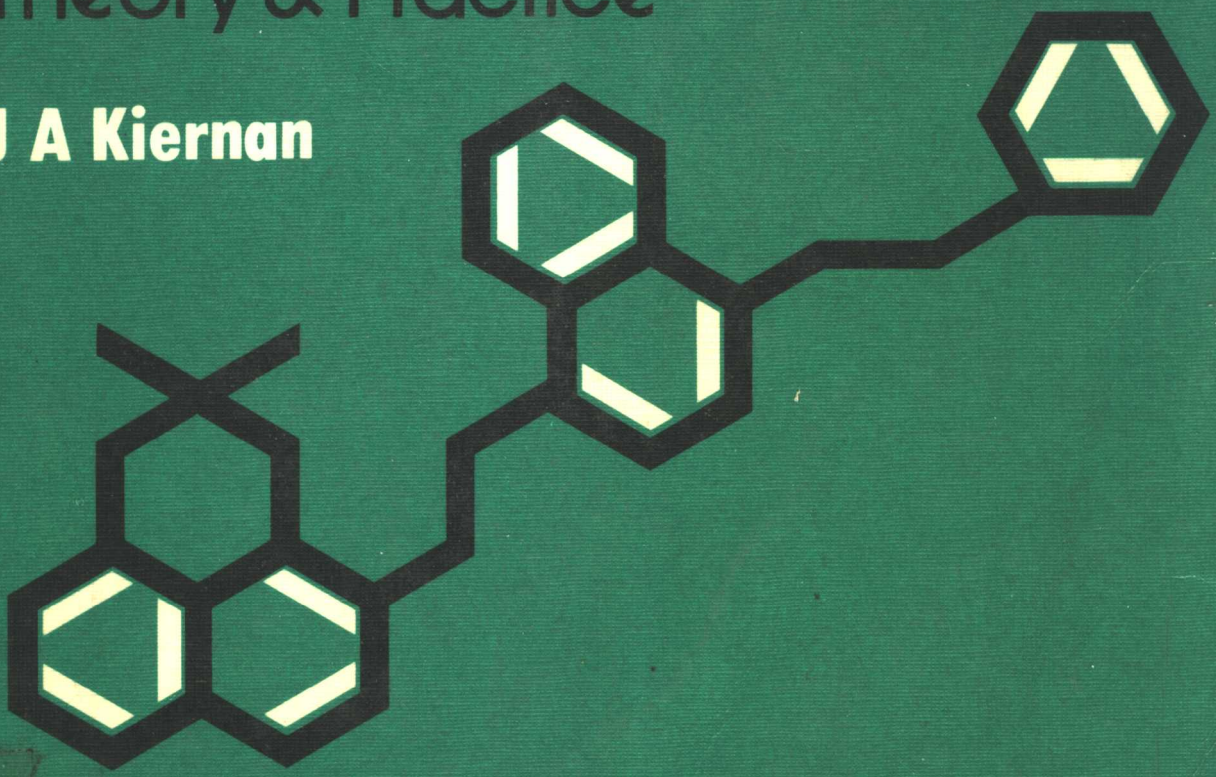
Histological & Histochemical Methods:

Theory & Practice

J A Kiernan



Histological and Histochemical Methods

Theory and Practice

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Preface

IN THIS text it is my intention to explain the major groups of methods used in the preparation of biological specimens for examination with the optical microscope. The scientific principles involved are illustrated by detailed descriptions of selected practical procedures. The reader should be in a position to understand exactly what he is doing and why he is doing it when carrying out any of the techniques.

It is not feasible to explain everything from first principles, so I have assumed that the reader's education in science includes the subjects of chemistry and biology to standards a little below those of the Advanced Level of the General Certificate of Education in Great Britain or a little above those of first-year university courses in North America. An elementary knowledge of biochemistry will also be helpful, and the interpretation of stained sections will obviously be easier for the reader who knows some histology. The methods discussed in this book are all applicable to the tissues of vertebrate animals. Most of them may also be used for invertebrates and plants.

The number of references to the literature has been kept fairly small. Those cited fall into three categories:

(a) Books and review articles, to which the reader is referred for more information. References of this type are given both for the methods discussed in detail and for others which are mentioned only briefly.

(b) Original papers of recent date when the material is not adequately covered in books or review articles.

(c) Original papers concerned with controversial issues when the interested reader may wish to evaluate the evidence for himself.

Anyone proposing to use a histological or histochemical method in research or for any other important purpose is urged to find out as much as possible about the underlying physics and chemistry. Few of the methods used for staining tissues are fully understood, but sound scientific principles govern both the choice of the technique for a particular purpose and the practical conduct of the procedure. This book is not a collection of recipes to be followed unthinkingly: the student will have to read the "theory" in order to carry out the "practice" intelligently. Although the text is set out in such a way that it may, with advantage, be read from beginning to end, many readers will want to refer only to single chapters. Numerous cross-references are given in order to avoid repetition of explanatory material contained in the various parts of the book. No student taking a course in histological techniques could reasonably be expected to memorize the whole book. Indeed, much of the content is included solely for purposes of reference: much of the material in Chapters 5 and 20 and all the detailed practical instructions, for example, fall into this category. It is not unreasonable that the text be available for consultation by students while sitting written or practical examinations.

The "theoretical" exercises at the end of each chapter should help the student to assess his own progress. These are mostly questions requiring deductive reasoning based on the information presented. The "practical" exercises may serve as a guide to teachers in planning laboratory classes. A few of the suggested experiments involve live animals: in Great Britain these must be carried out by a person holding a Home Office licence with the appropriate certificates. Universities and colleges in most other countries have their own regulations, which should be observed.

Various colleagues have commented on parts of the manuscript. I am especially grateful to Drs R. C. Buck, M. G. Cherian, B. A. Flumerfelt, P. Haase, and J. R. Sparrow. Their advice has resulted in several improvements to the text, but I remain responsible for any errors or failures to explain subjects clearly enough. Comments on the content and style of this book will be welcomed, especially from those readers who are making their first encounters with practical histology and histochemistry. My own understanding of these subjects has been acquired from having to use the methods in research, which has been supported by grants from several organizations. Thanks are due to the British Medical Research Council and the National Fund for Research into Crippling Diseases, and to the J. P. Bickell Foundation (Toronto), the Canadian Medical Research Council, the Multiple Sclerosis Society of Canada, and the Ontario Thoracic Society.

