# IMPROVED TECHNIQUES FOR THE PREPARATION OF ULTRATHIN FROZEN SECTIONS

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ABSTRACT

Ultrathin frozen sections of biological tissues for electron microscopy provide certain advantages in cytochemical studies in which the penetration of cells by large molecules is necessary and in morphological studies of cellular constituents which are dissolved by the reagents employed in routine plastic embedding. The recent introduction of several types of commercially available cryo-ultramicrotomes makes it possible for many laboratories to employ this valuable tool. This paper summarizes recent improvements in the methods developed in this laboratory for preparing ultrathin frozen sections and reviews some of the inherent problems involved in their use. These procedures may serve as a baseline for other investigators who can then modify or adapt them for their specific purposes.

## INTRODUCTION

In a series of earlier papers (2–5) we described a method for obtaining ultrathin sections of frozen biological tissues for electron microscopy. Although the ultrastructural preservation of the cells was not always satisfactory and the quality of the sections varied, it was shown that this technique could be used successfully for cytochemical (14, 24, 25) and immunological (20) studies, allowing an entirely different approach as compared with other procedures. All of these initial studies were based on the use of an improvised installation which consisted of a Porter-Blum MT-1 microtome mounted in an ordinary commercial deep freeze unit (2, 4). The absence of standardized equipment may explain in part why so few attempts have been made in other laboratories to develop further this technique. A major exception is the work of Christensen, in which a method was devised to obtain ultrathin frozen sections of unfixed tissues without the use of liquids for spreading or staining purposes in order to avoid extraction of soluble substances (6, 7). Following his indications, Ivan Sorvall Inc. (Norwalk, Conn.) has developed a cryokit applicable to the MT-2 microtome. A comparable approach has been made by Hodson and Marshall (11). Other interesting devices for cutting ultrathin frozen sections have been built by C. Reichert Optische Werke AG, Vienna. based on the studies by Dollhopf and collaborators (8, 9), and by LKB Produkter, Stockholm, according to Appleton's (1) and Persson's (19) designs. The instruments in use in this laboratory are those of Sorvall and Reichert, but we have also successfully tested the LKB cryo-ultramicrotome (12).

In this paper we recommend a routine technique which we believe to be considerably improved compared with our earlier proposals. Our immediate purpose is not to reach the theoretically ideal situation where the tissues would be neither fixed nor dehydrated nor spread on any liquid, but to obtain a reasonable number of high-quality sections of aldehyde-fixed cells which will be useful for a variety of cytochemical applications. Further improvements will certainly be necessary, but the technique presented here is a

workable one and can serve as a start for using the commercial cryo-ultramicrotomes.

## MATERIAL AND METHODS

### *Fixation*

Normal adult rat liver, pancreas, kidney, duodenum, adrenal, lymph nodes, heart muscle, and cerebellum were used for this study. In addition, tissue cultures infected with Herpes simplex and Adenovirus 12, and pellets of *Bacillus subtilis* were also examined. The fresh tissues were cut into small cubes 1–1.5 $mm^3$ and fixed for 1 hr at room temperature in 2.5% *glutaraldehyde* buffered at pH 7.2 with 0.2 M sodium cacodylate buffer. We maintained this type of fixation for most of our studies except where otherwise indicated in the text. In a few instances, 4% depolymerized *paraformaldehyde* in cacodylate buffer for 24 hr at 4°C was also used, particularly with lymph nodes for the localization of specific proteins by means of the enzyme labeled antibody method (20). Muscle has been fixed in formalin vapors for 5-30 min. *Fresh, unfixed* liver, heart, and cerebellum were also used and immediately frozen in isopentane at liquid nitrogen temperature.

### *Embedding*

This step is not indispensable, but is strongly recommended if large tissue areas are to be examined. Ordinary pure gelatin is used for this purpose. A 20% or 10% solution is prepared with distilled water at 37°C. The tissue blocks are immersed in this solution for 5–30 min, with frequent stirring, and then cooled, At the present time we generally embed for either 5 min or 20 min in a 20% solution. The thiogel embedding with cross-linking agents used in earlier studies did not give better results and has now been abandoned (2). The sectioning qualities of the blocks are generally improved if the blocks are cut 2 or 3 days after embedding in gelatin. The same favorable effect is obtained if gelatin is postfixed in glutaraldehyde after embedding and before sectioning. Unembedded tissues cut as well but the sections are more fragile and tend to disintegrate easily when spread.

### *Freezing*

Whereas gelatin-embedded blocks were originally placed for 15–30 min in a 50% solution of glycerol before freezing, we now recommend a 30% solution for 5–15 min. These concentrations and the shorter time of treatment are sufficient to reduce considerably ice crystal damage. With a higher percentage of glycerol the sectioning becomes more difficult because of the stickiness of the sections. It is also possible to cut the tissue without an antifreeze agent. The results are sometimes excellent, but the tendency for ice crystal formation is increased. The tissue blocks soaked in glycerol are first placed on the object holder at room temperature. Their moisture is sufficient to provide adhesion. They are then immersed in liquid nitrogen together with the holder, and frozen. Alternatively, the tissue is put in contact with a copper disc at the temperature of liquid nitrogen in order to avoid bubbling (7). The use of *isopentane* cooled with liquid nitrogen as the freezing medium avoids the bubbling of nitrogen around the object to be cooled.

### *Sectioning*

Before sectioning, the frozen blocks are trimmed with a precooled razor blade under the binocular microscope of the Sorvall microtome or with the special trimming device attached to the Reichert equipment. This step is very difficult with the Sorvall instrument because the entire tissue block frequently becomes detached. Trimming mainly consists of removing the excess of the peripheral gelatin bed and frozen glycerol. The object holder is then fixed with the object onto the microtome and kept at a temperature of around −70°C, maintained in our homemade equipment by means of dry ice (4), and in the Sorvall, Reichert, and LKB cryokits by means of liquid nitrogen vapor. As described earlier (3), the temperature of the knife and the trough in our original device was kept between −20° and −25°C and the air in the deep freeze was about −35°C. In the Sorvall cryokit, both the tissue and the knife have the same temperature of −70°C; in the Reichert microtome, the knife temperature is kept at −50°C. This is advantageous as the spreading medium freezes more slowly. We originally emphasized the importance of cutting the tissues very rapidly in order to obtain very thin sections, but with the three commercial instruments we have tested, high speed is no longer required. The cutting speed is nevertheless higher than that normally used for plastic-embedded tissues. The quality of the sections from the two instruments in our laboratory is the same, but the number of sections obtainable per day is somewhat higher with the Sorvall although it seems easier to work with the Reichert.

If, for some reason, contact of the sections with a spreading liquid has to be avoided, the sections can be picked up one by one at the knife edge with a thin steel needle or a tiny brush, and put directly on a Formvar-coated grid (Belden Mfg. Co., Chicago, Ill.). The more or less wrinkled sections are then flattened by means of the polished end of a cooled copper rod (7). Otherwise, it is better if they are allowed to spread in a trough on a 50% solution of dimethylsulfoxide (DMSO) before being picked up with a plastic Marinozzi ring (16) dipped in the usual way into the spreading solution. If the use of DMSO is not

desirable, e.g. for cytochemistry of enzymes, one can use a 50% solution of glycerol as antifreeze for the trough. After this preliminary spreading, the sections are transferred to distilled water and allowed to float in the plastic rings for further spreading until required for cytochemical reactions.

### *Processing of the Sections for Cytochemical Use*

Two important points of procedure have to be respected carefully:

(*a*) To avoid precipitates of reaction products on the copper grid it is preferable to keep the sections floating in plastic rings throughout the reactions.

