A SIMPLE METHOD FOR OBTAINING INCREASED CONTRAST IN ARALDITE SECTIONS BY USING POSTFIXATION STAINING OF TISSUES WITH POTASSIUM PERMANGANATE

D. F. PARSONS. From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee

Epoxy resin appears to be the best embedding material for preserving tissues for electron microscopy. Its low linear coefficient of contraction[1] ensures that no appreciable distortion can occur owing to polymerization damage or excessive shrinkage (1). This plastic also is exceptionally stable in the electron beam, so that surface damage to the section is slight. Although Araldite blocks are often more difficult to cut than those of methacrylate, the introduction of the diamond knife (2) with its superior cutting abilities over the glass knife has considerably reduced this difficulty. The greatest disadvantage of Araldite is the low contrast of sections of osmium tetroxide–fixed material in the electron microscope. In the author's experience, satisfactory lead staining (3, 4) of Araldite sections is difficult to obtain. More contrast is achieved by staining Araldite sections with potassium permanganate (5, 6), but a fine precipitate on the stained sections is troublesome.

Potassium permanganate–fixed tissues (7) show satisfactory contrast in Araldite, but permanganate fixation appears less perfect than osmium tetroxide fixation in several respects (8). Mitochondria are nearly always swollen, and some structures, *e.g.*

[1] The linear contraction is less than 1 per cent, an amount even lower than that of the polyester resins like Vestopal W, which contract about 2.5 per cent (author's measurements, unpublished data).

RNA granules and fat vacuoles, either are not stained or are oxidized or leached away during fixation. On the other hand, permanganate has proved especially useful for the study of membranous structures of the cell (9). In recent experiments to study the effects of ionizing radiation on cell membranes, many attempts were made to prevent the swelling and breaking-up of mitochondria during fixation by permanganate solutions. The combined use of a modified osmium tetroxide fixative, Araldite embedding, and staining of the tissue in an acetone solution of potassium permanganate was found to give good preservation and increased contrast of these structures.

MATERIALS AND METHODS

Mouse heart muscle, liver, pancreas, kidney, and ovary were fixed for 20 to 90 minutes in a buffered osmium tetroxide fixative. In some cases, a specially prepared fixative, which preserved mitochondria better than veronal-acetate-buffered osmium tetroxide fixative, was used. The solution was prepared by dissolving 1.90 gm of sucrose in approximately 15 ml of 2 per cent osmium tetroxide; 0.78 ml of 0.5 M Na_2HPO_4 and 0.08 ml of 0.25 M citric acid were added (to give a pH of 7.2 to 7.3). The fixative then was made up to 20 ml with 2 per cent osmium tetroxide. Palade's osmium tetroxide was also used for some tissues (10).

After fixation, the tissue was rinsed with 30 per cent acetone and dehydrated by 10 minute immersions in 30, 70, and 90 per cent acetone solutions. The tissue was then soaked twice for 10 minutes in reagent grade acetone and stained for 10 to 15 minutes in a 1 per cent solution of potassium permanganate in the same acetone. After immersion in the permanganate solution, the tissue was washed with reagent grade acetone containing a reducing agent, methyl acrylate. This step was found to be essential to obtain high contrast in the sections and also to prevent free permanganate from interfering with the solidification of the epoxy resin. Two drops of methyl acrylate[2] (Eastman Organic Chemicals, Rochester, New York, containing hydroquinone inhibitor) were added to 25 ml of acetone. The tissue was washed twice briefly in acetone and then immersed for 10 minutes. The Araldite mixture contained the following: 10 ml Araldite 6005; 10 ml dodecenyl succinic anhydride; 2 ml dibutyl phthalate; 0.30 ml benzyldimethylamine.

The specimens were soaked for 40 minutes in a 1:1 mixture of Araldite and acetone at 40°C and then given two changes in the Araldite mixture without benzyldimethylamine catalyst and three changes with the catalyst, each immersion lasting 40 minutes and all at a temperature of 40°C. The tissue was embedded in gelatin capsules, left overnight at 40°C, and then left for 24 to 48 hours at 65°C.

Sections were cut with a 1.6 mm, 47° cutting angle diamond knife (Instituto Venezolano de Investigaciones Científicas, Venezuela) and examined in a Siemens Elmiskop I electron microscope (operated at a voltage of 60 kv) on a thin Parlodion supporting film that had been stabilized with carbon. A single condenser and a 50 μ objective aperture were used. The time of staining was adjusted to give adequate contrast using a 50 μ objective aperture. Somewhat more contrast can be obtained using a 20 μ aperture. The exposures of the negative and positive photographs were controlled by exposure meters and all prints were made on F4 Kodabromide paper.

