

# A TECHNIQUE FOR ULTRACRYOTOMY OF CELL SUSPENSIONS AND TISSUES

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## ABSTRACT

Ultracryotomy of fixed tissue has been investigated for a number of years but, so far, success has been limited for several reasons. The simple technique herein reported allows the ultracryotomy not only of a variety of tissues but also of single cells in suspension, with a preservation and visualization of ultrastructural detail at least equivalent to that obtained with conventional embedding procedures. In this technique, sucrose is infused into glutaraldehyde-fixed tissue pieces before freezing for the purpose of controlling the sectioning consistency. By choosing the proper combinations of sucrose concentration and sectioning temperature, a wide variety of tissues can be smoothly sectioned. Isolated cells, suspended in a sucrose solution, are sectioned by sectioning the frozen droplet of the suspension. A small liquid droplet of a saturated or near-saturated sucrose solution, suspended on the tip of an eyelash probe, is used to transfer frozen sections from the knife edge onto a grid substrate or a water surface. Upon melting of the sections on the surface of the sucrose droplet, they are spread flat and smooth due to surface tension. When the section of a suspension of single cells melts, individual sections of cells remain confined to the small area of the droplet surface. These devices make it possible to cut wide dry sections, and to avoid flotation on dimethyl sulfoxide solutions. With appropriate staining procedures, well-preserved ultrastructural detail can be observed. The technique is illustrated with a number of tissue preparations and with suspensions of erythrocytes and bacterial cells.

## INTRODUCTION

Bernhard and Leduc (1) made a major contribution to the development of ultracryotomy of fixed tissue by describing the method of floating frozen sections on a dimethyl sulfoxide (DMSO) solution and washing them with water after they melted. Since then, several workers have adopted this method and have described their results (2-5).

In principle, sections of freshly fixed and frozen tissues should retain structures which are free from chemical and structural artifacts produced during the dehydration and embedding procedures of conventional ultramicrotomy. However, structural delineation in frozen sections has not generally

been up to the standards of conventional sections. One important problem is the control of the sectioning consistency of frozen tissues. The plasticity which allows a frozen tissue to be sectioned is decided by the intrinsic nature of each tissue. Temperature variation alone cannot compensate for this variability. For instance, ultrathin sections of pure water cannot be obtained at any temperature. To improve the sectioning quality, Bernhard and Viron (2) embedded aldehyde-fixed tissue pieces in a gelatin solution at 37°C and Iglesias and coworkers (3) used a methyl cellulose solution for embedding with or without preembedding in a

gelatin solution. Tissue can also be embedded in a bovine serum albumin (BSA) solution (6, 7) with or without cross-linking with aldehyde, a method which may have an advantage over the above two methods in that the embedding can be performed near 0°C. All these macromolecules, however, do not penetrate into cells (1-3, 7) and such embedding methods do not improve the intrinsic sectioning quality of cellular interiors or cytoplasmic organelles.

A method to control the plasticity of frozen tissue pieces in a more positive manner is to infuse chemically inert, hydrophilic substances of appropriately low molecular weights into fixed tissue pieces before freezing them. This probably also reduces ice crystal damage. In the present study, sucrose was found to be satisfactory for this purpose. Tissue pieces infused with sucrose or suspensions of single cells in sucrose solutions can be smoothly sectioned while frozen.

Another cause of difficulty in obtaining wide and flat sections in the previous methods, in which flotation on 50-60% DMSO is used to recover the sections (2-5), is likely to be the high viscosity of the flotation liquid at -45° to -55°C. It prevents smooth spreading of sections and the sections are often wrinkled. In addition, from the cytochemical point of view, the chemical effects of DMSO on macromolecules have not been fully explored and they may be undesirable. Bernhard and Viron (2) used a 50% glycerol solution to float sections which were to be used for cytochemical purposes. The viscosity of this solution, however, is even greater than that of 50% DMSO at low temperature and restricts the performance of ultracryotomy. In the present technique, a small droplet of a near-saturated or saturated sucrose solution, which remains in a molten state for a short but adequate period at or above -120°C, is used to collect frozen sections. Sections on the edge of the knife adhere to the droplet, melt on the droplet when removed from the cryokit bowl, and can then be transferred onto grids.

The most commonly used substances for positive staining are uranyl acetate (UA) and lead citrate (Pb), which are either quite low or high in pH. Possible damaging effects of such substances on tissue structures need to be seriously evaluated in ultracryotomy. In fact, our experience is that a long exposure of sections to UA often caused a disintegration of the structures. It appears that melted sections of frozen tissues are more susceptible to the

harmful effect of surface tension than are conventionally embedded sections, even when tissues are heavily prefixed. The combination of these effects could also be a basic cause of observing inferior morphological definition in frozen sections. In the present study, negative staining is found to be more suitable for ultrastructural preservation in frozen sections.

