J D BANCROFT

HISTOCHEMICAL TECHNIQUES

SECOND EDITION

BUTTERWORTHS

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2nd Edition

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FOREWORD

Histochemistry continues to play an increasingly important role in its application to the Biological Sciences. Its technology, increasing in volume and to some extent in complexity, is handled in this volume essentially in the light of practicability.

The author has had long experience of the techniques of which he writes and he speaks with the authority conferred by this experience.

I have no doubt that this worthy successor to the first edition, will be equally acceptable and, as is proper, of greatly enhanced value to its users.

A. G. Everson Pearse

PREFACE

In the time since the First Edition of this book was published, the field of histochemistry has expanded considerably and many more reliable histochemical methods are now available. The best and most useful of these methods I have included in this Second Edition, and I have omitted a few methods which I included in the First Edition in a rush of youthful enthusiasm, but which I have subsequently found unreliable or technically difficult.

Dr Alan Stevens has written an introductory chapter discussing the problems and values of histochemistry, and the chapter on carbohydrates has been increased in size to incorporate new concepts and methods in carbohydrate histochemistry. New chapters include a separate and distinct chapter on amyloid, in which there has been a considerable increase in knowledge in the last seven years, and a new chapter in which the theoretical concepts in enyzme histochemistry are discussed. The final chapter is an introduction to a fast-expanding facet of histochemistry, namely ultra-histochemistry, the application of histochemistry to electron microscopy.

In the production of this edition I am indebted to Alan Stevens who read the entire manuscript and played an important role in its revision; to Professor A. G. E. Pearse for his Foreword; and to Professor I. M. P. Dawson for his encouragement and tolerance. For secretarial assistance thanks are due to Miss Colleen Peel and to my wife Stella.

J. D. Bancroft

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Introduction

by A. Stevens

Histochemical techniques enable the identification and localization of specific substances within tissues. The methods depend on chemical reactions between the substance to be identified and localized in a tissue section, and one or more reagents in which the tissue section is incubated. The histochemist tries to arrange matters so that the end product of the chemical reaction is both coloured and insoluble, and therefore easily visible on microscopy. Histochemical methods such as the PAS reaction and the Perls' Prussian Blue reaction are well known to histopathologists, but until recently the scope of histochemistry has been limited by one big problem. To cut thin sections of tissue it is necessary for it to be made rigid and to be embedded in a firm supporting medium; the embedding medium used in routine histopathology is paraffin wax. The process of embedding the tissue is a violent one, involving the immersion of the tissue in hot molten wax; it is essential, to minimize the distortion and disruption of the tissue during processing, that the tissue is adequately fixed beforehand. Furthermore the tissue must be completely dehydrated with alcohol and finally infiltrated with an organic solvent (usually toluene) with which the embedding wax is totally miscible. The major limiting factor in histochemistry is that all these processes, so essential for the successful cutting of thin sections, inevitably chemically modify or completely destroy or remove the very substances we are attempting to demonstrate. Lipids in the tissue are dissolved out by the alcohols and xylene used in dehydration and clearing of the tissue. Many tissue enzymes are destroyed by the fixative and by the heat necessary in the embedding of the tissue in wax, and the accurate intracytoplasmic

localization of soluble substances such as glycogen is impossible in tissue exposed to aqueous fixatives.

TABLE 1.1

Different methods of tissue processing and their application to histochemical procedures

Processing method	*Histochemical method*
(1) Routine paraffin processing	Carbohydrates Proteins Pigments
(2) Special paraffin processing	Proteins Hydrolytic enzymes (if no other method available)
(3) Freeze drying	Carbohydrates Proteins
(4) Freeze drying formol-vapour fixation	Carbohydrates Proteins Fluorescence techniques
(5) Freeze drying with double embedding	Hydrolytic enzymes Proteins Carbohydrates
(6) Frozen sections formalin-fixed	Fat stains Amyloid stains
(7) Cryostat sections fresh unfixed	Oxidative enzymes Hydrolytic enzymes Almost all techniques after suitable fixation
(8) Cryostat sections from fixed block	Hydrolytic enzymes

Histochemical techniques in some cases may be applied to sections produced by different methods than recommended above. The above Table is included as a guide.