(*b*) Never let the sections dry until all steps of the final staining are completed. Sections of glutaraldehyde-fixed tissues which are not to be used immediately are floated on distilled water, where they can be kept at 5°C for 24 hr without losing a significant amount of diffusible material. However, one has to check carefully in each case to find out whether soluble enzymes or other components are rapidly extracted. It should be realized that each current cytochemical procedure has to be specially adapted as far as the concentration of substrate or other reagents and time of treatment are concerned. In addition, the strongly reduced thickness of our sections and the absence of a plastic matrix give them properties that are quite different from those of semithin or thick sections. After processing, the sections are mounted in the usual way on Formvar-coated copper grids. It is not within the scope of this paper to describe particular cytochemical applications of ultrathin frozen sections.

### *Staining*

The originally proposed *negative staining* for 15 sec either with 2% phosphotungstic acid (PTA) or, better, with 4% silicotungstate (ST) at 37°C, can still be used alternatively as a routine stain (3). All of the membranes are well visualized in negative image. However, the sections look homogeneously gray and usually do not show preferential contrast. We now prefer to use a *positive stain* based on the classical uranium and lead salts. The staining times are much shorter than for plastic sections: 0.5% uranyl acetate in distilled water for 1 min, followed by lead citrate for 5 sec up to a maximum of 1 min.

### *Drying of the Specimens*

After staining of the sections on the grids, they are usually dried in air. In order to avoid shrinkage artifacts which occur at the section surface around certain organelles, it may be useful after staining to rinse the grids with a solution of 2.5–5% polyethylene glycol (Carbowax M 600, Union Carbide Corp., Chemicals & Plastics, New York) or 5–10% glycerol for 1–3 sec. It is essential that the sections not be permitted to dry between staining and the application of Carbowax or glycerol. Because the stain is partially removed by both solutions, higher concentrations of uranium acetate, and longer staining times (5% for 2–5 min followed by 1 min poststaining in lead citrate instead of 5 sec) are used to compensate partially for the resulting loss of contrast. Attempts have also been made to dry the sections by sublimation at low temperature (−50°C), but this has not improved the results.

### *Examination in the Electron Microscope*

After staining and drying of the sections, they are examined in the usual way without further treatment. A Siemens Elmiskop I electron microscope was used at 80 kv, with an objective aperture of 50 $\mu$.

## RESULTS

### *Methodology*

As a general rule, it can be said that, the longer the *fixation* the harder the tissue and the easier the cutting. Structural preservation of large areas of the tissue is also improved. With shorter fixation times, the well-preserved cellular areas are smaller, although it is always possible to find single cells with very well-preserved organelles even after 5 min of fixation. The proposed fixation time of 1 hr in glutaraldehyde at room temperature has the advantage of giving the best average results, for all the tissues we have studied, with respect to the regularity of sectioning, the thinness of the sections, and the fine structural preservation over a relative large area. The thinnest parts of the sections are perfectly comparable to those of plastic-embedded tissues (Figs. 2, 3 *b*, 6, 8). Fixation in glutaraldehyde can easily be reduced to 15 min, with comparable fine structural preservation if the tissue blocks allow total penetration of the fixative. Paraformaldehyde, 4% for 15 min at 20°C has given inferior results, but the same fixative used for 24 hr at 4°C was found acceptable for lymphoid tissue; the sections often had the tendency to split into fragments. However, such fragments are useful if the cytochemical or immunological applications require very slight fixation. *Unfixed tissue* which was simply frozen without contact with liquids either before or after sectioning was also examined. In such cases the general ultrastructural preservation was found to be poor, particularly because of mechanical compression and excessive folding of the section. In addition,

the lack of contrast of such unstained specimens made the interpretation of the results very difficult. Sections of unfixed tissues, when spread on liquid in the cooled trough, rapidly disintegrate.

The question of whether or not the tissues should be embedded in *gelatin* depends on the tissues to be examined. Fragile, loosely bound tissues, e.g. lymph nodes, and, obviously, pellets of isolated cells or bacteria have to be embedded before sectioning. Such embedding, first proposed by Gilev (10) for thin sectioning, is not necessary for compact epithelial tissue such as liver, kidney, and pancreas. Nevertheless, we recommend brief gelatin embedding, in all cases, for supporting the otherwise extremely fragile sections. The gelatin molecules, the molecular weight of which may vary between 100,000 and 1,000,000 daltons (23), are believed to penetrate not into the cytoplasm, but only into the intercellular space. This is sufficient to improve the sectioning quality of tissue and to prevent disruption when sections are spread on various liquids. Most of the results presented here have been obtained after embedding for 20 min at 37°C. There has not been an important difference when the embedding time was shortened to 5 min.

The use of an *antifreeze agent* to prevent ice crystal formation is not always necessary and depends on the tissue to be studied. Ice crystals are particularly frequent in kidney, and are found in fewer numbers in other tissues.

Freezing in *liquid nitrogen* has been the general procedure used. Immersion in *isopentane* precooled with $N_2$ as recommended for freeze-drying of tissues did not improve the fine structural preservation of the tissues. *Trimming* of the frozen blocks is of particular importance. If the excess of gelatin around the tissues is not cut away, sections will show marked local stickiness and will not spread evenly.

*The sectioning temperature* of the tissues seems to be optimal around −70°C. Sections of good quality have been obtained at −80°C, but still lower temperatures increase the fragility of the sections and are not recommended for the types of tissues and the methods of fixation used in this study. However, experimentation with much lower temperature may be rewarding for unfixed tissues with different elasticity. Temperatures higher than −50°C diminish the cutting efficiency as the stickiness of the tissue increases.

*The speed of cutting* is at present at the upper limit of the commercial Reichert and Sorvall MT2 microtomes. As the results are quite satisfactory, there is no reason to change this parameter in the immediate future. It might be interesting, however, to increase the speed of cutting for tissues whose physical properties are different from those hitherto studied.

*Glass knives* were used exclusively and were prepared with an LKB knife-maker with the usual angle at the cutting edge. We have had no experience so far with diamond knives, but it seems to us that they are not as essential as for cutting plastic. Frozen tissues are relatively soft compared to Epon blocks. The lifespan of a glass knife is thus much longer. Whereas the *section thickness* was variable in our prototype microtome, regular ribbons of remarkably even sections can now be obtained with the commercial microtomes tested, provided of course that the work is carried out under optimal conditions of tissue processing.

How should the sections be mounted on grids? From the point of view of general morphological preservation, immediate spreading at low temperature seems to be the best procedure. Although folding never can be totally avoided, it is then greatly diminished. In addition, compression of the sections practically disappears. Both of the *spreading liquids* that we used, either DMSO or glycerol, were satisfactory. The latter has to be replaced frequently because it freezes quite rapidly at the cryostat temperatures employed. Both liquids allow not only floating and spreading of the section but also observation of the section under the binocular microscope. However, no interference colors are visible and thus we have no precise criterion for an estimate of the section thickness, although we believe that under good conditions of cutting the average thickness is close to that of plastic-embedded material. The possible *loss of diffusible substance* into these spreading liquids is difficult to evaluate. It is believed to be low, if the sections are directly mounted on grids at low temperature. The solubility of lipids and low molecular weight proteins at −30°—50°C in the liquids used seems to be negligible. However, it may be increased if, after the first low temperature spreading, the sections are carried with plastic rings into distilled water for washing at room temperature; e.g., most liver glycogen then disappears. Otherwise, fine structure remains unchanged.

