A Double Imbedding Method Giving Water-Permeable Ultrathin Sections for Electron Microscopy. By G. W. Grigg and H. Hoffman. (*From the Commonwealth Scientific and Industrial Research Organization, Animal Genetics Section, Department of Genetics, University of Adelaide, Adelaide, Australia.*)*

Improved techniques of tissue preparation have substantially eased the application of electron microscopy to biological material. With the use of butyl and methyl methacrylates for imbedding (Newman, Borysko, and Swerdlow, 1949), the glass knife for cutting (Latta and Hartmann, 1950), and buffered osmium tetroxide solution for fixing (Palade, 1952), it is possible to prepare cellular material well enough to obtain detailed pictures at high resolution. The development of the diamond knife by Fernández-Morán (1953) is a further significant advance in technique, making possible the routine preparation of sections of consistent quality. As a result, descriptive electron microscopy may be considered to have reached the definitive stage. The need for analytic methods seems indicated.

Methacrylates, though used with great success, have certain disadvantages. If the plastic is left in, it tends to lower the inherent contrast of the section because of its own relatively high electron density; this can be slightly reduced by raising the beam intensity in the electron microscope. Cellular elements that are of low inherent electron density and that are not stained by osmium tetroxide are almost invisible when viewed in methacrylate sections. Removal of the methacrylate from the section by solution introduces tremendous surface tension stresses during drying which result in marked distortion (Anderson, 1954). Sjöstrand (1956) suggested that the thickness of the supporting films normally used to mount the sections on grids was such as to reduce resolution and contrast, and he mounted his material on metal-coated fenestrated membranes, attempting to view important structures through the holes in the membranes. This fails to deal with the lack of inherent contrast, and little improvement resulted. What is required is an imbedding medium appreciably lower in density than the methacrylate polymers, but able to preserve spatial arrangement within ultrathin sections.

The rapid development of descriptive electron microscopy makes chemical identification of cell elements in ultrathin sections highly desirable. Methods so far adopted such as Bradfield's silver Feulgen modification (1954), and the phosphatase techniques of Sheldon, Zetterquist, and Brandes (1955), can locate chemical substances or enzymes in cellular structures only crudely; metal precipitation appears to be an inadequate technique for identification at the electron microscopic level. Indirect methods of chemical identification have been more successful. Palade and Siekevitz (1956) identified the ribonucleoprotein (RNP) particles of the cytoplasm by homogenisation, differential centrifugation, and digestion. Such methods are cumbersome and limited to major cellular components. A simple method, which identifies cell constituents by treating the sections with specific reagents, is obviously desirable. One of the most specific methods of identification is the use of enzymic digestion, with morphological comparison of the specimen before and after treatment. Such a technique requires an imbedding medium which is permeable to aqueous solutions containing large molecules, but which preserves fine structure in the specimen. It is reported here that double imbedding of the material, first in celloidin and then in methacrylate, satisfies the requirement for an imbedding medium of higher permeability and low density, and at the same time produces a block of suitable physical characteristics for cutting into ultrathin sections. This technique is essentially a modification of the classical celloidin-paraffin double imbedding first used for electron microscopy by Pease and Baker (1948). After cutting, the sections are mounted on copper grids covered with formvar film, and the methacrylate polymer in the sections is removed with carbon tetrachloride, leaving the sections in their celloidin support.

Technique

Imbedding.—A schedule, which we have found satisfactory, is presented below. Dehydration is accomplished in an automatic dehydrator over a period of usually about 1 hour (see appendix 1 and Text-fig. 1). A graded series of alcohols can be substituted for this procedure.

Schedule:

1. Fix small pieces of specimen (about 1 mm. cube) in Palade's buffered osmium tetroxide (1 per cent pH 7–7.4, at +4°C.).

