

ULTRATHIN FROZEN SECTIONS

I. Methods and Ultrastructural Preservation

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ABSTRACT

A relatively simple method for obtaining ultrathin, frozen sections for electron microscopy has been developed. Tissues, cultured cells, and bacteria may be employed. They are fixed in 1.25–4% glutaraldehyde for 1–4 hr, are washed overnight in buffer at 3°C, and are embedded in 20% thiolated gelatin or pure gelatin. Before sectioning they are partially dehydrated in 50% glycerol, frozen in liquid nitrogen on a modified tissue holder, and subsequently maintained at –70°C with dry ice. Finally, they are sectioned very rapidly with glass knives on a slightly modified Porter-Blum MT-1 microtome in a commercial deep-freeze maintained at –35°C and are floated in the trough of the knife on a 40% solution of dimethylsulfoxide (DMSO). The sections are picked up in plastic loops and transferred to distilled water at room temperature for thawing and removal of the DMSO, placed on grids coated with Formvar and carbon, air-dried, and stained with phosphotungstic acid, sodium silicotungstate, or a triple stain of osmium tetroxide, uranyl acetate, and lead. Large flat sections are obtained in which ultrastructural preservation is good. They are particularly useful for cytochemical studies.

INTRODUCTION

The freezing of tissues in preparation for electron microscopy has been used either as a method of preservation which avoids chemical fixation or as a means of rendering the tissue rigid enough for thin sectioning without the use of embedding plastics. In both instances the aim is to maintain cellular structure with as little denaturation and dislocation as possible. Although the cytoplasm is immobilized, it should retain sufficient reactivity of its enzymes and specificity to the action of various agents to be of particular usefulness in cytochemical studies. Ultrastructural cytochemistry has already made considerable progress with the use of various procedures involving both chemical fixation and plastic embedding (11, 13,

14, 17, 27–29). Nevertheless, the use of cells that have undergone less alteration is desirable both to control and to extend the results obtained with other methods.

A major preoccupation of most studies of cells preserved by freezing, that is by freeze-drying or freeze-substitution followed by plastic embedding, has been the evaluation of the relative degree of conservation of ultrastructure (7–9, 24, 25, 30). However, cytochemical investigations with such material have been published. For example, Nelson has demonstrated adenosine triphosphatase (21) and succinic dehydrogenase (22) in frozen-dried spermatozoa, and Mundkur has localized polysaccharides (18), nucleic acids (19), and

sulphydryl groups (20) in frozen-dried yeast. Both investigators carried out their reactions on the frozen-dried cells before final plastic-embedding and sectioning. Chase (3) demonstrated alkaline phosphatase activity directly on ultrathin sections of frozen-dried intestine that was embedded by impregnation with dissolved polymerized methacrylate. Recent advances in the freeze-substitution method (24, 25) promise fruitful new investigations in ultrastructural cytochemistry with tissues preserved by drying and freezing.

The preparation of frozen sections for electron microscopy, of both fresh cells and chemically fixed cells, was first attempted by Fernández-Morán in 1952 (5, 6). His sections were either air-dried or frozen-dried immediately after sectioning, and subsequently cytochemical studies, such as enzymatic digestion and microincineration, were carried out directly on the dried sections. Many of the important technical procedures that are necessary for obtaining frozen, thin sections were already proposed at this time by Fernández-Morán but the method was not pursued further, either in his laboratory or in others, probably because the advent of plastic-embedding media introduced greater ease of tissue preparation and better preservation of ultrastructure. Interest in frozen sections was renewed in this laboratory when it became apparent that thin sections of tissues embedded in epoxy resins or water-miscible methacrylates could not be used for the localization of enzymes or specific antigens and antibodies. Preliminary results (1, 2) with aldehyde-fixed, gelatin-embedded tissues were promising, but the harvest of good sections was rather poor. As a result of recent modifications in the method, large numbers of frozen sections can now be obtained so that extensive experimentation (12) can be carried out with them.

MATERIAL AND METHODS

Fixation

The liver, kidney, and pancreas of the rat were employed as test tissues. Other material occasionally employed were mouse mammary tumors, cultured cells, and bacteria. Cultured mammalian cells, bacteria, and, in one trial, isolated virus particles were centrifuged into pellets during fixation and subsequently handled like blocks of tissue. It was not advantageous to place isolated cells in agar as in embeddings with plastics. Blocks of tissues or pellets 1–1.5 mm³ were routinely fixed for 1 hr at 3°C in

2.5% glutaraldehyde buffered to pH 7.2 with 0.1 M sodium-cacodylate buffer and then were washed overnight in two changes of the cacodylate buffer on an agitator at 3°C. A few other fixing schedules were tried, including 1.25% glutaraldehyde for 15 min and 1 hr, 4% glutaraldehyde for 4 hr, and 4% formaldehyde, also maintained at pH 7.2 with 0.1 M sodium cacodylate buffer, for 1 and 30 hr.

Embedding

Several embedding procedures are possible. Most embedding was carried out in Thiogel¹ (Schwarz Bio Research, Inc., Orangeburg, N. Y.), a thiolated gelatin (26), primarily with a mixture containing 10% Thiogel A, molecular weight 10,000, plus 10% Thiogel B, molecular weight 100,000. These Thiogels, obtained as both anhydrous powders and gels, were dissolved and mixed with considerable stirring in distilled water heated to approximately 80°C. Only a few milliliters of the Thiogel mixture were prepared at one time, as needed. Once dissolved, this mixture was placed in a 37°C oven. When it had attained 37°C, usually after 30 min, the tissue blocks were added. In most cases the tissues were left in the Thiogel for impregnation at 37° for 1 hr. Then, 5% dimethylsulfoxide (DMSO) was stirred into the Thiogel. DMSO acts as an oxidative cross-linking agent, and in 5–10 min the Thiogel changes from a liquid to a soft, highly elastic gel. The jars containing the Thiogel and tissue blocks were stored at 3°C. The tissues could be more easily sectioned 1 or 2 days after embedding than immediately afterwards. In a few trials the tissues were not impregnated in the Thiogel but merely immersed in it briefly before the DMSO was added. In other trials the DMSO was not added, and the Thiogel was hardened by cooling. Other embedding media employed included 20% concentrations of Thiogel A alone and Thiogel B alone, both cross-linked with DMSO, and a purified, nonthiolated gelatin (Rousselot, Paris, France) (2) hardened simply by being stored at 3°C. The nonthiolated, pure gelatin had a firmer consistency and was less elastic than the Thiogels.

Sectioning

In preparation for sectioning, a tissue block was cut out of the hardened Thiogel or gelatin and was trimmed so that only a thin layer of gel remained around the block. It was then placed in 50% glycerol for 15 min at room temperature. This step is

¹ We are indebted to Schwarz Bio Research, Inc. for samples of Thiogel and, especially, to Dr. Norman A. Rosenthal, Director of the Department of Applied Research and Development, for his advice and interest.

absolutely essential in order to obtain flattened, relatively unwrinkled sections.

