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SOME IMPROVEMENTS IN ZOOLOGICAL MICROTECHNIQUE FOR ELECTRON MICROSCOPY

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The results we achieve in scientific endeavour are only as good as our methods allow us to obtain. Not only is it necessary to scrutinise these methods to find ways of getting better results, but it is incumbent on us to find better ways of getting these results. Apart from improving the quality and accuracy of the end-products we can make our methods more efficient by lowering cost of materials involved, eliminating wastage, reducing time taken to complete a routine, and in general trying to simplify procedure as much as possible. In the case of some more empirical techniques, one should strive to rationalise the steps as far as possible to remove mystique which too often prevails where accurate knowledge is lacking.

Recently we have been involved in setting up an electron microscope and have had to endure the usual period of manipulative ineptitude and failures before familiarity has bred some skill and useful results. But from this experience we have been able to evolve several improvements in microtechnique for the preparation of zoological material for electron microscopy. These enable us to make new knives, and to cut, stain and photograph sections in $2\frac{1}{2}$–3 hours from already prepared blocks. Furthermore, all staining is performed in an ordinary laboratory atmosphere without the troublesome formation of lead carbonate crystals on the grids. We do not use any artificial atmosphere such as nitrogen, and because of this are not encumbered either with extra apparatus involved while using nitrogen or with the necessity of having to work under hoods.

The following are the sections of this technique in which we think improvements on existing methods have been effected:

the cleaning of staining solutions, wash liquors and embedding materials from particles of suspended material;
the preparation of glass knives;
the preparation and use of a new stain.

Each of these will now be discussed.

Cleaning of Staining Solutions, etc.

Particles of extraneous "inert" material must surely be regarded as one of the main sources of annoyance to an electron microscopist working at the magnifications usually employed. These have the perverse habit of depositing themselves in the

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most undesired of places. It is cause for wonder that methods seen in reference works do not mention the necessity for filtering staining solutions, embedding materials and wash liquids to rid them of this type of contamination. We reckoned there were enough problems in the technique without having to endure this added burden which could be so easily eliminated. Maybe it is unwritten law in electron microscope laboratories to filter solutions before use. If this is the case, the textbooks are of little help to the beginner if they omit the necessity for filtration. Some idea of the amount of suspended matter can be got by putting a strong pin-point light source under the container of stain or other liquid being used. This is the test we employed to evaluate the quality of our filtration.

For this reason, we filter all solutions except the staining fluid through a sintered glass filter of porosity grade 4, making sure that the container collecting the filtrate is the only one used for storing its contents. The staining fluid is filtered through a sintered bacterial filter. The filtering apparatus is shown in figure 1. It is simple to assemble and dismantle since all joints are standard taper glass joints. Under no circumstances should filter paper be used.

The embedding material we use is Ciba Araldite and we have adopted the following method for removing suspended foreign matter from it. The Araldite is diluted with an equal volume of benzene, and this mixture is passed several times through the filter. The diluted Araldite is collected in a flask which can then be attached to a rotary evaporator. The benzene is recovered under vacuum, leaving the Araldite at about the same consistency that it had initially—but very much cleaner. The hardener is treated in the same way. Benzene was chosen because of its capacity to form an azeotropic mixture with water. We thought that traces of water left in the Araldite after manufacture might be responsible for much of the early difficulty we had in getting Araldite-embedded sections to remain stable under the electron beam. The washed Araldite has proved to be so stable that no supporting film of Formvar is needed: and this automatically enables high resolution and magnification of any part of the field.

The water used to wash the grids after staining is also filtered rigorously.

PREPARATION OF GLASS KNIVES

Several books mention the use of "float", "drawn" or Libbey-Owen plate glass for the making of high quality knives. This glass gets its name from a process whereby freshly-rolled plate glass is passed over the surface of molten lead before cooling. The idea behind this is to anneal the surface of the glass to relieve the strain formed during cooling. We wondered if it was better to subject the glass to a treatment which would give a more deep-seated release of strain, since less strain in the body of the glass seemed to us more important than a stress-free surface.

Glass and toffee share few similarities, but there is one which in our opinion seemed most important. Both these substances are essentially amorphous solids that on heating melt to form viscous liquids and on cooling again become solid without crystallisation. If one wants to crack toffee easily and cleanly, it should be cooled in a refrigerator. We applied the same idea to the glass plates from which the knives are made, and found that much better knives could be made from cold glass than from glass held at room temperature. So we now keep all glass to be used for knives in a refrigerator at a temperature of —5°C.

