found in histological sections.

Types of Birefringence

Certain crystals or tissue structures show more than one index of refraction for a given wavelength of light and are therefore said to be doubly refracting or birefringent. As was shown above, this is due to some sort of asymetrical and orientated spatial arrangement of particles. These particles carry resonating charges capable of interacting with the oscillations of light waves. Birefringent material may show one or more than one type of such arrangement; the more common types may be characterized as follows.

Intrinsic or crystalline birefringence. – This refers to a type of anisotropy due to an asymetrical alignment of chemical bonds, ions or molecules. Many crystals display this type of birefringence, it is also common in biological objects such as collagen and muscle fibres, and chromosomes.

Intrinsic birefringence in a specimen is independent of the refractive index of the immersion medium which is probably due to the fact that the orientated elements are of close structure between which the medium does not penetrate.

Form birefringence. – This is found in mixed bodies, wherein asymetrical particles of one refractive index are dispersed in a specially oriented manner in a medium having a different refractive index. At least one dimension of the particles must be small in relation to the wavelength of light employed. These dispersed particles may be filaments, sheets, and so on, and they may be dispersed in a liquid, gas or solid; they can give rise to birefringence even if separately either or both are isotropic. Tests for form birefringence depend upon causing media of varying refractive index to penetrate between the particles when, at the appropriate R.I., form birefringence will disappear.

(Examining objects mounted in a variety of mountants with differing R.I., for example, water, glycerol, HSR, and so on.)

Strain birefringence. – When a dielectric substance is subjected to mechanical stress, the bonds within the substance can be distorted and give rise to a pattern which will result in birefringence. This is most simply demonstrated by twisting clear plastic (Perspex) between crossed polaroids when a birefringent spectrum of colour is produced. Similarly, glass or elastic tissue fibres under stress show birefringence.

Positive and negative birefringence. – An object that appears bright on a dark field, when viewed between crossed Nicol prisms (or polaroids), is said to be birefringent *(see above).* This does not allow the determination of direction of the fast and slow axes of the doubly refracting material; having two different R.I.s one light ray will be retarded (slow) in relation to some distinguishing dimension of the object.

In a collagen fibre the slow axis of transmission is parallel to the long axis of the fibre, the fibre is thus said to show positive birefringence with respect to its long axis; conversely, a chromosome shows negative birefringence with respect to its long axis.

To determine the sign of birefringence in a fibre or crystal (for example, to differentiate between the urate crystals of gout from calcium pyrophosphate dihydrate (CPPD) (*see* page 389) one needs, in addition to a pair of Nicol prisms (or polaroids), a first order red quarter-wave plate. Focus the object to be examined between crossed polaroids (or Nicol prisms) and insert the quarter-wave plate between the object and the analyser (upper prism) and rotate the plate until its slow axis of transmission (usually indicated by a stamped line or arrow) is parallel with the fibre or long axis of the crystals (E–F in *Figure 33.7*). The field will now be uniformly red(from its interference effect) and if the fibre or crystal appears blue then the slow axes of the plate and object are parallel and this is positive birefringence, if the object appears yellow it indicates negative birefringence (the slow axis of the plate is parallel with the fast axis of the object). In *Figure 33.7*, if the slow axis of the quarter-wave plate is parallel with E–F and the crystal appears blue on a red background this is positive birefringence, if it appears yellow on a red background then it displays negative birefringence (with respect to its long axis). Dichroism is also detected by the use of a polarizing microscope (*see* page 309).

Summary

An over-simplification of the theory of polarization, but one which illustrates its normal use in histology, is as follows *(Figure 33.8).*

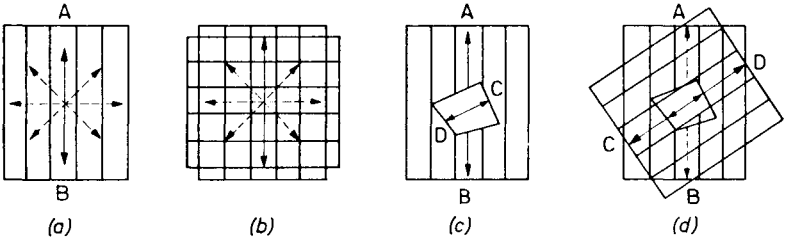


Figure 33.8 – Diagrammatic summary of the theory of polarization

The Nicol prisms (or polaroids) act as grilles, and allow only light vibrating in a single plane (*Figure 33.8a* AB) to pass through; by inserting a further prism above the first one (*(Figure 33.8b)* all light is stopped. If the light from such a prism passes through certain crystals its direction is rotated from AB to CD *(Figure 33.8c)* and a further Nicol prism placed above the crystal in the direction CD *(Figure 33.8d)* will stop all the light from the lower prism except that which has passed through the crystal and is vibrating in the optical path of the upper prism, so the crystal will be light on a black background.

Chapter 34

The Phase-Contrast Microscope

The introduction of phase-contract microscopy is probably the greatest single advance in biological laboratories in this century. For the first time, living organisms and cells may be examined in detail without previous treatment, and the image produced a true one.

All living cells and organisms, although appearing almost homogeneous when examined unstained by ordinary microscopic methods, are composed of minute structures having slight differences in refractive index. The phase-contrast microscope, by converting these slight differences in refractive index into changes of amplitude (or brightness), produces an image that may be accurately focused and one that is easily seen or photographed.

Professor Zernicke, who was awarded the Nobel prize for his work on phase contrast, first applied his original work on telescopes to the biological microscope in 1935, but it was not until 1945 that a commercial model was available in Great Britain, although Burch and Stocks described a method for the conversion of an ordinary microscope in 1942.

These microscopes are now being widely used, and there seems little doubt that they will in the future replace what we now know as the routine microscope, since they may be used either for phase-contrast microscopy or for routine purposes.

Principles of Phase-contrast Microscopy

Without going too deeply into the theory of phase contrast, the first part of this chapter is intended to explain the broad principles underlying its use. To understand these principles it is necessary to recall some of the properties of light rays.