The tissue was then simultaneously frozen and affixed to a modified support or holder which fits into the chuck of an MT-1 Porter-Blum microtome (1). This is accomplished by placing the tissue on the tip of the holder, slightly moistened with cacodylate buffer, and then by immersing the whole into liquid nitrogen. The holder plus tissue were then placed in a cryostat at -35°C and the reservoir of the tissue holder, a metallic, $4 \times 3 \times 1$ cm trough between the tissue and the chuck of the microtome (1), was filled with chips of dry ice. It is necessary to wait a minimum of 15 min for the temperature of the holder and tissue to reach that of the dry ice but much better sections are obtained when they are cut 1–2 hr after introduction of the tissue into the cryostat. Thus, it is useful to have several holders if sections of several different tissues are needed in a single day. The holders with affixed tissues can be stored on top of blocks of dry ice lying on the floor of the cryostat beside the microtome. The block of tissue which has been affixed to the holder is discarded after sections have been cut from it.

The mounting of the microtome in the cryostat, a modified commercial deep freeze, was exactly as described by Bernhard in 1965 (1). Glass knives were used at the usual angle of approximately 45° for gelatin-impregnated tissues and were tilted forward toward the tissue block at a somewhat greater angle for Thiogel-impregnated tissues. The trough of the knife was filled with 40% DMSO, a solution which does not freeze at the temperature employed and on which sections will float. Under the conditions employed in this study, the temperature of the DMSO in the trough and that of the knife edge were -23°C , that of the tissue block was -70°C , and that of the air within the cryostat at the level of the knife was -35°C .

The block of tissue was trimmed further with a razor blade precooled on dry ice to remove all enveloping gelatin. The microtome setting was at 0; the advance of the arm bearing the tissue does not correspond to that at room temperature. Even at this setting, only very thick sections were obtained at normal cutting speeds. In order to obtain sections thin enough for electron microscopy it was essential that the sections be cut by turning the microtome handle very rapidly. Since this was done manually, it was inevitable and, incidentally, very useful that sections of varying thicknesses were picked up on each grid. It was not possible to determine section thickness on the basis of light refraction since the embedding medium and the lighting of the microtome differed from those usually employed. Indeed, the floating sections are barely visible.

The sections were picked up from the trough in a

drop of the DMSO solution with the plastic rings designed by Marinozzi (13), were removed from the cryostat, were floated for a few seconds on distilled water at room temperature for removal of the DMSO and for thawing and flattening the sections, and finally were picked up on Formvar-carbon-coated grids and air-dried.

Staining

Frozen, thin sections of aldehyde-fixed tissues had low contrast and were difficult to stain (2). Best results were obtained by negative stains, either 2–3% phosphotungstic acid (PTA) neutralized with *N* NaOH to pH 7.0, at 37°C for 5–10 sec, or 4% sodium silicotungstate (ST) pH 7.0 at 37°C for 15–20 sec. The grids were dried without rinsing. Faint positive staining of some cell components were obtained by treating the sections with a triple stain, 5 min with 2% osmium tetroxide, pH 7.2, followed by saturated, aqueous uranyl acetate for 30 min to 2 hr, then by lead citrate for 5 sec. The grids were rinsed with distilled water and dried after each of these three steps. This is a positive stain primarily with respect to ribosomes and chromatin and is unsatisfactory for other cell constituents. Other positive staining methods are being sought.

Cytochemical Reactions

Endogenous enzymes remain active in ultrathin sections prepared by this procedure, and they are described in an accompanying paper (13).

Specific enzymatic digestion of cell constituents is also possible as in plastic-embedded tissues (13–17). The procedures tested included 0.1% RNase for 30 min and 1 hr and pepsin at dilutions of 0.1, 0.01, 0.001, and 0.0001% for 5, 15, and 30 min. Control sections were incubated in the medium without the enzyme. After being rinsed in distilled water, the sections were stained with the triple stain.

Microscopy

Electron micrographs were taken with a Siemens Elmiskop I at 80 kv with $50\text{-}\mu$ objective apertures.

RESULTS

Technical Procedures

Unfixed tissue and formaldehyde-fixed tissues are poorly preserved, whereas glutaraldehyde at all concentrations and fixation times gave acceptable ultrastructural details. A standard fixation of 2.5% glutaraldehyde for 1 hr is recommended for most procedures.

Unembedded tissues can be sectioned, but they become badly fragmented. Furthermore, they offer

no advantage either with respect to stainability, digestability by ribonuclease or pepsin, or activity of endogenous enzymes.

With the exception of Thiogel B alone, all embedding media, namely 20% Thiogel A, 10% Thiogel A plus 10% Thiogel B, and 20% non-thiolated, pure gelatin, gave good and equivalent support to the mammalian tissues employed. The mixture of Thiogel A and Thiogel B was superior to pure gelatin for cultured cells and bacteria.

The initial problems in this technique were an extensive wrinkling or folding of the sections during cutting and the failure of the sections to flatten out on the solution in the trough. These difficulties were considerably reduced by introducing the step of impregnating the gelatin-embedded blocks with 50% glycerol before freezing. Now, large flat sections can be obtained, with folds only on the leading edge of each section. Serial sections can sometimes be obtained but with difficulty since they adhere to each other very loosely. Some extremely thin sections remain folded. Some of these wrinkles may be due to rapid dulling of the knife. We have not yet tried diamond knives in the cryostat.

The only difficult step in the procedure is the cutting of the sections. As long as sectioning is done manually, it will require a certain amount of

practice and skill. As pointed out above, it is necessary to turn the microtome handle as rapidly as possible. It appears that with the slightest hesitation the sections become thicker. In practice, with manual cutting we usually obtain sections of varying thicknesses on each grid.

Sections that are floated on the top of various incubating media can be left in plastic loops without drying, or first picked up and dried onto Formvar-carbon-coated grids. Results are the same with either procedure. In fact, with several cytochemical techniques the air-dried sections can be stored on the grids overnight and can be incubated the following day.

Ultrastructural Preservation

Frozen sections of glutaraldehyde-fixed, gelatin- or Thiogel-embedded tissues which are stained with PTA or ST resemble sections of tissues which are fixed in glutaraldehyde and embedded in glycolmethacrylate (GMA)² or hydroxypropylmethacrylate (HPMA) (13), unfixed but dehydrated in glycerol and embedded in HPMA (23), or partially dehydrated prior to freeze-substitution and embedding in methacrylates or epoxy resins

² Leduc, E. H., and W. Bernhard. 1967. *J. Ultrastruct. Res.* 19: 196.