With these devices, we have found that definition of many structures in frozen sections is comparable to, or sometimes better than, that seen in conventionally embedded sections.

## MATERIALS AND METHODS

### Fixation

Mammalian tissues used for this study were cerebral cortex, kidney, liver, pancreas, sciatic nerve, skeletal muscle, spleen, and testis of normal adult rat, mouse, guinea pig, rabbit, or cow. In addition, human and rabbit erythrocytes, *Drosophila melanogaster* testis, spinach leaf, and bacteria, *Escherichia coli* and *Bacillus subtilis*, were also examined.

All mammalian tissues were cut into blocks with sides of 0.5-1 mm and fixed with 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 30-60 min, with the exception of sciatic nerve which was sometimes fixed more extensively with 5% glutaraldehyde for 3 h. Fruit fly testis was fixed intact in the same manner. Spinach leaf was dissected into 0.2-1 mm squares or rectangular shapes and fixed in 1% glutaraldehyde for 2 h, under a slight vacuum of 100-300 mm Hg to accelerate the removal of air from the intercellular spaces of mesophyll and the penetration of the fixative into the pieces. Erythrocytes and bacteria were fixed usually with 1% and sometimes with 0.5% glutaraldehyde for 10-60 min. The fixation was always carried out in an ice bath. Fixed tissue pieces stored in 0.1 M phosphate buffer, pH 7.4, at 4°C for 0.5-1 day generally showed somewhat better results than those sectioned immediately after the completion of fixation.

The structural preservation in ultracryotomy, which lacks the embedding process, seemed to be strongly dependent upon the freshness of tissue pieces at the time of fixation, more so than in conventional ultramicrotomy.

### Infusion

Fixed tissue pieces were placed in solution of sucrose or glucose in 0.05-0.1 M phosphate buffer, pH 7.4, in small bottles in an ice bath. The sucrose and glucose concentrations were 20-150 and 20-70 g/100 ml of buffer, or 0.5-2.3 and 1-2.7 M in molar concentration, respectively. In the present paper,

the molar concentration is used to express the sugar concentrations.

Sections of infused and frozen tissues as well as frozen droplets of the solutions themselves indicated that sucrose yielded better results than glucose, although glucose could replace sucrose if required for some specific reason. The hardness of the frozen tissue piece was inversely related to the sugar concentration within a certain range. DMSO and glycerol, in 20–30 and 20–60% (vol/vol), respectively, were also tried but were inferior to the sugars.

The range of sucrose concentration for optimum sectioning seemed to be 0.6–1.6 M. The range of glycerol concentration used in the past studies (2, 3) was 25–50% (vol/vol), which is 3.4–6.9 M in molar concentration. Physicochemically, therefore, sucrose infusion might have less drastic effects than glycerol infusion.

The infusion of sucrose was carried out in an ice bath usually for 10–30 min, depending on the sucrose concentration and the size of tissue pieces, and sometimes for 1 h when sucrose concentrations higher than 1.6 M were tried. In order to detect possible dehydration of tissue pieces due to a concentration gradient during the infusion, a series of sucrose solutions of graded concentrations were used and the result was compared with that of the one-step infusion. No appreciable difference was recognized between the two.

We have also performed experiments with 20–30% BSA solution, with or without the addition of sucrose. This is an excellent medium in which to suspend cells or minute tissue pieces in order to section them in a frozen droplet. After melting these sections and mounting them on the grids, BSA can be readily removed by washing. If it is necessary to maintain the relative positions of isolated cells or to preserve the configurations of fragile tissues such as the fruit fly testis, BSA can be cross-linked with aldehyde to a gelatinous state to hold cells or tissues in place (6, 7). However, the electron micrographs reproduced in this paper, unless otherwise indicated, were obtained with sucrose-infused samples.

### *Freezing*

The Sorvall MT-2 microtome (Ivan Sorvall, Inc., Newtown, Conn.) and its cryokit attachment which was originally developed by Christensen (8, 9) were used in this study. Tissue pieces or droplets of the suspensions of isolated cells were placed on the specimen holders, short copper rods with flat ends. They were frozen either by direct immersion in liquid nitrogen or by first bringing them into light contact with a precooled copper block and immediately immersing them in liquid nitrogen, as recommended by Christensen. No significant difference was found

between the two methods, and the direct immersion method, which was much simpler than the other, was most often used in this study. The sucrose in the tissue pieces most probably prevented ice crystal formation even at a relatively slow freezing rate.