London, Ontario J. A. KIERNAN

Conventions and Abbreviations

CONVENTIONS

It is important that the reader be familiar with the conventions listed here before attempting to follow the instructions for any practical procedure.

[ ] (i) Enclose a complex, such as $[Ag(NH_3)_2]^+$ or $[PdCl_4]^{2-}$.

(ii) Indicates "concentration of", in molar terms. Thus, $[Ca^{2+}]^3$ = "the cube of the molar concentration of calcium ions".

Accuracy. Solids should be weighed and liquids measured to an accuracy of ±1%. With quantities less than 10 mg or 1.0 ml, an accuracy of ±10% is acceptable.

Alcohol. Unqualified, this word is used for methanol, ethanol, isopropanol, or industrial methylated spirit (which is treated as 95% v/v). When the use of a specific alcohol is necessary, this is stated. "Absolute" refers to the commercially available "100%" ethanol, which really contains about 1% water and may also be contaminated with traces of benzene. Absolute ethanol is hygroscopic and should be kept in securely capped bottles, which should be only three-quarters filled to allow for evaporation. In an ordinary covered staining tank, ethanol does not remain acceptably "absolute" for more than about 5 days.

When diluting alcohols for any purpose, use distilled or de-ionized water.

Concentrations expressed as percentages. The symbol % is used in various ways:

(i) For solids in solution, % = grams of solid dissolved in 100 ml of the final solution.

(ii) For liquids diluted with other liquids, % = number of millilitres of the principal component present in 100 ml of the mixture, the balance being made up by the diluent (usually water). "70% ethanol" means 70 ml of absolute ethanol made up to 100 ml with water.

(iii) For gases (e.g. formaldehyde), % = grams of the gas contained in 100 ml of solution.

(iv) Where doubt may arise, the symbol v/v, w/v, or w/w is appended to the % sign. For dilution of acids and ammonia, see Chapter 20.

Formalin. This word refers to the commercially obtained solution containing 37–40% (w/v) of formaldehyde in water. The shortened form "formal" is used in the names of mixtures such as formal–saline and formal–calcium. The term "formol" will be found in some books, but this is wrong because the ending -ol suggests, incorrectly, that formaldehyde is an alcohol.

Safety precautions. The precautions necessary in any laboratory, especially the prevention of fire, should be observed at all times. Some reagents used in histology and histochemistry have their special hazards. These are mentioned as they arise in the text.

Concentrated mineral acids (especially sulphuric) must be diluted by adding acid to water (**not** water to acid) slowly with stirring.

Formaldehyde and hydrochloric acid should not be thrown down a sink together: their vapours can react together in the air to form *bis*-chloromethyl ether, a potent carcinogen. Each substance should be flushed down the drain separately, with copious running tap water.

Concentrated nitric acid must not be allowed to come into contact with organic liquids, especially alcohol: the strongly exothermic reaction may result in an explosion.

Salts—water of crystallization. The crystalline forms of salts are shown in instructions for mixing solutions. If the form stated is not available, it will be necessary to calculate the equivalent amount of the alternative material. This is simply done by substitution in the formula

$$\frac{W_1}{M_1} = \frac{W_2}{M_2}$$

in which W = weight, M = molecular weight, and subscripts 1 and 2 refer to the prescribed and the alternative compounds respectively.

For example: 125 mg of cupric sulphate ($CuSO_4$) are prescribed, but only the hydrated

salt, $CuSO_4.5H_2O$, is available. Molecular weights are 223.14 and 249.68 respectively. Then

$$\frac{125}{223.14} = \frac{W_2}{249.68}$$

$$W_2 = \frac{125 \times 249.68}{223.14}$$

$$= 139.9$$

It will therefore be necessary to use 139.9 (i.e. 140) mg of $CuSO_4.5H_2O$ in place of 125 mg of the anhydrous salt.

Solutions. If a solvent is not named (e.g. "1% silver nitrate"), it is assumed to be water. See also under **Water** below.

Structural formulae. Aromatic rings are shown as Kekulé formulae, with alternating double bonds. Thus benzene is

Modern practice favours the designation

which indicates the equivalence of all the bonds in the ring. The Kekulé formulae are used in this book because, with them, it is easier to understand structural changes associated with the formation of coloured compounds, such as the formation of quinonoid from aromatic rings.

A few deviations from standard chemical notation (e.g. in formulae for lipids) are explained as they arise.

Temperature. Unless otherwise stated, all procedures are carried out at room temperature, which is assumed to be 15–25°C. The other commonly used temperatures are 37°C and about 60° C. A histological laboratory should have ovens or incubators maintained at these temperatures.

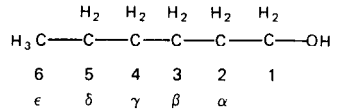
If an oven containing melted paraffin wax is used as a 60°C incubator, make sure that any aqueous or alcoholic solutions put in it are covered. Water or alcohol vapour may otherwise contaminate the wax.

Water. *When "water" is prescribed in practical instructions, it means distilled or deionized water.* When water from the public supply may be used, it is specifically mentioned as "tap water".

ABBREVIATIONS

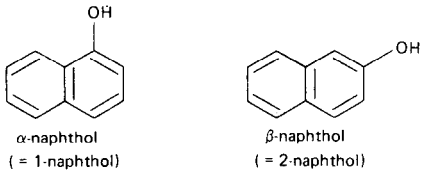
α, β (i) Used to indicate the configuration at position C1 in glycosides (see Chapter 11).

(ii) In aliphatic compounds the α-carbon atom is adjacent to the carbon atom bearing the principal functional group (i.e. α is carbon number 2). The use of numbers and Greek letters is shown below for *n*-hexanol:



(iii) In glycerol and its derivatives, the middle carbon atom is designated as β and the carbons on either side as α and α'.