All of the material illustrated in these plates was fixed in 2.5% glutaraldehyde for 1 hr and washed overnight in cacodylate buffer before impregnation with Thiogel or pure gelatin.

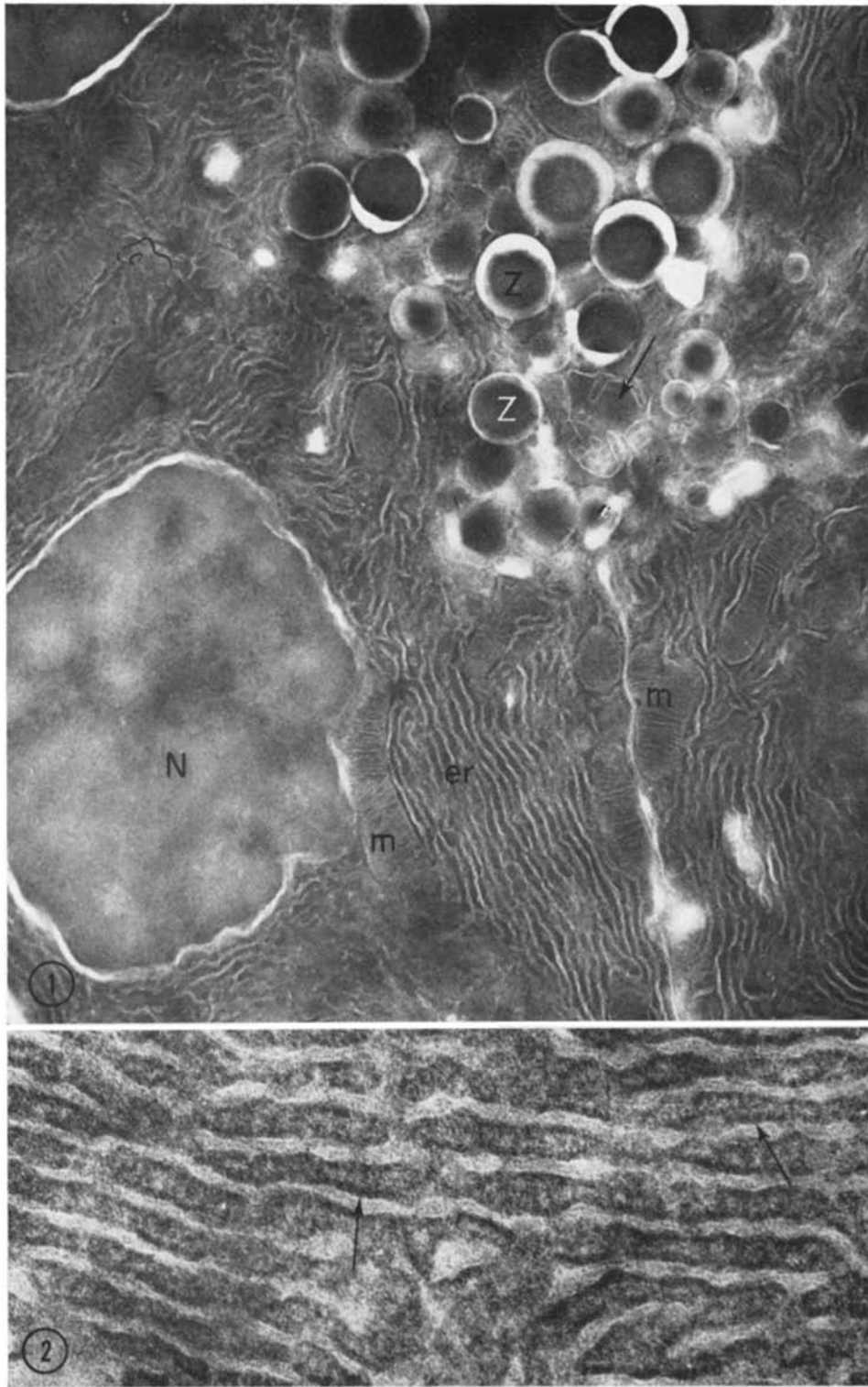
Abbreviations

bm, basement membrane
er, ergastoplasm
m, mitochondrion
mb, microbody
n, nucleoid

N, nucleus
p, protein droplet
s, sinusoid
v, virus particles
z, zymogen granule.

FIGURE 1 Low magnification electron micrograph of an ultrathin frozen section of exocrine pancreas cells of the rat embedded in pure gelatin and stained with PTA. Because the section is relatively thick and because PTA was used, there are retraction artifacts or fissures around the nucleus (*N*) and most of the zymogen granules (*z*). With this negative stain the ribosomes on the ergastoplasmic membranes (*er*) are not denser than the surrounding hyaloplasm, but they are visible with other stains (see fig. 2). Arrow points to the lumen of an acinus in which microvilli are well preserved. $\times 18,000$.

FIGURE 2 Higher magnification of the ergastoplasm of the same pancreas as that in Fig. 1, which was stained with the osmium tetroxide-uranyl acetate-lead sequence which gives a faint positive stain of some organelles. In this material the ribosomes can be discerned in a few places (arrows) but they do not stand out clearly. $\times 40,000$.



(24). All cytoplasmic membranes are clearly defined and appear in negative image against a background of varying density depending on the region of the cell or organelle in question, the thickness of the section, and the duration of staining time (Figs. 1, 3-5). The core of the microbodies also appears in negative image (Fig. 3). Microbodies tend to exhibit maximum density, the matrices of mitochondria are less dense but variable, and the hyaloplasm binds still less stain (Fig. 3). Glycogen is usually dissolved out of the sections. The ribosomes are poorly discernible with negative stains (Figs. 1 and 3). Nucleoli and interchromatic granules are stained. The chromatin is not stained by PTA (Fig. 1) but is faintly positive with ST (Fig. 3). In contrast, very faint positive staining of the ribosomes (Fig. 2) is obtained after triple staining with osmium tetroxide (5 min), uranyl acetate (1-2 hr), and lead (5 sec) and the chromatin becomes moderately stained. Longer exposure to osmium tetroxide (1-2 hr) followed by lead, or to reduced osmium tetroxide (12) will give a faint positive staining of the membranes. Thus, unlike staining in sections of plastic-embedded tissues, positive staining is difficult, and even negative staining is faint in very thin, frozen sections. For this reason, some finer details of ultrastructure have not yet been discerned in frozen sections, such as the vesicles and multivesicular bodies of the Golgi complex. Thus, further work will be necessary to obtain a better routine procedure for positive staining.