RESULTS

Fig. 1 shows the typical low contrast obtained with untreated Araldite tissue. Figs. 2 through 4 demonstrate the degree of contrast obtained by staining different tissues with a 1 per cent solution of potassium permanganate in acetone for 12 minutes, followed by reduction of the permanganate *in situ*. The effect of staining a mouse ovary for 12 minutes in the permanganate solution is shown in Fig. 2. Compare Fig. 2 with Fig. 1, which shows parts of two interstitial cells of the ovary. Staining for 12 minutes produces a general enhancement of contrast of nearly all structures, including the RNA granules. Staining for 20 minutes gives very high contrast, especially of membranes, but the latter are sometimes fragmented. RNA granules are indefinite or absent in some areas after 20 minutes or more of staining. Fig. 3 shows part of a pancreatic cell. The marked affinity of the RNA granules for the stain, when staining times of 12 minutes or less are used, can be seen to the best advantage. However, there is also a general enhancement of contrast.

The described staining procedure did not produce fine structural damage in the other tissues examined (mouse kidney and heart muscle). However, scattered small light areas in the cytoplasmic matrix of liver cells were suggestive of the removal of some component, perhaps the osmiophilic droplets described by de Harven (11). Under the described conditions, glycogen is not selectively stained as is the case when thin sections are stained with permanganate (6).

[2] Methyl methacrylate is also suitable.

Recently, another type of epoxy resin, Shell Epon 812, has been introduced (12, 13) which appears to have certain advantages over Araldite. Limited use, in our hands, indicates that its lower viscosity does allow more complete penetration of the tissue and more rapid embedding. Epon-embedded tissues and compared with other staining techniques. Figs. 4 through 6 illustrate the different contrast and character of permanganate– and phosphotungstic acid–stained tissue blocks and also of lead-stained (50 per cent saturated lead subacetate (14)) sections for

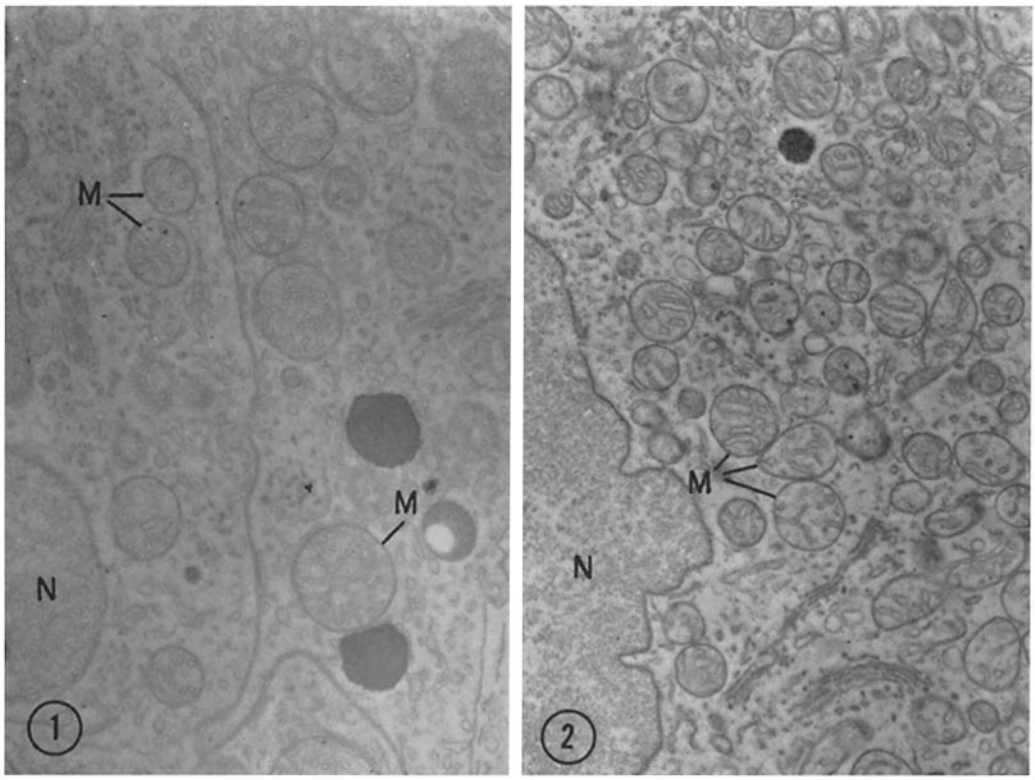

FIGURE 1

Mouse ovary fixed in the described citrate-buffered osmium-sucrose fixative and embedded in Araldite. No permanganate was used and the figure shows the typical low contrast of untreated Araldite sections. Parts of two interstitial cells are shown with characteristic mitochondria (*M*). *N*, nucleus. × 14,400.