(iv) In derivatives of naphthalene, indicate the position of a substituent relative to the site of fusion of the rings:



α-naphthol (= 1-naphthol) β-naphthol (= 2-naphthol)

△	Symbol used to indicate double bonds in lipids (see Chapter 12).
ε-	Indicates carbon number 6 or a substituent on this atom, as in the case of the amino group at the end of the side-chain of lysine.
μg	microgram ($= 10^{-6}$ g or 10^{-3} mg).
μm	micrometre ($= 10^{-6}$ m or 10^{-3} mm); also sometimes called a "micron".
ACh	Acetylcholine.
AChE	Acetylcholinesterase.
APUD	Amine precursor uptake and decarboxylation.
Ar	An aryl radical (in formulae).
AThCh	Acetylthiocholine.
ATP	Adenosine triphosphate.
ATP-ase	Adenosine triphosphatase.

bis-	Twice (in names of compounds).
B.P.	British Pharmacopoeia; Boiling point.
BuThCh	Butyrylthiocholine.
B.W. 284C51	1,5-*bis*-(4-diallyldimethylammonium-phenyl)-pentan-3-one. (The initials are for Burroughs Wellcome, the manufacturer).
°C	Degrees Celsius (Centigrade).
ChE	Cholinesterase (= pseudocholinesterase).
C.I.	*Colour Index* (see Chapter 5).
cis-	Indicates a geometrical isomer in which two substituents lie on the same side of the molecule.
CNS	Central nervous system.
Con A	Concanavalin A.
CPC	Cetylpyridinium chloride.
cyt.	Cytochrome (with identifying letter, a, b, c, etc.).
D-	Indicates a compound, usually a sugar, of the D-series. The compound itself is not necessarily dextrorotatory.
DAB	3,3′-diaminobenzidine.
dansyl	The 5-(dimethylamino)-1-naphthalenesulphonyl radical.
DFP	Diisopropylfluorophosphate.
DMAB	*p*-dimethylaminobenzaldehyde (also known as Ehrlich's reagent).
DMP	2,2-dimethoxypropane.
DNA	Deoxyribonucleic acid.
DN-ase	Deoxyribonuclease.
DOPA	β-3,4,dihydroxyphenylalanine.
DPN	Diphosphopyridine nucleotide (an obsolete name for NAD^+, q.v.).
DPX	A resinous mounting medium. The initials stand for its three components, distrene (a polystyrene), a plasticizer, and xylene.
E_0, E_0'	Symbols for oxidation–reduction potentials (see Chapter 16).
E600	Diethyl-*p*-nitrophenyl phosphate.
E.C.	Enzyme Commission (see Chapter 14).
EDTA	Ethylenediaminetetraacetic acid. Also known as versene, sequestrene, edetic acid, and (ethylenedinitrilo)-tetraacetic acid. Usually used as its disodium salt.
Fab	Part of the immunoglobulin molecule (see Chapter 19).
FAD	Flavin adenine dinucleotide (see Chapter 16).
FAGLU	A fixative containing formaldehyde and glutaraldehyde (see Chapter 17).
Fc	Part of the immunoglobulin molecule (see Chapter 19).
FITC	Fluorescein isothiocyanate.
FMN	Flavin mononucleotide (see Chapter 16).
GBHA	Glyoxal-*bis*-(2-hydroxyanil).
H-acid	8-amino-1-naphthol-3,6-disulphonic acid.
H-chain	Part of the immunoglobulin molecule (see Chapter 19).
H. & E.	Haematoxylin and eosin (see Chapter 6).
HNAH	2-hydroxy-3-naphthoic acid hydrazide. May equally be named 3-hydroxy-2-naphthoic acid hydrazide.
HRP	Horseradish peroxidase.
IgG	Immunoglobulin-G.
L-	Indicates a compound (usually a sugar or an amino acid) of the L-series. The compound itself is not necessarily laevorotatory.
LVN	Low viscosity nitrocellulose.
M	(as in 0.1 M) Molar (= moles per litre).
m-	*meta-* (in names of benzene derivatives).
MBTH	3-methyl-2-benzothiazolone hydrazone.
mole	The molecular weight, expressed in grams.
M.W.	Molecular weight.
N	(as in 0.1 N) Normal (= gram-equivalents per litre; see Chapter 20).
N_A	Avogadro's number: 6.022×10^{23} molecules per mole.
n-	Normal, indicating an unbranched chain, as in *n*-butanol.
NAD^+	Nicotinamide adenine dinucleotide.
NADI	Naphthol-diamine (reaction).
$NADP^+$	Nicotinamide adenine dinucleotide phosphate.
NANA	*N*-acetylneuraminic acid.
Nitro-BT	Nitro blue tetrazolium.
nm	nanometre (= 10^{-9} m or 10^{-3} μm).
NQS	1,2-naphthoquinone-4-sulphonic acid.
NSM	Neurosecretory material.

o-	*ortho-* (in names of benzene derivatives).
OPT	*o*-phthaldialdehyde.
p-	*para-* (in names of benzene derivatives).
PAN	Perchloric acid–naphthoquinone (method; see Chapter 12).
PAP	Peroxidase–antiperoxidase (reagent; see Chapter 19).
PAS	Periodic acid–Schiff (method; see Chapter 11).
PCMB	*p*-chloromercuribenzoate.
pH	The logarithm (to base 10) of the reciprocal of the molar concentration of hydrogen ions.
PMA	Phosphomolybdic acid.
pg	picogram ($= 10^{-12}$ gram).
PMS	Phenazine methosulphate.
PNS	Peripheral nervous system.
PTA	Phosphotungstic acid.
PVA	Polyvinyl alcohol.
PVP	Polyvinylpyrrolidone (also called "povidone").
R, R′	Indicate alkyl or aryl radicals, in formulae.
RNA	Ribonucleic acid.
RN-ase	Ribonuclease.
S.G.	Specific gravity (also = density, in g per cm^3).
Susa	A fixative mixture introduced by M. Heidenhain: short for *Sublimat-Säure* (see Chapter 2).
t-	Tertiary, as in *t*-butanol: $(CH_3)_3COH$.
TCA	Trichloroacetic acid.
TMB	Tetramethylbenzidine.
TPN	Triphosphopyridine nucleotide (an obsolete name for $NADP^+$, q.v.; see Chapter 16).
TPP	Thiamine pyrophosphate.
TPP-ase	Thiamine pyrophosphatase.
trans-	Indicates a geometrical isomer in which two substituents lie on opposite sides of the molecule.
TRIS	*Tris*(hydroxymethyl)aminomethane.
UDPG	Uridine-5-diphosphate glucose.
UQ	Ubiquinone.
U.S.P.	United States Pharmacopoeia.
v/v	Volume ÷ volume.
w/v	Weight ÷ volume.
w/w	Weight ÷ weight.