Besides animal cell structures, bacterial cells (*Bacillus subtilis*, *Escherichia coli*), and virus particles (reovirus, adenovirus, Bittner's milk factor, and mouse leukemia viruses) have also been examined in ultrathin, frozen sections after negative staining. No systematic study has been carried out as yet, but preliminary results indicate an excellent preservation of their fine structure, which allows even investigations at high resolution (Figs. 6-9). The bacterial cells appear extremely dense. This is probably due to the fact that the sections were rather thick, because pellets of bacteria were the most difficult to cut among all our specimens. There is no evidence of swelling or extraction. Like mammalian cells, ribosomes are not differentially or specifically stained by PTA or ST; so the cytoplasm is homogeneously and finely granular (Fig. 6). The nucleoplasm is less dense, and without fibrillar structure (Figs. 6 and 7). In order to show RNP particles and DNA fibers, it may be

necessary to change the fixation schedule or to extract enzymatically diffuse proteins which may mask these structures. On the other hand, a dense component of the cell membrane, including the mesosome (Fig. 7), is particularly well preserved. As far as the study of virus particles is concerned, now, for the first time, it is possible to apply negative staining directly on sectioned virions; this allows us to avoid superposition effects (Fig. 8). Fine structural details of the virus surface are thus visible in viruses preserved *in situ* in infected cells (Fig. 9).

The major disadvantages of the frozen sections lie in the appearance of fissures and holes in some parts of some tissues. The fissures were most pronounced between cells in the kidney. Occasionally the cells of the pancreas also were pulled apart, but this was extremely rare in liver or mammary tumors. Fissures also appeared around certain structures, in particular the protein droplets of the kidney and zymogen of the pancreas (Fig. 1). Holes sometimes appeared among the microvilli of brush borders or of bile canaliculi.

Enzymatic Digestion

Ribonuclease, at the same concentration and the same incubation times, had the same effect on frozen sections as on GMA sections (14). After a 30 min incubation in 0.1% solution the ribosomes were completely attacked and the nucleolus partially digested. Concomitantly, there was the usual increase in stainability of the mitochondria, zymogen granules, and chromatin. Pepsin, on the other hand, attacked frozen sections much more vigorously than GMA sections. In glutaraldehyde-fixed, GMA sections (14) a 0.5% solution was used; zymogen granules were completely digested in 5 min and mitochondrial matrix, in 15 min. In glutaraldehyde-fixed, frozen sections 0.01% pepsin attacked both zymogen granules and mitochondria in 5 min, and even a 0.001% solution had a detectable effect after 15 min. That in pepsin-digested sections the ribosomes stood out more clearly, suggests that the pepsin may also have removed some of the protein of the hyaloplasm.

DISCUSSION

Although there are similarities between our method and that of Fernández-Morán (5, 6) in his early investigation of frozen sections, several innovations have led to a far better preservation of ultrastructure. The first is the use of glutaralde-

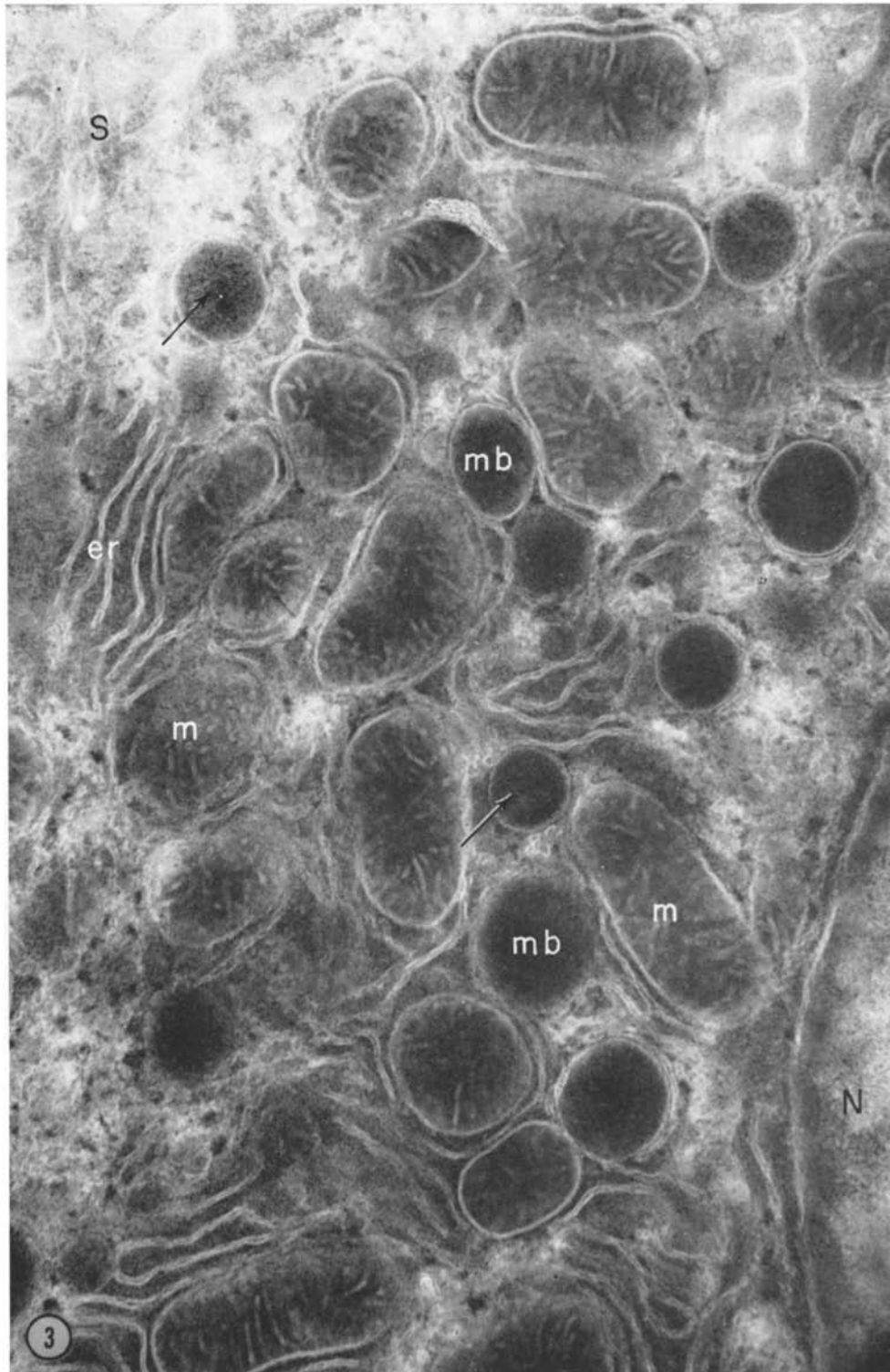


FIGURE 3 Frozen ultrathin section of rat liver embedded in Thiogel and stained with sodium silicotungstate. Contraction artifacts are absent; indeed, they are rare in frozen sections of the liver. Arrows point to negative images of the crystalloid core of microbodies (*mb*). $\times 30,000$.

