On colouring epon-embedded tissue sections with Sudan black B or Nile blue A for light microscopy

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With one plate (fig. 1)

Summary

Sections of osmium-fixed tissues embedded in epon 812 colour with either Sudan black B or Nile blue A solutions to reveal a variety of detail by direct microscopy with normal apochromatic or semi-apochromatic objectives. The clarity of the coloration gives a picture fully comparable to that seen by phase-contrast microscopy. The plastic is not removed. 1-μ sections or thinner sections down to green or gold, are mounted on clean glass slides by drying down from 20% acetone/water after flattening on a hot plate. Colouring is carried out at room temperature in Sudan black B (saturated solution in 70% alcohol) for 1 to 2 h. The result is a reversed or negative effect, for the epon plastic takes the stain avidly, but dense elements of the tissue do not, and appear white against a blue background of stained plastic. Lipid droplets retain a capacity to colour, becoming dark blue to blue-black. Nile blue sulphate (1% aqueous solution) colours thin sections of tissue in 1 to 2 h at 60° C, acting apparently as a basic dye on most cell elements, and also colours lipid droplets dark blue. After both techniques the sections are mounted in Farrant's medium.

Introduction

The recent publication by Luft (1961) of a simple and reliable method for the embedding of tissue for electron microscopy in epoxy resin, epon 812, and the work of Glauert, Rogers, and Glauert (1956), Glauert and Glauert (1958), Kushida (1959), and Finck (1960), mark a most significant step forward in

technique, and many laboratories have all but eliminated from their procedures the use of methacrylate. In the last decade various staining methods have been applied to tissues embedded in methacrylate, since it is possible to achieve some staining of structures without removal of the plastic; a greater number of techniques succeed after removal of the plastic, which is comparatively simple. It is not, however, so easy to remove epoxy resins from sections of tissue.

Routine inspection of 1- to 2-μ sections with the light microscope is an important part of the technique of thin sectioning. It is very satisfactorily carried out using positive phase-contrast objectives and 'anoptral' contrast objectives (commercially made by Reichert after Wilska, 1953), see Palay and others (1962). However, not all laboratories are equipped for this type of observation, and it is desirable that there should be available some simple staining techniques for use on epon-embedded material, without removal of the plastic, which will yield the same type of information when studied with ordinary objectives by direct microscopy. We have established two such techniques: either colouring with Sudan black B, or with Nile blue A, which give results of such clarity that they can be highly recommended. The information to be derived from their use is fully comparable to that obtained by phase-contrast microscopy and, indeed, it has already proved possible to detect, by the use of Nile blue A, structures known to exist from electron micrographs but not noticed by phase contrast.

Material and methods

Sections of material embedded in epon 812 (obtained as epikote 812, Shell Chemical Company Limited, 15/17 Great Marlborough Street, London, W. 1. batch 3 and batch 10) according to the procedure of Luft (1961), were cut on 'Huxley' ultramicrotomes (Huxley, 1957) at a thickness of 1 μ or less using glass knives. This was done more quickly and easily on a modified prototype (McGee-Russell and others, 1962) but could also be done satisfactorily on the commercially obtainable machine (Cambridge Instrument Company). Two principal types of tissue were used: either Krebs II ascites cells pelleted by centrifugation in buffered osmium tetroxide, or cerebral ganglion of the snail *Helix pomatia*, fixed by immersion fixation in buffered osmium (exact constitution of the fixative, and the proportion of added calcium chloride as recorded by Palay and others, 1962). With both types of material results were similar. The sections were floated on to a droplet of 20% acetone/distilled water, and warmed for 5 to 10 sec on a hot plate in order to flatten them. The fluid was drained off and the sections were allowed to dry down on to the warm, clean, glass slide. With no further treatment, the slides were placed in staining solutions for different lengths of time. Results were unsatisfactory with toluidine blue and Sudan IV for periods of staining up to 22 h, no striking differentiation of elements being obtained. However, the following two techniques, arrived at after similar tests, can be recommended.
**Colouring epon sections with Sudan black B**

**Solutions required**

Sudan black B in 70% alcohol, saturated solution prepared by refluxing in the normal way. Filter before use. (Sample used in the tests: B.D.H. standard reagent); Farrants’s medium (Baker, 1960).

**Schedule**

Place epon sections of osmium-fixed tissues (thickness preferably i μ or less) mounted on clean glass slides directly into the solution of Sudan black B in a Coplin jar. Allow to stain for 1 to 2 h at room temperature.

Remove the slide from the Coplin jar and rinse off the remaining Sudan black with a gentle stream of distilled water. Drain off the excess water and mount beneath a coverslip in Farrants’s medium. View with an intense light source.