### *Sectioning*

After freezing, the specimen holder was quickly attached to the precooled head of the microtome arm by means of either the precooled standard tool or a pair of hemostats. The most frequently used range of temperature was  $-50^{\circ}$  to  $-90^{\circ}\text{C}$ . The sectioning was mostly carried out by hand. The average thickness of a series of sections was estimated from the thickness setting of the microtome, which was varied from 300 to 1,000 Å, and the continuity of serial sectioning. Experience gained in observing the sections in the electron microscope soon made it possible to estimate the approximate thickness. The plasticity of infused and frozen specimens was such that, at the optimum conditions, extremely slow sectioning often gave rise to “cellophane-like” sections, which showed patches of interference colors which correlated approximately with the color-thickness relation known in conventional ultramicrotomy. The absence of a floating medium permitted a very slow sectioning speed. Both glass and diamond knives were used in this study.

### *Mounting Sections on Grids*

A 2.3 M or a saturated sucrose solution was used for mounting sections on the grids. A 0.5–1 mm droplet of the sucrose solution was picked up on the tip of an eyelash probe. When the droplet was introduced into the interior of the cryokit bowl held at  $-50^{\circ}$  to  $-120^{\circ}\text{C}$ , it stayed in a molten state for 5–15 s, before freezing to a “crystal-clear” state, depending on its size and the bowl temperature. This delay of freezing was long enough to bring the droplet into light contact with the frozen sections lying at the knife edge. Frozen sections adhered to the bottom of the droplet. Their original shape did not change at this step.

When the droplet was brought out of the cryokit bowl, it momentarily frosted over and regained a clear appearance, as it melted. After being held for an additional few seconds to ensure the spreading of sections by surface tension, the droplet was transferred to the substrate of a grid by touching it lightly to the surface. The sections, which were floating on the bottom surface of the droplet, attached firmly to the substrate. The grid was then floated on the surface of a water or buffer droplet, face down, to wash out the sucrose. Individual sections of isolated cells or minute tissue pieces, sectioned while “embedded” in frozen droplets of

sucrose solution, stayed near their original location on the sucrose droplet at the time of melting and were readily transferred to the grid substrate. The substrate, collodion-carbon or Formvar-carbon, was made hydrophilic before mounting sections by ionization in a vacuum evaporator.

If, for some reason, sections were to be collected on an air-buffer interface rather than directly on the grid, the frozen sucrose droplet with sections adhered was turned upside down inside the cryokit bowl to bring the sections to the top and, before melting, brought into light contact with the buffer surface. The sucrose droplet then melted and the sections floated onto the interface. An alternative method was to touch the melted sucrose droplet, with sections floating on its bottom surface, to the bottom surface of a buffer droplet suspended over a hole in a piece of thin polypropylene sheet. The plastic piece was turned upside down to bring sections to the top and then floated on the surface of a larger volume of buffer. If necessary, sections confined within the hole of the plastic piece could be easily transferred from one solution to the next by transferring the plastic piece. Such a procedure of transferring sections was described by Marinozzi (10).

### Staining

Sections were stained either negatively with 0.2–0.5% neutral phosphotungstic acid (PTA) or 0.5% UA, or positively with one or a combination of the reagents, 2% osmium tetroxide, 0.5–2% UA, and 0.5% Pb. For negative staining, a PTA or UA solution was left in contact with the sections for 1–15 s before removing most of the staining solutions. A 0.2% sucrose-0.5% PTA solution was also used for negative staining. Periods of positive staining

were 5–60 min for OsO<sub>4</sub>, 5 s to 10 min for UA, and 5 s to 1 min for Pb.

Sections were usually dried in air at room temperature. The positively stained, very thin sections, particularly those stained with OsO<sub>4</sub>, showed damaging effects of surface tension during the drying. Freeze-drying the sections or embedding them in a very thin layer of collodion, Formvar, BSA, or Epon before drying did not help. Negative staining, on the other hand, seemed to prevent such damage, perhaps by filling voids in the sections and giving a rigid support to cellular components. For a uniform negative staining, it was a prerequisite to use the grids covered with hydrophilic substrates. The negative staining with PTA was usually more uniform than that with UA.

A Philips EM-300 electron microscope was used for examination, usually at 60 kV and sometimes at 80 kV.

### RESULTS AND DISCUSSION

The results presented here are all representative of readily reproducible observations. Although the cryo-ultramicrotome needs to be further improved, particularly in the precision of temperature regulation, results were obtainable without much experience.