### *Yield of Sections*

Sectioning with the cryo-ultramicrotomes and, in particular, handling the sections, requires considerable skill. Tissues usually can be sectioned more readily after they have been frozen for several hours; hence, blocks mounted and frozen in the morning are best sectioned during the afternoon. The yield of sections is also affected by the hardness of the tissue, either inherent or induced by fixation, and by the persistence of excess frozen gelatin or glycerol around the tissue block.

### *Staining*

The introduction of a simple *positive stain* has greatly facilitated our work. Cell structures appear better differentiated than after negative staining, and the simple positive stain is much easier and better than the practice of the triple positive stain mentioned in a previous paper (2). In particular, nucleic acid–bearing structures are densely stained and proteins show different degrees of density instead of their uniform aspect after PTA or ST. The limitation of the short uranyl-lead stain, however, lies in the fact that membranes are usually not visible. Only exceptionally do they appear with a faint negative contrast. This is a particular handicap for the study of mitochondria where the cristae are only rarely visible (see Figs. 1 and 2). The positive stain is least satisfactory for sections of kidney, muscle, and brain which are better stained with PTA or silicotungstate (Figs. 3, 4, 6, 8). Attempts to combine positive staining with PTA, applied simultaneously with uranyl-lead stain or as a separate negative stain afterwards, have failed so far.

To avoid possible *extraction* of substances during the staining procedure, osmium vapor was also tried as a means of obtaining differential contrast of cell structures, but without success.

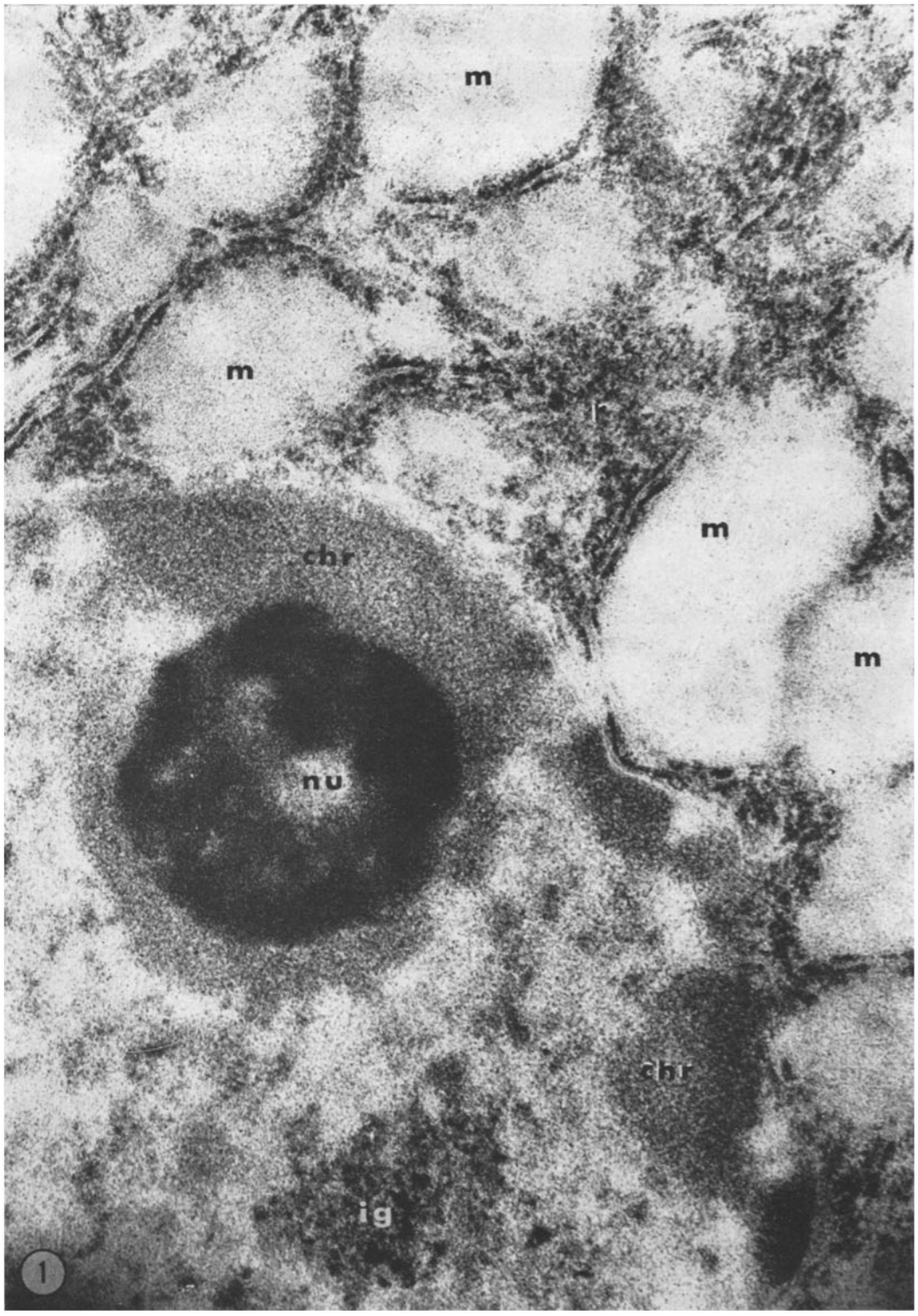
### *Drying*

*Air drying* of the stained sections mounted on grids is the last step in the processing of the specimens. We have not found an ideal method for totally avoiding retraction artifacts, but in most tissues they are minimal. The thinner the sections, the less the distortion that occurs. Retraction and local splitting frequently occur between the mitochondrial rods in convoluted tubules of the kidney and particularly around zymogen granules (Fig. 5 *b*) and the secretion granules of Langerhans Islets in the pancreas. Moreover, distortion of macromolecular structures such as ribosomes is probably also due to drying. *Sublimation* of the water at low temperature has not given consistent results. Freeze-drying under vacuum of the processed sections has not been investigated. However, a brief rinsing of the sections with a solution of Carbowax or glycerol gives a clearly protective effect. These molecules are known to be very hydrophilic, and they delay considerably the drying of the grids. Zymogen granules can thus be completely prevented from shrinking (Fig. 5 *a*). There is a concomitant disadvantage, however, because an increase in the uranium concentration and prolongation of staining is necessary before such treatment, and the residual contrast is then quite irregular. For the time being, therefore, we suggest that staining first be carried out without posttreatment and that the Carbowax or glycerol treatment be employed only if shrinking artifacts are too numerous.

### *Fine Structural Preservation*

Although we were able to obtain high-quality sections in our earlier attempts at cryo-ultramicrotomy (Fig. 2), the commercial instruments that we now use improve the preservation of large areas. Folding, wrinkling, and chattering have diminished, although a similar preparative technique is used. Quite frequently, our present sections allow electron micrography at low magnifications (2000–10,000).