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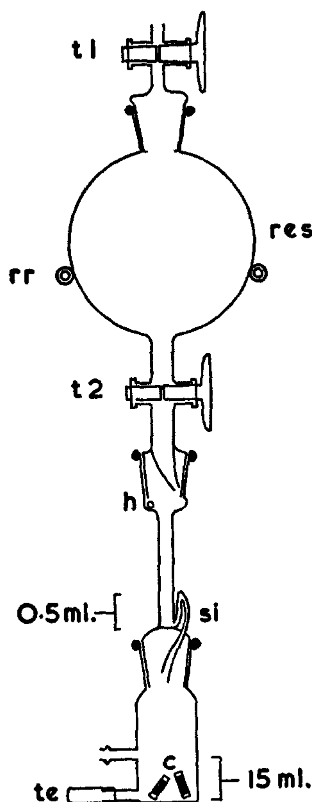

TEXT-FIG. 1. Automatic tissue dehydrator. *c*, stainless steel mesh capsules; *h*, "breathing" hole for siphon; *rr*, rubber covered retort ring; *res*, 1 litre reservoir for absolute alcohol; *si*, siphon; *t1*, glass tap; *t2*, glass tap with fine opening; *te*, rubber teat.

2. Dehydrate, after two rinses in distilled water (5 minutes), from distilled water to absolute alcohol in about 1 hour. Complete the dehydration with two changes of absolute alcohol (5 minutes each).

3. Transfer to alcohol-ether mixture (1:1) for 10 minutes.

4. Transfer into 4 per cent celloidin solution in alcohol-ether mixture, 6 to 12 hours under pressure in imbedding "bomb" (see appendix 2 and text-fig. 2) at 60° C. A suitable celloidin is Gurr's histological celloidin.

5. After infiltration under pressure, transfer the tissue blocks in a thick film of celloidin solution to the lid of a slide staining jar half filled with chloroform; invert the lid over the liquid. After about 10 minutes, the blocks are sufficiently set to drop into liquid chloroform where they remain for about half an hour.

6. Cut the hardened celloidin blocks down to minimal size, and drain them of chloroform; then drop into gelatin capsules containing methacrylate monomer with catalyst (benzoyl peroxide, 2 per cent).

7. Expose the gelatin capsules filled with methacrylate monomer to a cooled mercury vapour lamp. They will polymerise in the ultraviolet light at about room temperature. A suitable mixture of monomers, which produces blocks of requisite hardness, consists of 15 per cent methyl methacrylate, 85 per cent butyl methacrylate. Blocks containing doubly imbedded tissue tend to be softer than normal methacrylate blocks; hence the necessity to harden with an increased amount of methyl methacrylate.

8. Section the blocks in the usual way for methacrylate, transfer sections to formvar-coated grids, and dip in carbon tetrachloride for about 10 seconds, to remove the methacrylate from the sections.

A schedule for treatment of sections with enzyme solutions is provided below: the procedure to be followed is similar for most reagents.

1. Transfer sections on formvar films over copper grids to enzyme solutions at 35–50°C. for appropriate periods.

2. Wash grids in tubes of distilled water for 5 minutes, repeat washing 5 times.

3. Transfer grids to N/5 HCl for 15 minutes.

4. Wash again 5 times in distilled water.

Sections have been treated successfully with the enzymes papain, pepsin, trypsin deoxyribonuclease and ribonuclease, and with staining solutions such as phosphotungstic acid and ferric chloride. Enzymic digestion at the appropriate pH was carried out by submerging the grids carrying the sections in enzyme solution in a small test tube. In this way, it is possible to work with microgram quantities of expensive or rare enzymes, 0.05 to 0.1 ml. of solution only being required. Sometimes a fine precipitate, resulting from interaction between buffer solution and grid, obscures the sections. This can usually be removed by treating the grids with N/5 HCl, for about 15 minutes.

Using the procedures set out above, ribbons of sections treated with a reagent such as an enzyme may be compared with untreated ones on separate grids. As the sections used in electron microscopy are extremely thin, those on different grids may well be cut through the same cells.