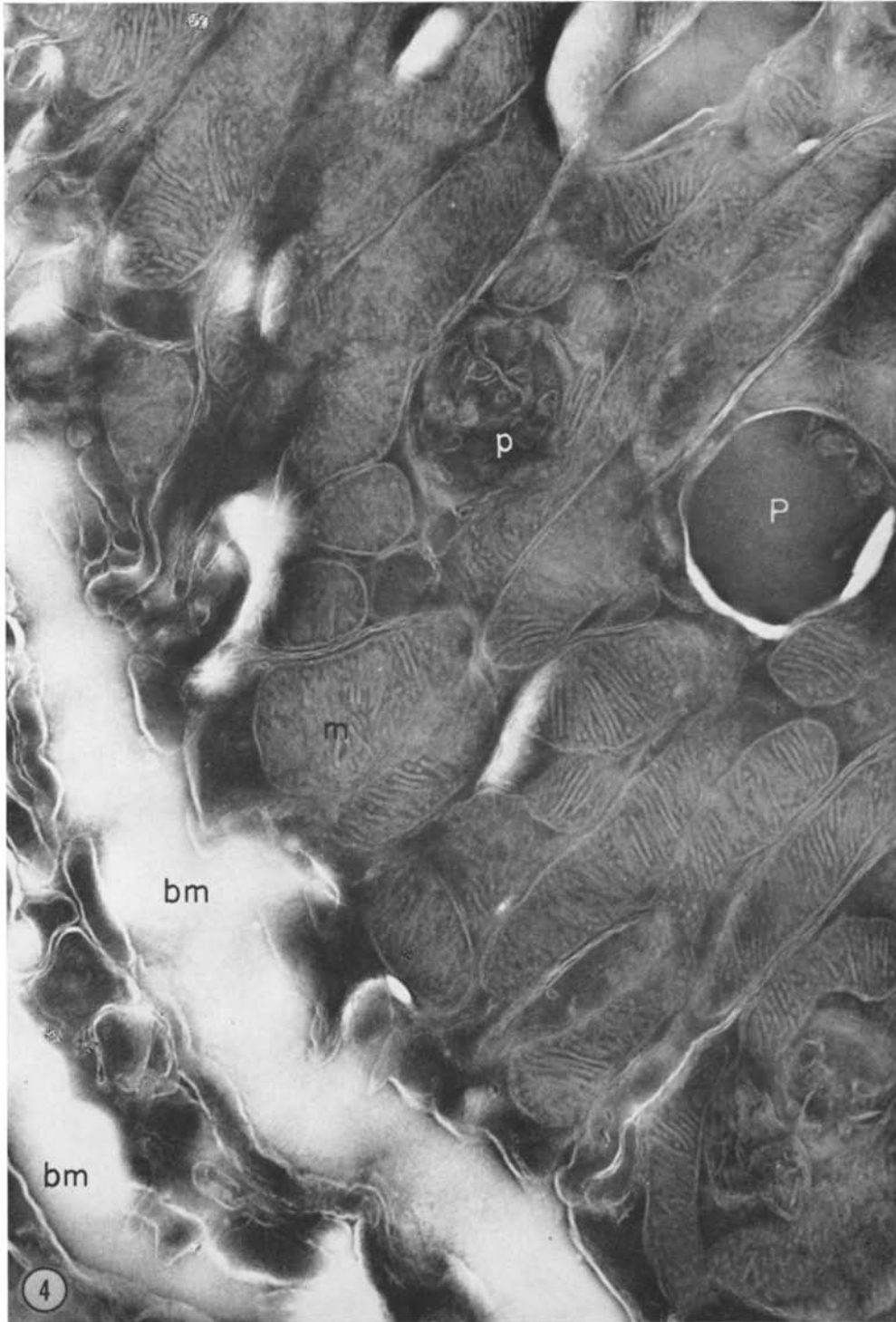


FIGURE 4 Frozen ultrathin section of rat kidney embedded in pure gelatin and stained with PTA. Although this is a thick and heavily stained preparation, the basement membranes (*bm*) remain unstained. Cell membranes and cristae mitochondriales stand out sharply in negative image. Within the protein droplets (*p*) or lysosomes irregular accumulations of membranes are readily detected. Retraction artifacts, probably caused by the stain, have produced a fissure around the protein droplet (*p*) at the right hand side of the plate. $\times 30,000$.