*Liver* cells generally have a well-preserved nucleus with all components structurally defined: chromatin, nucleolus, inter- and perichromatin granules (Fig. 1). Nuclear pores are clearly seen in tangential sections. Large areas of the cytoplasm are structurally intact with the exception of glycogen which frequently, but not always, is dissolved and leaves holes in the section. Areas of ergastoplasm are well visualized with positive stain (Fig. 2). Mitochondria are well preserved, are mostly clear without being swollen, but reveal no cristae with the positive staining method (Fig. 1). However, negative staining demonstrates that the cristae are quite intact. Microbodies with uricase crystals are easily identified, as are bile canaliculi, and the rather fragile space of Disse. If cytochemical studies are to be carried out on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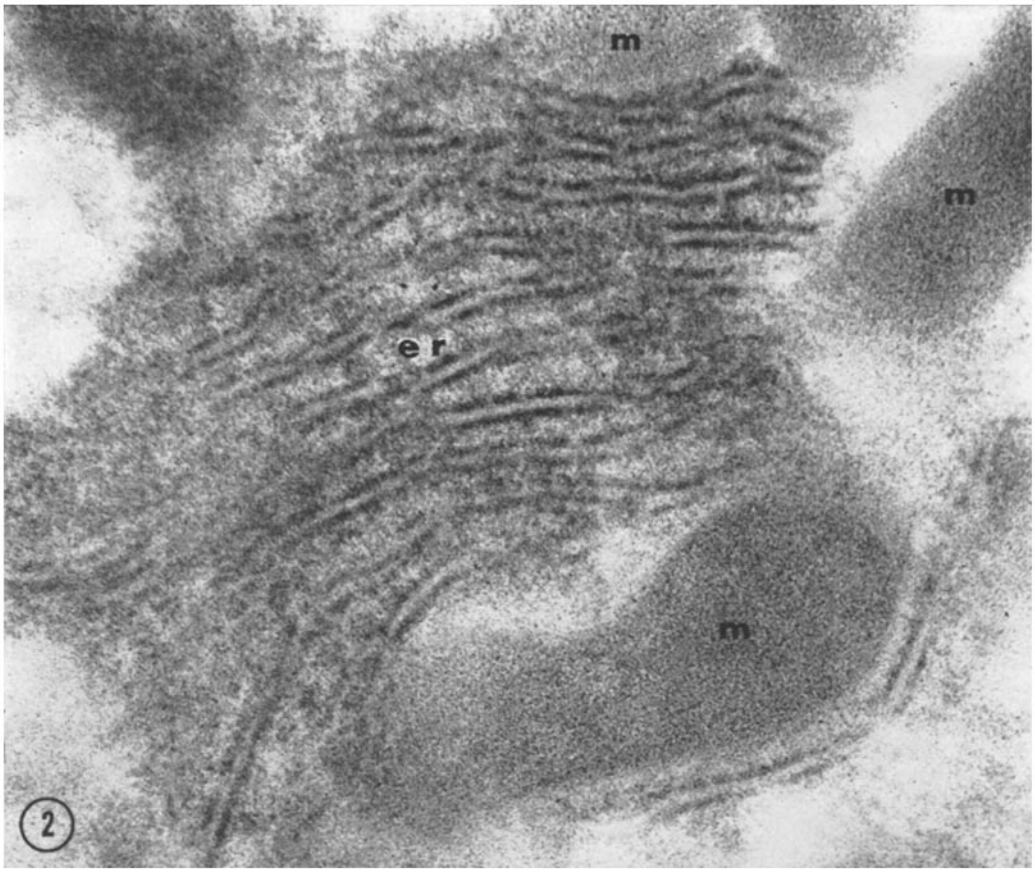


FIGURE 2 Portion of the cytoplasm of a liver cell; positive stain (uranyl acetate 5%, 2 min; lead citrate, 1 min). Ergastoplasmic lamellae (*er*) with ribosomes and mitochondria (*m*) are visible. The membranes are not visualized. Ribosomes in rodlike aggregates also appear. After staining, the section was rinsed for 2 sec in a 5% solution of Carbowax to reduce retraction artifacts due to drying. × 60,000.

liver, it is recommended that the animals be sacrificed after one night of starvation in order to decrease the glycogen content of the hepatocytes, which are then uniformly preserved.

*Kidney* is relatively easy to cut, and some fine structural details show an excellent preservation, particularly the glomerulus with the processes of its podocytes (Fig. 4) and the brush border of the convoluted tubules (Figs. 3 *a* and 3 *b*). Mitochondria may sometimes be well preserved, but may

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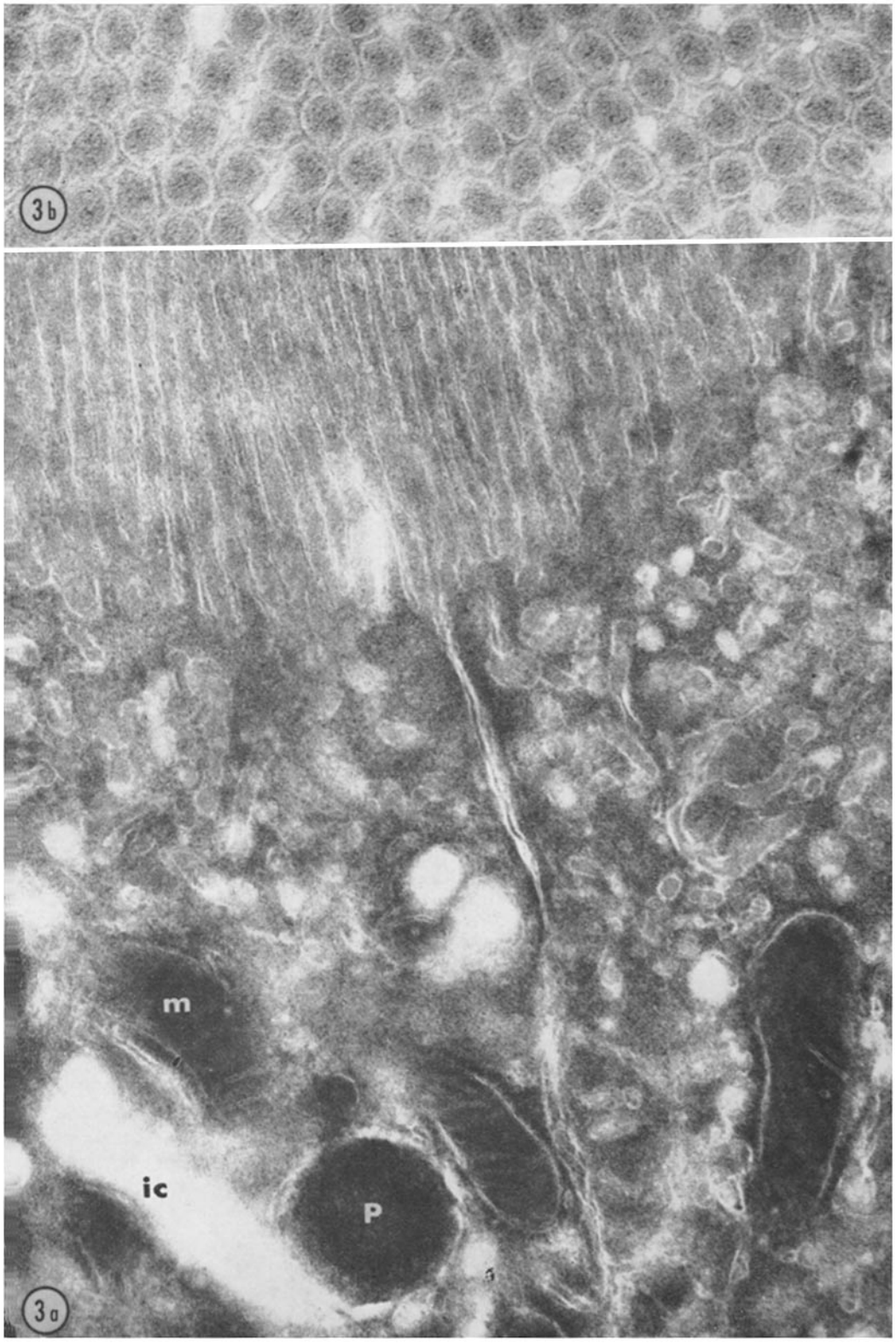
All the tissues presented here have been fixed for 1 hr in glutaraldehyde, embedded in gelatin, soaked in 30% glycerol and frozen in liquid nitrogen. The sections were made either with our own prototype equipment (Fig. 2), or with the Reichert cryo-ultramicrotome (Figs. 1, 3–5, 6 *b*, 8), or with the Sorvall MT2 (Figs. 6 *a*, 7).