It is possible also by the following procedure to treat alternate sections or groups of sections on the same grid, leaving the remaining group untreated as a control. Since methacrylate polymer, that has been "beamed" in the electron microscope, becomes insoluble in carbon tetrachloride, masking sections before "beaming" preserves the methacrylate film in some, and not others. A mask, constructed by soldering together halves of Sjöstrand type grids (using the side with the widespaced bars) is placed between the grid carrying the sections and the electron beam. The specimen is then exposed to a beam of medium intensity for several minutes, and thereafter removed and treated with solvent. The "unbeamed" portion of the sections is then devoid of methacrylate, and

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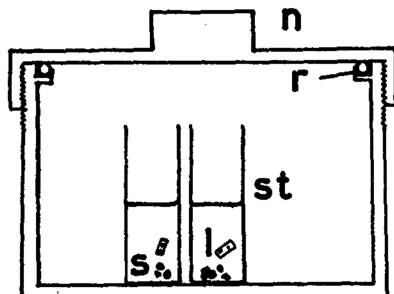
permeable to enzyme solutions; while the beamed part, still imbedded in its impermeable methacrylate, is immune to digestion. In certain cases it is possible to treat sections that have been viewed and photographed in the electron microscope. This, however, is more feasible with electron stains of a metallic nature because beaming may modify specimens so as to render them immune to enzymatic digestion.

OBSERVATIONS AND DISCUSSION

The double imbedding method described was devised primarily to make possible the treatment of ultrathin sections with enzyme and other aqueous solution; it also gives a higher contrast, owing to the low density of the residual celloidin, and hence better resolution, with any given thickness of sections. Fine detail and orientation are as well preserved as in conventional imbedding. This can be seen by treating one-half of the sections with solvent, and leaving the methacrylate in the others. Preservation of detail and orientation are well illustrated in Fig. 1.

These new methods introduce no technical difficulties, require only reagents easily obtained, and the procedure is as straightforward and simple as conventional methacrylate preparation. The blocks are essentially similar to normal methacrylate ones, and the sections cut and handle like the conventional ones. When the methacrylate has been removed, the sections are permeable to aqueous solution.

The most important feature of the technique is this ready permeability to aqueous solutions of sections so prepared. Full advantage has not yet been taken of this fact, and the full range of uses has not yet been realised. We have analysed the chromatin filaments of the nucleus, distinguishing their protein and nucleic acid elements by enzymatic digestion and establishing their relationships (Grigg and Hoffman, 1958). The scope for further application of such cytochemical methods to the analysis of cell ultrastructure is obviously large.



TEXT-FIG. 2. Imbedding bomb. *l*, small labels; *n*, hexagonal nut; *r*, rubber "0" ring; *s*, specimens; *st*, specimen tubes filled with celloidin solution in alcohol-ether.

SUMMARY

A method has been described which enables the cutting of ultrathin sections embedded in methacrylate, after which the methacrylate may be removed, leaving sections supported in celloidin.

Sections obtained in this way possess enhanced contrast and are readily accessible to enzyme and reagent solutions. Detail in the specimens is fully preserved. By these methods sections may be enzymically digested, enabling the identification of specific substances or groupings.

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APPENDIX 1

Automatic Tissue Dehydrator.—The automatic tissue dehydrator in use in this laboratory is illustrated in Text-fig. 1. In operation, the fixed and washed specimens are placed in small capsules of wire mesh, closed with polythene plugs. They are then transferred into distilled water in the bottom cell of the dehydrator with tap *T1* open. The reservoir, *res*, is filled with absolute alcohol. Tap 2 is then adjusted so that alcohol flows through it at approximately 1 drop per second. When about 0.5 ml. of alcohol collects in the tube, it siphons over through *si* into the lower vessel containing the specimens in water, ensuring agitation and good mixing. Complete dehydration takes approximately 1 hour, as indicated by a small glass hydrometer which

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sinks to the bottom. Thereafter, the rubber teat, *te*, on the lower outlet tube is removed, and the liquid in the lower container is quickly drained off and replaced with fresh absolute alcohol by opening tap *T2* wide.

It was found convenient to insert small pencilled paper labels into the capsules; these labels remain with the specimens throughout the imbedding procedures. This dehydrator has proved simple, reliable, and labour-saving.

APPENDIX 2

Imbedding Bomb.—A convenient container, used to impregnate tissues with celloidin under pressure, at 60°C. is illustrated in Text-fig. 2. It is constructed of steel, is 3 inches in diameter, and 2½ inches high. The lid seals to the body by means of a rubber "0" ring, *r*. This ring has to be replaced after about four imbedding operations. Neoprene "0" rings, with longer life may be substituted here.

