THE PREPARATION OF FROZEN-DRIED TISSUE FOR ELECTRON MICROSCOPY*

By ISIDORE GERSH, Ph.D.

(*From the Department of Anatomy, The University of Chicago, Chicago*)

The purpose of this article is to describe the methods developed in my laboratory for the study of frozen-dried preparations with the electron microscope. The main objects of such methods are to retain the advantages of fixation by freezing and drying in the study of fine structure and at the same time to expand the technical possibilities so that a larger variety of morphological and cytochemical stains should be available.

In this paper only the basic technical details will be given, together with a justification for their use. Fuller details with a more extensive justification will be published separately in several papers dealing with morphological and cytochemical studies of liver cells. The methods described were developed with the collaboration of Dr. I. Isenberg, Dr. J. L. Stephenson, Dr. W. Bondareff, and Dr. H. Finck. Some of the results have been presented in abstract form in the proceedings of the American Association of Anatomists (1955), the Sixième Congrès International d'Anatomie (1955), and the Troisième Congrès International de Biochimie (1955).

Morphological Method

(*a*) *Freezing and Drying.*—The object is to avoid the formation of ice crystals during the manipulations. Liver slices 0.1 to 0.2 mm. on a side are prepared in a moist box (Fig. 1). The process is accelerated by the use of a double-bladed modified Valentine knife (Fig. 2). Two or three of the minute liver slices are pushed with a tungsten filament probe on an aluminum foil about 3 mm. square and 0.05 mm. thick. The foil and tissue are immersed and vigorously shaken in liquid propane cooled to about −175°C. by means of liquid nitrogen. The liquid propane is condensed from the gaseous state in a long-necked flask immersed in liquid nitrogen and transferred to a beaker for easier access (Fig. 3). The frozen specimen is transferred to a gelatine capsule in liquid nitrogen and the capsule is stoppered loosely with cotton. The specimens may be stored indefinitely in liquid nitrogen, or dried. For this purpose, the capsule containing the specimen is transferred to the vacuum chamber at about −30°C. After 1 day, the vacuum chamber is brought to room temperature and air is leaked slowly through a silica gel desiccant.

(*b*) *Postfixation in 95 Per Cent Alcohol.*—The object is to denature the proteins sufficiently to prevent as far as possible significant structural reorganization attributable to subsequent

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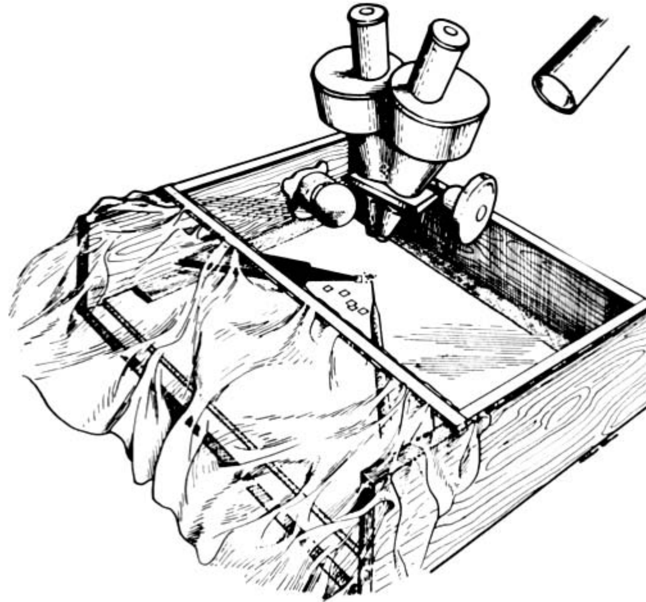

FIG. 1. Diagrammatic representation of moist box used for the preparation of thin pieces of organs and tissues for freezing.

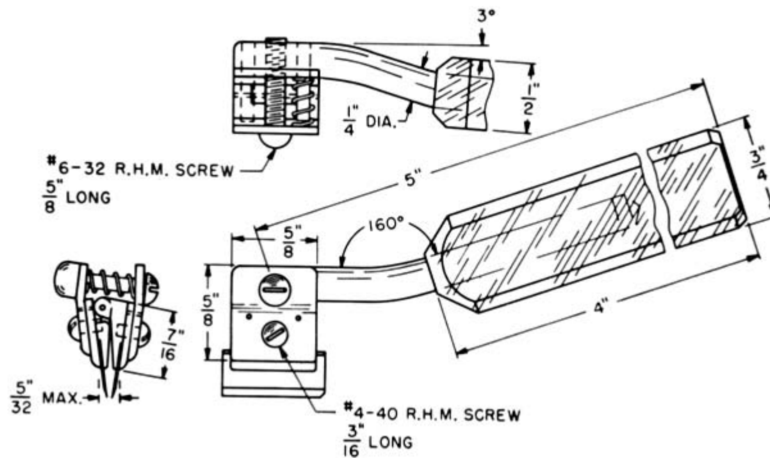

FIG. 2. Drawing of double-bladed modified Valentine knife for the preparation of thin pieces of liver in the moist box.