### Sectioning

The most basic factor affecting the quality of sections is the plasticity of the block. When tissues are frozen, water probably becomes the most brittle component, whereas proteins will provide a plasticity to frozen tissue pieces. Indeed, a droplet

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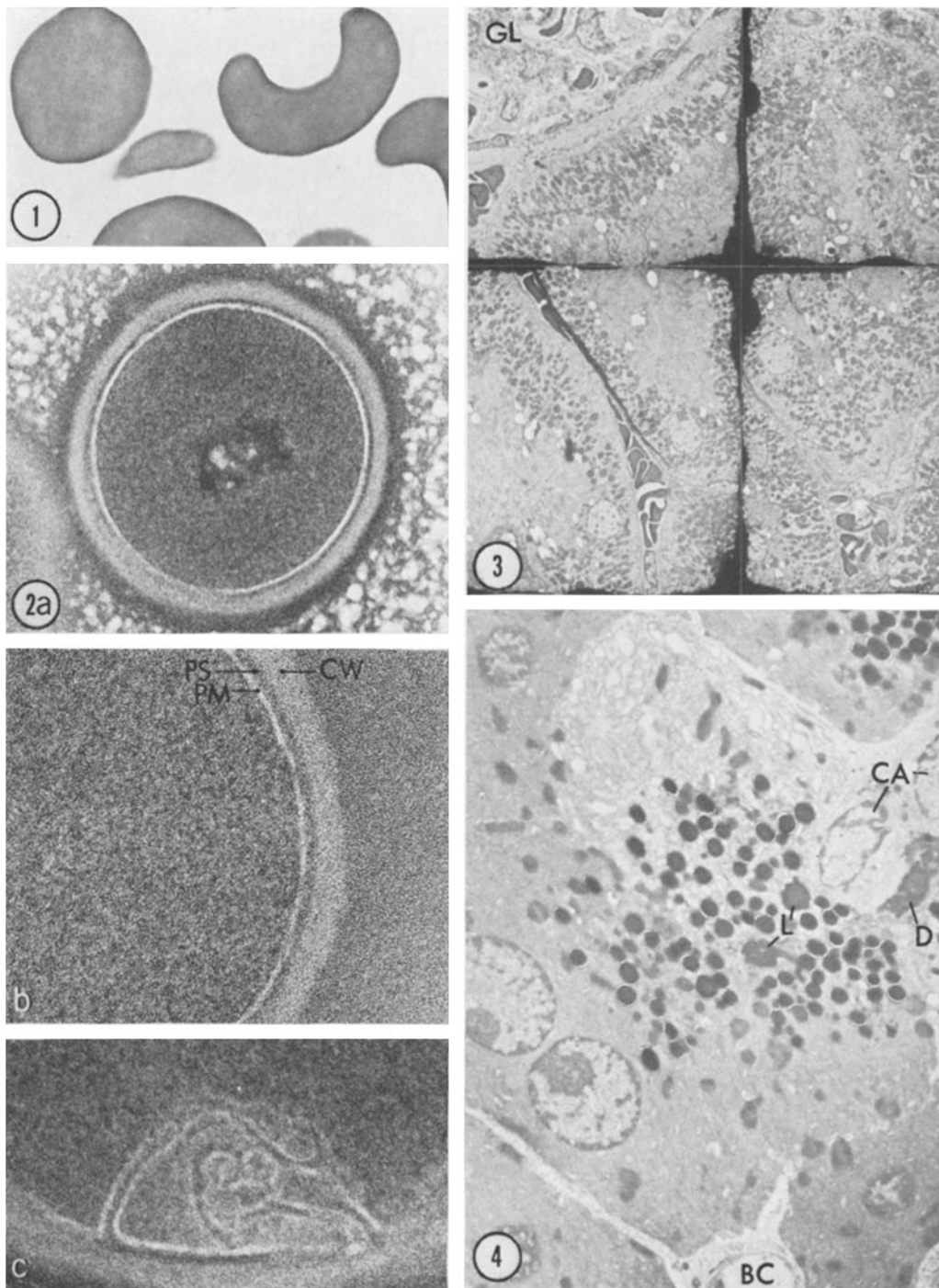
All electron micrographs are of frozen sections of tissues fixed with glutaraldehyde and infused with sucrose. Unless otherwise stated, all sections were negatively stained with a 0.2–0.5% neutral PTA solution as described in the text. Symbols are explained in the legend of each figure.

FIGURE 1 Rabbit erythrocytes, fixed in 1% glutaraldehyde for 30 min and sectioned at  $-70^{\circ}\text{C}$  while embedded in a frozen, 1.1 M sucrose solution.  $\times 2,800$ .