SPECIALIZED ABBREVIATIONS

The following are used only for special purposes and are explained in the appropriate parts of the text.

Chapter 11. Monosaccharide residues: Fuc, Gal, GalNAc, Glc, GlcNAc, GlcUA, IdUA, Man, Xyl. Lectins: A, DBA, LA, LCA_1, PHA, RCA_1, RCA_{120}, SBA, UEA_1, WGA.

Chapter 16. Tetrazolium salts: BSPT, BT, DS-NBT, INT, MTT, Nitro-BT, NT, TNBT, TTC, TV.

Chapter 17. Biogenic amines: ADR, DA, HIS, 5HT, NA.

Contents

Each chapter is preceded by a detailed table of contents.

1

Introduction to Microtechnique

Many theoretical explanations and practical instructions are contained in this book. The present chapter concerns various aspects of the making of microscopical preparations that are fundamental to all the techniques described in the later chapters. It cannot be over-emphasized that unless the student or technician understands the rationale of all that is to be done, he will not do it properly. Some purely practical information relevant to the manipulations discussed here will be found in Chapter 4 as well as in Section 1.6 of this chapter.

1.1. THICKNESS AND CONTRAST

In order to be examined with the microscope, a specimen must be sufficiently thin to be transparent and must possess sufficient contrast to permit the resolution of structural detail. Thinness may be an intrinsic property of the object to be examined. Thus, small animals and plants, films and smears of cells, macerated or teased tissues, and spread-out sheets of epithelium or connective tissue are all thin enough to be mounted on slides directly as **whole mounts**. In the study of histology and histochemistry, one is more often concerned with the internal structure of more substantial, solid specimens. These must be cut into thin slices or **sections** in order to make them suitable for microscopical examination.

Freehand sections, cut with a razor, are rarely used in animal histology but are still sometimes employed for botanical material. Though some expertise is necessary, sectioning in this way has the advantage of requiring little in the way of time or special equipment. When sections of human or animal tissues are needed in a hurry, **frozen sections** are commonly used. The ordinary **freezing microtome** is used for fixed material, while the **cryostat**, essentially a microtome mounted in a freezing cabinet, may be used for cutting sections of fixed or unfixed tissue. More skill is called for in the operation of a cryostat than for a freezing microtome. Another advantage of cutting frozen sections, aside from speed, is the preservation of some lipid constituents, which are dissolved out during the course of dehydration and embedding in paraffin wax. A disadvantage of the freezing microtome is that the sections are commonly too thick (e.g., 20–100 μm) for the resolution of fine structural detail within cells. Much thinner sections can be obtained by using a cryostat. The **vibrating microtome** (Vibratome) can be used to cut sections of unfixed, unfrozen specimens. The blade of this instrument passes with a sawing motion through a block of tissue immersed in an isotonic saline solution. The cutting process is much slower than with other types of microtome, so it is not feasible to prepare very large numbers of sections.

When the preservation of lipids and of heat-labile substances such as enzymes is not important, the specimens are **dehydrated**, **cleared** (which means, in this context, equilibrated with a solvent which is miscible with paraffin), **infiltrated** with molten paraffin wax, and, finally, **embedded** (blocked out) in solidified wax. **Paraffin sections** are most commonly cut on a **rotary microtome**, though a **rocking microtome** or a **sledge microtome** can also be used. The sections come off the knife in ribbons, and with sufficient skill it is possible to obtain **serial sections**, which may be as little as 4 μm thick, through the whole block of tissue. **Polyester wax** (Steedman, 1960), which is miscible with 95% alcohol, is handled in much the same way as paraffin. Its lower melting point (about 40°C as compared with 55–60°C for most paraffin waxes) is an advantage for

some tissues and histochemical methods. When relatively thick sections of large specimens are required, it is more convenient to embed in celloidin or low-viscosity nitrocellulose. **Celloidin sections**, 50–200 μm thick, are usually cut on a sledge microtome. Various **synthetic resins** are sometimes used as embedding media for light microscopy, though their main application is in the cutting of extremely thin sections for examination in the electron microscope. Resin-embedded tissue is usually sectioned with an **ultramicrotome**, using a glass or diamond knife. Sections 0.5–1.0 μm thick, suitably stained for optical examination, are valuable for comparison with the much thinner sections used in ultrastructural studies.

The optical contrast in a thin specimen is determined partly by its intrinsic properties but largely by the way in which it is processed. If the specimen is not stained, contrast will be greatest when the mounting medium has a refractive index substantially different from that of the specimen. Differences of this type are emphasized in the **phase-contrast microscope**. This instrument is especially valuable for the study of living cells, such as those grown in tissue-culture. In histology, the natural refractility of a tissue is usually deliberately suppressed by the use of a mounting medium with a refractive index close to that of the anhydrous material constituting the section (approximately 1.53). Almost all the contrast is produced artificially by **staining**.

Fluorescence is the property exhibited by substances which absorb light of short wavelength such as ultraviolet or blue and emit light of longer wavelength, such as green, yellow, or red. The phenomenon can be observed with a **fluorescence microscope** in which arrangements are made for the emitted (long wavelength) light to reach the eye while the exciting (short wavelength) light does not. Fluorescing materials therefore appear as bright objects on a dark background. The fluorescence microscope can be used to observe **autofluorescence** due to substances naturally present and **secondary fluorescence** produced by appropriate chemical treatment of the specimen. The autofluorescence of a tissue, which is due to various endogenous compounds, notably flavoproteins (Benson *et al.*, 1979), frequently interferes with the interpretation of secondary fluorescence.