manipulations. The dried specimens are removed from the aluminum carriers and transferred rapidly to glass-stoppered Feigl tubes (approximately 0.2 ml. capacity (Fig. 4)), in which they remain for 24 hours.

(c) *Staining en Bloc.*—The object is to increase selective electron scattering by increasing

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the mass of various sites, with minimal disturbance of the fine structure retained by post-fixation in 95 per cent alcohol. Fresh solutions of the following list of reagents were made up in 95 per cent alcohol (5 per cent or saturated). The 95 per cent alcohol in the Feigl tubes is removed with a micropipette and replaced with the reagent solution. This is removed after 15 minutes to 18 hours, depending on the reagent. The specimens are washed with several

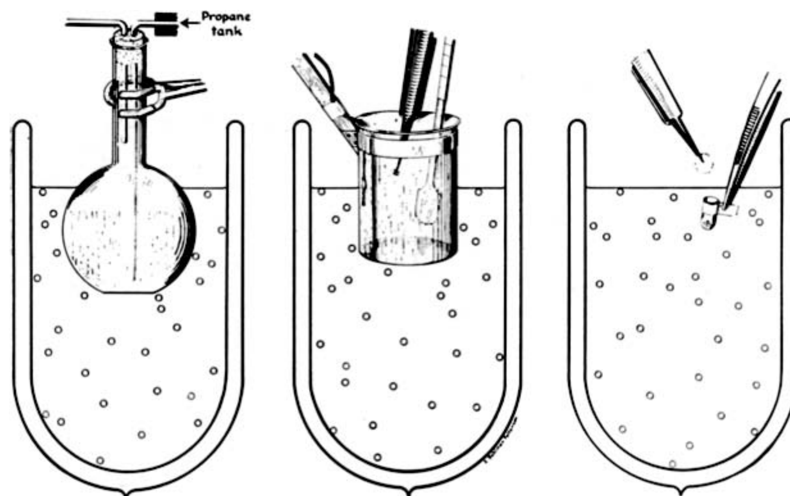

FIG. 3. Diagrammatic representation of collection of liquid propane and its use in the freezing of specimens; subsequent transfer to gelatine capsules for indefinite storage or drying is also shown.

changes of 95 per cent alcohol, and remain in the last change for 1 day. The following is an alphabetical list of reagents tested:

Arsenic pentoxide
Barium bromide
Barium iodide
Bismuth chloride
Cadmium acetate
Cadmium iodide
Cerium chloride
Cerium nitrate
Cesium chloride
Chromium trioxide
Cobalt acetate
Cobalt chloride
Cobalt nitrate
Cupric acetate
Cupric bromide
Gallium nitrate
Gallium sulfate
Gold chloride
Indium nitrate
Iridium tetrachloride
Iron chloride
Iron sulfate
Lanthanum chloride
Lanthanum nitrate
Lead acetate
Lead nitrate
Manganese acetate
Mercuric acetate
Mercuric chloride
Nickel bromide
Nickel chloride
Osmium tetroxide
Osmium trichloride
Phosphomolybdic acid
Phosphotungstic acid
Platinic tetrabromide

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Potassium mercury cyanide
Potassium nitroprusside
Potassium selenocyanide
Sodium cacodylate
Tantalum pentachloride
Tellurium tetrachloride
Thallium acetate
Thorium nitrate
Tin chloride
Tin iodide
Uranyl nitrate
Vanadyl chloride
Yttrium nitrate
Zinc acetate
Zinc chloride
Zirconium tetrachloride

(*d*) *Infiltration and Embedding.*—The object is to embed in methacrylate with minimal distortion of the fine structure of the stained specimen. The specimens are infiltrated with