Tissue to be investigated histochemically must therefore be processed in an entirely different manner to tissue for normal histological stains, although certain very stable substances can be demonstrated by histochemical means in paraffin sections. These are discussed in Chapter 2. For the majority of histochemical reactions, however, an entirely new method of tissue processing and section cutting has had to be devised, and most of the improvements in histochemistry have only emerged in recent years after the development of satisfactory methods of tissue preparation. The most important problem to overcome was that of producing high quality thin sections of tissue without the use of a supporting medium such as paraffin wax, and without having to subject the tissue to the necessary pre-treatment with organic solvents and heat implicit in normal tissue embedding. In normal histology the tissue is partly protected from the ill-effects of such treatment by the process known as fixation. For a histochemist's purposes however the protection offered by fixing the tissue is totally inadequate. As we have seen, the very process of fixing the tissue, while it preserves to a certain extent the architectural integrity of the tissue, inevitably destroys many of the substances which the histochemist wishes to demonstrate.

The solution to this problem lay in the development of techniques for producing thin sections from a piece of tissue which has been frozen hard. The intrinsic water content of the tissue, when frozen, acts as a very suitable supporting medium for the tissue and renders the tissue rigid enough for thin sections to be cut from it using specialized microtomes such as the freezing microtome and cryostat. Although the formation of ice within the tissue when it is frozen, and the subsequent dissolution of the ice when the section is thawed, may lead to some architectural distortion and other artefact, the substances in which the histochemist is interested remain largely unaltered and are capable of being demonstrated by histochemical reactions. Sections produced in this way contain the full complement of substances, and the tissue has been exposed to no damaging agent other than a low temperature; this seems to matter very little from a practical point of view. The ability to consistently produce high quality thin frozen sections, using both a freezing microtome and a cryostat, is the first requirement of a technologist involved in histochemical work; the procedures are dealt with in the relevant chapters later in this book.

It can be seen that the tissues sectioned in this way do not need the protection offered by preceding fixation. However this protective effect is not the only function of fixatives; they are also important in fixing the chemical substances in place within the cell cytoplasm and preventing diffusion (therefore facilitating accurate localization), and also may act as mordants, enhancing the staining characteristics of certain substances and structures. In certain circumstances fixation has an important part to play in histochemistry, although it is avoided completely if any of the oxidative enzymes are to be demonstrated. Paradoxically, accurate localization of sites of hydrolytic enzyme activity is considerably enhanced if the tissue has been properly fixed; fixation reduces the amount of diffusion of the enzyme which inevitably occurs when the frozen section thaws. Although the process of fixation reduces by a small amount the quantity of demonstrable hydrolytic enzyme present, this is of no consequence practically and the improved localization makes fixation well worthwhile. The most widely used fixative for histochemical purposes is formol calcium, fixation usually being carried out at 4°C. Although fixation is valuable in the demonstration of hydrolytic enzymes, it must be for a limited time only (usually about 12 hours). The longer the tissue is kept in fixative, the more appreciable will be the loss of demonstrable enzyme activity.

Two more sophisticated methods based on the freezing of tissue are *freeze drying* and *freeze substitution*. In freeze drying, the tissue is frozen, and the water (in the form of ice) is completely removed in

conditions of partial vacuum and at sub-zero temperatures. In freeze substitution the water in the tissue is replaced by an organic solvent, again at sub-zero temperatures, but at normal atmospheric pressure. These two techniques, although they require special equipment and some expertise, are most valuable in the histochemical identification and localization of carbohydrates and proteins. Freeze-dried tissue blocks are best fixed in formalin vapour before embedding and sectioning; in freeze substitution fixation can be effected by incorporating a fixative such as picric acid in the substituting fluid.