1.2. STAINING AND HISTOCHEMISTRY

The histologist stains sections in order to facilitate the elucidation of structural details. The histochemist, on the other hand, seeks to determine the locations of known substances within the structural framework. The disciplines of histology and histochemistry overlap to a large extent, but one consequence of the two approaches is that the staining techniques used primarily for morphological purposes are sometimes poorly understood in chemical terms. Histochemists, not wanting to be thought of as mere dyers, frequently avoid calling their procedures "staining methods". Nonetheless, the science of histochemistry has contributed several techniques to the art of histology: it is obviously most desirable to be able to demonstrate a structural component by "staining" for a substance which it is known to contain. Conversely, there are many empirically derived histological techniques of immense value which do not depend upon well-understood chemical principles.

1.3. SOME PHYSICAL CONSIDERATIONS

The intelligent handling of microscopical preparations requires familiarity with the physical properties of several materials which are used in almost all techniques. All too often, the beginner will ruin a beautifully stained section by forgetting that two solvents are immiscible, or by leaving the slides overnight in a liquid which dissolves the coloured product. The following remarks relate mainly to sections mounted on slides, but are also applicable to blocks of tissue or to smears, films, wholemounts, or free-floating sections.

Water is completely miscible with the common alcohols (methanol, ethanol, isopropanol, methylated ethyl alcohol). Water is immiscible with xylene, benzene, chloroform, and most other clearing agents. These clearing agents are, however, miscible with the alcohols in the absence of water. Melted paraffin wax and the resinous mounting media (Canada balsam, Xam, Permount, DPX, etc.) are miscible with the clearing agents but not with the alcohols or with water. One mounting medium, euparal, is notable for being miscible with

absolute alcohol as well as with xylene. Because of these properties of the common solvents, a specimen must be passed through a **series** of liquids during the course of embedding, staining, and mounting for examination.

For example, a piece of tissue removed from an aqueous fixative, such as a formaldehyde solution, must pass through a **dehydrating agent** (such as alcohol) and a **clearing agent** (such as chloroform) before it can be infiltrated with paraffin wax. Ribbons of paraffin sections may be floated on warm water, which will remove wrinkles, when they are being mounted on glass slides. A thin layer of a suitable **adhesive** (see Chapter 4) may be interposed between the slide and the sections, but this is not always necessary. The slides must then be **dried** thoroughly in warm air before being placed in a **clearing agent**, usually xylene, to dissolve and remove the wax. The slides will now bear sections of tissue which are equilibrated with the clearing agent. Passage through alcohol (or any other solvent miscible with both xylene and water) must precede immersion of the slides in water. Sudden changes are avoided if possible, so a series of graded mixtures of alcohol with water is used. Most staining solutions and histochemical reagents are aqueous solutions. If a permanent mount in a resinous medium is required, the slides carrying the stained sections must be **dehydrated**, without unintentionally removing the stain, in alcohol or a similar solvent, **cleared** (usually in xylene), and, finally, **mounted** in the resinous medium.

The physical properties of some commonly used solvents are given in Chapter 4, where some practical guidance for their use will also be found.

Since resinous media are themselves dissolved in clearing agents, a mounted preparation will not be completely transparent for a few hours. The resin has to permeate the section and the solvent has to evaporate at the edges of the coverslip. When these events have taken place, the specimen will be equilibrated with the mounting medium and should have almost the same refractive index as the latter. Consequently, most of the observed contrast will be due to the staining method.

Frozen sections are collected into water or an aqueous solution. They may be affixed to slides and dried in the air either before or after staining. The frozen section on the slide is, therefore, at first equilibrated with water and must be dehydrated and cleared before mounting in a resinous medium. If the products of staining would dissolve in organic solvents, as is the case with the Sudan dyes and with the end-products of some histochemical reactions, it is necessary to use a water-miscible mounting medium. Several such media are available (e.g. glycerol jelly, Apathy's, Farrant's, polyvinylpyrrolidone) but they usually do not suppress the intrinsic refractility of the specimen as completely as do the resins.

Celloidin sections require special handling owing to the properties of the embedding medium. Nitrocellulose is soluble in a mixture of equal volumes of ethanol and diethyl ether, commonly called ether–alcohol. Celloidin is hardened by 70% alcohol, chloroform, or phenol. Absolute alcohol makes celloidin swell but does not dissolve it. Aqueous solutions penetrate freely through the matrix of nitrocellulose, so it is not necessary to remove the latter in order to stain the contained section of tissue. Molten paraffin wax can also permeate a nitrocellulose matrix without dissolving it. Specimens which are expected to be difficult to section are often infiltrated with celloidin, cleared, and then infiltrated with and embedded in wax. This procedure is known as **double embedding**.

More infrequently used embedding media are the water-soluble polyethylene glycol waxes and glycol methacrylate resins. The specialized techniques for the use of these substances are also based on simple physical principles, but will not be discussed here.

Many of the dyes used in histology can be removed from stained sections by alcohol. This property is useful for the extraction of excesses of dye, a process known as **differentiation**, but it can also be a nuisance. Since a stained preparation must be completely dehydrated as well as adequately differentiated, the timing and rate of passage through graded alcohols is often critical. It is one of the arts of histological technique to obtain the correct degree of differentiation.

1.4. PROPERTIES OF TISSUES

Freshly removed cells and tissues, especially those of animals, are chemically and physically

unstable. The treatments to which they are exposed in the making of microscopical preparations would damage them severely if they were not stabilized in some way. This stabilization is usually accomplished by **fixation**, which is discussed in Chapter 2. For some purposes, especially in enzyme histochemistry, it is necessary to use sections of unfixed tissues. As has already been stated, such sections may be cut with a cryostat or a vibrating microtome. Unfixed sections are stable when dried onto glass slides or coverslips but become labile again when wetted with aqueous liquids that do not produce fixation. Many histological staining methods do not work properly on unfixed tissues.