Some books recommend that slow fracture of glass produces better knives; but a slow fracture is not easy to achieve. It can be accomplished much more readily if the glass is cold when placed under strain in the knife-maker and

allowed to stand. A slow rise in temperature allows the glass to expand and thus apply the final strain to induce slow fracture. Using this method, we can make first-class knives from plate-glass sold by glass merchants.

In our opinion the edge of a knife made from cold glass lasts longer than one made from glass held at room temperature, and shows evidence of being sharper. The making of the knives is much simpler and easier and there are virtually no rejects because the glass fractures so cleanly. Consequently, there is a great saving not only in time but also in glass. We use a Japanese Messer knife-maker. Each knife is washed in absolute alcohol and then 100% acetone to remove water of condensation.

PREPARATION OF STAINING SOLUTIONS AND THE SCHEDULE FOR THEIR USE

The lead citrate and lead hydroxide solutions normally employed in electron microscopy suffer from the drawback that if used in the ordinary atmosphere their alkaline nature permits the formation of lead carbonate crystals. We have eliminated this problem by using an acid lead stain—with results we think are the equal of those achieved with lead hydroxide or citrate.

Although we cannot find in reference works the reason why uranyl salts are used, we presumed their use stems from the fact that insoluble uranyl compounds would be opaque to electrons. Being a heavy metal, uranium would react with protein and thus become localised. In an electron beam the uranyl-protein compounds would therefore be recognisable as the darker areas in a clear field. If this were so, one might expect an increase in the concentration of the uranyl salt to lead to better staining. From some trials we did, it seemed quite definite that the concentration of uranyl salt had a marked effect on the efficacy of the staining. Methods already employed by electron microscopists use uranyl acetate; but this has a limited solubility in water compared with the nitrate. We therefore chose to use uranyl nitrate.

To escape the formation of lead carbonate crystals, one has to use an acidic lead salt solution. We decided to work with lead nitrate since solutions of this in water are normally acidic without pH adjustment—sufficiently so to exclude the possibility of lead carbonate formation. Initially, we stained sections in a solution of uranyl nitrate and then in a solution of lead nitrate. The results were very heartening—sufficiently so to indicate that these chemicals could be relied on to give us consistent results. Because the nitrates of these two elements are very soluble in water and the common anion effect would not cause precipitation if both were mixed together, we combined the two into one stain solution. Only analytical grade reagents were used.

We also examined the effect of pH on the staining and seemed to get the best effect when the pH was brought to 1.5 with ammonia—using a glass electrode to measure this adjustment.

The formula of the stain is:

uranyl nitrate AR	4.0 gms
lead nitrate AR	0.4 gms
citric acid AR	0.25 gms
distilled water	5.0 mls
pH	1.5

The staining time is ½ an hour at 35°C.

The following advantages accrue from using this mixed stain. There is no precipitation of lead carbonate (or any other chemical) while using this stain mixture. All staining is done on the bench top in an ordinary atmosphere without

taking any extra precautions other than excluding atmospheric dust from the grids at all times. The overall staining time is shorter with this combined stain.

By using this stain from which lead carbonate cannot be precipitated and filtering all solutions that come into contact with the grid, we find it commonplace to be able to photograph large areas of a section at low magnification (see Plate 1). One is not forced to pick areas whose suitability is dictated by the absence of lead carbonate crystals or other inert material, because now the whole of the grid is usable. In Plate 2, note the freedom from microcrystals originating from the stain. Because of the nature of our stain we find that a high magnification study of an area viewed at low magnification can be made with confidence, knowing that this type of artefact will be absent. Where we did get contamination, we found it coming from the forceps—which we now clean regularly with '400' emery paper.

Up to this point the main fixative used has been osmic acid, although glutaraldehyde has been tried on several occasions—producing results in staining comparable with those obtained after osmic fixation. We do not know if fixatives other than these will permit a similar quality of staining.

We wish to thank Mr. Alan Hoverd for preparing the accompanying figure. The microscope used throughout this work is a Zeiss EM9A.