Now that the histochemist is equipped with adequate tools for his job, the number of substances for which histochemical methods have been devised is increasing rapidly. Adequate histochemical methods now exist for the demonstration of carbohydrates, proteins, lipids, nucleic acids, pigments, amines and a very wide range of enzymes. However, we must accept that most of the histochemical reactions which we use are embarrassingly non-specific and that we are unable to identify substances with the accuracy of the biochemist with the reagents at our disposal. At this juncture it is perhaps relevant to mention the very important part which the chemical manufacturing industry, and a handful of firms in particular, have played in the development of histochemistry. They have continually striven to produce reagents, and particularly enzyme substrates, of such purity and specificity that there is now some hope of increasing the specificity of our histochemical reactions. Histochemists have used considerable ingenuity in devising methods to increase the specificity of their reactions, and the use of such techniques as specific enzyme inhibition has led to more accurate identification of certain enzymes. A good example of this is in the elucidation of the various types of 'esterase'.

A very recent development in histochemistry has been the application of a limited number of histochemical methods (mainly involving the demonstration of enzymes) to blocks of tissue which are subsequently to be processed for electron microscopy. In this way we can study the ultrastructural localization of enzyme activity within cell organelles.

Histochemistry is fast gaining respectability; it has long been used as a research tool in all branches of the biological sciences, and is now being increasingly used in diagnostic histopathology. If we wish this to continue we must ensure that any new methods are simple enough to be performed in a routine laboratory and that they are consistently reproducible.

2

Fixation

The requirements of a fixative vary, depending upon whether the tissue is to be used for the demonstration of enzymes, or for other purposes. The main functions of a fixative are:—

(1) to preserve the tissue
(2) to prevent diffusion
(3) to protect the tissue from subsequent treatment.

For tissue that is processed for paraffin wax embedding the following factors are also relevant.

(1) The hardening of the tissue by the fixative
(2) The rate of penetration of the fixative
(3) The conversion of semi-liquids—gels—to semi-solids
(4) The effect on staining reactions.

PRESERVATION OF THE TISSUE

The first consideration of fixation must be to preserve the tissue as near as possible as it was in life, whilst at the same time not altering the chemical composition or localization of the constituents of the tissue. This with present day fixatives is not wholly possible; however, thought must be given to what is required of the tissue before a means of fixation is chosen.

Tissue on removal from the body, if left in the air, will dry, shrink and undergo bacteriological changes and autolysis (chemical reactions taking place by the enzymes within the tissue cells). If the tissue is placed in water, the tissue swells rapidly and is unrecognizable. Upon death, autolysis and putrefaction (the tissue being affected by bacteria from an outside source) take place. To avoid these changes, fixation must be carried out as rapidly as possible

if the tissue is to be preserved in a state comparable with that in life. The preserving fluid (fixative) must not cause swelling of the tissue, it must stop chemical reactions taking place with the autolytic enzymes, whilst not removing all other enzymes from the tissue. The preservative must also cause as little shrinkage of the tissue as possible.

Preservation by freezing

Tissues frozen rapidly to temperatures of −70°C and below are well preserved. There is no major loss of enzyme activity and diffusion is prevented. The chemically reactive constituents remain unaltered until the tissue is thawed.

Prevention of diffusion or loss of substance

For the majority of histochemical procedures, it is necessary that the substances to be demonstrated are in their true position. Despite all the fixatives to hand today, this is not always possible. In some instances, the substances are affected and moved by the fixative itself, e.g. the diffusion artefact shown in glycogen. It is possible to avoid this effect with glycogen by using freeze drying, followed by formalin vapour fixation. In other cases, the fixative by its action on cell constituents will render the substances insoluble to other reagents and hence demonstrable by histochemical techniques. The correct choice of a fixative is vital. Formaldehyde does not act upon lipids, and is therefore ideal for their demonstration, whereas any of the many alcohol-containing fixatives cannot be used, because of the extraction of lipid material by the alcohol.

Protecting the tissue from subsequent treatments

After suitable fixation, the tissue is generally processed either through to paraffin wax or as an alternative, it is quenched and frozen sections are cut. In both of these procedures, the tissue is subjected to fluids or actions (thawing) which will cause loss of substances in the first technique, or diffusion of substances in both techniques. These can be overcome to a certain degree by the correct fixation at the start of the process for paraffin-embedded material, and by fixation before freezing when demonstrating lysosomal enzymes.