Most methods of fixation make the tissues harder than they were in the living state. Provided that it is not excessive, hardening is advantageous because it renders the tissues easier to cut into sections. However, some tissues such as bone are too hard to cut even before they have been fixed. These have to be softened after fixation but before dehydration, clearing, and embedding. Calcified tissues are softened by dissolving out the inorganic salts that make them hard, a procedure known as **decalcification** (see Chapter 3). Other hard substances such as cartilage, chitin, and wood require different treatments. Exceptionally robust microtomes equipped with massive chisel-like knives of tungsten steel have become available in recent years. These will cut sections of undecalcified bone and other hard tissues.

Even initially soft specimens sometimes become unduly hard by the time they are embedded in wax. These can be softened by cutting sections to expose the interior of the tissue at the face of the block and then immersing for a few hours in water. Although the solid wax is present in all the interstices of the tissue, materials such as collagen can still imbibe some water and be made much softer. Various proprietary "softening agents" are marketed for the same purpose, but they are, in my experience, no better than plain water. Another important factor in microtomy is the hardness of the embedding mass relative to that of the tissue. This is determined by the composition of the former and by the ambient temperature. Obviously, the proper use of the microtome is also necessary if satisfactory sections are to be cut.

1.5. BOOKS AND JOURNALS

There is a profusion of books, large and small, that give directions in practical microtechnique. Some of the more modern ones also briefly explain the rationales of the different methods described. The texts of Baker (1966), Humason (1972), Bradbury (1973), Culling (1974), Cook (1974), and Bancroft & Stevens (1977) can all be recommended, but there are many others equally valuable. Some of them will be cited in the following chapters. Books such as these should always be at hand for the practising microscopist. For histochemistry, the major treatise in English is that of Pearse (1968, 1972); other important ones are Barka & Anderson (1963), Thompson (1965), and Lillie & Fullmer (1976). In these works it is usually assumed that the reader is familiar with the chemical principles underlying the explanations of how the methods work.

Some journals are devoted largely to the publication of papers on methodology. The major ones are *Stain Technology*, the *Journal of Histochemistry and Cytochemistry*, *Histochemistry* (formerly *Histochemie*), the *Histochemical Journal* and the *Journal of Microscopy* (formerly *Journal of the Royal Microscopical Society*). Relevant papers appear in other journals too, but by scanning the ones listed above it is not difficult to keep up with the major advances in the field.

1.6. ON CARRYING OUT INSTRUCTIONS

THIS IS IMPORTANT. READ THIS SECTION BEFORE ATTEMPTING TO PERFORM ANY OF THE TECHNIQUES DESCRIBED IN LATER CHAPTERS. See also "Conventions and Abbreviations" for methods used to express concentrations of solutions and for the correct interpretation of such terms as "alcohol" and "water" and for guidance on accuracy of measurement of weight, volume, and temperature.

In this and other texts, practical schedules are given for many techniques. Since the number of methods described in this book is relatively small, it has been possible to be quite explicit, so that the methods should all work properly if the instructions

are followed exactly. There are, however, some general rules applicable to nearly all staining methods. These will therefore be given now in order to avoid tedious repetition in the following chapters.

1.6.1. De-waxing and hydration of paraffin sections

Place the slides (usually 4–12 of them) in a glass or stainless steel rack and immerse in a rectangular tank containing about 400 ml of xylene. This is the most useful size of tank for most purposes. Smaller ones are available, but when they are used their contents must be renewed more often. A "commercial" or "technical" grade of xylene (mixed isomers) is satisfactory. Agitate the rack, up and down and laterally, three or four times over the course of 2–3 min. If for some reason it is inconvenient to agitate the slides, they should be left to stand in the xylene for at least 5 min. A single slide is de-waxed by moving it slowly back and forth in the tank of xylene for 1 min. Individual slides should be held with stainless steel forceps.

Lift the rack (or individual slide) out of the xylene, shake it four or five times and touch it onto bibulous paper (three or four thicknesses of paper towel, or filter paper) and place in a second tank of xylene. Agitate as described above, but this time 1 min is long enough. The purpose of this second bath of xylene is to remove the wax-laden xylene from the initial bath, thereby reducing the risk of precipitation of wax upon the sections when they are passed into alcohol, in which wax is insoluble. The removal of excess fluid by shaking and blotting is very important and is done every time a rack or slide is passed from one tank to another. If it is not done, the useful life of each tankful of xylene or alcohol will be greatly shortened. The instruction "drain slides" refers to this shaking-off of easily removed excess liquid.

After the second bath of xylene, drain the rack of slides and place in a tank containing about 400 ml of absolute ethanol. Agitate at intervals of 10–20 s for 1½–2½ min. Drain slides and transfer to 95% ethanol and agitate in this for about 1 min. Drain slides and transfer to 70% alcohol. Agitate for at least 1 min. For an individual slide, it is sufficient to move it about with forceps for about 20 s in each change of alcohol. If the slides have to be left for several hours, or even for a few days, they should be immersed in 70% alcohol. This will prevent the growth of fungi and bacteria on the sections but will not make them come off the slides or become unduly brittle. If some sections do detach from the slides during de-waxing or hydration, more will certainly be lost in later processing. If attachment of the sections appears to be precarious, a film of nitrocellulose should be applied, as described in Chapter 4.

Hydration of the sections is completed by lifting the rack (or individual slide) out of the 70% alcohol, draining it, and immersing in water. Agitation for at least 30 s is necessary for removal of the alcohol. Without agitation, this takes 2–3 min. A second rinse in water is desirable if all traces of alcohol are to be removed.

It is possible to use small volumes of xylene and alcohol by carrying out the above operations in coplin jars (which usually hold up to five slides) or rectangular staining dishes (usually for 8–12 slides). The xylenes and alcohols are poured into these vessels and the slides agitated continuously with forceps. To change the liquid, pour it out (without losing the slides) and replace it with the next one in the series. Working in this way, each lot of xylene or alcohol should be used only once. When tanks holding 400 ml are used, the liquids can be used repeatedly. They should all be renewed when traces of white sludge (precipitated wax) appear in the absolute ethanol. This commonly occurs after 10–12 racks of slides have been de-waxed and hydrated. In order to minimize evaporation, contamination by water vapour and the risk of fire, all tanks containing alcohol or xylene should have their lids on when not in use.