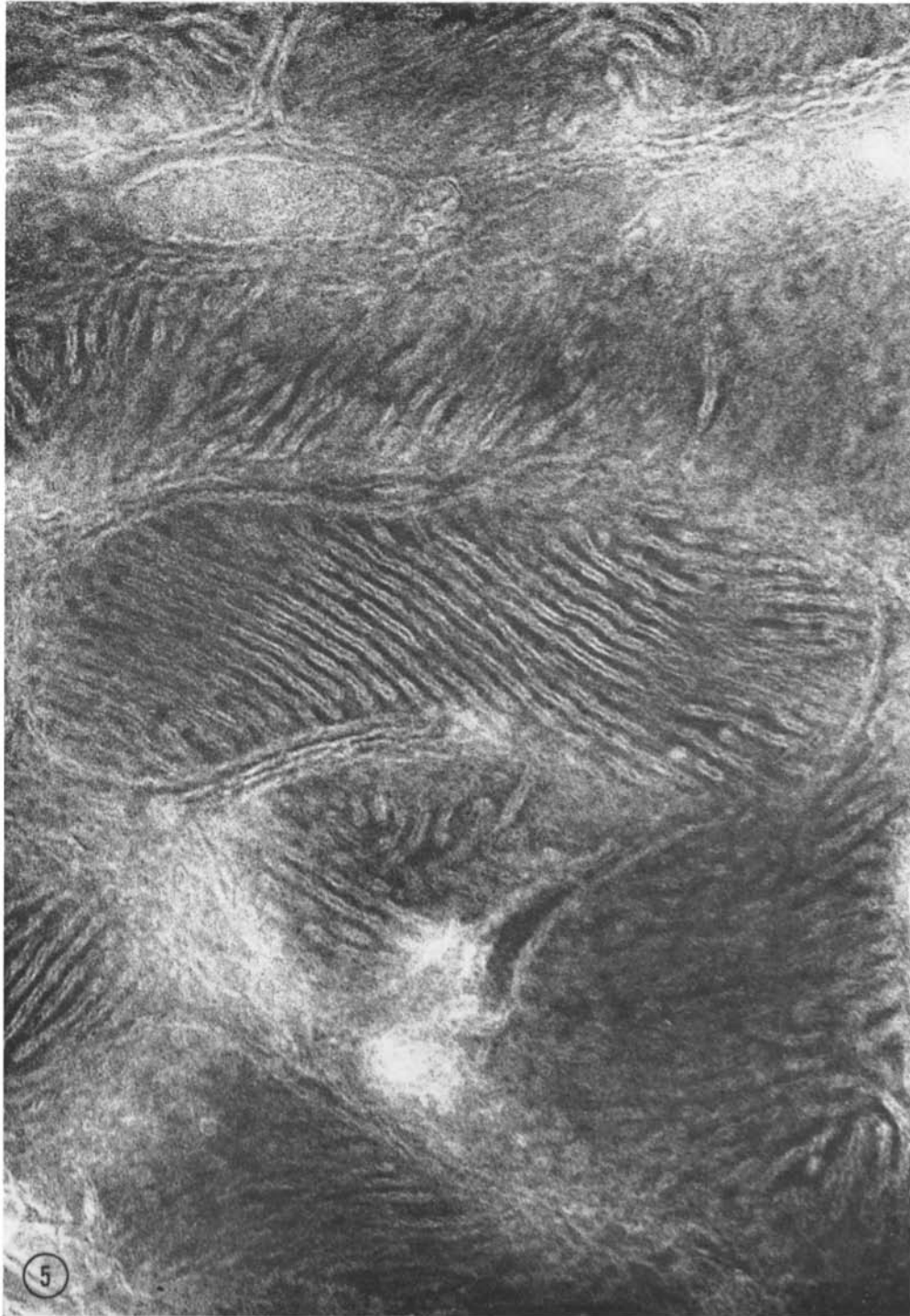


FIGURE 5 Rat kidney cortex embedded in gelatin and stained with PTA. The infolded cell membranes and the mitochondrial membranes stand out sharply in negative image against the densely stained mitochondrial matrix and hyaloplasm. With this technique elementary particles are not evident. $\times 90,000$.

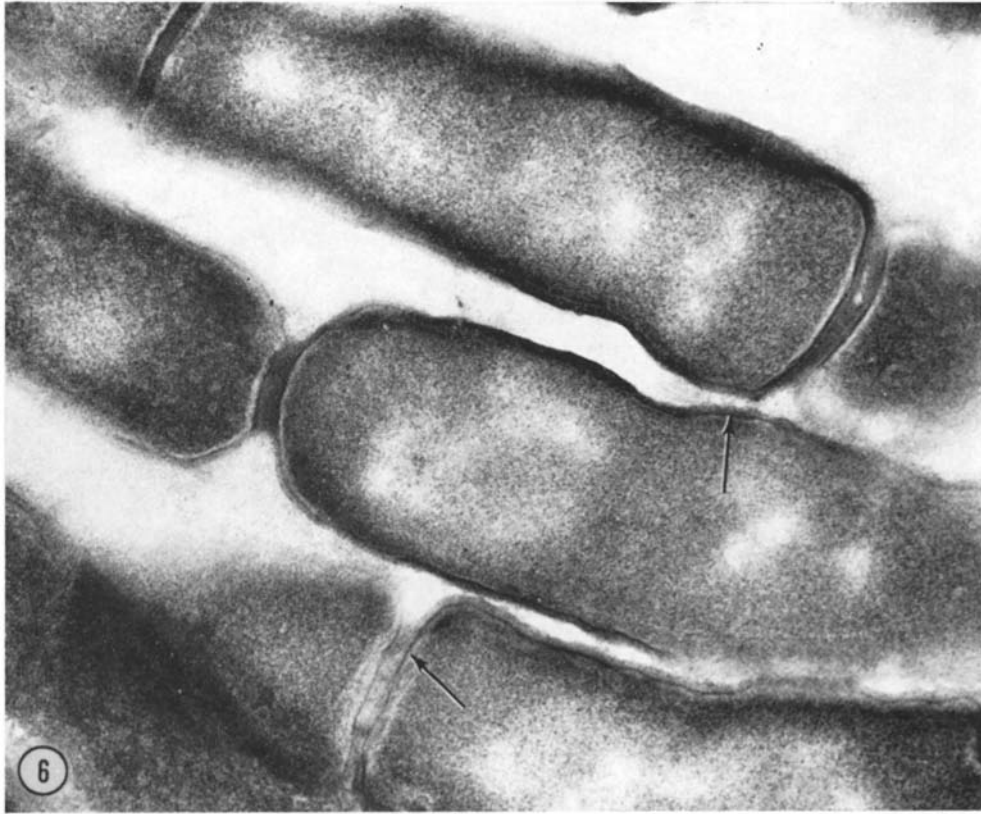


FIGURE 6 Frozen section of *Bacillus subtilis* embedded in Thiogel and stained with PTA. The nucleoplasm, like the chromatin of mammalian cells, binds little or no PTA. Also like mammalian cells, the cytoplasm is homogeneously dense and ribosomes are not specifically stained. The cell wall exhibits two layers of different densities, an inner, very dense, and narrow line (arrows) and an outer, less dense, and wider zone. $\times 44,000$.

hyde (27), which preserves both ultrastructure and reactivity of tissue proteins. Like Fernández-Morán, we had poor results with unfixed or formaldehyde-fixed material. He found that OsO_4 -fixed tissue gave the best sections with his method, but we avoided osmium tetroxide as it inhibits most tissue reactions. The second difference is the routine embedding of tissues in gelatin or Thiogel and the subsequent impregnation of the embedded blocks of tissue with 50% glycerol. Whereas Fernández-Morán mentions that gelatin-embedding is possible and that tissues are easier to cut when soaked in glycerol or glycol-physiological saline, he gives no details and it is not clear whether he tried both steps on the same tissue blocks. In our hands both are essential. Temperature control in our work was improved by placing

the microtome in a cryostat and by having a reservoir for dry ice built onto the support for the tissue. Hence, the tissue was constantly maintained at -70°C . We obtained poor sections when we used a block that was undergoing temperature changes, that is, one that was warming to -70°C after prior freezing in liquid nitrogen. We also originally floated our sections on distilled water or physiological saline, but this is technically difficult with a microtome in a cryostat because the trough must be repeatedly emptied and refilled to avoid freezing. The use of 40% DMSO eliminates this difficulty. A large number of other solutions were tried without success (1). A final difference is that Fernández-Morán used a motor-driven microtome that delivered 50–80 rpm. Because sectioning must be very rapid, this is a modifica-