Hardening of material for paraffin processing

To obtain a suitable consistency for cutting paraffin sections, the tissue must be hardened by the fixative. All the fixatives in routine use will harden the tissue. The problem is that some will overharden

the material, if it is left in too long. Hardening also allows very soft and friable tissues to be handled without undue damage during the treatment before embedding.

Rate of penetration of fixative

Before a tissue can be considered fixed, the fixative must have fully penetrated it. The rate of penetration varies with different fixatives; formaldehyde, for instance, has a rapid rate of penetration compared with picric acid, which penetrates at a moderate rate. Osmium tetroxide has a slower rate than either. The faster the rate of penetration of a fixative the better, as autolytic changes may still occur in the centre of a specimen despite it being in a fixative. The question of penetration is more complicated in the case of compound fixatives. Here, if one agent causes swelling, another usually causes shrinkage, so that uneven fixation sometimes results.

Effect of fixation on staining reactions

The choice of fixative usually depends upon three factors: (1) which stain or reaction is to be applied to the section; (2) whether there is any urgency; and (3) the size of the piece of tissue.

If the reason for processing the tissue is to demonstrate glycogen, then cold Bouin's or Gendre's would be used. However, if the Feulgen reaction was to be applied, Bouin's would not be used, as it causes overhydrolysis (see Feulgen reaction).

Some fixatives act as a mordant, a constituent of the fixative making a chemical linkage between the dye and the tissue. When this type of fixative is used, more specific staining occurs. An example of this is the use of mercury in a fixative, which gives the tissue an affinity for trichrome stains.

Freezing instead of fixing

For some histochemical procedures, it is necessary to use unfixed material. When this is required, the tissue is rapidly frozen on removal from the body. The freezing of the tissue preserves it by stopping the autolytic enzymes from reacting and, whilst the tissue remains frozen, it remains preserved. On thawing, the tissue must be placed in a fixative. As well as stopping the chemical reactions, freezing lso brings the tissue to a solid state, the ice in the tissue acting as the embedding medium when the sections are cut.

The preservation of enzymes is difficult. The optimal method for hydrolytic enzymes is fixation in formol calcium or formol saline at 4°C. The enzymes are less affected by the fixatives at low temperatures, and little loss of enzyme activity occurs. After this fixation,

the tissue is placed in gum sucrose for 24 hours. The blocks are then blotted dry and frozen. Sections are cut in a cryostat and the histochemical method applied. The prefixation of the block stops the diffusion of the enzymes from their true localization when the section is thawed before incubating.

GENERAL CONSIDERATIONS

The fixation of proteins also needs careful consideration, as the majority of fixatives react with protein groupings. Some fixatives precipitate proteins, e.g. alcohol, while others undergo chemical reactions with proteins, e.g. formaldehyde.

Fixatives produce the best results when used at temperatures just above freezing point. For obvious reasons, however, this is not possible in routine histology laboratories.

The size of the piece of tissue to be fixed should be as small as possible (a thickness of 0·5 cm is ideal for routine use). As the fixative penetrates rapidly, the time of fixation can be kept to a minimum. To obtain rapid fixation, the tissue should be covered by 10 to 20 times its own volume of fixative. Fixatives as a general rule tend to be acid, so for histochemical methods it is usually necessary to adjust the pH to neutrality.

FIXING SOLUTIONS

Formaldehyde

This is the most popular fixing agent in histology. It may be used on its own or in other compound fixatives. Formaldehyde, which is a gas, is normally available as a 40 per cent solution in water. When solutions of formalin are prepared, this 40 per cent solution must be taken as 100 per cent, e.g. to prepare a 10 per cent solution of formalin, 10 parts of 40 per cent formaldehyde are added to 90 parts of water.

Solutions of formalin are invariably acid, due to the formation of small amounts of formic acid in the formaldehyde. For all histochemical, and the majority of histological, techniques it is necessary to bring the pH of formalin to neutral. This can be done by employing either a buffer (sodium phosphate), or by placing calcium carbonate at the bottom of the container holding the formaldehyde solution. Fixation in acid formalin over a lengthy period produces formalin pigment, especially in blood-containing tissues (*see* page 182). If the tissue has been fixed in acid formalin over a long period of time, difficulty may be encountered in making acid dyes such as eosin stain correctly.