FIGURE 1 Portion of a rat liver cell showing a typical aspect after brief positive stain (uranyl acetate 0.5%, 1 min; lead citrate, 5 sec). Ribonucleoprotein (RNP)-carrying structures are strongly stained. nucleolus, *nu*; interchromatin granules, *ig*; cytoplasmic ribosomes, *r*. The chromatin (*chr*) has a medium contrast. Mitochondria (*m*) are practically unstained. The membranes are not visible. Air drying without rinsing in Carbowax solution. × 56,000.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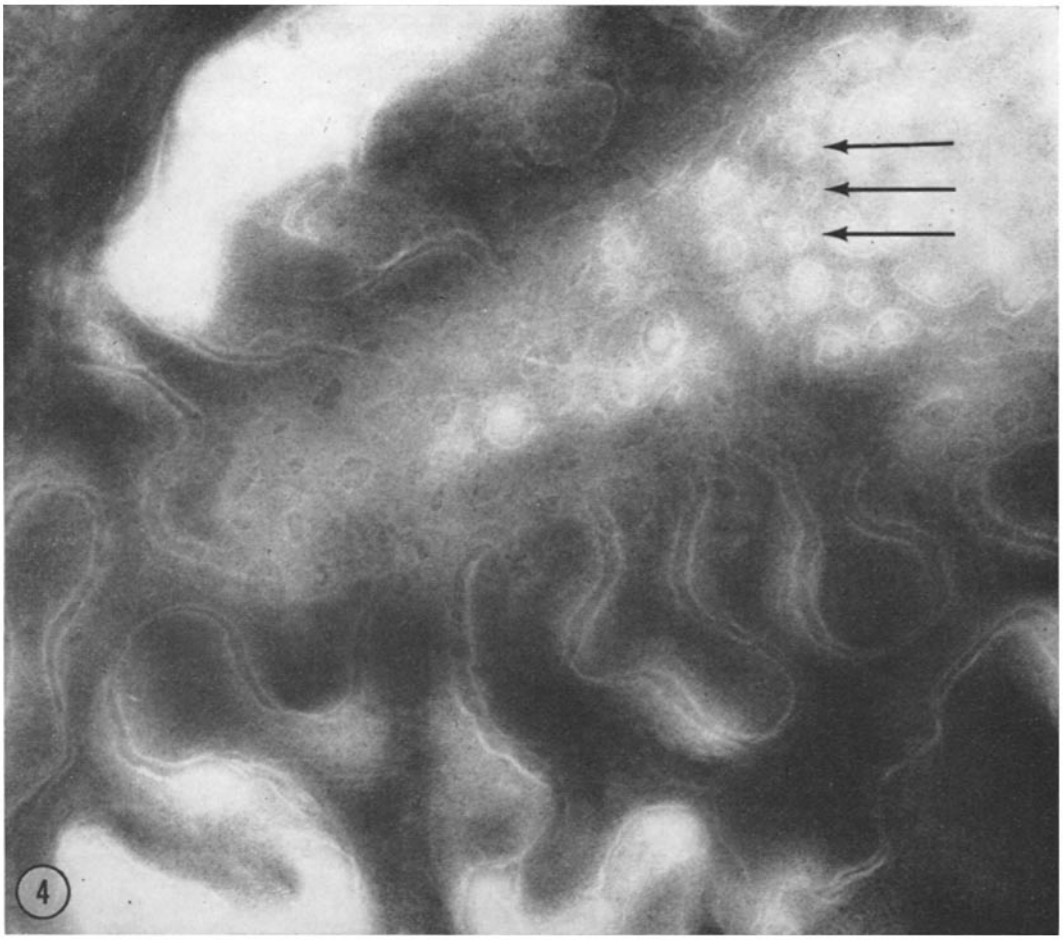


FIGURE 4 Kidney; tangential section of a glomerular capillary; negative stain. The intertangled processes of podocytes are well visible, as is the sievelike structure of the endothelial membrane (arrows). × 60,000.

show a tendency to contract. Moreover, large spaces frequently appear between the mitochondrial rods, probably due to ice crystal formation between contiguous cell walls. Holes are also frequently observed in the cytoplasm in close proximity to the brush border (Fig. 3 *a*). The basement membrane and the underlying capillaries are extremely resistant to the effects of freezing and cutting.

*The exocrine pancreas* cells reveal well-preserved lamellar ergastoplasm and elongated mitochondria. As already mentioned, zymogen granules are mostly retracted (Fig. 5 *b*) from the surrounding ergastoplasm unless the sections are covered, immediately after positive staining, with a thin layer of polyethylene glycol or glycerol. The protective effect of these solutions may be spectacular (Fig. 5 *a*). The same effect is observed on the beta granules of the Langerhans cells, which otherwise are extremely sensitive to air drying.

FIGURE 3 Fig. 3 *a*, kidney cell from convoluted tubule; negative stain, 15 sec in ST; portion with brush border and cytoplasm. Mitochondria (*m*) and protein droplet (*p*) are shown, as well as damage due to ice crystal formation (*ic*). × 30,000. Fig. 3 *b*, Cross-section of a brush border; negative stain; extremely thin section. × 60,000.