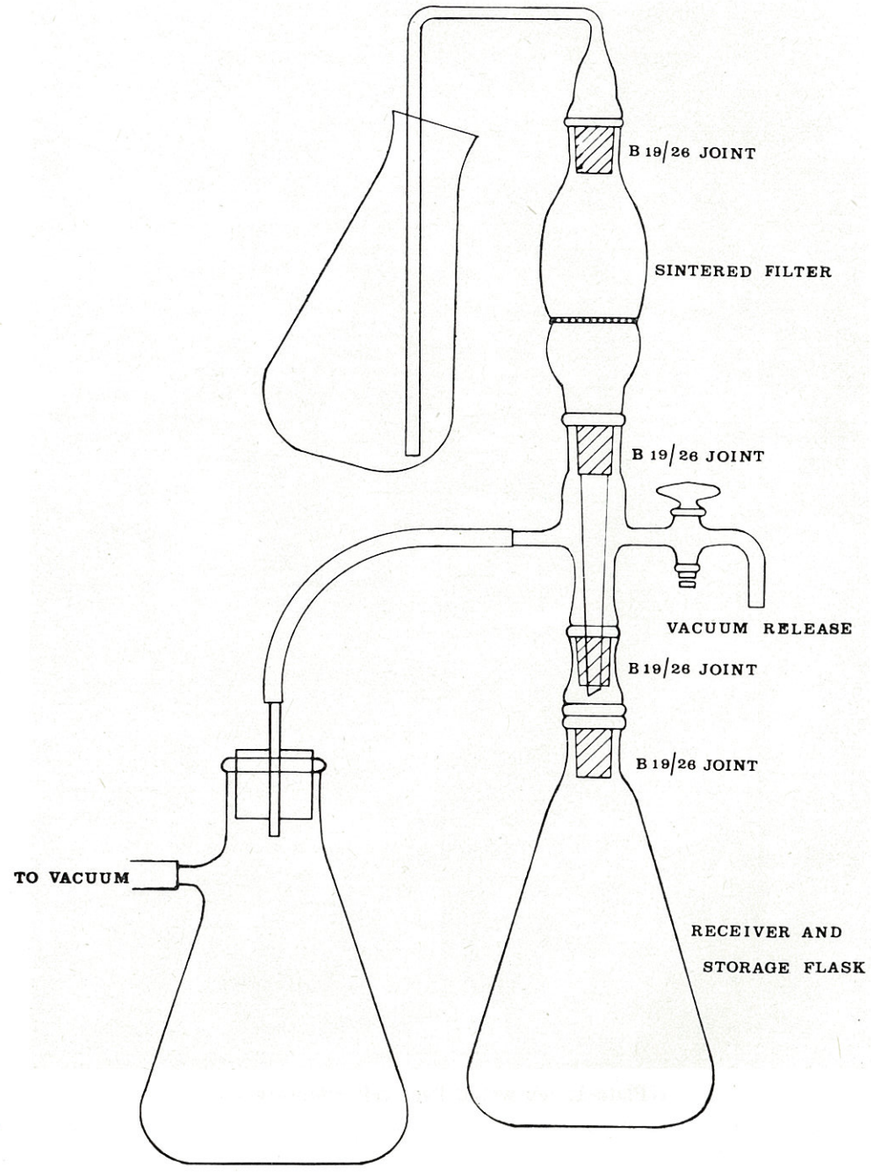

Fig. 1. Diagram of filtering apparatus.