FIGURE 2 *Bacillus subtilis*. A cross section in (a), a part of cross section in (b), and a mesosome in (c); sectioned at  $-70^{\circ}\text{C}$  while suspended in a 1.1 M sucrose solution. All sections are negatively stained with a 0.2% sucrose-0.5% PTA solution. Formation of pits in (a) is due to the evaporation of sucrose. In contrast to (a), pits are absent in (b), which is an extremely thin section only very lightly negatively stained. In all pictures, particularly in (b), the surface structure, consisting of cell wall (CW), periplasmic space (PS), and plasma membrane (PM), is clearly defined. (a),  $\times 65,000$ ; (b),  $\times 130,000$ ; (c),  $\times 130,000$ .

FIGURE 3 Rat kidney. A part of a  $0.3 \times 0.5$  mm section, covering four neighboring grid holes. This illustrates the wide areas available in sectioned tissue. One glomerulus (GL) and several proximal convoluted tubules are seen.  $\times 950$ .

FIGURE 4 Rat pancreas. Several acinar cells with numerous zymogen granules and two centroacinar cells (CA) defining one side of the duct (D) are seen. L, acinar lumina; BC, blood capillary.  $\times 3,000$ .



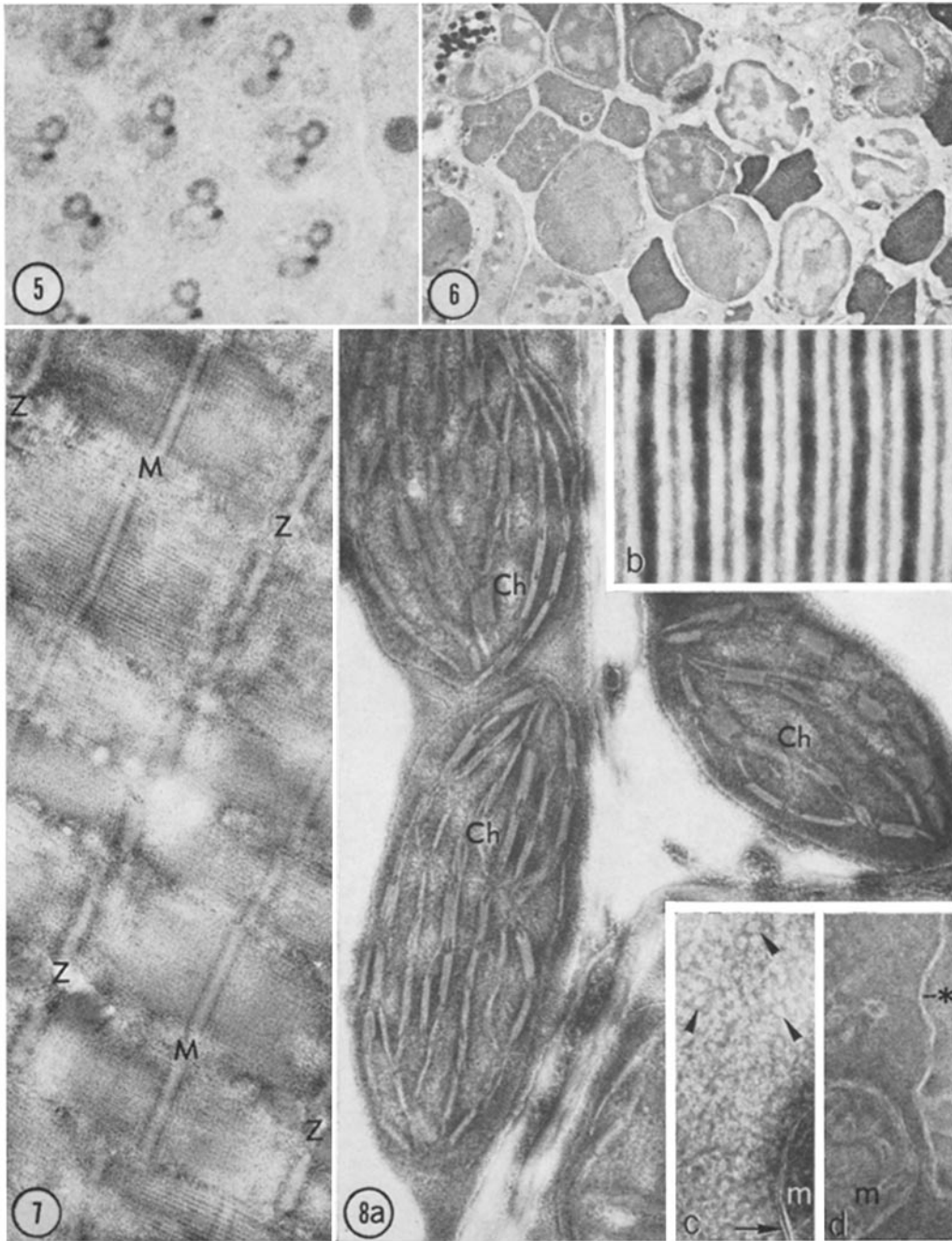


FIGURE 5 Part of a spermatid bundle in *D. melanogaster*. The testis was embedded in cross-linked BSA before sucrose infusion.  $\times 16,000$ .