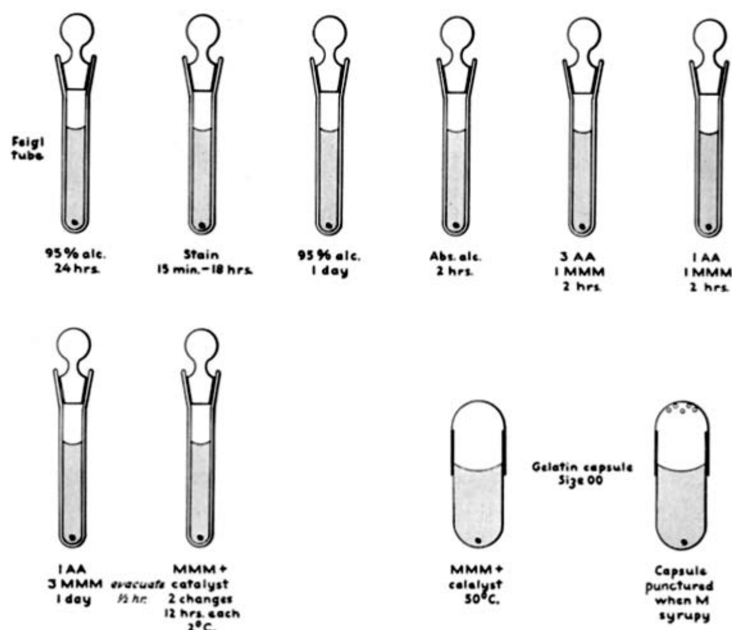

FIG. 4. Diagrammatic representation of the schedule used in morphological studies for postfixation, staining, infiltration with methacrylate, and polymerization.

methacrylate monomer according to the schedule (Fig. 4), where AA is absolute alcohol, MMM is methacrylate monomer mixture (95 per cent butyl and 5 per cent methyl methacrylate), and catalyst is luperco. The specimens are now transferred from the Feigl tubes to clean gelatine capsules and polymerized as in the schedule. The gelatine capsule is softened in water and peeled off.

(*e*) *Sectioning.*—The blocked specimen is trimmed, and adjusted in the microtome chuck. A specially designed microtome is used which depends only on the elastic properties of certain metals and is independent of oil films, sliding surfaces, or gears. The specimen is cut with a glass knife, floated on water, and oriented on the specimen grid.

(*f*) *Observation and Photography of Sections.*—Thin sections are viewed at a magnification of 60,000 with a Philips EM 100A unit, and photographs were made on 35 mm. Adox film with a reduction of approximately 3½ times. The

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film was developed for 16 minutes at 20°C. with microdol. Negatives are enlarged about 5 times on printing.

(*g*) A number of control studies were made to test the effectiveness and the margin of safety of the method.

1. Pieces of liver of similar size were frozen in propane at temperatures ranging from −76°C. to −152°C. Ice crystals appeared regularly at the highest temperature only.

2. Pieces of liver of thicknesses ranging from 0.05 to 0.75 mm. were frozen in propane at −175°C. Ice crystals appeared regularly only in the largest specimens. (See also the article by Stephenson in this Symposium for a more rigid analysis of the problem of freezing water in a vitreous state.)

3. Pieces of liver were found to be dry after only 30 minutes in the drying apparatus customarily used.

4. Frozen specimens were dried at −78°C. The morphology was the same as after drying at about −30°C.

5. Frozen specimens were stored at about −30°C. for 15 days and then dried at the same temperature. The morphology was undisturbed by the long storage period.

6. The basic morphological pattern after postfixation in 95 per cent alcohol persisted after postfixation by other reagents. These include: heating *in vacuo* at 100°C. for 12 hours or more, followed by immersion in 95 per cent alcohol; immersion in buffered alkaline formaldehyde-dioxane; immersion in a mixture of zinc acetate and phosphotungstic acid in cold acidified alcohol; immersion in 5 per cent *aqueous* platinum tetrabromide and phosphomolybdic acid; and immersion in 5 per cent solutions of the latter reagents in 80 per cent alcohol.

7. The basic morphological pattern remained the same also after postfixation and staining in *aqueous* osmium tetroxide.

8. The basic morphological pattern remained the same after all methods of staining, and could be recorded also in unstained specimens, though the images of the latter lacked the contrast that could be found in favorably stained specimens.

9. The basic morphological pattern remained the same after embedding in other media, such as candelilla wax, paraffin-celloidin, and diglycerol distearate.

10. The basic morphological pattern remained the same whether the section was mounted dry and viewed, or whether it was floated on water, mounted, dried, and viewed.

Cytochemical Methods

The methods employed are for glycogen, desoxyribonucleic acid, and ribonucleic acid. As the stains used contain primarily C, H, N, and O, it was necessary to develop the theoretical aspects of the use of such stains for creating significant contrast for viewing with the electron microscope. This analysis will be presented separately by D. I. Isenberg.