FIGURE 2

The ovary was fixed and dehydrated as in Fig. 1, but the tissue was soaked for 12 minutes in a 1 per cent solution of potassium permanganate in acetone before infiltration with Araldite. Part of an interstitial cell is shown. There is a considerable general increase in contrast. *M*, mitochondria; *N*, nucleus. × 14,400.

Improved section cutting ability was obtained by reducing the hardener-to-resin ratio, as suggested by Wood and Luft (13), and the resin shows a much greater permeability to lead and light microscope stains.

Because of these advantages the permanganate staining procedure has been carried out with Epon-embedded tissues. In Fig. 4 mitochondria of the mouse oocyte are shown after the permanganate staining procedure (12 minutes). Fine structure appears intact and there is no precipitation of the stain (see the electron light areas, *V*). The staining is uniform throughout the block.

While satisfactory phosphotungstic acid staining

of Araldite-embedded tissue blocks was readily obtained by soaking in 0.2 per cent (saturated) phosphotungstic acid ($P_2O_5 \cdot 24WO_3 \cdot XH_2O$) in reagent grade acetone for 5 to 10 minutes, weak and uneven staining was obtained when the tissue was embedded in Epon. Soaking the dehydrated tissue block in 0.2 per cent phosphotungstic acid in acetone for 20 to 40 minutes followed by two brief acetone washes appeared to give somewhat better results (Fig. 5) than the alkaline ethanol treatment that has been suggested (13). The contrast obtained was considerably less than that given by permanganate or lead staining, but fine structure was preserved and no precipitation was seen.

Fig. 6 illustrates the effect of lead staining (50 per cent saturated lead subacetate (14)) of Epon sections for 1 minute (4). The membranes of the oocyte mitochondria and the RNA granules (*P*) show very dense staining. Fine precipitation of the stain was fairly frequently seen with both lead hydroxide (3) and lead subacetate (14) staining and appeared to be due partly to very fine particles suspended in the stain and partly to the staining of dirt passed onto the section from the microtome trough. To avoid this it appears necessary to use an ultrafine sintered glass filter in place of the fine-pored filter paper originally suggested (4) for filtration of the staining solution. Frequent washing out of the microtome trough (especially if a diamond knife is used) is advantageous.

DISCUSSION

The method described can be carried out with only a slight modification of the routine embedding procedure. The inconvenience and difficulty of staining sections are also avoided. Some caution is necessary in the use of potassium permanganate, since it is a strong oxidizing agent and may destroy some structures. The RNA granules first take up the stain strongly, but if the staining is continued for more than 20 minutes, many of the granules disappear. If the fixation and staining of aqueous solutions of permanganate can be compared with the action of permanganate in acetone, it would appear that RNA granules are not visible in permanganate-fixed tissues because they are destroyed during fixation.

The use of a wash solution consisting of a reducing agent in acetone is an important feature of the technique. Apparently the permanganate is reduced *in situ* and thus is more firmly bound to cellular structures. If the reducing acetone is not used, the staining is weaker and the epoxy resin mixture solidifies less readily inside the tissue. The increase in contrast is general with a slight selectivity for membranes. Probably selectivity for membranes can be increased in some cases by using shorter osmium tetroxide fixation times.

The principal advantage of the permanganate staining technique is that high contrast can be obtained in both Araldite and Epon epoxy resins without the inconvenience of staining each section. In lead staining of sections, fine precipitates have frequently been obtained by the author with both lead hydroxide and lead subacetate stains. These are often not visible at lower magnifications (less than 40,000 times). If the use of section stains is anticipated, then some of the blocks may be processed for this purpose without permanganate staining. However, if Araldite embedding is used, staining of the tissue block is almost a necessity since the sections are nearly impermeable to lead and other stains (lead staining requires 1½ hours). After the permanganate treatment Araldite sections take up lead subacetate stain in 5 to 10 minutes. This may be useful if extra high contrast is required.