1.6.2. Staining

An instruction such as "stain for 5 min" means that the sections must be in intimate contact with the dye solution for the length of time stated. Slides (alone, or in racks, coplin jars or staining dishes, as convenient) are immersed in the solution, agitated for about 10 s, and then left undisturbed. Free-

floating frozen sections are transferred to cavity-blocks, watch-glasses, or the wells of a haemagglutination tray containing the staining solution. Folds and creases in such sections must be straightened out if uniform penetration of the dye is to occur. The best instrument for handling frozen sections is a glass hook or "hockey stick", fashioned by drawing out a piece of glass rod in the flame of a Bunsen burner. Sections of nitrocellulose-embedded material, if not affixed to slides, are manipulated in the same way as frozen sections.

In some techniques of enzyme histochemistry and immunocytochemistry, only one drop of a scarce or costly reagent can be applied to each section. This is done with the slide lying horizontally. The slide bearing the section, covered by the drop, is placed on wet filter paper in a closed petri dish: the drop will not evaporate if the air above is saturated with water vapour. Horizontal slides are also used in staining methods for blood films. In this case the reagents are not expensive, so the slides are placed, film upwards, on a pair of glass rods over a sink. The staining solution is poured on to flood the slides and later washed off by a stream of water or of a suitable buffer.

Since sections of tissue take up only minute quantities of dyes and other substances, there is no need for the volume of staining solution or histochemical reagent to be any greater than that required to cover the sections. Exceptions to this general rule are rare and are mentioned in the instructions for the methods concerned.

1.6.3. Washing and rinsing

The excess of unbound dye or other reagent is removed from the stained sections by washing or rinsing, usually with water. A "wash" is a more prolonged and vigorous treatment than a "rinse". Agitation of slides for a minute or more in each of three changes of water constitutes an adequate wash. When tap water is suitable, the slides are placed for about 3 min in a tank through which the water is running quickly enough to produce obvious turbulence. A rack of slides should be lifted out of the running tap water and then replaced every 20–30 s in order to ensure that all the slides are thoroughly washed. A rinse, rather than a wash, is prescribed when excessive exposure to water would remove some of the dye specifically bound to the sections. Rinsing is done in the same way as washing, but the slides are agitated continuously and the total time of exposure to water is only about 15 s.

With unmounted frozen or nitrocellulose sections, it is more difficult to control the process of washing. The sections are carried through three successive baths (50 ml beakers are convenient) of water and are kept in constant motion for 20–40 s in each. Free-floating sections should not be allowed to fold or to crumple into little balls. Stains that are easily extracted by water should not be applied to sections of this kind.

1.6.4. Dehydration and clearing

For stains or histochemical end-products insoluble in water and alcohol, dehydration is a simple matter. The slides are agitated continuously for about 1 min in each of the following: 70% alcohol, 95% alcohol; two changes of absolute ethanol, methanol, or isopropanol. There is no objection to taking them straight into 100% alcohol (in which case, three or four changes will be needed), but the use of lower alcohols will protect the more expensive anhydrous liquids from excessive contamination with water. **Slides must be drained** (see Section 1.6.1) as they are transferred from one tank to the next. Clearing is accomplished by passing the slides from absolute alcohol into xylene (two changes, 1 min with agitation in each). They may remain in the last change of xylene for several days if mounting in a resinous medium cannot be carried out immediately. Used as described the alcohols and xylenes, in 400 ml tanks, can be used repeatedly for 10–12 racks of slides. The contents of all the tanks must be renewed when the xylene becomes faintly turbid.

Many dyes are extracted by alcohol, especially if water is also present. When this is the case, the instruction will be to "dehydrate rapidly". For rapid dehydration, drain off as much water as possible and then transfer the slides directly to absolute alcohol. Agitate very vigorously for 5–10 s in each of three tanks of this liquid, draining between

changes, and then clear in xylene as described above. The alcohol used for rapid dehydration should be renewed after processing four racks of slides. It is not possible to dehydrate free-floating sections rapidly. These should be mounted onto slides after washing, allowed to dry in the air, and then passed quickly through three changes of absolute alcohol into xylene.

Some cationic dyes are much less soluble in *n*-butanol than in lower alcohols. An alternative method of dehydration consists of blotting of the slides between three thicknesses of filter paper and then immersing them (using a clean, dry staining rack) in *n*-butanol, two changes, each 5 min with agitation once or twice in each. Resinous mounting media are miscible with *n*-butanol, but clearing in xylene allows optical clarity to develop more rapidly.

1.6.5. Understanding the methods

There is a reason for everything that is done in the making of a microscopical preparation. Before trying out a technique for the first time, the student should read about and understand the underlying physics and chemistry. He should then read through all the practical instructions and make sure that he understands the purpose of every stage of the procedure.

Some technical methods can be learned only by practice. These include the cutting of sections, the mounting of sections onto slides, and the application of coverslips to sections. In this book, no attempt is made to teach these skills, which must be acquired under the guidance of more experienced colleagues. Excellent descriptions of these procedures are to be found in the works of Krajian & Gradwohl (1952), Culling (1974), Gabe (1976), and Bancroft & Stevens (1977), but there is no substitute for practice in the laboratory. Success in microtechnique requires the integration of craftsmanship with intelligent appreciation of scientific principles.

1.7. EXERCISES

Theoretical

1. What type of preparation would you make in order to investigate the populations of cells in (a) the mesentery of the rat, (b) the articular cartilage of the head of the human femur?

2. A frozen section of skin is stained with an oil-soluble dye in order to demonstrate lipids. What would be an appropriate procedure for making a permanent microscopic preparation of the stained section?

3. If alcohol that has been used to dehydrate a fairly large piece of animal tissue is mixed with water, the resulting liquid is turbid. Why?

4. Some staining methods are applied to whole blocks of tissue which are subsequently embedded, sectioned, cleared, and mounted. To what artifacts would this type of technique be especially prone? How could the artifacts be minimized?

5. It is sometimes necessary to remove the coverslip from a section mounted in a resinous medium. Devise a reasonable procedure by which such a section could be made ready to stain in an aqueous solution of a dye.