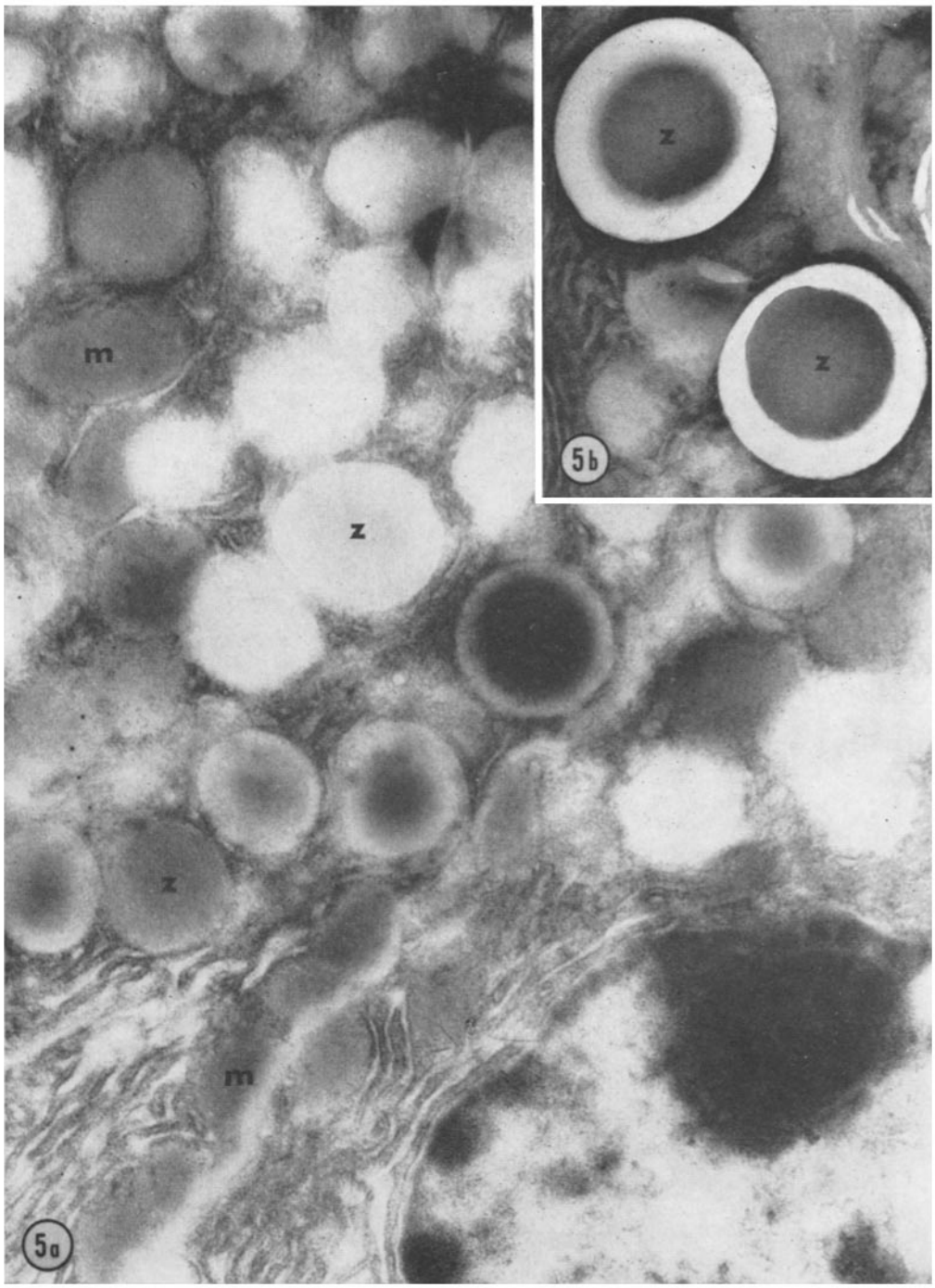


FIGURE 5 Exocrine pancreas cell; positive stain. Fig. 5 *a*, good preservation of an extended area; protection of the section with 10% Carbowax solution before drying. Zymogen granules (*z*) are not retracted, but show different densities. Mitochondria, *m*. × 15,000. Fig. 5 *b*, typical retraction artifact of zymogen granules (*z*) without Carbowax protection. × 30,000.

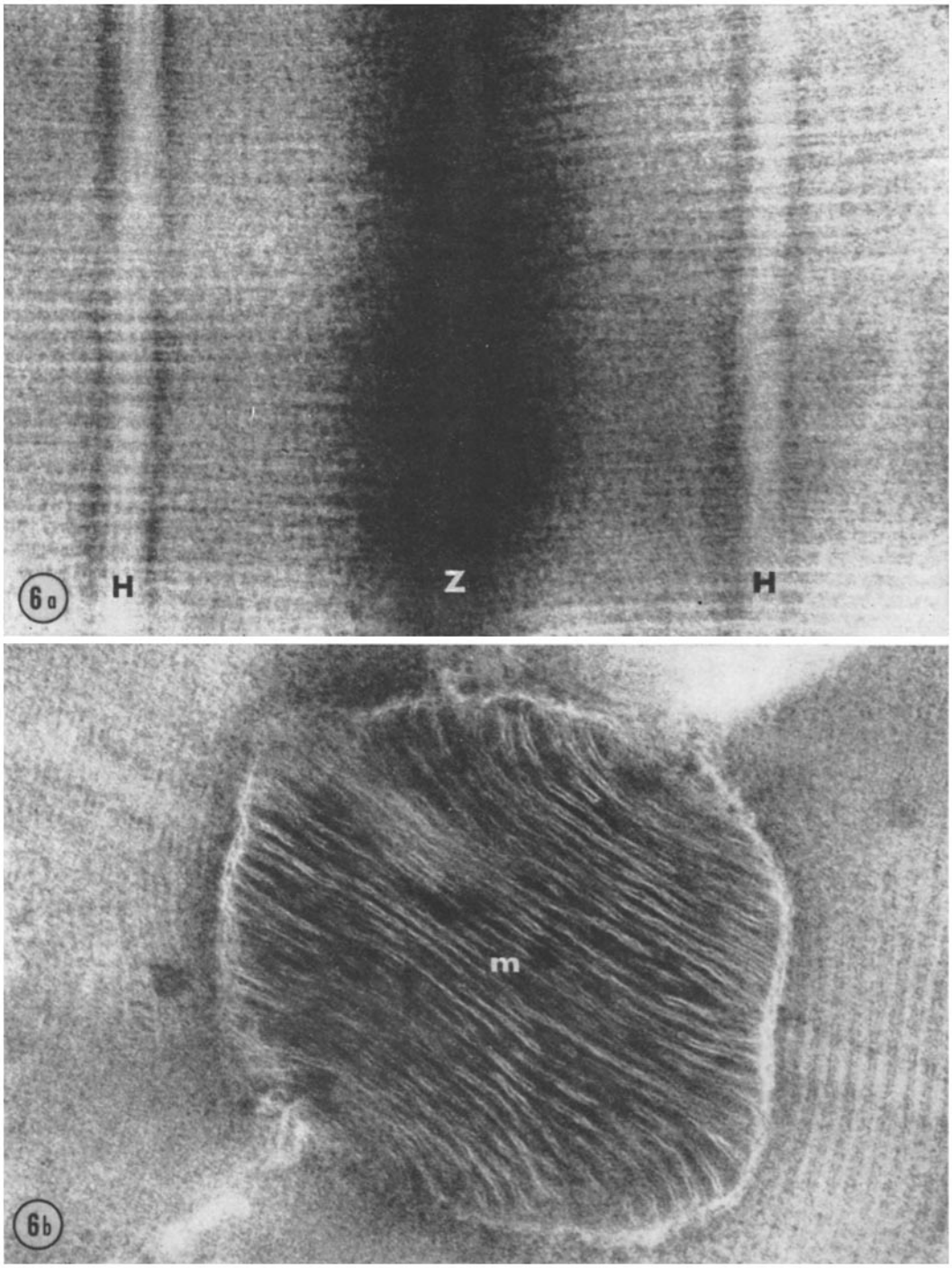


FIGURE 6 Fig. 6 *a*, heart muscle, very thin section, negative stain, portion of a myofibril which is in contracture. Myofilaments are visible, *H* and *Z* bands are shown. × 60,000. Fig. 6 *b*, mitochondrion (*m*) with numerous cristae. × 60,000.

*Muscle*, as a tissue relatively resistant towards osmotic or mechanical damage, is easy to cut and shows excellent preservation of the muscle fibrils with regular banding (Fig. 6 *a*). Mitochondria in heart muscle appear beautifully preserved after staining (Fig. 6 *b*), even after very short fixation with formaldehyde vapors.

*Lymph node* sections are very fragile and easily disintegrate during spreading. However, cellular fine structure is well preserved and it is easy to identify the whole series of immunologically competent cells.

*The adrenal cortex* contains extensive, well-preserved areas of cytoplasm containing mitochondria with tubular cristae visualized by negative stain (Fig. 7 *a*). Many of these mitochondria show a small, electron-opaque crystal-like inclusion composed of regularly spaced lamellae (Fig. 7 *b*). Concerning the preservation of the lipids, most liposomes disappear, leaving a hole in the very thin sections. As is well known from light microscopy, lipid droplets that are larger than the thickness of the section tend to drop out of aldehyde-fixed, frozen sections. Ultrathin frozen sections probably show a similar effect.