Light, arising from a point source, may be represented by straight lines or, since it is propagated in waves, by *sine* curves. These curves are a useful method of representation since they can be made to show not only amplitude and wavelength but the retardation of one ray in relation to another. *Figure 34.1* shows the method by which amplitude and wavelength may be represented using the *sine* curve. Retardation of one light ray in relation to another is shown in *Figure 34.2* where the lower ray, having passed through a block of glass, is retarded by half a

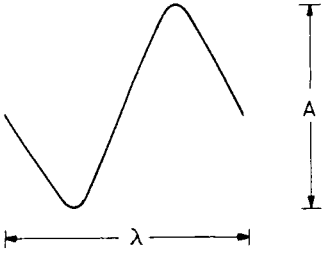


Figure 34.1 – Sine curve representing a ray of light

A Amplitude or brightness

λ Wavelength or colour

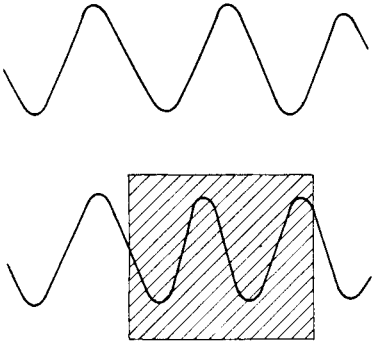


Figure 34.2 – Retardation of lower light ray by a block of glass

wavelength. It will be appreciated that the eye is sensitive to changes in amplitude (or brightness) and to changes in wavelength (which are changes in colour) but not to changes in phase, where one wave is retarded in relation to another.

One further property of light that must be considered is that of interference *(see Figure 34.3).* If two rays of light strike a screen at the same point, as in *Figure 34.3a,* the resultant light on the screen will be the sum of the amplitude of the two rays (*b* and *c*) as shown in the *sine* curve (*d*). If one of the two rays (arising from the same point source) had passed through a block of glass, of an exact thickness to retard that ray by half a wavelength, instead of getting an increase in light there would be no light. This is because coherent light rays have the property

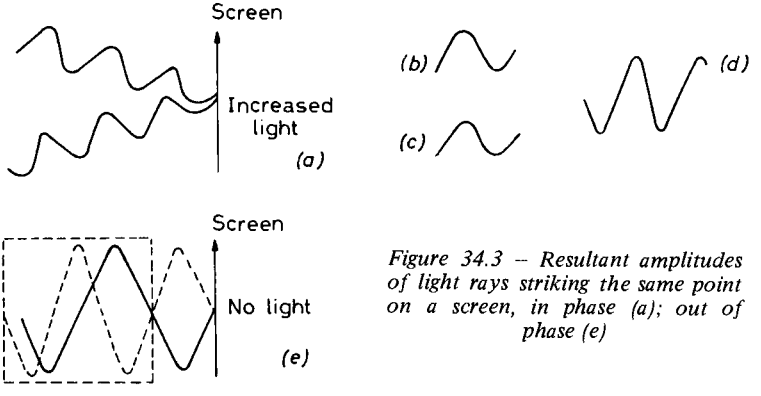


Figure 34.3 – Resultant amplitudes of light rays striking the same point on a screen, in phase (a); out of phase (e)

in interfering with each other. This interference may (as shown in *Figure 34.3e)* result in complete extinction of the light or in a reduction of it depending on the relative amplitudes of the light rays. If, for instance, in *Figure 34.3* the direct ray in (*e*) had the amplitude of (*d*) and the retarded ray the amplitude of (*b*) instead of extinction of the light, there would have been light of the amplitude of (*c*). For practical purposes it may be said that when coherent light rays interfere the amplitude of the resultant ray can be obtained by subtracting the amplitude of one from the other. Fractional phase differences (for example, ¾ or ¼ of a wavelength) between rays will result in partial interference and in this way an image of an unstained object may be built up.

By almost closing the iris diaphragm of the substage condenser of the normal microscope an image of this small aperture is formed at the back focal plane of the objective *(Figure 34.4b).* This image, which can be seen by removing the eyepiece, may be focused with an auxiliary eyepiece. If a phase grating is placed in the object plane (on the stage), instead of only one image being seen at the back focal plane of the objective three images are visible, the original bright one in the centre,

and a further two, one at each side, which are less bright *(Figure 34.4a)*. A phase grating consists of alternate strips of material of differing refractive index and the additional images have been formed by the light diffracted by the object. These images, known as diffraction images, may, by this method, be differentiated from the direct image. One other effect that has taken place, which is not apparent but can be proved, is that the diffraction images are out of phase with the direct rays by ¼ of a wavelength (¼λ), that is, they have been retarded by a ¼λ in relation to the direct rays *(Figure 34.4c)*.

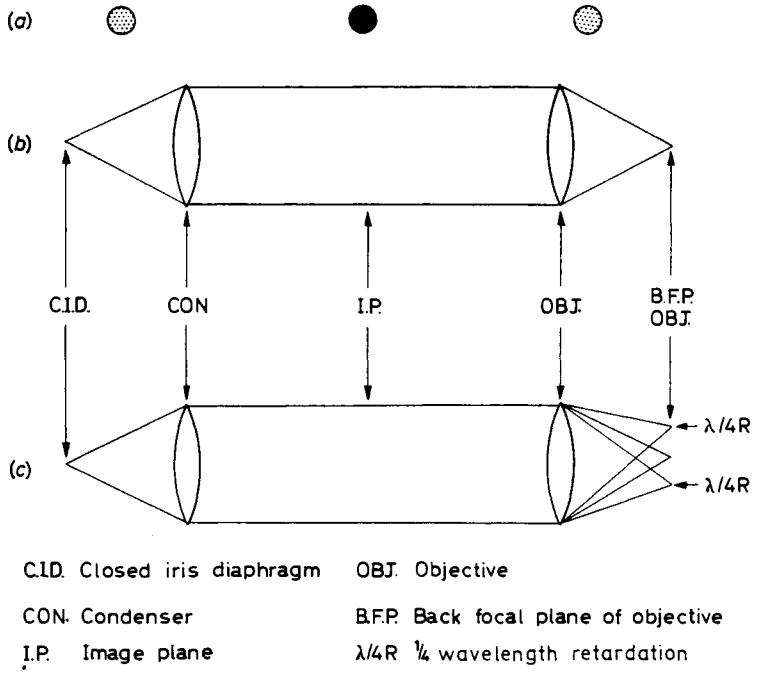


Figure 34.4 – Formation of diffraction images

If an annulus is placed in the substage condenser an image of that annulus will be formed in the back focal plane of the objective, and an object possessing slight non-homogeneities (such as unstained living cells) placed in the object plane will produce a halo of light both inside and outside the annular image. This halo is composed of light rays which have been diffracted by the object and are ¼λ out of phase with the direct light rays.