Practical

6. Kill a rat (by overdosage with ether vapour) and make spreads of its mesentery. Stain the spreads with polychrome methylene blue (Chapter 6) and make permanent mounts in a resinous medium. Mount a stained spread from water into an aqueous mounting medium. What happens? Deliberately pass one of the slides too quickly through the dehydrating and clearing agents and observe the consequences of doing this.

7. Stain a spread of mesentery with Sudan IV or Sudan black B (Chapter 12) and make a permanent preparation. The Sudan dyes colour lipids by dissolving in them.

2

Fixation

It is not sufficient for the histologist that a specimen be transparent and that it possess adequate optical contrast. The cells and extracellular materials must be preserved in such a way that there has been as little alteration as possible to the structure and chemical composition of the living tissue. Such preservation is the object of **fixation**. Without being spatially displaced, the structural proteins and other constituents of the tissue must be rendered insoluble in all of the reagents to which they will subsequently be exposed. "Perfect" fixation is, of course, theoretically and practically impossible to attain.

Biological material may be fixed in many ways and some of these will now be discussed.

2.1. PHYSICAL METHODS OF FIXATION

The simplest physical method is the application of **heat**. This results in the coagulation of proteins and the melting of lipids. The resemblance to the living state is not very close after such treatment, but the method is often used in diagnostic microbiology. The shapes and staining properties of bacteria and some other micro-organisms are preserved sufficiently well to permit identification. Heating of large specimens is accomplished by immersion in boiling fixative solution or by cooking in a microwave oven.

Animal tissues are sometimes processed by the techniques known as **freeze-drying** and **freeze substitution**, though only the former is purely physical in nature.

For freeze-drying, the piece of tissue, which should be no more than 2 mm thick, is frozen as quickly as possible (usually by immersion in isopentane, cooled by liquid nitrogen) and then transferred to a special chamber. Here the tissue is maintained *in vacuo* at about −40°C until all the ice it contains has sublimed and has been condensed in a vapour trap maintained at a yet lower temperature. Alternatively, the water vapour may be absorbed into a tray of phosphorus pentoxide. The dried specimen may then be infiltrated with paraffin wax and sectioned in the usual way, though the sections cannot be flattened on water. Freeze-drying does not insolubilize proteins, so it is not, strictly speaking, a method of fixation. However, the morphological preservation is sometimes excellent, and water-soluble substances of low molecular weight are not lost. A freeze-dried block may be chemically fixed in a suitable gas, such as formaldehyde, thus combining the advantages of the two procedures.

In freeze substitution the frozen specimen is dehydrated by leaving it in a liquid dehydrating agent, usually ethanol or acetone, at a temperature

below −40°C. The liquid dissolves the ice but does not, at the low temperature, coagulate proteins. When dehydration is complete, the temperature is raised to 4°C for a few hours in order to allow chemical fixation by the alcohol or acetone to take place. The block is then cleared and embedded in paraffin. Sections of unfixed tissue, cut on the cryostat, can be freeze substituted much more quickly than blocks. The relative merits of freeze-drying and freeze substitution are discussed in great detail, together with many technical aspects not mentioned above, by Pearse (1968).

2.2. CHEMICAL METHODS OF FIXATION

For most histological and histochemical purposes, liquid fixatives are used. These substances affect the tissues both physically and chemically. The principal physical changes produced are shrinkage or swelling, and many of the fixatives in common use are mixtures of different agents, formulated so as to balance these two undesirable effects. Most fixatives harden tissues. Moderate hardening is desirable for sectioning with a freezing microtome, or if embedding is to be in nitrocellulose, but can lead to difficulty in cutting wax-embedded material. Dehydration and infiltration with paraffin always produce some further shrinkage and hardening, whatever may be the state of the tissue when it comes out of the fixative. The volume of a fixed, paraffin-embedded specimen is commonly 60–70% of what it was in life. Another important property of a chemical fixative is its rate of penetration. This rate will determine the duration of fixation and the maximum permissible size of the specimen. These physical aspects are reviewed by Baker (1958). Many chemical reactions are involved in fixation, and some of these will now be described. The chemistry of fixation has been reviewed by Baker (1958), Pearse (1968), and Hopwood (1969).

2.3. GENERAL PROPERTIES OF FIXATIVES

The structure of a tissue is determined largely by the configuration of its contained proteins, especially the lipoproteins, which are major components of the plasmalemmae and membranous organelles of cells, the fibrous glycoproteins of such extracellular elements as collagen and basement membranes, and the globular proteins which are dissolved in the cytoplasm and extracellular fluid. In some tissues, extracellular mucosubstances (e.g. chondroitin sulphates) also contribute substantially to the local architecture. All of these substances must be stabilized by fixation. The nucleic acids and their associated nucleoproteins should also be preserved, as should the macromolecular carbohydrates (mucosubstances: comprising glycoproteins, proteoglycans, and at least one polysaccharide—glycogen) and, if their histochemical demonstration is required, the lipids. Fortunately, most of the commonly employed fixatives render insoluble the proteins, nucleic acids, and mucosubstances, though some may be more completely preserved than others by particular agents. Many fixatives do not directly affect lipids and the preservation of these substances depends largely upon the avoidance of agents which dissolve them.

2.3.1. Physical considerations

The rate of **penetration** will determine the size of a block to be fixed by immersion. Rapidly penetrating agents will usually fix in 24 h a specimen whose least dimension is 3–5 mm. For slowly penetrating fixatives the thickness of the block should not exceed 2 mm. The **duration** of fixation should not exceed 24 h except in the case of formaldehyde which takes a week to cause full stabilization of histological structure. Distortion due to slow penetration can be offset by perfusion of the fixative through blood-vessels or by injection into thin-walled cavities. **Shrinkage and swelling** are not, *per se*, detrimental to the quality of fixation, but must be allowed for in quantitative work. The overall change in size is easily determined by measuring appropriate dimensions of the fresh specimen and of the stained, mounted sections. It must not be assumed, however, that all the components of an organ or tissue will shrink or swell equally. Empty spaces due to unequal shrinkage of cells or larger regions of tissues are common artifacts, especially