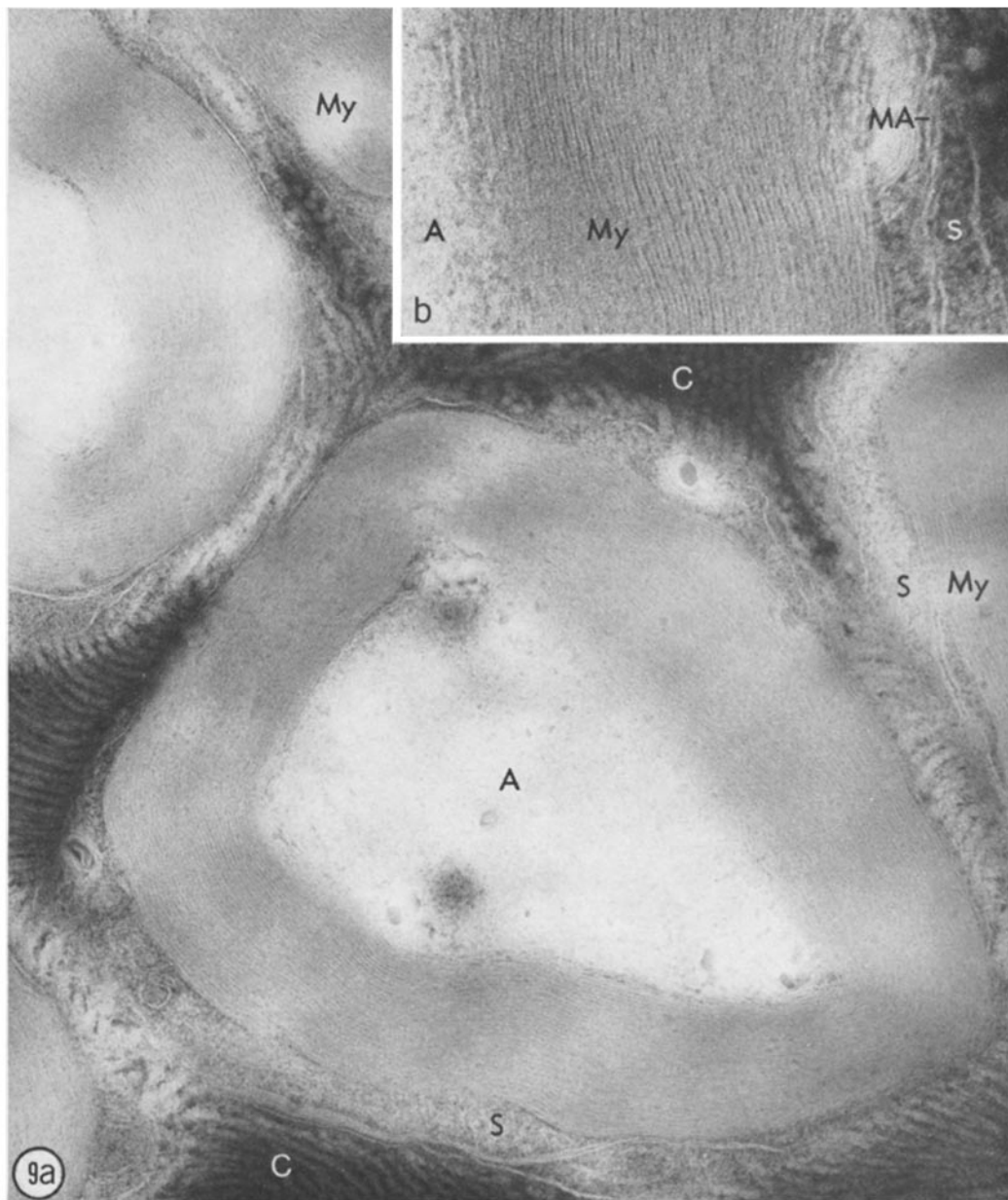
FIGURE 6 Mouse spleen, including various blood cells.  $\times 2,500$ .