It will follow that if the diffracted rays of light could be retarded a further ¼λ, then the phase difference between the direct and diffracted rays would be ½λ and interference (as in *Figure 34.3e*) would take place in the final image plane, building up a picture, in light and shade, of an unstained specimen. Zernicke devised the 'Z' plate, now known as the phase plate, which, placed at the back focal plane of the objective, brought this about. The phase plate consists of an optically plane glass disc out of which is cut a channel to coincide with the image of the light annulus. The depth of the channel must be the exact depth to retard the diffracted rays, which travel through the full thickness of the plate, by a ¼λ in relation to the direct rays which travel through the channel *((Figure 34.5).*

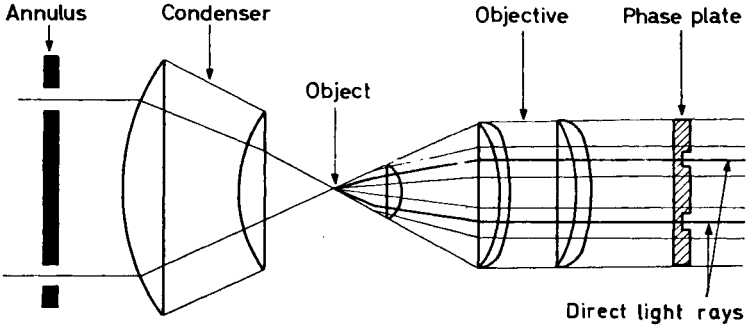


Figure 34.5 – Passage of light rays through the optical components of the phase-contrast microscope

Although interference will now take place, the great difference in amplitude (or brightness) between the two sets of rays will prevent the maximum contrast from being obtained. To overcome this factor light-absorbing material is deposited in the area of the channel which reduces the amplitude of the direct light without affecting the diffracted light, this permitting the maximum contrast to be obtained.

A broad summary of these principles is illustrated in *Figure 34.6; (a)* a ray of direct light from the annulus, on passing through the object (*b*) gives rise to a diffracted ray (dotted line), which is retarded by ¼λ; (*c*) on passing through the phase plate the diffracted ray, retarded by a further ¼λ is now in a position to interfere with the direct light ray: (*d*) the amplitude of the direct light ray is reduced after passing through the light-absorbing material and better contrast is obtained. Although in the illustration, for the sake of clarification, (*c*) and (*d*) take place

separately, in practice they occur almost simultaneously, and interference, of course, does not take place until the images are once again combined at the real image plane in the eyepiece.

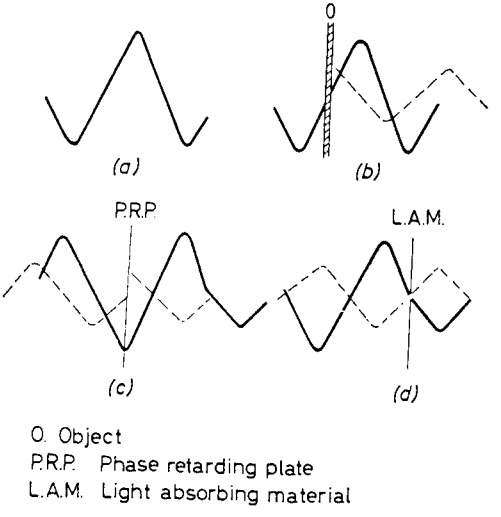


Figure 34.6 – Diagrammatic summary of the theory of phase-contrast microscopy

The foregoing theory should only be true if monochromatic light is used as an illuminant, since white light would be split into its component colours when diffracted; in practice, however, white light may be used but better contrast is obtained by using a mercury green filter in conjunction with a compound high intensity lamp.

Equipment

Phase-contrast equipment need not be very expensive and several papers have been written describing methods of adapting normal microscopes for phase contrast at a small cost (Kempson, Thomas and Baker, 1948; Culling, 1950). The performance of such a converted microscope is not usually equal to a specially designed commercial model, but will suffice for routine purposes. The microscope described in this chapter will be that available from Vickers Ltd. *(Figure 34.7).*

There are several other models available commercially which give equally good results by using a cross-shaped or slit-shaped aperture

instead of an annulus in the substage, but since the underlying principle is the same they are not described.

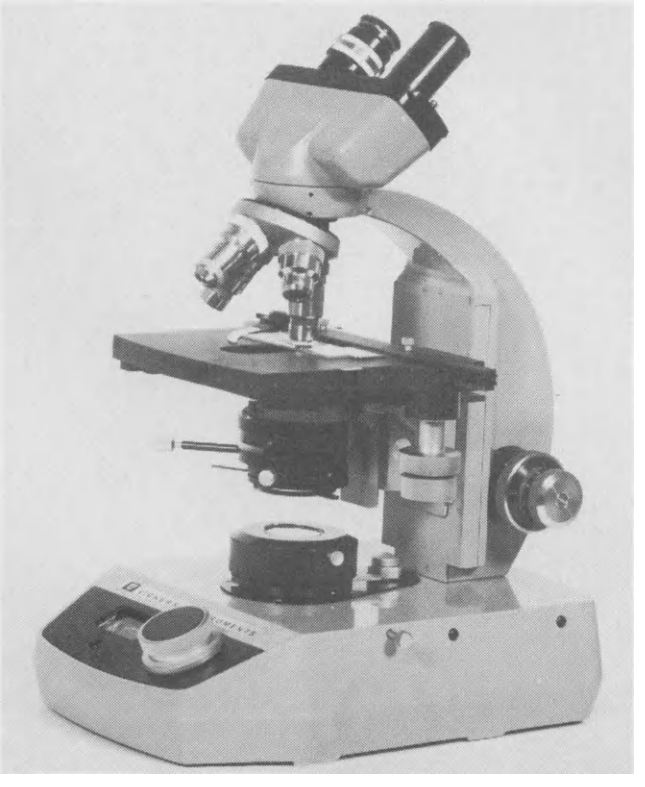


Figure 34.7 – The phase-contrast microscope (reproduced by courtesy of Vickers Limited)

Lamp

An intense source of illumination should be used, such as a high intensity compound lamp, with a mercury green (Wratten 62) filter.

Annulus

A different sized annulus will be required for each objective. These may be inserted separately, but a rotary changer carrying a set of

