PRACTICAL HISTOCHEMISTRY

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INTRODUCTION

RATIONALE OF THIS BOOK

Over the past ten years several hundred academic and applied biological and medical scientists have visited us to ask how they could apply histochemistry to their particular studies. Often their questions were purely technical: they wanted to know how to stain for a particular enzyme or substance. They saw histochemistry as an extension of histology and they wanted a simple, well established recipe; they did not want to go into the minutiae of the science underlying the staining method any more than they did when applying special stains in histology. For such enquirers we saw our task as twofold: we had to give them a reliable technique that they could follow as simply as a routine histological method but it had to be a method which, as far as we could ascertain, had a real scientific basis. Other workers came with problems in tissue metabolism and chemical dysfunction. For these, histochemistry was an extension of biochemistry. Its special advantages were that it allowed them to relate a specific activity to a particular tissue component and it was a form of biochemistry that could be done on relatively minute pieces of tissue such as could be removed safely by biopsy, or could be kept either in maintenance or proliferative culture. Some of these queries related to the mode of action of drugs, or of hormones, or of potentially toxic food additives; others were concerned with the biochemistry of disease. Such workers were primarily concerned with metabolic biochemistry; but they too did not want to delve into the minutiae of the many variants that can be found for almost every histochemical reaction (and which are discussed in detail in the specialist text-books of histochemistry such as those by Pearse and by Barka and Anderson).

Hence, over the years, we have tested a range of histochemical reactions so as to provide simple techniques which, if followed precisely, will yield reactions where the reactive compounds or enzymes are present; which will give a measure of the degree of activity actually shown in the tissue at the time of its removal; which can also indicate the total activity of which the tissue could be capable; but all of which have a rational basis. These methods were collected in a laboratory manual and they have been used by the many visitors to our laboratory. They have now been put together, in rather more detail, first of all for the many visitors who have complained that they could not copy out all the manual, and then for wider use by whoever

wishes to apply histochemistry in his studies, whether as an extension of histology or of biochemistry. **No attempt has been made to include techniques of electron histochemistry. This is a special field of its own and is completely outside these terms of reference.** The main criterion we have used in selecting these methods is their reliability (for a more complete review of all methods which have been described, the reader must be referred to the comprehensive books on histochemistry); provided the instructions are followed, and provided the tissue contains the substance or enzyme to be tested, then you should obtain a visible reaction, the intensity of which should indicate the effective concentration or activity of the substance tested. We do not guarantee that these methods will give the most 'beautiful' stained preparations (although we believe that they often will); we have aimed at providing methods which will give the most beautiful preparations which are also rational and scientifically meaningful, should such meaning become required. For this is one of the hazards of histochemistry: initially a method may be selected deliberately and solely because it yields an excellent histological stain but there seem to be few histochemists who can then resist the temptation to interpret tissue metabolism in the lurid light of such staining.

WHAT IS STAINED IN HISTOCHEMISTRY?

Histochemical staining can answer two questions: it can tell you what is the gross chemical composition of cells or of a tissue, or it can tell you what is the degree of activity of the cells or tissue studied. Before launching into the methods which in fact answer these questions, it is worth considering what each of these questions implies, and consequently how the results obtained by the methods may be interpreted.

(*i*) **Histological histochemistry.** To understand the advantages of this type of histochemical enquiry, we will consider two cases which were presented to us. Pathologists were concerned over the diagnosis of a tumour in the neck of a patient. On histological examination the tissue seemed to be composed of either ganglion cells (i.e. to be diagnosed as a carotid-body tumour) or of fibroblasts (i.e. in a fibrosarcoma). Expert opinion was divided. All that was required, histochemically, was to show that the qualitative histochemistry was characteristic for one or the other cell type. Although the methods were somewhat complex (they are given in the techniques section of this book) they showed conclusively the presence of high concentrations of gangliosides, so showing that this was a carotid-body tumour. In the second case, a secondary growth was found in a lung. From the histology alone, it was difficult to be certain whether the primary growth was in the liver or adrenal. The high concentration of steroids in the cells

of the secondary growth indicated strongly that the primary tumour was to be found in the adrenal.

In both these examples, histochemistry was used (in the first case with extensive controls) to provide histochemical 'stains' which indicated the chemical characteristics of the tissue and in this way histochemistry materially aided histological diagnosis. Another example of this use of histochemical 'stains' for the characteristic chemistry of a tissue is the well-known work of Willighagen on Hirschsprung's disease, which will be quoted because it involves enzyme histochemistry, not structural histochemistry as in the previous two examples. This disease is a congenital malformation which results from the absence of ganglion cells in the large bowel. Consequently that part of the bowel lacking ganglia is resected and the surgeon needs to know the upper limit of the resection. Willighagen tests the pieces of bowel that are removed and the surgeon continues resection until the latest pieces show the enzymatic activity (very high dehydrogenase activity) which characterizes these ganglion cells.