Formaldehyde will not fix lipids, nor will it remove them, which allows for their demonstration. The following formaldehyde solutions are amongst those used in histochemistry and histology:

(1) 10 per cent formol saline
(2) 10 per cent formalin (aqueous)
(3) 10 per cent neutral formalin
(4) 10 per cent formol calcium
(5) 10 per cent formalin in alcohol
(6) 10 per cent formol sucrose
(7) Formaldehyde vapour.

As in routine histology, formaldehyde is probably the most used fixative in histochemistry. It is an ideal fixative for tissue in which lipids are to be demonstrated, as lipids are chemically unaltered by formaldehyde fixation. Phospholipids are well preserved by formaldehyde fixation when calcium has been added to the fixative. In enzyme histochemistry, the majority of hydrolytic enzymes are well preserved with fixation in formol calcium at 4°C. The amount of enzyme activity is reduced if the fixation time is extended or if it is carried out at room temperature. Formaldehyde vapour is recommended for the fixation of freeze dried tissues and produces excellent demonstration of mucosubstances including glycogen (*see Plate 1*) and proteins. Nucleic acids are also well preserved after vapour fixation.

(N.B. The formulae for these solutions, and other compound fixatives that contain formaldehyde, will be found at the end of this chapter.)

Other aldehyde fixatives

Other aldehydes, besides formaldehyde, may be used as fixatives. They are especially useful when enzyme histochemistry is followed by electron microscopy. Sabatini, Bensch and Barrnett (1963) produced a paper dealing with nine different aldehydes. They buffered the fixatives to between pH 5·5 and 7·6. The hydrolytic enzymes worked satisfactorily after most of the fixatives, as did NADH and NADPH diaphorases. Flitney (1966), using seven different aldehydes (*see* Table 2.1), investigated the rate of fixation of these fixatives with albumen, and their effect on enzyme activity in cryostat sections. In regard to the fixation of cryostat sections, he found that aldehydes which fixed the albumen most rapidly also destroyed most enzyme activity. This could be overcome, however, by using these fast acting aldehyde fixatives for very short times, i.e. for an 8 micron cryostat section, 30 seconds to 1 minute was usually sufficient. The two aldehydes that gave good fixation

with reasonable morphological preservation and with only a small amount of enzyme loss were glutaraldehyde and acrolein when used for 60 seconds. These aldehydes have not gained popularity as

TABLE 2.1

Aldehyde fixatives

Aldehyde	%	*Buffer*	*Final* pH
Acrolein	10		6·8–7·2
Glutaraldehyde	6		7·2
Crotonaldehyde	10		7·4
Formaldehyde	10	Phosphate buffer	7·2–7·4
Hydroxyadipaldehyde ..	12		7·4
Acetaldehyde	10		7·2–7·4
Glyoxal	4		6·8–7·2

block fixatives because of the time taken to penetrate to the centre of the tissue. The advantage to be gained by short fixation is lost.

Alcohol

This is rarely used on its own in histology because of the damage it causes by shrinkage and excessive hardening of the block. It is, however, used more often in histochemistry, especially for fixation of cryostat sections, to demonstrate enzymes. Alcohol is a good fixative as the enzymes are almost unaffected by cold alcohol (4°C) with the exception of the esterases. For the demonstration of glycogen, alcohol is occasionally used as an 80 per cent solution. This, while being an ideal fixative for glycogen, has drawbacks from a morphological point of view. When used in compound fixatives, however, alcohol will give acceptable results. It will precipitate proteins, and will remove lipids. It also makes the freezing of tissue and the subsequent sectioning difficult. The following are the more common fixatives which contain alcohol.

(1) Formol alcohol
(2) Carnoy
(3) Clarke's
(4) Gendre's fluid
(5) Acetic alcohol formalin (AAF)
(6) Wolman's fixative.

(The formulae for the above fixatives can be found at the end of this chapter.)

Osmium tetroxide

Osmic acid finds little use in routine histology and histochemistry, with the exception of the demonstration of some lipid-containing structures, although it is in constant use in electron microscopy where it is used in a 1 per cent buffered solution.