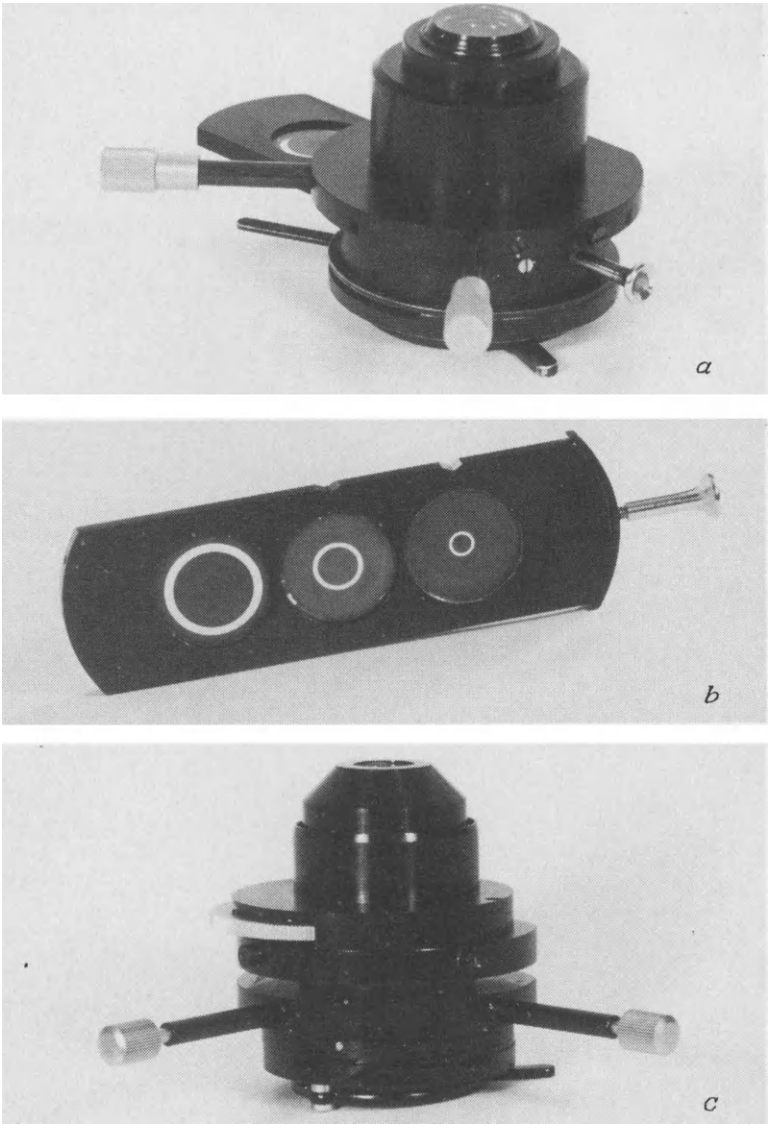


Figure 34.8 – The phase-contrast microscope; (a) and (b) show one way of inserting the phase rings into the light path, whilst (c) shows the alternative, more popular way

annuli, one for each objective, mounted below a special condenser is more convenient; each of these annuli can be centred by means of two centring screws.

Objectives

Objectives are supplied with phase plates already fitted, and since the phase plates affect their performance only slightly when used in a normal manner, they may be used without an annulus for routine microscopy.

Auxiliary Microscope

The auxiliary microscope is used for examining the back focal plane of the objective and ensuring that the objective phase plate and the condenser annulus are properly adjusted.

Setting up the Microscope

(1) The microscope is set up in the usual way (page 589), ensuring that there is no annulus in the substage.

(2) Focus on the object, closing the iris diaphragm if necessary.

(3) Rotate the annulus changer until the appropriate annulus is in position. Without disturbing the focus remove the eyepiece and replace it with the auxiliary microscope.

(4) Adjust the auxiliary microscope to bring the image of the phase plate into sharp focus.

(5) If the image of the light annulus does not coincide with the grey ring of the phase plate *(Figure 34.8a)*, it is adjusted with the centring screws until its image is concentric with, and completely covered by, the grey ring of the phase plate; the condenser may need to be raised or lowered slightly to adjust the size of the image of the light annulus. If the light annulus is not evenly illuminated the light should be adjusted.

(6) The auxiliary microscope is replaced by the eyepiece and a phase contrast image will be observed.

Note. – This procedure should be repeated each time the objective is changed.

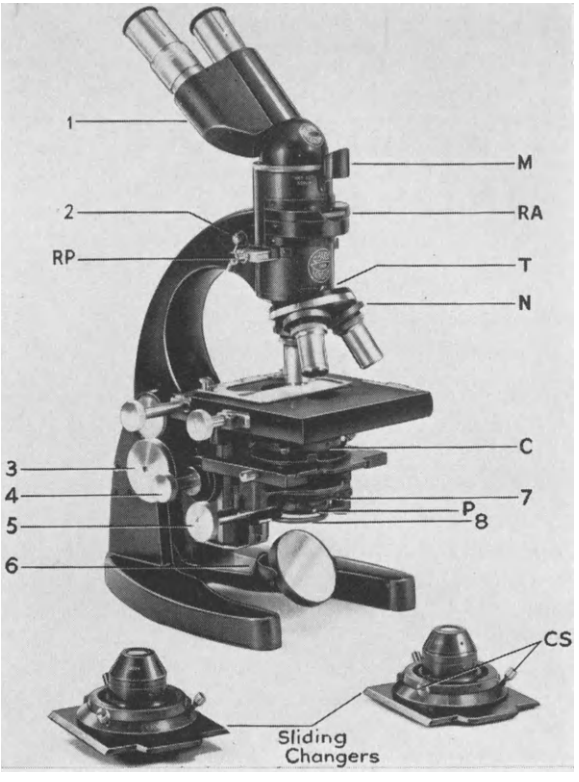
Chapter 35

The Interference Microscope

Although a form of interference microscope has been in use for some years, it is only since World War II that it has been developed for use in the biological laboratory with increased sensitivity. It will now detect and accurately measure phase changes in an object of as little as $^1/_{300}$ of a wavelength.

There are two types currently in use in Great Britain; the Baker interference microscope (designed by Mr. F. H. Smith), and the Dyson interference microscope (available from Cooke Troughton and Simms Ltd.). Since these microscopes are based on similar principles, only the Baker model *(Figure 35.1)* will be discussed.

The basic difference between the interference microscope and the phase-contrast microscope is that the former does not rely on diffraction by the object for interference, but generates mutually interfering beams which produce the contrast. It is this feature which enables such small phase changes to be seen and measured. The two rays, which eventually combine to produce the final image, are formed by a plate of birefringent material immediately above the condenser. These two rays, having passed through the condenser, are re-combined by a similar plate of birefringent material at the face of the objective *(Figure 35.2)*. Both these rays will have arisen from the same point of the light source, which is essential if interference is to take place in the final image, but the first will pass through one point in the image and the other through an area adjacent to it (the reference or comparison area). Consequently, each point in the final image is a compound one made up of two



1. Binocular eyepiece.
2. Body-tube lock.
3. Coarse adjustment.
4. Fine adjustment.
5. Substage focus.
6. Mirror bracket.
7. Substage iris.
8. Filter ring.

RP. Quarter-wave plate.
M. Scale magnifier.
RA. Rotating analyser.
T. Tubelength corrector (on left.)

N. Nosepiece changer.
C. Condenser.
P. Polarizer.
CS. Condenser-adjusting screws.

Figure 35.1 – Double-refracting interference microscope for transmitted illumination

superimposed mutually different views of the same point of the object. By using polarized light and an analyser the phase relationship between the two rays can be adjusted and measured. The goniometer analyser is

calibrated in degrees, and a magnifier fixed above enables small movements to be read accurately.