**Result**

The epon plastic colours intensely with Sudan black to give a bright blue colour, when viewed with high intensity transmitted illumination. This appears as a general background colour against which the densest elements of the tissue, such as the nucleoli, nuclear membrane, chromosomes, and so on (from which, presumably, the plastic is largely excluded) appear as clear white objects, almost as if viewed by dark ground microscopy. It is a startling image (fig. 1, A). Other, presumably less dense elements, partially penetrated by the plastic, such as the general cytoplasm and mitochondria, appear in pale shades of greyish blue. Granules such as the yellow granules of the neurone of the snail *Helix* (Chou, 1957 a, b), which have intrinsic colour, may remain completely uncoloured by Sudan black and stand out clearly (fig. 1, A). Large lipid granules, such as those observed in ascites tumour cells (Ross, 1961), or vertebrate liver cells, or the same snail neurones, retain a capacity to colour with the Sudan black, and become intensely blue-black. The histochemical specificity of this coloration has not been established firmly, but the site of coloration appears to be the same as that determined by valid histochemical techniques, using Sudan reagents.

**Comments**

Some difficulty may be experienced when using the Sudan black solution, as the sections are liable to float off in the liquid. This can be overcome by pre-coating the slide with a very thin film of ‘complete resin mixture’ (Luft, 1961) made extremely dilute with acetone. Such a film dries quickly and can be sufficiently hardened in a 60° C oven in 10 to 15 min. Sections dried down on to such a film from acetone/water become firmly attached, and will not be lost during treatment with Sudan black solutions. The thin film of plastic will, however, stain, and although this does not disturb the result so far as the
section is concerned, it gives a less pleasing slide. Clean slides normally ensure adhesion of the sections.

The unusual result of this use of Sudan black immediately makes one ask whether it is a property of this compound alone. Tests carried out with Sudan IV solutions showed that over periods up to 20 h the plastic colours slightly pink. There is no striking differentiation of cellular elements. There is no colouring of large lipid droplets. Hence the capacity of the two Sudan reagents to colour lipids would not appear to be related to their ability to colour the plastic. The slightly basic nature of Sudan black may be significant.

One may also inquire whether the result with Sudan black is restricted to this particular resin mixture. Tests were carried out on sections cut from blocks of tissue embedded in methacrylate, vestopal (Ryter and Kellenberger, 1958), araldite (Glauert and Glauert, 1958), and X 133/2097 (Stäubli, 1960). None gave as satisfactory a result as the epon-embedded material. The methacrylate-embedded tissue (newt intestine) showed slight coloration: the microvillar border of the intestinal epithelial cells coloured a pale blue, and the goblet cells developed some colour. The epoxy and polyester resins showed a fair degree of staining with Sudan black, but the time required for the uptake of the dye varied. Stäubli's X 133/2097 gave a result very similar to the epon, in 1 h. Vestopal took 3 to 4 h to achieve some coloration of the plastic. Araldite showed some colour only after immersion for 22 h, and even after

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**Fig. 1 (plate).** 1-μ sections of cerebral ganglion of the snail *H. pomatia* fixed in buffered osmium and embedded in epon 812. The photomicrographs were made on a Reichert Zetopan microscope with a Reichert 1.8 mm apochromatic ×100 oil-immersion objective, and plan eyepiece ×8, on polaroid Land type 55 P/N 4 × 5 in. film, using the polaroid film holder model 500 in a special camera mount manufactured in the workshop of these laboratories by Mr. B. Firmager, under the direction of Mr. R. Emery. The final prints used here were made by normal photographic enlargement from polaroid negatives.

A, epon section of snail nerve-cells coloured with Sudan black B. Dense elements of the cells such as the nuclear membranes, chromatin, nucleoli, and some cytoplasmic inclusions stand out in reversed contrast as white objects against the dark background of the plastic which is coloured deep blue. Lipid granules (lg) colour dark blue to blue-black. General cytoplasm is differentiated in shades of greyish blue. Certain cytoplasmic inclusions which have intrinsic colour are unstained, and stand out prominently because of their own colour, e.g. the complex yellow granules (yg), characteristic of these cells. The particle marked yg is a clear demonstration of the position of small dense particles within the body of the complex yellow granule. Some authors (e.g. Chou, 1957) have emphasized the occurrence of external 'satellites' associated with the cytoplasmic inclusions of snail neurones; internal densities would appear to be equally prominent. This photomicrograph demonstrates that the Sudan black technique enables one to detect sectioning artifacts easily: tramlines indicative of a deteriorating knife edge run from the top right to the bottom left of the picture.