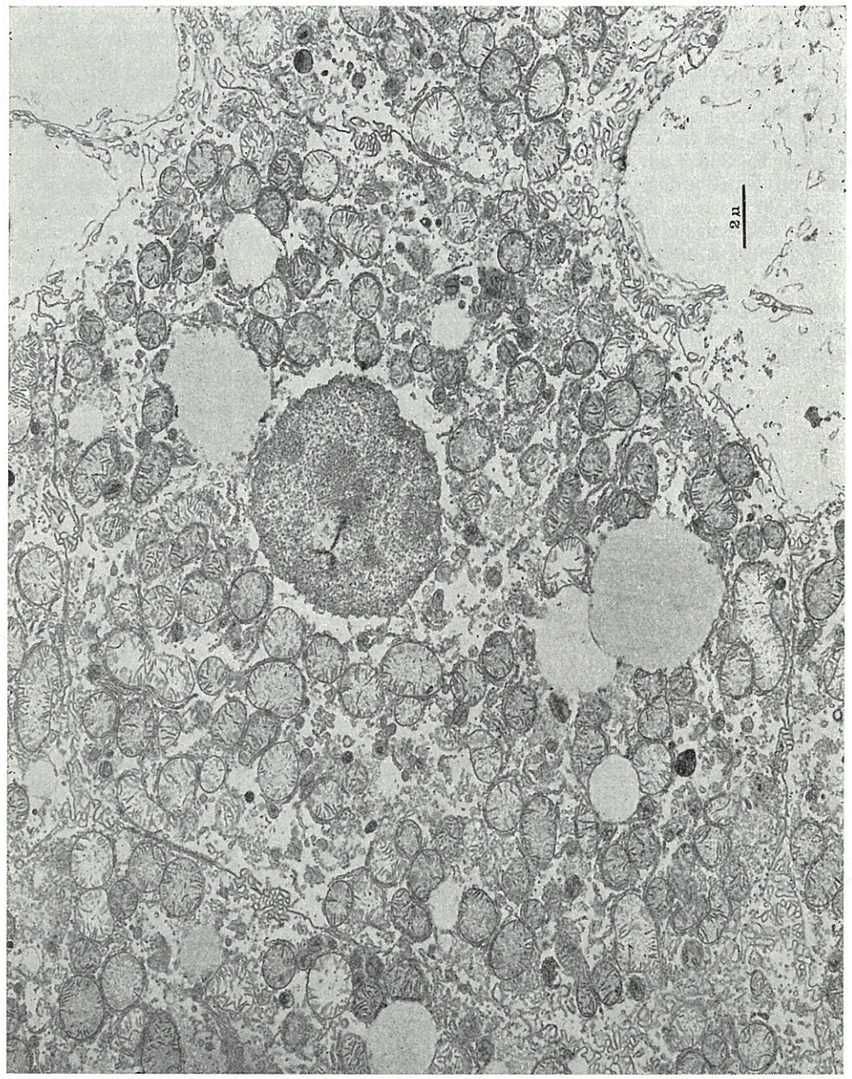

Plate 1. A whole liver cell (mouse).

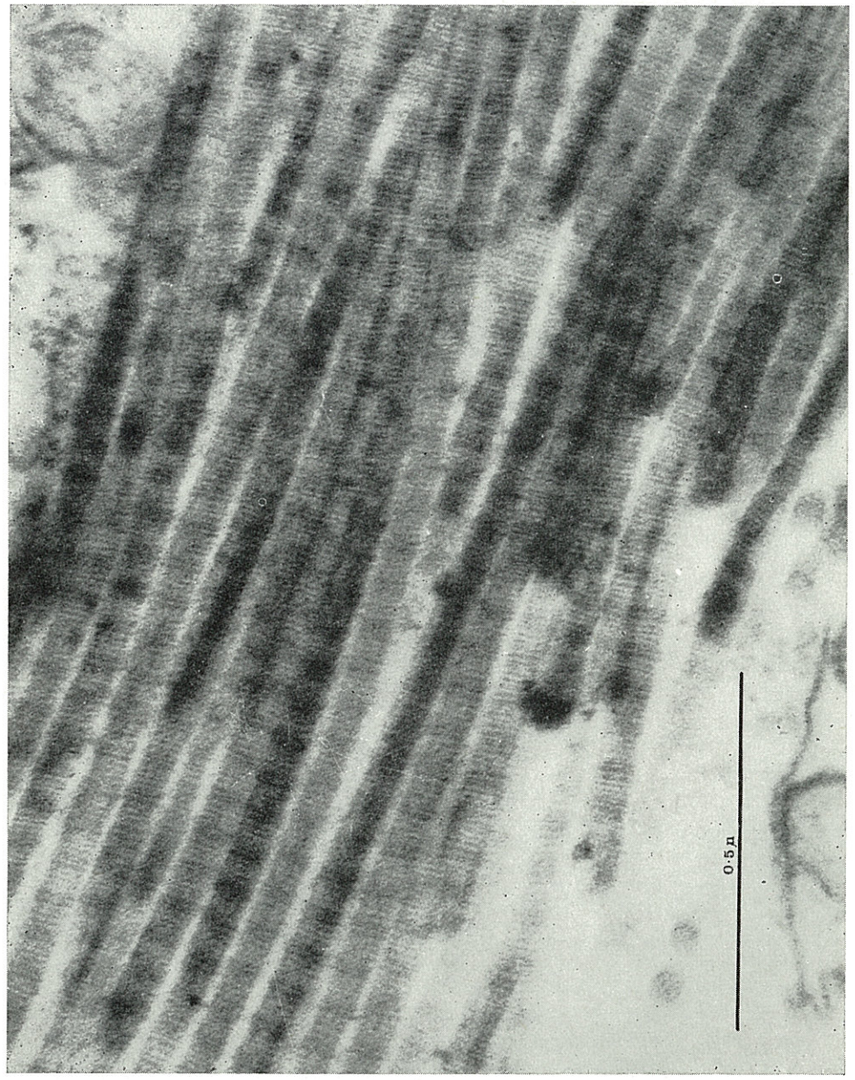

Plate 2. Collagen fibres from mouse testis.