If white light is used as an illuminant, the various phases appear as different colours which change as the goniometer is rotated. Monochromatic light produces an amplitude contrast image and by rotation of the goniometer the degree of contrast can be varied for different components.

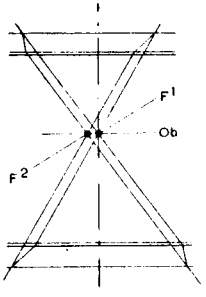
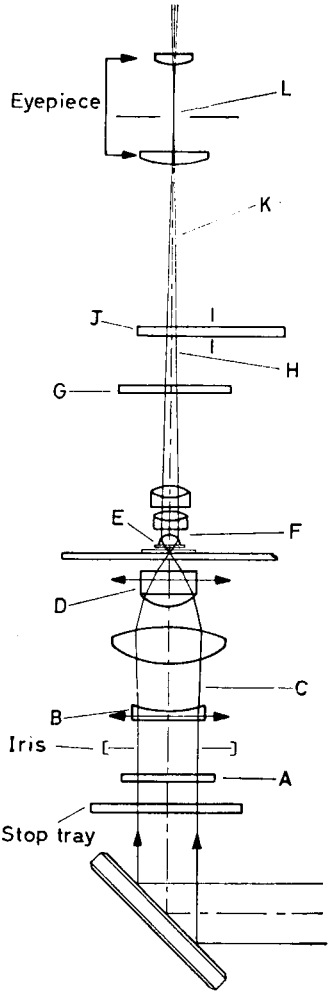


Figure 35.2 – Shearing system: Ob–object plane; F^1–object beam focus; F^2–reference beam focus

Uses

The microscope can be used for two purposes. (1) As an infinitely variable phase-contrast microscope with which individual parts of living cells may be studied with maximum detail; for this purpose there is little to be gained over the use of a good phase-contrast microscope, particularly since the condensers and objectives are matched and a change of objective also means changing and adjusting the condenser. A further disadvantage is that the high power (× 100) objective is a water immersion lens with the consequent limitation of resolution, and so on.

(2) As a highly accurate optical balance, it may be used for estimating dry mass down to 1×10^{-14} g. The discovery that an increase in refractive index of 0.0018 is due to a 1 per cent increase in concentration of the solid substances contained in cells, and the fact that the refractive index of cell components can be estimated from the phase difference between them and the reference area (usually the fluid in which they are suspended) has made this possible. It is this aspect of interference microscopy that is at present being developed by many research workers.



A. Swing-out polarizer. The rotation of this element controls the intensity relationship between the double-refracted beams, permitting the out-of-focus image to be extinguished for normal transmitted light conditions.
B. Double-refracting negative lens.
C. The double-refracted rays entering the Abbé condenser.
D. Double-refracting plate cemented to the front lens of the condenser, rendering it bi-focal.
E. Double-refracting plate rendering the objective bi-focal.
F. The re-combined double-refracted rays.
G. Quarter-wave plate.
H. The re-combined rays circularly polarized in opposite directions by the quarter-wave plate.
J. Rotatable analyser, with swing-out section, calibrated in degrees.
K. The phase relationship between the circularly polarized rays is adjusted by the analyser.
L. Final image exhibiting interference between the in-focus image of the object superimposed upon the out-of-focus image.

Figure 35.3 – Simplified diagram of the double refraction interference microscope for × 100 double-focus system

(Figures. 35.1, 35.2 and 35.3 are reproduced by courtesy of Messrs. C. Baker. Ltd.)

Construction

Figure 35.3 is a diagram of the component parts of the Baker interference microscope and it will be seen that the instrument is an extremely complicated one, the use and adjustment of which requires a great deal of practice.

Chapter 36

The Electron Microscope

Introduction

The light microscope lens systems have been perfected to such a degree that resolution is limited by the physical properties of image formation rather than the properties of the lenses themselves. From the discussion of optical theory it was seen that the resolution of any optical system is limited by the wavelength of light employed, and that an object that is smaller than this wavelength will cause so little perturbation of the light beam that it will not be resolved in the image. The best light microscopes are therefore limited to a resolution of about 2,000 Å*. The ultraviolet microscope, by using wavelengths about one-half that of white light, achieves a resolution of 1,000 Å.

By using an electron beam instead of light rays, the electron microscope gives much better resolution. The wavelength of moving electrons depends on their velocity. At an acceleration of 50,000 volts they have a wavelength of about 0.01 Å, and one may expect to resolve images of about this order. Due to lens defects which can be corrected in the light microscope but have not so far been corrected in the electron microscope, the resolution is limited to about 4 Å, which is still several orders of magnitude better than the best optical microscopes.

Tissue to be examined in the electron microscope must be processed so that sections of less than 0.1 μ thickness can be cut. These very thin sections are necessary because of the poor penetrating properties of the electron beam; the usual sections prepared for light microscopy would be completely opaque. Techniques of tissue preparation have now been developed so that it is relatively easy to cut thin enough sections, and

*1 mm = 1,000 microns; 1 micron = 10,000 Angstrom (Å).

basically these techniques are the same as those used in routine histological laboratories. Briefly they consist of fixation, dehydration in alcohol, embedding in special plastics, and sectioning with a specially

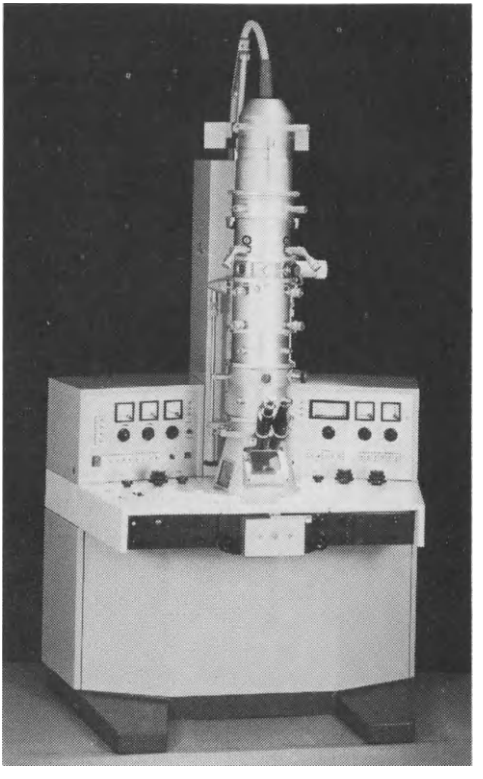


Figure 36.1 – Sieman's electron microscope

designed microtome, using knives made from broken glass. The thin sections are picked up on small copper grids for examination in the electron microscope.