FIGURE 7 Rat skeletal muscle, negatively stained with 0.5% UA. Z and M, Z and M lines, respectively.  $\times 32,000$ .

FIGURE 8 Parts of small spinach leaf pieces, sectioned at  $-75^{\circ}\text{C}$  while embedded in a frozen droplet of 1.6 M sucrose. Smoothly sectioned chloroplasts (*Ch*) are seen in (a). A part of a granum is shown at high magnification in (b). Ribosomes are observed in (c) (arrowheads) but not in (d). The double membranes of a mitochondrion (*m*) in (c), at the portion indicated by an arrow, and the plasma membrane (\*) in (d) are clearly seen in negative contrast. (a),  $\times 14,000$ ; (b),  $\times 400,000$ ; (c),  $\times 50,000$ ; (d),  $\times 50,000$ .

of 20–30% BSA, frozen in liquid nitrogen, can be sectioned at  $-60^{\circ}$  to  $-80^{\circ}\text{C}$  very successfully. This quality of a protein solution remains essentially the same when the solution is fixed with

glutaraldehyde. The gelatinous block resulting from cross-linking of BSA shows a sectioning consistency which seems to be superior to that of an “unfixed” solution. These observations suggest that



**FIGURE 9** Rabbit sciatic nerve, sectioned at  $-80^{\circ}\text{C}$  after infusing with 1.5 M sucrose. In (a), myelin sheaths (*My*), Schwann cells (*S*), collagen fiber bundles (*C*), and axons (*A*) are all satisfactorily sectioned, although certain distortions are still recognized in the myelin sheaths. In (b), the myelin sheath shows the familiar pattern of alternately repeating dense and faint lines. The membrane portion indicated by *MA* may be the mesaxon. The periodicity of myelin is seen to be 150–170 Å. (a),  $\times 40,000$ ; (b),  $\times 100,000$ .

highly hydrated tissues or tissue regions are generally more difficult to section in the frozen state than those rich in protein. The aim of sucrose infusion is to overcome this difficulty and, at the same time, to prevent the formation of ice crystals.

The frozen droplet of 1.1–1.6 M sucrose can be smoothly sectioned at  $-50^{\circ}$  to  $-80^{\circ}\text{C}$ . The smooth nature of sections of individual cells is demonstrated in Figs. 1 and 2. When cells are suspended in 0.6 M sucrose, 1.2–2.1 M glucose, or 50% glycerol, sections of frozen droplets of suspensions tend to fragment due to the lack of the proper plasticity of the block. Even then, sections of cells can be obtained but they are usually less smooth than those obtained at the higher sucrose concentrations. The smoothness of these sections is also affected by the concentration of cells in the frozen droplet. Either the concentration of sucrose or the cryokit temperature should be increased when sectioning dispersed cells, vis-a-vis concentrated cells.

In order to section tissue blocks smoothly, a proper combination of sucrose concentration and cryokit temperature is essential. For instance, at a temperature of  $-70^{\circ}\text{C}$ , the optimum sucrose concentration is 0.9–1.1 M for rat kidney (Fig. 3), pancreas (Fig. 4), liver and testis, or for the fruit fly testis (Fig. 5), but 0.6 M for mouse spleen (Fig. 6) or rat skeletal muscle (Fig. 7). Droplets containing small pieces of spinach leaf which consist of large vacuolar space and much smaller cytoplasmic volume are smoothly sectioned when the sucrose concentration is 1.4–1.6 M and the cryokit temperature is  $-70^{\circ}$  to  $-80^{\circ}\text{C}$  (Fig. 8).

An interesting example of the choice of a proper combination of conditions is sciatic nerve. Besides the difficulty of dissecting the tissue into small pieces without causing damage and the problem of slow penetration of glutaraldehyde, the nerve is an extremely heterogeneous structure consisting of a large number of collagen fibers, lipid-rich myelin sheaths, and water-rich axons, making it one of the hardest to section. When pieces of nerve infused with 1.1 M sucrose are frozen and sectioned at  $-70^{\circ}\text{C}$ , the sections are compressed in the direction of sectioning, and the compression is most extensive in the regions of myelin sheaths. This indicates that, under such conditions, the myelin sheaths are softer than the axons or the extracellular spaces filled with collagen fibers. The sheaths differ from the other components in that they are much more hydrophobic and sucrose

infusion will least affect the hardness of these structures. Lowering the temperature while simultaneously increasing the sucrose concentration should, therefore, increase the hardness of the sheath only and provide a uniform consistency to the entire nerve. Indeed, satisfactory results are obtained by sectioning at  $-80^{\circ}\text{C}$  after infusing with 1.4–1.5 M sucrose (Fig. 9). A similar combination is appropriate for sectioning cerebral cortex.