Epon (12, 13) appears to be the most suitable epoxy resin at present available. It is unfortunate that phosphotungstic acid staining is impaired by the resin since this stain in Araldite and methacrylate brings out fine detail. The uniform staining of permanganate giving high contrast, and the absence of precipitation or structural damage under the described conditions, suggest that permanganate is the most suitable tissue stain for Epon-embedded materials.

The Oak Ridge National Laboratory is operated by Union Carbide Corporation for the United States Atomic Energy Commission.

The author gratefully acknowledges the technical assistance of Miss M. P. Edwards and Miss S. R. Graves.

Received for publication, May 31, 1961.

REFERENCES

1. Parsons, D. F., and Darden, E. B., Jr., Optimal conditions for methacrylate embedding of certain tissues and cells sensitive to polymerization damage, *Exp. Cell Research*, in press.
2. Fernández-Morán, H., Applications of a diamond knife for ultra-thin sectioning to the study of the fine structure of biological

tissues and metals, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, No. 4, suppl., 29.
3. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals. II. Application of solutions containing lead and barium, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 727.
4. PARSONS, D. F., and DARDEN, E. B., JR., A technique for the simultaneous dirt-free lead staining of several electron microscope grids of thin sections, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 834.
5. LAWN, A. M., The use of potassium permanganate as an electron-dense stain for sections of tissue embedded in epoxy resin, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 197.
6. DROCHMANS, P., Mise en évidence du glycogène dan la cellule hépatique par microscopie électronique, *J. Biophysic. and Biochem. Cytol.*, 1960, **8**, 553.
7. ZEBRUN, W., and MOLLENHAUER, H. H., Electron microscope observations on mitochondria of rat testes fixed in potassium permanganate, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 311.
8. LUFT, J. H., Permanganate—A new fixative for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1956, **2**, 799.
9. PORTER, K. R., and MACHADO, R. D., Studies on the endoplasmic reticulum. IV. Its form and distribution during mitosis in cells of onion root tip, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 167.
10. PALADE, G. E., A study of fixation for electron microscopy, *J. Exp. Med.*, 1952, **95**, 285.
11. DE HARVEN, E., and FRIEND, C., Electron microscope study of a cell-free induced leukemia of the mouse: A preliminary report, *J. Biophysic. and Biochem. Cytol.*, 1958, **4**, 151.
12. FINCH, H., Epoxy resins in electron microscopy, *J. Biophysic. and Biochem. Cytol.*, 1960, **7**, 27.
13. LUFT, J. H., Improvement in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
14. DALTON, A. J., and ZEIGEL, R. F., A simplified method of staining thin sections of biological material with lead hydroxide for electron microscopy, *J. Biophysic. and Biochem. Cytol.* 1960, **7**, 409.

FIGURE 3

Part of a mouse pancreatic cell after 12 minutes of staining with potassium permanganate. The RNA granules attached to the membranes of the endoplasmic reticulum show a very high contrast. *N*, nucleus. × 14,400.

FIGURE 4

Mitochondria of mouse oocyte fixed in Palade's osmium tetroxide presented at higher magnification to show the preservation of fine structure and absence of precipitation of the stain. Stained for 12 minutes with potassium permanganate and embedded in Epon. Membranes and RNA granules (*P*) are dense. The spaces (*V*) inside the mitochondria are free of precipitate. × 50,000.

FIGURE 5

Mitochondria of mouse oocyte fixed in Palade's osmium tetroxide and stained for 40 minutes with a saturated solution (0.2 per cent) of phosphotungstic acid in acetone following acetone dehydration. Embedded in Epon. Contrast is not so great as with the permanganate stain. The RNP granules (*P*) are not very dense, but no precipitation of the stain is visible in the spaces (*V*) inside the mitochondria. × 50,000.

FIGURE 6

Mitochondria of mouse oocyte fixed in Palade's osmium tetroxide and embedded in Epon. The section was stained for 1 minute with a 50 per cent saturated solution of lead subacetate (14). RNP granules (*P*) and membranes have a high density. The fine fibrillar and granular structures present in the mitochondrial spaces (*V*) may be due to slight precipitation of the stain or may represent real structures. Precipitation sometimes occurs if the stain is not filtered through a fine enough filter or if dirt from the microtome trough is picked up by the section. × 50,000.