B, epon section of snail nerve-cells coloured with Nile blue A. A delicate coloration of most of the cellular elements is achieved. Nuclear membrane, chromatin, filamentous elements in the cytoplasm, cytoplasmic granules, and cell membranes are all differentiated in shades of blue. Background plastic is colourless. Note that the large lipid granules (lg) are coloured a dark blue, and that, also, the complex granules (yg) are coloured blue. A tiny punctate granule (pg) about 0.6 μ in diameter can be detected easily in this type of preparation. These granules can barely be distinguished in unstained plastic sections of similar cells by phase-contrast microscopy, but are prominent in electron micrographs (observations to be published). They are not mitochondria, which in electron micrographs of these cells show profiles which would indicate that the majority of the mitochondria are filamentous.
Fig. 1

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50 h araldite did not colour as darkly as the epon after 2 h. However, a similar reversed effect was achieved, and dense elements such as sperm-heads and basement membranes showed up white against the pale blue plastic. It remains to be shown which components in the embedding media are responsible for these colorations with Sudan black.

Colouring epon sections with Nile blue A

Solutions required

1% aqueous solution of Nile blue A. Filter before use. (Sample used in this work: Nile blue sulphate, Hopkin and Williams Ltd., Chadwell Heath, batch no. 88624, kindly given to us by Dr. G. David); Farrants's medium.

Schedule

Place the epon sections (1 μ or less in thickness) of osmium-fixed tissue, mounted on glass slides, directly into the solution of Nile blue A in a Coplin jar. Allow to colour in a 60° C oven for 1 to 2 h.

Remove the slide from the warm Nile blue solution and rinse with a gentle stream of distilled water.

Drain and mount in Farrants's medium.

Result

The majority of the elements of the cells of the tissue are dyed with delicacy and clarity in different shades of blue. The plastic is almost uncoloured, although a very faint pink may be discerned. The nuclear membrane, chromatin, chromosomes, cell membrane, mitochondria, and cytoplasmic inclusions are all well shown. There is a remarkable correspondence between the stained image and the image seen by phase-contrast microscopy of the same stained section; the Nile blue in fact enhances the phase-contrast image, and it is a useful exercise to go rapidly from phase contrast to bright field, and back again, whilst viewing the material. The colouring of lipid inclusions is worthy of comment. In snail neurones (fig. 1, B) and in ascites tumour cells, it is apparent that the lipid droplets coloured by the Sudan black technique are coloured by the Nile blue, but that the Nile blue also colours other lipid-containing granules, for example the yellow granules of complex character found in the cytoplasm of the Helix neurone (yg in fig. 1). It colours more generally than Sudan black, when used on plastic sections, and gives a most satisfactory morphological picture.

Comments

We have experienced no difficulty with sections detaching during staining with Nile blue A solutions, and no special precautions need be taken.

Brief experience suggests that it is possible to dehydrate the Nile-blue-coloured sections rapidly in acetone, pass them through xylol, and mount them in Canada balsam to achieve what may be a permanent preparation.
This is not possible after Sudan black. The degree of permanence, however, has still to be established by the passage of time.

The questions posed for the effect obtained with Sudan black must be considered again for Nile blue A. Is the colouring effect a special property of this dyestuff? Does Nile blue A produce a coloration with tissue supported in other embedding mixtures? Coloration with Nile blue is well known to be a complex process. The oxazine component of the dyestuff can act as a basic dye. Resistance of the stained sections to acetone, mentioned above, suggests that it is the basic property of the dyestuff which is significant in the reaction with epon-embedded tissue. We therefore tested the ability of another well-known basic dye, toluidine blue, to stain sections of this kind. Ascites tumour cells are intensely basiphilic after normal methods of preparation either as smears, or in paraffin sections, and stain heavily with toluidine blue. This property cannot be shown after fixation in osmium tetroxide and embedding in epon 812, and we were unable to obtain any satisfactory staining of the plastic sections with toluidine blue. The basic character of the dyestuff would not, therefore, appear to be the cause of the colouring effect with Nile blue A.

We tested the same variety of tissues and embedding media to determine the effect upon the Nile blue colouring. The Nile blue method produces pleasing results with methacrylate, araldite, and vestopal sections in 1 to 2 h, and acts as an excellent general colouring agent for most of the cellular elements. With Stäubli’s X 133/2097 (tissue: digestive gland of Helix) in 1 to 2 h there is some uptake of Nile blue on to the plastic, which colours pink, but the elements of the tissue, nuclear membrane, chromatin, and complex cytoplasmic granules, dye in well-differentiated shades of blue, and stand out distinctly. The background pink coloration does not obscure the informative dyeing effect. These results would appear to indicate that the colouring with Nile blue A is dependent upon the properties of the embedded tissue rather than upon the nature of the embedding plastic. The Nile blue technique can be recommended as an excellent general method for the study of plastic embedded tissues.

It is a pleasure to acknowledge the hard work of Dr. W. C. de Bruyn, of the University of Leiden, who co-operated with the senior author (S. M. M. R.) in a survey of embedding media, and prepared the blocks of material embedded in araldite, vestopal, and X 133/2097, whilst visiting the Department of Zoology, Birkbeck College, University of London, during 1961. It is also a pleasure to thank Dr. F. K. Sanders for his enthusiasm and initiative in encouraging work in electron microscopy in these laboratories.

References

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