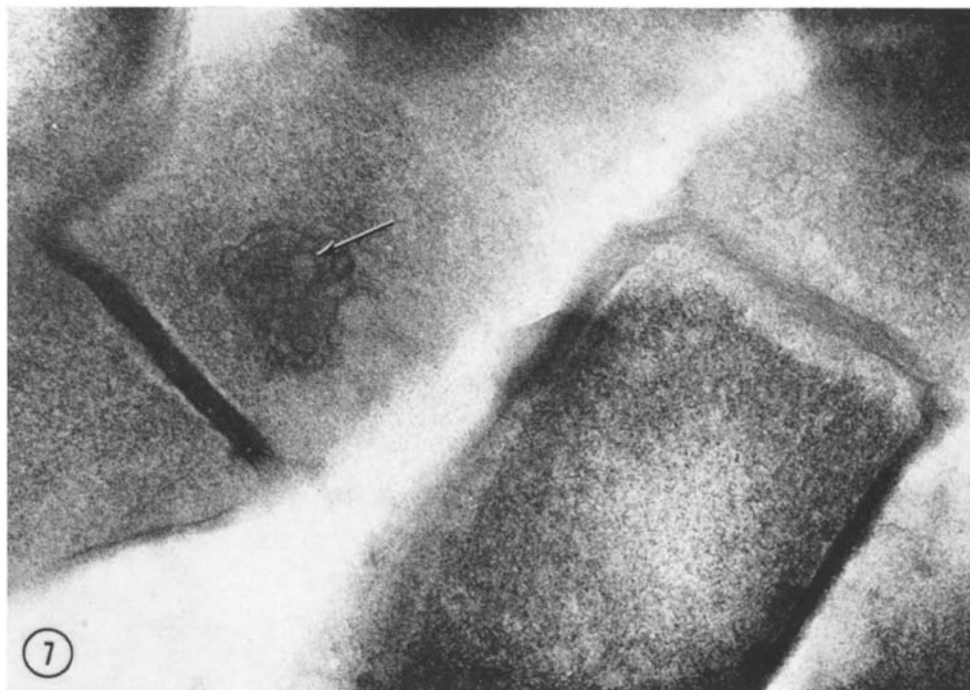


FIGURE 7 Higher magnification of the preparation in Fig. 6. The arrow points to a chondrioid or mesosome. The stained portion of the chondrioid corresponds in width and density to the inner layer of the cell wall. $\times 76,000$.

tion which might give us sections of more uniform thickness. The current automatic microtomes are too slow. Another improvement in our method might be that of using diamond knives to reduce or eliminate wrinkling of the sections.

The procedure described in this paper for obtaining frozen sections is difficult only with respect to the actual process of cutting sections, that is, as compared with the sectioning of plastic-embedded tissues at room temperature. Indeed, the preparation of the tissue is otherwise much simpler than the methods of freeze-drying, freeze-substitution, and plastic embedding.

We are not certain to what degree the Thiogels and pure gelatin penetrate into the tissues. It seems most likely that they may form a supporting matrix in the intercellular spaces but that they do not penetrate the cells. There are two reasons for this deduction. First, although Thiogel A has a low molecular weight and might be able to penetrate the cells, the sections we obtained from tissues which were simply immersed in the Thiogel immediately before it was cross-linked with DMSO

were as good as those from tissues that were impregnated with Thiogel for a full hour before gelation. Second, frozen sections of unembedded tissues exhibited the same cellular ultrastructure as those of embedded tissues, but only fragments and not whole sections of the tissue could be obtained. We therefore assume that the main function of gelatin may be to provide a scaffolding for the whole mass of tissue.

The reason for the importance of glycerol impregnation of the tissue is not clear. It is possible that ice crystals are smaller or absent after freezing (24, 25) so that better sections are obtained. Since our sections are always thawed before they are dried and examined, small vacuoles produced by ice crystals would not be apparent. It is our impression that the chief effect of glycerol is to reduce the "stickiness" of the sections, that is the gelatin or Thiogel in the sections, and allow the folds to flatten out. Gelatin embedding of osmium tetroxide-fixed tissue followed by partial dehydration has been used to obtain ultrathin sections at room temperature and these were reported to be

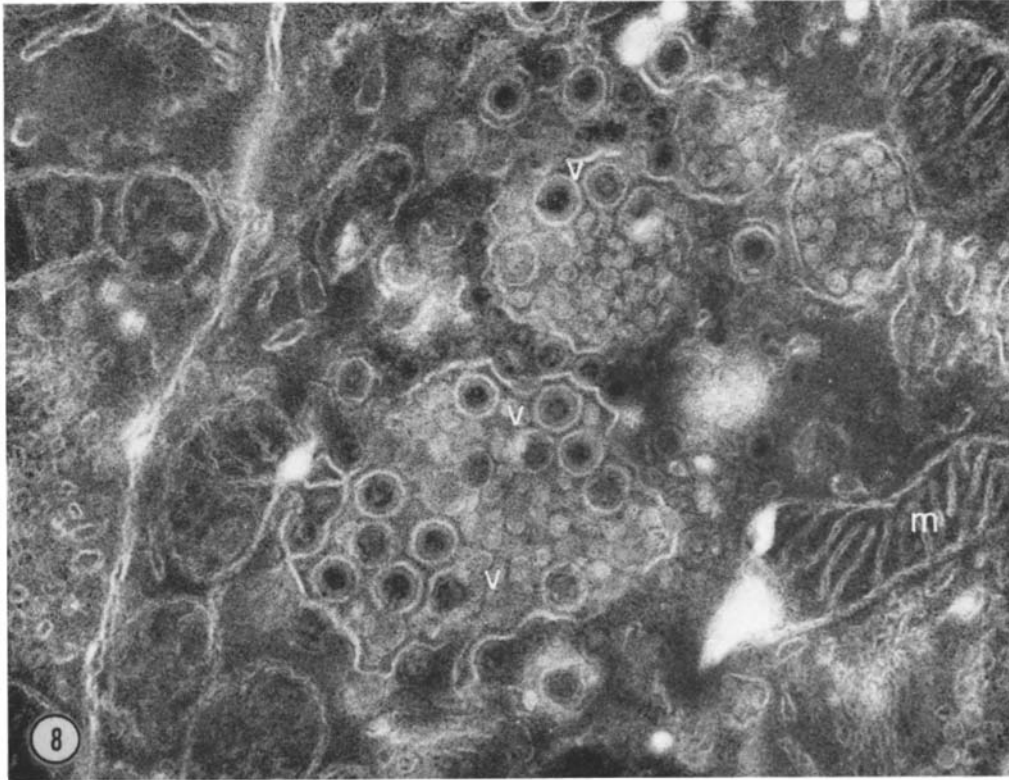


FIGURE 8 Frozen ultrathin section of a C3H mouse mammary tumor, embedded in Thiogel and stained with PTA. Virus particles (*v*) are visible both in the cytoplasm (type A) and in intracellular vacuoles (type B). $\times 60,000$.

sticky (10). Even when gelatin-impregnated tissues were thoroughly dried in a vacuum before sectioning at room temperature, posttreating with as little as 2% glycerol facilitated sectioning (7).