(ii) **Active groups and molecules.** It has often been claimed that a limitation of histochemistry is that it can show only those active groups which are *available* to the dye or reaction. In fact this need not be true, but more significantly, this is not a limitation but a major advantage. Except in the type of case quoted above, the major differences between cells lie not so much in their gross characteristics but in the degree to which their active groups or enzymes are being used. This is particularly true for different physiological and pathological conditions occurring within a single tissue or organ. The student of metabolism is well aware that cells and tissues have a great chemical or physiological reserve on which they can draw in times of stress; for such workers it is important to know to what extent the reserve is being called into play at the time of biopsy. For these studies histochemistry can show how much activity, or how many reactive groups, are manifestly available and, by suitable unmasking procedures, it can show how much (latent) activity can be made available (that is, it can give an approximation to the total gross activity).

The histochemist must therefore define his question and use the techniques to answer that specific question. The methods described in this book are designed generally to allow him to show the degree of activity manifested in the tissue at the time of biopsy; they can also yield 'total' activity if the latent reserve of activity is unmasked.

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PART I: THE PREPARATION OF SECTIONS

INTRODUCTION

One of the main reasons why histochemistry is bedevilled by controversy is because the tissue sections used by different workers have been prepared in very different ways. The same staining method, used on the same tissue, may yield very different results, depending solely on how the tissue and sections have been processed. It is frequently concluded, therefore, that histochemistry is an art ('histo-alchemy') and a fickle art-form at that; in fact, more often, the trouble lies in the lack of standardization of the matter to which the histochemistry is applied.

Before sections can be cut, the tissue has to be hardened and autolysis stopped. This can be done in the following ways, each of which has its peculiar limitations.

1. By chemical fixation and embedding in a hard matrix. The whole point of chemical fixation is that the fixative should react with specific active chemical groups; it must be remembered that it is just these active groups which you want to stain in histochemistry and which are the active sites of enzymes. Consequently chemical fixation cannot avoid altering the chemical—and so the histochemical—nature of the tissue. Treatment with alcohol, or with acetone, whether for fixation or as part of the process of embedding, will cause denaturation (and so also change the active chemical sites which are available for the staining reactions) and it will affect lipid–protein bonds which stabilize membranes and control enzymatic activities (e.g. as discussed by Green, 1959, and by Mazanowska *et al.*, 1966). Other fat-solvents, such as xylene or chloroform, will not only remove free fats, but will enhance the splitting of lipid–protein complexes of the protoplasm (see Lovern, 1957).

These effects and dangers of chemical fixation are well documented (e.g. Danielli, 1953; Wolman, 1955; Barka and Anderson, 1963; the effect on enzymes has been detailed by Nachlas *et al.*, 1956; Schnitka and Seligman, 1960; Holt, 1959; Holt *et al.*, 1960).

2. By freeze-drying. In this technique autolytic processes are stopped by 'quenching' tissue in liquefied gases at very low temperatures (e.g. −190°C); this necessitates the use of very small specimens (preferably 1–2 mm square in cross-section). Water is removed (at about −40°C, drying proceeding for a few days) and the dried tissue is then embedded in paraffin wax. This sets

the tissue in a hard matrix so that sections can be cut with a conventional microtome. The wax is then removed (e.g. with xylene) and the tissue is then fixed, in the dried state, with absolute alcohol. The fact that the alcohol acts on dry protoplasm is said to be the reason for the improved preservation of the protoplasm over what would occur when the fresh tissue is plunged into absolute alcohol.

The drawbacks to this technique are (1) that different workers have different criteria for deciding when the tissue is 'dry' and so may obtain very different results; (2) the fact that fat solvents and alcohol must be used (and these introduce artifacts, as discussed above); (3) that the 'quenching' entails the use of liquefied gases, which are not easily available, and the use of very small specimens (and even these may show some zones which are well preserved and others which are obviously distorted). Many workers have not appreciated that a gas like nitrogen or air, when liquefied, will boil around the warm specimen and the gas so liberated will insulate the specimen from the cooling effect of the liquid gas (e.g. as was pointed out by Moline and Glenner, 1964). It is necessary to immerse the specimen in a gas which has a relatively high boiling point, like propane (or better still, a mixture of butane and propane) which has been cooled to −190°C by means of an outer bath of liquid nitrogen. These hydrocarbons have a good thermal conductivity and do not vaporize around the tissue, but they add to the technical difficulties.