At 60°C., in the presence of excess celloidin solution, pressure is approximately two atmospheres.

EXPLANATION OF PLATE 188

FIG. 1. A group of reticulum cells from the popliteal lymph node of mouse, osmium tetroxide-fixed and doubly imbedded in celloidin and methacrylate. Methacrylate polymer has been removed from the section with carbon tetrachloride, leaving the section supported in celloidin. Note the preservation of detail in membranes, mitochondria, and cytoplasmic granules.

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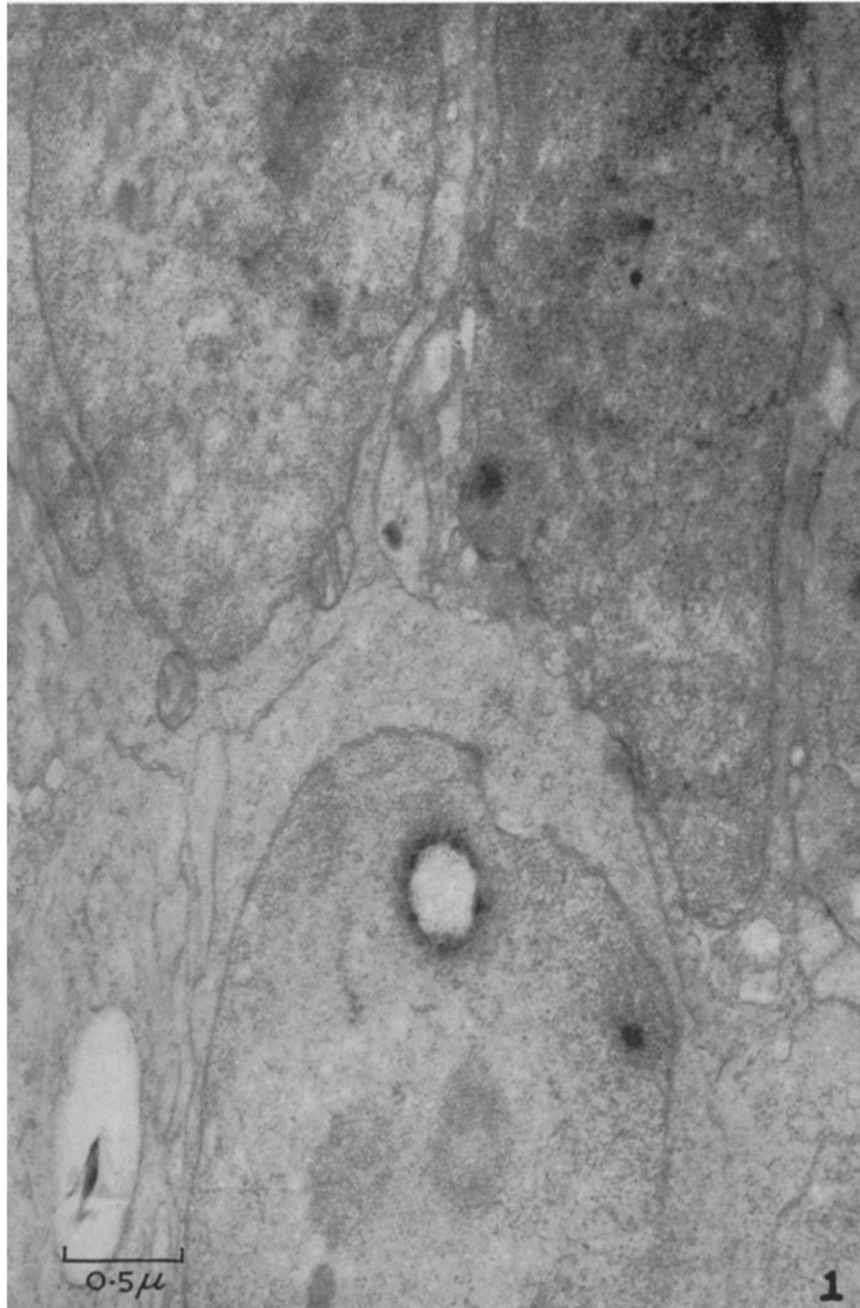

(Grigg and Hoffman: Double imbedding method)

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