Before describing briefly these new techniques, some elementary principles of the electron microscope will be reviewed.

Theory and Construction of Electron Microscope

The convergence of a light beam by a convex glass lens has its counterpart in the convergence of an electron beam as it passes through

the core of a circular magnetic field. Most electron microscopes use electromagnetic lenses. The convergence of an electron beam is shown producing an image; the image and object distances are related to the focal length of the lens in exactly the same way as in light optics (*see* page 571). The electron microscope is therefore constructed on the same optical principles as the light microscope, and the same formulae can be used to correlate magnification and focal distances *(Figure 36.2).*

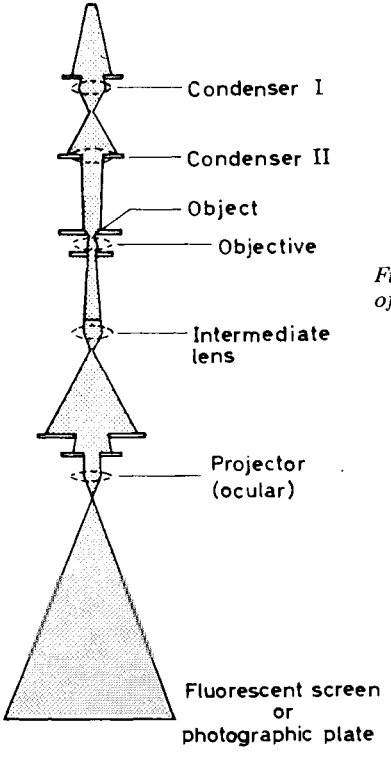


Figure 36.2 – Diagrammatic illustration of the optical path of the electron microscope

The electron beam is obtained from a heated tungsten filament which is surrounded by a metal cylinder known as the Whenalt cap. This cap serves to shape the electron beam. Just beyond the Whenalt cap is the anode which has an aperture through which the electron beam passes. A large voltage is applied between the cathode (the tungsten filament) and the anode, this gives the electrons their high velocity. They pass through the rest of the microscope without any further

acceleration. The Whenalt cap is given a voltage slightly lower than the filament, and this voltage is usually variable so that the flow of electrons from the cathode can be controlled. This is known as the bias voltage.

The electron beam first passes through the condenser lens. As in the light microscope this lens serves to focus the beam on to the object, and so provide 'illumination'. One must remember that the magnetic lens of an electron microscope can have different powers depending on the amount of current flowing in the electrical coils. In the light microscope the lens powers are, of course, fixed but the lenses are made movable with respect to the object so that the image can be focused and proper conditions of illumination obtained. In the electron microscope all of the lenses are rigidly fixed, but their focal points are variable by adjusting the lens currents. Thus, the 'illumination' of the object is achieved by varying the current in the condenser lens.

The imaging system of the electron microscope usually consists of three lenses; the objective, intermediate and projector lens. This gives three stages of magnification and makes it possible to achieve high magnification in a reasonable amount of space. The objective lens is placed with its focal point close to the object. Intermediate images are formed between each lens. The projector throws its image on to a fluorescent screen which may be substituted by a photographic plate to make a permanent record *(Figure 36.3)*. The entire illuminating and imaging system is usually referred to as the microscope column and is constructed upside down compared to the light microscope; that is, the electron gun and condenser lens are placed above and the image is formed below *(Figure 36.1* and *36.2)*. The column is very rigidly constructed and is maintained in a high vacuum since air molecules would deflect the electron beam. Because the specimen must be placed inside the vacuum it is not possible to examine living material in the electron microscope.

Electron optics are essentially similar to light optics. One important difference, however, is that the formation of the image is due to scattering of electrons by the molecules of the specimen and this scattering depends solely on the mass densities. Elements of high atomic weight such as lead or uranium cause marked electron scatter and appear very dense in the electron image. The lighter elements such as carbon, oxygen and nitrogen cause little electron scatter and have poor contrast. In the light microscope the image is due to absorption of light which depends more on molecular structure than atomic weights. Histological stains depend on absorption of certain wavelengths of light due to their molecular structure and are composed mainly of carbon, nitrogen and hydrogen atoms. Since these are all of low atomic weight they

have little electron scattering power and are not generally useful as stains for electron microscopy. Unstained tissues have very poor contrast in the electron microscope, but may be stained by a variety of heavy metal salts. Most such 'electron stains' are relatively non-specific and one does not have the battery of stains which are so useful in studying tissues in the light microscope.

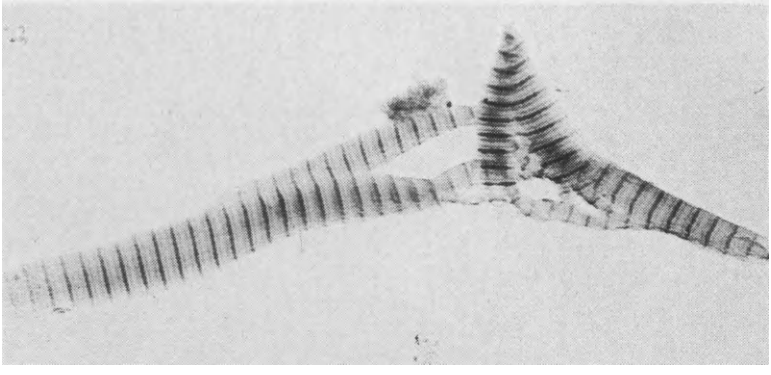


Figure 36.3 – Electron micrograph of re-precipitated collagen fibres from rat skin which have been shadowed with platinum. The white striations are approximately 2,400 Å apart

PREPARATION OF TISSUES FOR ELECTRON MICROSCOPY

The general principles are the same as those in the routine histological laboratory, and consist of fixation, dehydration, embedding, sectioning, and staining. The following pages will describe the methods which are in common use in most electron microscope laboratories. Many special and more or less experimental techniques are also available. For enzyme histochemistry the reader is referred to the papers by Holt and Hicks (1961), for antibody staining the paper of Singer and Schick (1961), for the preparation of frozen-dried tissues, the papers of Gersh (1956) and Grunbaum and Wellings (1960), and for techniques of freeze etching to the papers of Moor and his colleagues (1963, 1964).

Fixation

The most commonly used fixative is a buffered solution of osmium tetroxide originally described by Palade and often referred to as Palade's fixative. The preparation is given overleaf.