This subject has been extensively reviewed (see Bell, 1956; Danielli, 1953; *Symp. Inst. Biol.*, 1952).

3. By freeze-substitution in which the tissue is quenched, as for freeze-drying, but the water is then removed by substituting methanol for the water at low temperatures (such as −70°C). The tissue can be embedded in paraffin wax, or in ester wax, provided that either it is substituted again into butanol and then taken into mixtures of butanol and paraffin wax in the oven at 60°C or if the original substitution is done in acetone and the tissue embedded in ester wax (e.g. by the method of Chayen and Gahan, 1959).

However, many of the objections discussed above apply at least as strongly to this technique.

For fuller details of freeze-substitution reference should be made to the articles of Simpson (1941); Ostrowski *et al.* (1962a, b and c); and Davies (1954).

4. By freeze-sectioning techniques. It has long been apparent that autolysis can be stopped, and tissues hardened simultaneously, by the simple expedient of freezing the specimen and cutting sections of the frozen block. The primary objection to fresh frozen sections has been the practical one: the histology is obviously distorted. The secondary objections are just as

decisive even though they are more theoretical: when tissue freezes ice forms, first in the less concentrated fluid in the extracellular spaces and then even inside the cells (depending on the rate of cooling). The intracellular dehydration produced by the extracellular ice crystals increases the ionic concentration of the cytoplasm to a level which causes denaturation of the protoplasm and ruptures lipid–protein complexes (see Lovelock, 1957) whether these occur in the general cytoplasm or in cellular or subcellular membranes. During the process of cutting, the frozen tissue thaws because of the heat liberated by the impact of the block on the knife and freezes again (see Pearse, 1960, p. 21).

This process of chilling and sectioning tissue has been investigated in some detail by the present authors. They have shown that these dangers can be avoided: measurements with thermocouples under controlled conditions showed that tissue can be supercooled (i.e. no ice is formed) and this supercooled state can be maintained even during sectioning provided that this process is done at sufficiently low temperature, and with the knife cooled further to −70°C to dissipate the heat generated by the cutting (see Lynch *et al.*, 1966; Silcox *et al.*, 1965). The section is removed from the knife by apposing to it a glass slide taken from the ambient temperature of the laboratory and held by hand. The section jumps the gap between the knife (at −70°C) and the slide (at e.g. +20°C) and an imprint of the section is left as liquid water on the knife (it freezes rapidly of course). In this way the section is 'flash-dried' by distillation of the supercooled water over a temperature gradient of nearly 100°C operating over a gap of a few millimetres. Such sections are then stable.

Ice damage is undetectable in such tissue and sections. The detecting device may be a thermocouple in the tissue (as studied by Lynch *et al.*, 1966); it may be simple microscopic examination for ice crystals (or for the holes and damage produced by them, as in the study by Silcox *et al.*, 1965); it may be dark-ground illumination for investigating the degree of denaturation-aggregation of the protoplasm or it may involve a study of lipid–protein associations (as in Chayen, 1968a) or of the permeability of subcellular membranes (see Chayen, 1968b).

The actual rate at which tissue cools in the hexane bath recommended for chilling the tissue is shown in the thermocouple trace (Fig. 1). It will be seen firstly that the rate of cooling is twice as fast as that recorded by Moline and Glenner (1964) when chilling in liquid nitrogen (see remarks above); and secondly that there is no sign of ice-formation (compare with Fig. 2).

It is recommended that this procedure be used for all histochemical investigations. For certain reactions, to demonstrate particular substances, it is necessary to fix the sections specifically for the demonstration of these substances. Other substances or groups are better studied in unfixed sections. Details are given for each reaction. But the sections of tissue are standardized;

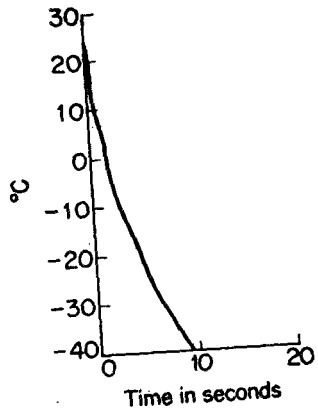

Fig. 1. The cooling curve of a piece of tissue immersed in hexane at −70°C. Note that the temperature reaches −40°C in about 9 sec and that there is no distortion (flattening) of the curve; therefore there is no indication of the formation of ice in this tissue.