*Brain tissue* has been examined for the purpose of studying the preservation of myelin sheets. Fig. 8 shows that the membrane layers are easily visualized with negative stain. Their preservation seems very good. Although cytoplasmic membranes are unstained in frozen sections, it is interesting that the typical periodicity of myelin can be seen.

*Cell cultures* are rather difficult to cut, but when a suitably embedded pellet is employed, they show a very well preserved ultrastructural architecture and also readily reveal fine structural details of infecting viruses.

*Bacteria* are now much easier to cut and very thin sections may be obtained, revealing details of the cell wall, the membrane with mesosomes, ribosomes, and DNA fibers.

## DISCUSSION

The results of ultrathin freeze-sectioning are much better than those presented earlier. First, it is relatively easy to obtain a number of sections of regular thickness in a short time. Secondly, the ultrastructural preservation, although not perfect, is improved and for the first time allows high resolution work. Artifacts of fixation, freezing, cutting, and staining still do occur, but they are disappointing only if one compares the results obtainable by this method with the high-level results obtainable with the now well-standardized plastic sectioning which was gradually improved over almost 20 years by many laboratories. It is our point of view that the purpose of preparing ultrathin frozen sections is to localize cytochemical reactions at the cell organelle level. This is possible now in spite of the presence of some artifacts. If well-chosen areas are examined, the fine structural preservation is amazingly good.

A criticism which can be made concerning the proposed procedure is linked with the use of chemical fixatives and of spreading media and stains, all of which may alter macromolecular structures and produce loss of diffusible substances. Thus, the main advantage of examining fresh frozen tissue in its natural medium is lost. As already pointed out, our main goal was to propose a workable, relatively simple, basic procedure. Modifications naturally will be invented for specific purposes. Each of the factors that possibly induce chemical or structural artifacts can be separately dropped and, therefore, its action can be controlled. *Fixation* in liquids can be reduced to a very short time, or can be replaced by fixation in formalin vapors which causes no loss of substance. *Embedding* in gelatin is not essential. *Spreading* of the sections, however, cannot be avoided easily. The solubility of lipids and proteins below $-50°C$ is believed to be negligible, but should be tested. A factor difficult to evaluate is the possible *chemical action of DMSO* upon the various cellular components. DMSO at high concentrations is known to break hydrogen bonds, and it might thus inhibit enzyme activity or prevent antigen-antibody reaction. We have no indication so far that such alteration indeed takes place. Should a direct chemical action of DMSO be suspected, DMSO can be replaced easily by the less offensive glycerol which, however, freezes very rapidly at the temperature of the cryostat and, therefore, must be changed frequently.

*Staining* does not seem harmful, as it is carried out after the cytochemical reactions. It may be omitted if the reaction products of a specific reagent allow the identification of the cell structure, and it has to be omitted if it affects the cytochemical reaction product. It seems highly desirable that new staining methods be developed which will allow visualization of cellular membranes as well as improved differential contrast of

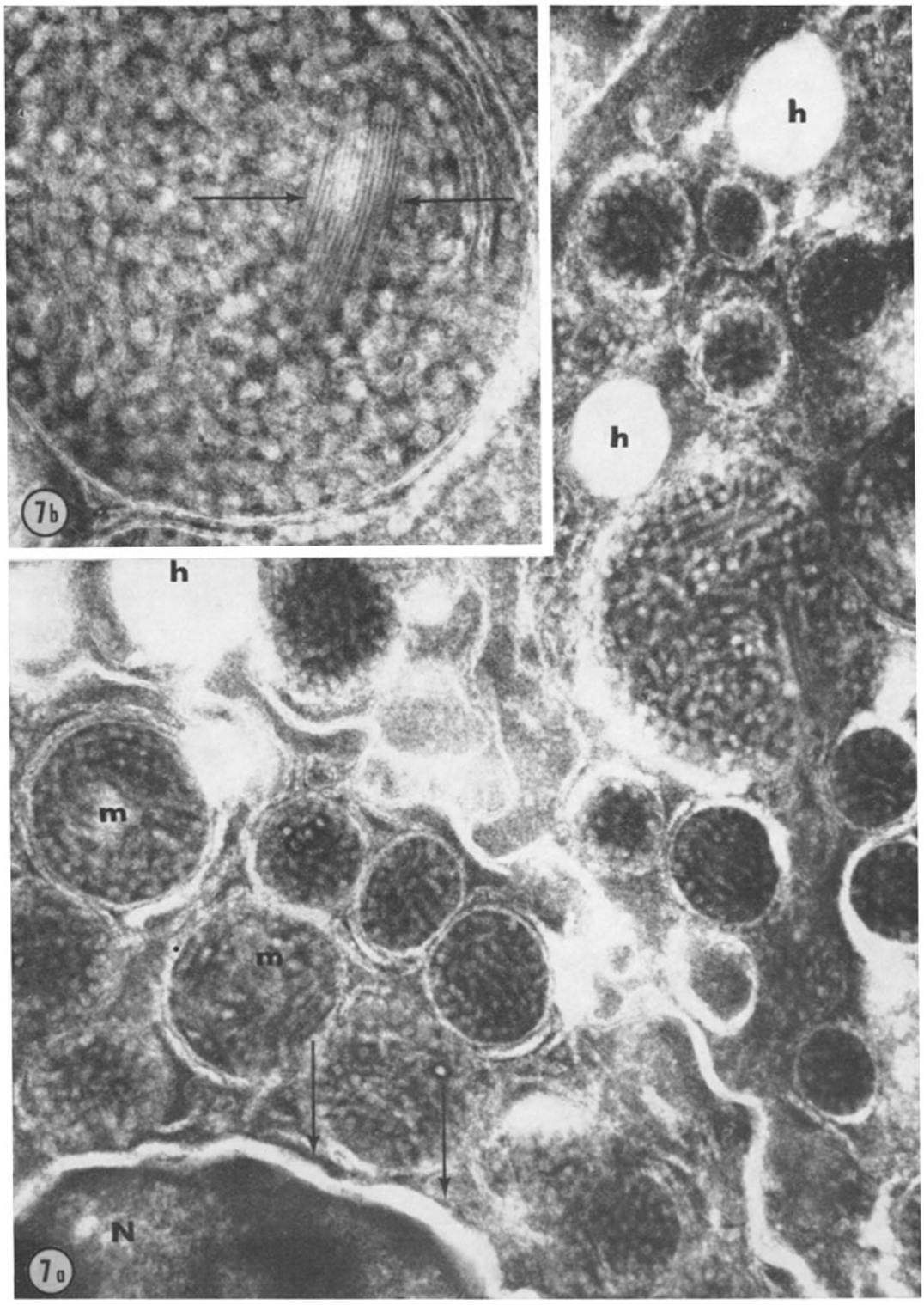


FIGURE 7 Fig. 7 *a*, adrenal gland, zona fasciculata of the cortex; negative stain. A nucleus (*N*) with retraction artifact (arrows) is visible. Cytoplasm and many mitochondria (*m*) with tubular inner structure. Holes (*h*) are frequently seen after lipid droplets or mitochondria have fallen out of the section. × 28,000. Fig. 7 *b*, higher magnification (× 60,000) of a single mitochondrion with crystal-like inclusion (arrows).