Palade's fixative

Stock buffer

Sodium barbitol	14.7 g
Sodium acetate	9.7 g
Distilled water to make	500 ml

Formula

Stock buffer	5 ml
0.1 N HCl	5 ml
Distilled water	2.5 ml
2 per cent aqueous osmium tetroxide	12.5 ml

The pH should be 7.4. The buffer solution should be kept in the refrigerator and is good for several months. The fixative should be used within a week or so and kept in a dark bottle in the refrigerator. *Osmium tetroxide fumes are extremely toxic. Avoid breathing the fumes or getting any solution on the hands.* The 2 per cent solution is best made by dropping a cleaned ampule into a glass-stoppered bottle containing the necessary quantity of water, and breaking the ampule by vigorous shaking. Solution of the crystals can be hastened by gentle heat.

Since Palade's fixative is hypotonic, many laboratories add sucrose, 0.045 g per ml. This is then known as Caulfield's fixative. Fixation in osmium tetroxide should not be longer than 60 minutes.

Glutaraldehyde Fixation

Glutaraldehyde fixation was introduced by Sabatini and colleagues (1963) as a method for electron microscopy. It is probably one of the most commonly used fixatives in this field at the present time, being customarily employed together with post-fixation in osmium tetroxide. Dr. W. H. Chase, in our laboratory, routinely fixes tissues in buffered glutaraldehyde for 4 hours in the refrigerator. He then rinses tissues in cold buffered sucrose following which they are post-fixed in Palade's osmium tetroxide for 1 hour. Tissues may be left in buffered sucrose solution in the refrigerator for longer periods if desired. The advantages of this method are improved penetration and preservation of some enzyme activity.

Buffered glutaraldehyde (pH 7.3–7.4) Karlsson and Schultz (1965)

Sodium dihydrogen phosphate ($NaH_2PO_4 . H_2O$)	3.31 g
Disodium hydrogen phosphate ($Na_2HPO_4 . 7H_2O$)	33.77g

Distilled water	925 ml
25 per cent Commercial glutaraldehyde	100 ml

Buffered sucrose solution (pH 7.3)

Sorensen's phosphate buffer pH 7.3–7.4 (page 171)	100 ml
Sucrose	4.5 g

The sucrose content is varied for different tissues, 4.5 per cent being a useful concentration for general use.

Since these fixatives penetrate very slowly into tissues, it is essential that the tissue blocks be cut into very small pieces, usually less than 1 mm in diameter. This must be done quickly, as soon as the tissues are removed, otherwise changes in all structures occur which although invisible in the light microscope, cause serious artifacts in the electron microscope. Clean razor blades are useful in dicing the tissue, care being taken to avoid compressing or otherwise distorting the tissues.

Fixation times depends on the size of the blocks and the type of tissue. Most tissues such as kidney and liver (in blocks less than 1 mm in diameter) will be well fixed in 1 hour. For special material such as brain, or botanical material, the reader should refer to papers by those working in these fields.

Dehydration

After fixation, the tissues are washed briefly in distilled water. Small glass vials or test tubes are suitable. Dehydration may be carried out in ascending concentrations of alcohol, starting with 30 per cent and progressing to 95 per cent in 3 or 4 steps of 5 minutes each. Acetone may also be used. While in 95 per cent alcohol the tissues should be examined under a dissecting microscope and further dissected if the blocks are too large. The tissues will be quite hard and easy to cut. They are transferred with Pasteur pipettes to 100 per cent alcohol and are now ready for embedding.

Embedding

In order to cut the very thin sections which will be transparent to the electron beam, it is necessary to embed the tissues in plastic. There are now two commonly used plastics for this purpose. It is recommended that one of these should be used routinely so that its characteristics become familiar, and faulty embedding can be recognized when it occurs. The methacrylates are easy to use, tissues are well infiltrated

and the blocks are easy to section. The plastic will evaporate in the heat of the electron beam, however, so that the section collapses and resolution suffers even although contrast is good. The epoxy resins are viscous solutions and penetration of the tissues may be difficult. The blocks are more difficult to section but the plastic is stable and resolution is good. Contrast is poor, because these plastics have high electron scatter and do not evaporate in the electron beam. It is usually necessary to stain the sections.

Polyester Embedding (Sjostrand, 1967)

The polyester, Vestopal W, may be used as an embeddant, and is thought by many to give superior resolution. For use, the Vestopal W is mixed with an initiator (tertiary butyl perbenzoate) and an activator (cobalt naphthenate). Acetone should be used for dehydration instead of ethanol, because Vestopal W is insoluble in ethanol.

The Vestopal, initiator and activator, must be stored in a refrigerator and protected from exposure to light. The Vestopal can be stored for several months, but neither the initiator nor the activator are usually stable for more than 2 months; should the finished blocks be soft it is most probably due to inactivation of the latter.

Method

Dehydration

30 per cent Acetone	15–30 minutes.
50 per cent Acetone	15–30 minutes.
75 per cent Acetone	15–30 minutes.
90 per cent Acetone	30–60 minutes.
100 per cent Acetone (dried over $CuSO_4$)	30–60 minutes.

Infiltration

1 part Vestopal W/3 parts acetone	30–60 minutes.
1 part Vestopal W/1 part acetone	30–60 minutes.
3 parts Vestopal W/1 part acetone	30–60 minutes.
Vestopal A/+1 per cent initiator +0.5 per cent activator	12–24 hours

Embedding

Transfer tissues to gelatin capsules filled with Vestopal W to which has been added 1 per cent initiator (tertiary butyl perbenzoate) and 0.5

per cent activator (cobalt naphthenate) and leave at 60°C for 24–48 hours to polymerize and harden. When preparing the mixture the initiator is first well mixed in the Vestopal W before adding the activator and it is important that the mixing is performed carefully and thoroughly to avoid irregular polymerization: the mixture does not keep for more than a few hours at room temperature.

Kurtz (1961) does use ethanol to dehydrate tissue, by transferring it from absolute ethanol to styrene (3 changes over a period of 1 hour, then into a mixture of equal parts of styrene and Vestopal W for 30 minutes followed by pure Vestopal W for 4–48 hours).

Embedding in Epoxy Resins

Epon 812 (Shell Oil Co.) is most commonly used, having replaced Araldite which has high viscosity. The following method is after Luft.

Solution A

Epon 812	62 ml
DDSA (Dodecenyl succinic anhydride)	100 ml

Solution B

Epon 812	100 ml
MNA (Methyl nadic anhydride)	89 ml

These solutions keep well in the refrigerator. The embedding mixture is made by adding together A and B in proportions depending on the degree of hardness desired. Seven parts of solution A to 3 parts of solution B will give a soft block, 3 parts of A to 7 of B will give a hard block. Intermediate mixtures may be used. It is best to experiment and find which is most suitable for the type of tissue and microtome being used. A suitable catalyst is added to the mixture, such as 1.5 per cent DMP–30*. Only sufficient plastic for immediate use should be prepared.