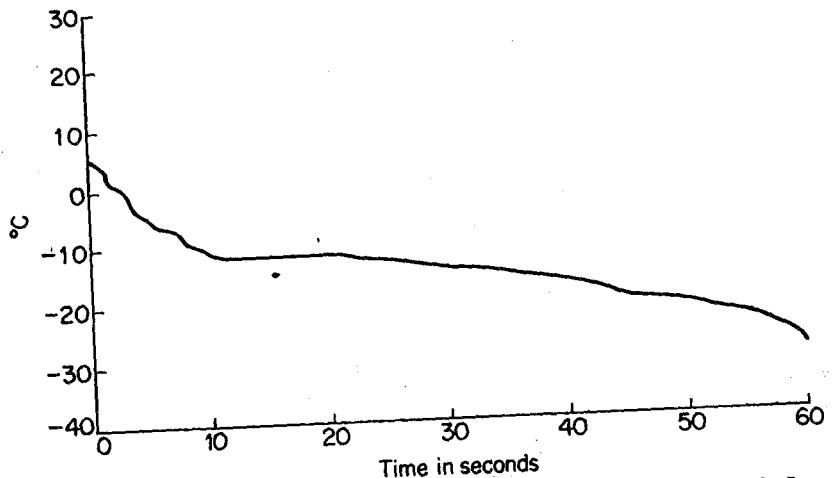

Fig. 2. The cooling curve of a piece of tissue chilled at about −20°C. In contrast to the curve in Fig. 1 there is extensive flattening of the curve, denoting ice-formation.

the preparative procedure has been shown not to introduce any apparent artifact, and the whole processing is under the rational control of the histochemist. Consequently, variations in histochemical reaction are due to true variations in activity (or in manifest activity) and so indicate metabolic differences.

METHODS

TO CHILL TISSUE

Pretreatment. One of the advantages of this procedure is that a wide range of tissues, from many animals and from plants, can all be chilled successfully by the same technique. Usually the tissue is chilled directly it is removed from the body or plant. Delicate structures, however, may benefit from immersion for 10–30 min in a 5% aqueous solution of polyvinyl alcohol (PVA; see Appendix 2) before being chilled.

Apparatus and materials. The main apparatus required is a chilling bath (a cheap polythene sandwich box is suitable if holes are cut in the lid). It should be heat-insulated by having expanded polystyrene or similar insulating matter put around it.

Also required are:

a beaker (about 50–100 ml capacity) which can be covered;
a low-temperature thermometer (recording down to −100°C);
CO_2 ice;
commercial spirit (or absolute alcohol);
hexane (B.D.H. Laboratory Reagent, boiling range 67–70°C, which must be free from aromatic hydrocarbons);
3 × 1 in corked specimen tubes;
Dewar flasks (the 1-gallon size is very useful for storage of tubes).

METHOD

The chilling bath is prepared by adding small chips of solid carbon dioxide (which cool quicker than do larger pieces) to alcohol in the chilling bath until a saturated solution is obtained. This state is obvious because (*i*) the alcohol–CO_2 mixture becomes viscous; (*ii*) addition of more solid CO_2 does not cause bubbling; (*iii*) the thermometer should record about −70°C.

The beaker, containing 30–50 ml of hexane, is inserted into the bath, preferably through a close-fitting space cut in the lid. More CO_2 ice is added to the bath to maintain the temperature. The temperature of the hexane should be at least −65°C before it is used. (Some workers prefer to use isopentane in place of hexane. We have no experience of this, but there is no reason to expect it to behave markedly differently from hexane.)

The tissue is cut into suitable pieces (which can be as large as 5 mm cubed in size) and these are dropped into the hexane at about −70°C. Small pieces (e.g. needle biopsies or material from curettage) can be blown into the hexane from a hollow spatula fitted with a large rubber bulb. Care must be taken to ensure that the tissue is plunged straight into the hexane without touching the sides of the beaker. The specimen is left in the hexane for at least 30 sec and preferably not longer than 2 min. It is then transferred, by

the use of pre-cooled forceps, to a dry tube at −70°C. (*Note: from this stage onwards, the chilled tissue should be handled only with cold instruments.*) It is often advisable to shake the surface hexane off the specimen while transferring it. Care must be taken to ensure that the tissue does not warm up during this process; the specimen, in the corked dry tube, should be encased in solid carbon dioxide in the Dewar flask for storage.

A variation of this method may be used with tissue like lung which floats on the hexane. This can be pressed gently (but firmly, with a scalpel or forceps, to eliminate air-pockets) against the inside of a glass specimen tube at about −70°C (preferably low down inside the tube to avoid the heating that occurs close to the hand). The temperature of the tube can be achieved by immersing it in the CO_2–alcohol bath, or by allowing it to equilibrate with solid CO_2 packed around it in a Dewar flask (method of Chayen *et al.*, 1960; Cunningham *et al.*, 1962).