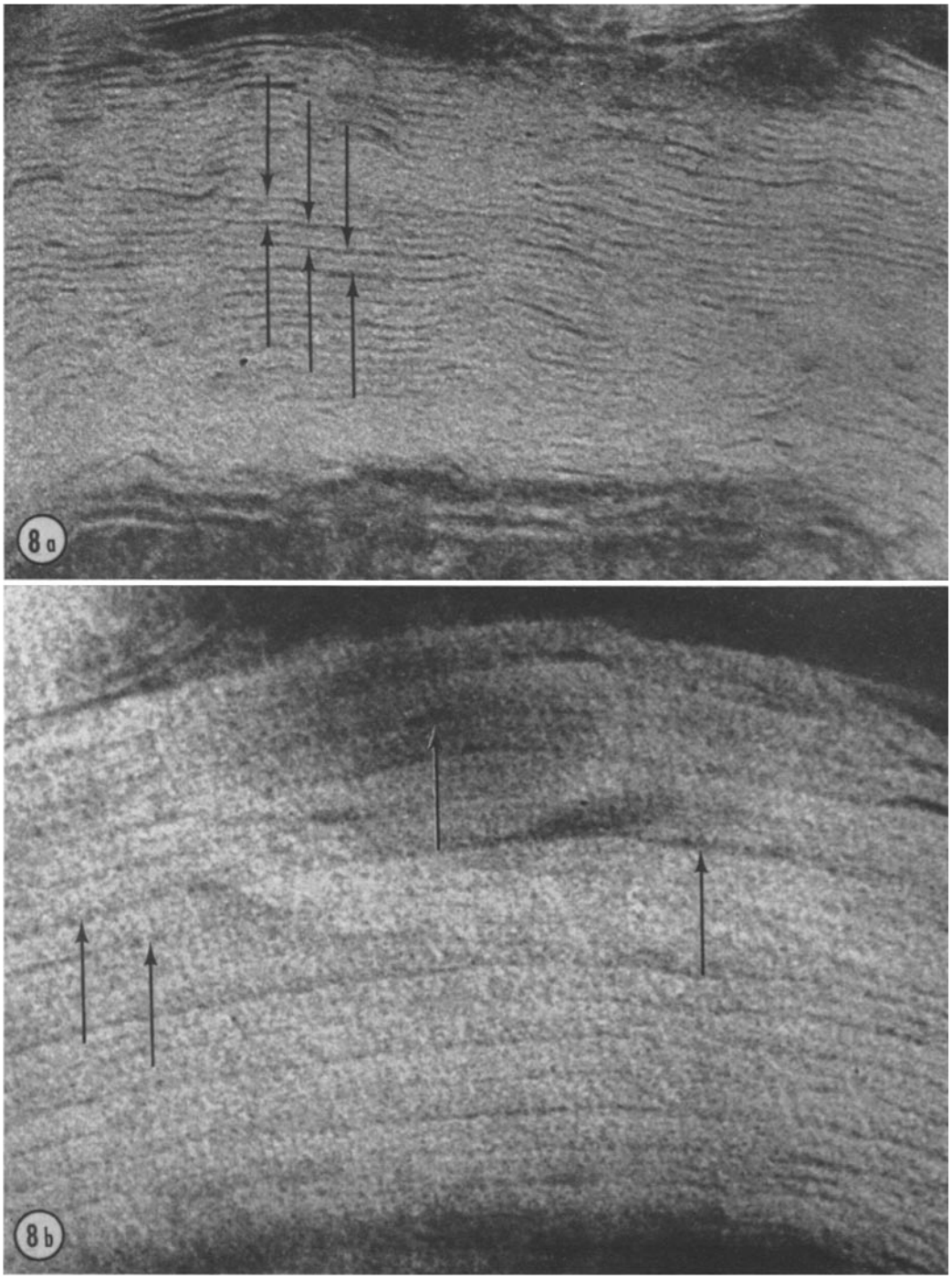


FIGURE 8 Fig. 8 *a*, cerebellum, cortex; myelin sheets; negative stain. Regular lamellar structures are well preserved (arrows). × 200,000. Fig. 8 *b*, regular granular aspect of the membranes is striking in some areas (arrows). Note the mesaxon in upper left corner. × 300,000.

other cell components, particularly nucleic acid–carrying structures.

It is noteworthy that the staining reactions of *membranes* in frozen sections are totally different from those of osmium-fixed and plastic-embedded material. Important molecular changes certainly take place in this latter case where fixation with osmium and dehydration with alcohol and acetone are probably the most corrosive steps. Lipids are at least partially lost, whereas in the case of frozen sections they are theoretically intact because no solvents for lipids are used. It is also known that the lamellar structure of myelin can be demonstrated in positive contrast even after total extraction of lipids (17). The staining properties of membranes are also different from those of glycol methacrylate (GMA)– or hydroxypropylmethacrylate (HPMA)–embedded material where no osmium fixation or alcohol dehydration is used (13, 18) but where lipids are probably dissolved by the GMA monomer. Membranes in frozen sections are not visible after the classical uranyl-lead stain but are shown in negative contrast after PTA or ST. Another interesting observation concerns the apparent *granularity* of membranes in frozen sections when studied at high magnification. This phenomenon is particularly visible in the lamellae of myelin sheets (Fig. 8 *a*, 8 *b*). The granular appearance of membranes has been repeatedly shown by Sjöstrand (21, 22) who insisted on the globular structure of mitochondrial and smooth-surfaced cytomembranes, which he visualized with methods different from ours. *Membrane research* may profit very much from the use of ultrathin frozen sections to study further some of the many unsolved problems with which it is dealing.

The commercially available equipment for cutting ultrathin frozen sections will now enable many laboratories to adapt the above methods to various cytochemical procedures for the study of specific problems. Sections without plastic embedding allow the free and rapid access of large molecules to the biochemically reactive groups within the cell and even within subcellular organelles. Those techniques in which the penetration of reagents into the cell was a problem in the past can now be examined in an entirely new system, and some methods that hitherto have been used successfully in blocks, thick sections, and isolated cells can now be controlled and possibly refined with exceedingly thin slices of biologically active tissue. When we began this work there was some concern that if sections were too thin, the cell constituents under investigation might be present in insufficient quantity to bring about the precipitation of a reaction product or to bind a marker molecule (14). However, preliminary observations with our thinnest frozen sections reveal a very delicate precipitate of lead phosphate at the site of adenosine triphosphatase (ATPase) activity in the Wachstein-Meisel reaction and an alkaline phosphatase or peroxidase reaction at the site of anti-enzyme antibody which was exposed to its antigen in the thin section (20). Other procedures under investigation in this laboratory which appear promising include the use of enzyme-labeled antibodies for the localization of intracellular antigens, a procedure that works erratically in fixed whole cells, and the use of ruthenium red (15) and concanavalin for specific binding to intracellular as well as extracellular polysaccharides.

Many other possible applications come to mind. Since phospholipids and triglycerides are theoretically unaffected by the procedures which we have outlined, *radioautographic studies* of some lipids may be envisaged. Christensen (7) is already applying the technology of ultrathin frozen sections to diffusible steroids, and his approach of employing sections that have not come in contact with liquids of any sort may also be applicable to diffusible ions and soluble proteins. Although we found that the removal of certain proteins and nucleic acids by specific protease and nuclease *digestions* proceeds with extreme rapidity in ultrathin frozen sections of even well-fixed tissue and, therefore, is difficult to control, *selected extractions* by salt solutions and buffers used routinely in biochemical procedures may be possible. Since controlled dissolution of some cell components may accentuate the remaining structures, this type of *chemical dissection* might be applied, for example, to the study of the organization of the myofilaments at the Z band, the orientation of the tonofilaments within the desmosomes, and the substructure of such organelles as the centriole and kinetochore. In spite of its present limitations, particularly with respect to the staining of lipids, we believe that the method presented here can become a useful tool with broad application in cellular topochemistry.

This work is dedicated to the memory of Nicole Granboulan.

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