Infiltration with the fairly viscous plastic solution is best done through propylene oxide which does not interfere with polymerization. After dehydration in absolute alcohol the tissues are transferred to propylene oxide for 1 hour with frequent swirling. Most of the propylene oxide is then decanted leaving a few ml in the bottom of the vial with the tissue. An equal amount of the prepared Epon mixture is added with continuous swirling until the solutions have completely

*Rhom and Haas Co., Philadelphia, Penna., U.S.A.

mixed. After half an hour the solution is decanted and replaced by the Epon mixture. This should be placed in the oven for an hour with frequent swirling. Suitable gelatin capsules are then filled with the plastic and the tissues are transferred with a Pasteur pipette to the top of the capsules. They are returned to the oven during which time the tissues settle through the plastic to the bottom of the capsules. They may be centered with a probe. Polymerization may be obtained overnight at 50–60°C. The excess plastic may be trimmed away from the hardened blocks with razor blades or, since the plastic is quite tough, with a dental burr.

Sectioning

There are many models of microtomes to choose from which have been designed to cut the very thin sections needed for electron microscopy. The operation of these instruments requires a good deal of skill which can only be obtained by practice, so that there is little to be gained by describing here the techniques: the main requirement, apart from well embedded tissues, is patience.

Diamond knives may be used for methacrylate blocks, but are unsuitable for epoxy resins unless the knife angle is less than 45 degrees. Glass knives are cheap and easily made by the following method.

Plate glass, $^{3}/_{16}$ or $^{1}/_{4}$ inches thick, is scored into strips about 1½ inches wide. A break is started by tapping the reverse side of the score line, and the strip is broken off by pressure over an applicator stick. The broken surface is examined and only those strips which have a smooth surface are selected; have a large waste-basket nearby. The good strips are scored into rhombic pieces with an angle of 45 degrees or 50 degrees. The score line should not be carried closer than ¼ inch to the good edge of the strip. The knives can be broken off with glazier's pliers, or by touching the centre of the score line with a Pyrex glass rod heated to white heat in an oxygen flame. Each knife edge should be examined in a low power microscope and the parts of the edge which are free of nicks or fine lines noted. A suitable water trough is attached to the knife to catch the sections as they are cut. The sections are then picked up on previously prepared grids *(see below)*.

Glass Knife Makers

The introduction of glass-knife-making machines, such as the LKB which we use, has greatly facilitated the preparation of first-class glass knives with very little practice. The use of these machines, together

with standardized glass strips, has done much to simplify the technique (and remove many of the frustrations) of ultra-thin sectioning.

Staining

The sections (supported by the grids) may be stained by a variety of heavy metal salts to increase the contrast. Most of these are non-specific, and indeed the nature of the combination with tissue constituents is not understood. One of the most useful is lead hydroxide. Lead hydroxide solutions may be prepared from sodium hydroxide and lead acetate, but are unstable and tend to cause heavy contamination due to deposits of lead carbonate. The method described by Karnovsky is recommended where the lead hydroxide is used in a highly alkaline solution which is easy to prepare and gives strong staining without contamination.

Lead Citrate Staining (Reynolds, 1963)

Lead citrate solution

Lead nitrate ($Pb(NO_3)_2$)	1.33 g
Sodium citrate ($Na_3C_6H_5O_7.2H_2O$)	1.76 g
Distilled water	30 ml

Place the above solution in a 50 ml volumetric flask, shake for 1 minute, then intermittently for 30 minutes. Add 8 ml of IN sodium hydroxide (NaOH), and make up to 50 ml with distilled water; mix by inversion. If the solution is turbid, centrifuge until clear immediately before use.

Staining the grids. – The grids are stained as described below for lead hydroxide. The stain is diluted from 1:5 to 1:1,000 with 0.01N NaOH and grids are stained for 4–5 minutes. After staining the grids are washed in 0.02N NaOH and distilled water.

Lead Hydroxide Stain (Karnovsky, 1961)

Add a spatula tip of lead oxide to 20 ml of 1 N NaOH and boil gently for 15 minutes. Cool and filter. Dilute the filtrate 50 or 100 times with distilled water. *The sections are stained conveniently on a paraffin surface* which is covered with a small Petri dish. A drop of the staining solution is placed on the paraffin and the grid floated on the drop with the section down in contact with the solution. Staining time

is 5–30 minutes. The grid is then washed by transferring rapidly through three beakers of distilled water using jeweller's forceps, and then dried on filter paper.

Uranyl Acetate Staining

A 1 per cent solution is prepared by adding 1 g of uranyl acetate to 100 ml double distilled water, which is shaken well and allowed to sit for a day or two; the solution should be centrifuged before use. Staining is carried out as described above for 2–60 minutes (we use 5 minutes). Grids are rinsed well in distilled water.

Increased contrast can be obtained by double staining with lead citrate *(above)*. The uranyl acetate is particularly good for fibrous proteins, it may also be used in alcoholic solution before the embedding process. Movat has described the use of silver proteinate (Protargol) and the silver methenamine stain which reacts with components of basement membranes. Many other heavy metals and organic dyes have been used in electron microscopy and many are useful in special circumstances. The original articles should be consulted.

Preparation of Electron Microscope Grids

The thin sections prepared for the electron microscope must be supported on plastic-covered grids to prevent their collapse in the heat of the electron beam. The grids are purchased and must be covered by a very thin film of plastic. Either formvar or collodion may be used and there are two principal methods of preparation.

Method I

Formvar is dissolved in ethylene dichloride at a concentration of 0.5 per cent, or collodion is dissolved in amyl acetate in a concentration of 1 per cent. Clean glass slides are placed for a few minutes in the plastic solution and then withdrawn and allowed to dry in a vertical position. A thin layer of plastic is formed over the surface of the glass slide as the solvent evaporates. The film, attached to the glass slide, is scored with a razor blade, into squares large enough to cover the electron microscope grids being used, and these are floated off on to the surface of a dish of distilled water, and then picked up one by one to cover one side of the grids. Each grid must be examined to ensure that it is completely covered by a smooth film.

Method II

A solution of 2 per cent collodion in amyl acetate is prepared. A large funnel is filled with water and a stainless steel screen is placed in the water near the top. As many as a hundred grids are placed on the screen. A drop of the collodion solution is allowed to fall on the surface of the water. This quickly spreads out and, as the solvent evaporates, a thin layer of collodion is left behind on the surface of the water. If the film is smooth with an even silvery colour it is probably suitable. The water is then drained out through the bottom of the funnel allowing the collodion film to settle over the screen and grids. After drying the grids are picked off and examined.

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