Sections may be picked up directly from the DMSO solution at -23°C in the trough of the knife onto precooled, Formvar- and carbon-coated grids, but they remain wrinkled, probably because they cannot spread while they are frozen. The ultrastructure of the portions of cells in the areas between the folds does not differ from that of cells which have been flattened by floating the sections on distilled water at room temperature; hence we have preferred the second procedure. Thawing the ultrathin sections at room temperature and air-drying them as they are placed on the surface of a carbon-coated film of Formvar is equivalent to the routine procedure of mounting thick cryostat sections for optical microscopy on glass slides. In both instances the frozen section is briefly thawed

and then simultaneously and rapidly affixed to a supporting surface and dried. The question which naturally arises is to what degree do drying artifacts occur. It would seem to us that the thinner the section, the more it resembles a two-dimensional structure and, therefore, the greater is the protection furnished by the supporting membrane against shrinkage and accompanying distortions. It will be necessary to confirm this, if possible, by directly examining both frozen and thawed sections that are not dried, by sandwiching them between protective membranes (4), and by using low temperature, specimen stages. Meanwhile, this concept is supported by the observation that in relatively thin, medium, and thick sections of pancreas that are all on the same grid and, hence, all received identical treatment, fissures around nuclei and zymogen granules are pronounced in the thickest sections (Fig. 1); they are limited to some of the zymogen granules in medium sections

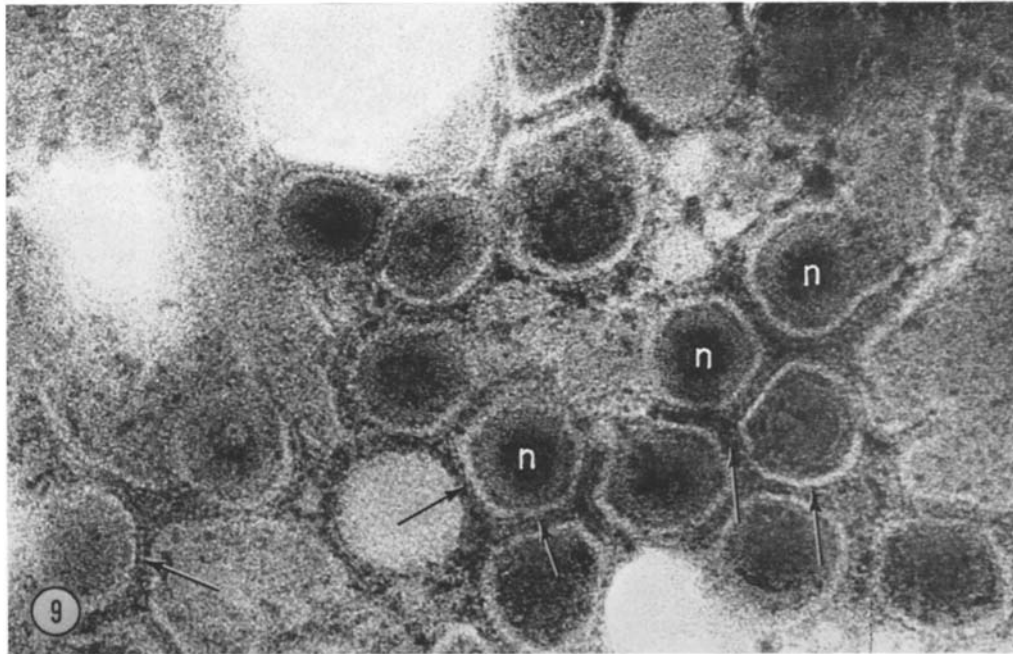


FIGURE 9 Higher magnification of the preparation in Fig. 8. The nucleoid (*n*) of virus particles (type B) is very dense, the surrounding protein coat is less dense, and the envelope is unstained. Arrows point to unstained spicules which cover the surface membranes of the virus particles and are revealed by negative staining. $\times 150,000$.

and are rare in the extremely thin sections. It must be emphasized, however, that the supporting film does not offer complete protection against distortion of the sections, even *after* drying, because fissures are more pronounced after staining with PTA than with ST and are more abundant after long incubation periods (3 hr at 0°C) than after shorter ones (5 min at 22°C). Thus, drying an ultrathin section onto a supporting film does not immobilize all of its constituents. Finally, fissures between cells were often found in kidney and to a much lesser extent in pancreas, but they did not occur in liver and mammary tumors. These fissures between cells might be related to relative cell adhesiveness because they are also more abundant in kidney than in other tissues after embedding in GMA.² Thus, the degree of distortion in the frozen sections depends in part on the nature of the tissue employed. Although the fissures are annoying, they do not interfere with the interpretation of cytochemical reactions carried out on the sections.

The question of what is the criterion of good

preservation of ultrastructure has been discussed succinctly by Rebhun (24). We can only affirm that the ultrastructure of glutaraldehyde-fixed, gelatin-embedded frozen sections is good, with the exception of the above mentioned fissures, in that it resembles that of tissues processed by several other methods now in use, namely, glutaraldehyde-fixed or unfixed, glycerol-dehydrated tissues in water-miscible plastics² (13, 23), or tissues dehydrated prior to freeze-substitution and embedding in epoxy resins (24, 25). The ultrastructure is much superior to that of frozen-dried material in which vacuoles produced by ice crystals are preserved. It is also true that unembedded glutaraldehyde-fixed tissues sectioned this way exhibit good structural preservation of organelles in fragments of tissues, but an additional requirement which we imposed was that of obtaining large sections as one does with plastic-embedded material.

The micrographs of *Bacillus subtilis* and of the Bittner virus are presented merely to illustrate that frozen sections of bacteria and viruses may be useful for ultrastructural studies. Applying nega-

tive stains directly on sectioned microorganisms, particularly on viruses, thus avoids the usual superposition effects, and may shed new light on the inner structure of these objects. But we have not yet made a systematic study of such sections with respect to either the identification of different structures with different stains or their cytochemical analysis.

In mammalian cells enzymatic digestion of frozen sections attacked the same components as in sections of tissue embedded in GMA and HPMA, but the concentration of enzyme and the time of incubation had to be modified. While this is a good control for sections of plastic-embedded materials, we feel that sections of GMA-embedded material are superior to frozen sections for this type of cytochemical study because GMA sections are easier to cut, more uniform in thickness, and, especially, much easier to stain. On the other hand, for the localization of sites of enzyme activity

directly on ultrathin sections (12), frozen sections prepared by the procedure described in this paper are superior. One of the future possible applications of this technique may be the localization of intracellular antigens by means of the immunoferritin method, which will avoid the problem of the conjugate penetrating into the cell. This investigation is commencing in our laboratory. It is our hope that the method of obtaining ultrathin frozen sections described here will be further improved and become one of many routine procedures available to the experimental biologists.

We are very grateful to Miss Annie Viron for her remarkable technical assistance, and we also wish to thank Miss Françoise Yven and Madame Marie Puech for the preparation of the manuscript.

This research was supported in part by United States Public Health Service Grant CA 00510-19.

Received for publication 26 January 1967.

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