(*a*) *Glycogen by the Periodate-Leucofuchsin Stain en bloc.*—All the reactions took place

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in glass-stoppered Feigl tubes. After freezing and drying, and postfixation in 95 per cent alcohol, the liver specimens were treated as follows:—

1. 70 per cent alcohol—1 hour.
2. Alcoholic periodic acid—30 minutes.
3. 70 per cent alcohol—6 to 8 changes—2 hours.
4. Leucofuchsin—3 hours.
5. Sulfurous acid—8 changes—4 hours.
6. 95 per cent alcohol—12 changes—24 hours.
7. Embed in methacrylate (see Morphological method).

A number of control specimens led to the conclusion that the method is as specific for glycogen when the preparations are viewed with the electron microscope as with the light microscope. These included the following preparations viewed with the electron microscope: buffered salivary control prior to staining, buffer control with no amylase prior to staining, and staining in the usual way, except for the omission of periodic acid or leucofuchsin. Finally, agreement of the image as viewed with the electron microscope with control preparations embedded in paraffin and stained in sections 6 μ thick was good. A fuller description of the method and results obtained with it will be published by Dr. W. Bondareff.

(*b*) *Cytoplasmic and Nuclear Basophilia* (*Ribonucleic and Desoxyribonucleic Acids*) *by the Use of the Gallocyanin-Chromalum Stain en bloc.*—Two new features enter into this description: (1) a glass unit which serves for purposes of drying at a low temperature, subsequent heating *in vacuo* at 100°C., and finally infiltration with 95 per cent alcohol *in vacuo* without transferring the specimens. This further denaturation by heat was found to be necessary to reduce structural disarrangements when aqueous media are used. (2) Use of a self-limiting, progressive stain.

After freezing and drying of liver specimens and their subsequent postfixation by heat and 95 per cent alcohol, the specimens were treated as follows:—

1. 70 per cent alcohol, 50 per cent alcohol, water—2 hours each.
2. Gallocyanin-chromalum stain—48 hours.
3. Water—8 changes, 2 hours.
4. 95 per cent alcohol—12 changes, 24 hours.
5. Embed in methacrylate by the platform method.

As with the method for glycogen, a number of control specimens led to the conclusion that this stain is very largely specific for ribonucleic and desoxyribonucleic acids, and also that each could be viewed separately by appropriate use of enzymes. These included the following preparations viewed with the electron microscope: ribonuclease digestion prior to staining, with the appropriate water control; desoxyribonuclease digestion prior to staining, with the appropriate adjuvant control; and chromalum without gallocyanin. Comparison with unstained specimens and with specimens stained with platinum tetrabromide after the various treatments showed that the gallocyanin-chromalum stain did not disrupt the morphology unduly. Comparison of the images viewed with the electron microscope with those of thicker sections from the same methacrylate blocks served to show that the method was as specific at the submicroscopic level as at the level of the light microscope.

(*c*) *Desoxyribonucleic Acid by the Feulgen Method en bloc.*—After freezing and drying, and postfixation by heat followed by 95 per cent alcohol *in vacuo*, the specimens were treated as follows:—

1. 70 per cent alcohol, 50 per cent alcohol, water—2 hours each.
2. N/1 HCL—30 minutes at 4°C.
3. N/1 HCL—15 minutes at 60°C.
4. Water—30 minutes.
5. Leucofuchsin—3 hours.
6. Sulfurous acid—8 changes, 3 hours.

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7. 95 per cent alcohol—12 changes, 24 hours.
8. Embed in methacrylate by the platform method.

As in the method for glycogen, a number of control specimens led to the conclusion that the method is as specific for desoxyribonucleic acid when the preparations are viewed with the electron microscope as with the light microscope. These included the following preparations viewed with the electron microscope: desoxyribonuclease preparations, with suitable adjuvant controls; preparations treated with enzyme but not hydrolyzed prior to staining; preparations not treated with enzyme and not hydrolyzed prior to staining, unstained preparations. However, it was evident from comparison with unstained as well as morphological preparations, that some swelling and diffusion took place at the submicroscopic level though these artifacts were not detectable with the light microscope. The methods for the nucleic acids as well as the results obtained will be published separately by Dr. H. Finçk.

SUMMARY

A method is described for the preparation of liver by freezing and drying which avoids the formation of ice crystals visible with the electron microscope. Such dried preparations after postfixation may be stained with numerous reagents. They may also be stained for cytochemical study of ribonucleic and desoxyribonucleic acids, and for glycogen. Details of the methods are given, together with a brief justification of them through a summary of the controls employed. Further details, as well as a description of the submicroscopic morphology following the use of the methods, will be described elsewhere.

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