TO MOUNT THE TISSUE

This is the most hazardous operation in the whole procedure. It should be effected expeditiously, and care must be taken not to allow the tissue to be warmed at any stage.

1. Prepare another alcohol–solid CO_2 bath at about −70°C.
2. Place the metal chuck in this bath with the top clear of the alcohol; leave it to equilibrate with the bath.
3. Place a drop of water on top of the chuck. The water should begin to freeze rapidly.
4. Remove the chilled tissue from the 3 × 1 in corked tube (in which it has been stored in the Dewar flask, packed with solid CO_2) and place it in a cavity in a piece of CO_2 ice so that its orientation can be determined before it is mounted, while keeping it chilled.
5. The water on the chuck is left to freeze until there is only a thin film of water, of comparable size to that of the specimen, left unfrozen. Then, expeditiously, transfer the specimen (with cold forceps) to this film of water. The residual water will freeze the tissue to the drop of ice on the chuck.
6. Remove the chuck (with the specimen) from the bath; wipe its sides free of adherent alcohol and stand it in the refrigerated cabinet of the cryostat.

TO PREPARE THE MICROTOME FOR SECTIONING

The angle of the knife is critical. The knife is positioned as follows:

On the knife-mounting of the microtome there are four screws: two threaded into lugs at the back and two into lugs at the front. The back pair are marked into eight divisions. Before the knife is placed in the mounting

the two back index screws should be unscrewed far enough to prevent them from protruding into the knife-mounting space.

The knife is then placed in the mounting and the two front screws are screwed up as tight as they will go, forcing the knife flat against the back of the mounting. The two index screws are then screwed forward until they touch the back of the knife. The number on each index screw opposite the line marked on the top of the respective lugs at the back of the mounting should then be read off and recorded.

The front screws are then unscrewed, releasing the knife. Both the index screws are then advanced one complete revolution until the same recorded number of each appears opposite the line on its respective lug. The front screws are then tightened back on to the knife so that it will be held firm and it will now be at the right angle.

Once it has been set, the knife must be cooled to $-70°C$ by packing CO_2 ice around the handle. We suggest that the knife be left, packed around with CO_2 ice for at least one hour before it is used. The ambient temperature in the cabinet must be maintained at $-30°C$ (Silcox *et al.*, 1965).

TO CUT SECTIONS

The chuck is locked on to the front of the arm of the microtome. It is positioned in such a way that the smallest edge of the block faces downwards. Blocks which have an epithelium on one edge should be positioned with this epithelium on one side and not at the top or bottom facing the knife. The screw which determines the thickness of the sections is set to the required size. In general, sections 8–10 μ thick are suitable for all histochemical work. However, tissue chilled and prepared in this way can be cut at any required thicknesses from 2–20 μ.

On the front of the microtome is the anti-roll plate assembly. As its name suggests it is designed to prevent the sections from curling up as they are cut. The new type of anti-roll plate is made of transparent Perspex and has two small nylon screws which allow you to adjust the gap between the anti-roll plate and the knife (Fig. 3). The angle of this plate to the knife is also critical but may have to be varied according to the tissue to be cut; consequently the correct angle setting will be found only by experience. However, whatever angle of the anti-roll plate is used, the top of the plate should never be lower than the cutting edge or high enough to touch the block.

TO PICK UP THE SECTIONS

The anti-roll plate keeps the sections flat against the knife while they are being cut. Then:

1. Swing the anti-roll plate away from the knife.

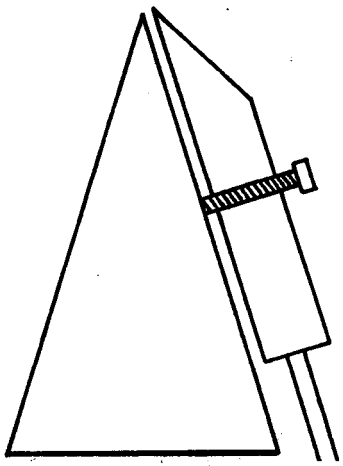

Fig. 3. Diagram of the side-view of the anti-roll plate in position against the knife, with the gap for the section adjusted by the screw.

2. Take a glass slide from the ambient temperature of the laboratory and bring it up to, and parallel to, the section on the knife. There should be no need to *press* the slide on to the section: it should move on to the slide.

3. Store the sections, on the slides, in the cryostat until used.