Another example of the importance of the proper hardness of the frozen tissue piece is shown in Fig. 10 *a*. In this section of a *D. melanogaster* spermatid tail, the sectioning direction was from right to left. The interior of the axoneme was probably of a proper hardness and supported the cross-sectioning of the peripheral elements as the knife entered the tail. After the tail was sectioned half way through, however, the surrounding cytoplasmic matrix was probably too soft to support the cross-sectioning of those peripheral elements at the far side and they were pushed outward, resulting in an oblique sectioning at this side of the tail.

In general, knowledge of conventional ultramicrotomy is applicable to ultracryotomy. For instance, a hard frozen specimen is better for cutting very thin sections than for cutting thick sections. Soft blocks tend to slip by the knife edge, resulting in sections of variable thickness. Thin sections of such specimens are often compressed in the direction of sectioning, while thick sections are not. Sometimes, even when the hardness of a specimen is so great that thick sections of the order of  $1\text{--}2\ \mu\text{m}$  crumble as they are cut, thin sections of  $500\text{--}1,000\ \text{\AA}$  can be smoothly cut. In such cases, extremely slow sectioning speed is advisable. The sharpness of the knife is of course important. When the knife edge is dull, thin sections are compressed and tend to pile up at the knife edge. As Bernhard and Viron (2) pointed out, the glass knife is more practical in ultracryotomy than in conventional ultramicrotomy but the quality of the sections still deteriorates if too many are cut at one part of the knife edge. Sometimes, deterioration is noticeable after cutting only about ten sections and, in such cases, the use of a diamond knife is required.

### Surface Tension

Ultrathin frozen sections of unfixed tissues are destroyed when they come in contact with water, although some organelles such as polysomes and



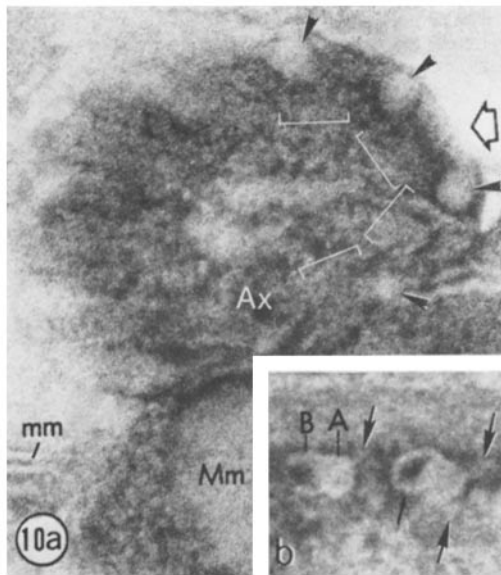


FIGURE 10 Portion of a *D. melanogaster* spermatid tail in (a) and that of a mouse spermatid tail, sectioned at the level of the end piece, in (b). Both sections represent the profiles in the head-to-tail direction. In (a), satellite microtubules (arrowheads) of four peripheral doublets of the axoneme (Ax) appear in cross section but the rest show oblique profiles. This variation is probably related to the direction of sectioning (large, short arrow). Cross profiles of peripheral doublets are expected to be seen at the locations indicated by brackets but are not clearly defined, suggesting a uniform coverage of PTA on them. In (b), two peripheral doublets, each consisting of A and B subfibrils (A and B), show fairly well-defined cross profiles in negative contrast, although a part of the A subfibril of the one at the left is apparently covered by PTA. At the doublet at the right, two arms (large arrows) are seen but at the one at the left, one of them is not clearly defined. It is of interest to note that the interiors of B subfibrils appear to be empty but those of A subfibrils do not, just as in conventional section. The substructure of the wall of A subfibril is visible (small arrow). Mm and mm in (a) are major and minor mitochondrial derivatives, respectively.  $\times 200,000$ .

certain membrane structures can still be observed. Therefore, cross-linking of the protein matrix is required for the maintenance of tissue structure. The degree of structural preservation during exposure of the section to the damaging force of surface tension or dissolution into water varies depending upon the tissue and the intracellular protein concentration and organelle population. This is not a problem in conventional microtomy

but is perhaps one of the most serious problems in ultracryotomy. Fixation should be as light as possible to preserve the chemical nature of the tissue but should be heavy enough to preserve structural organization.

On two occasions in the application of the technique, sections are exposed to surface tension: when they melt on the sucrose droplet and when they are dried on the grid substrate. The first one, which is a more basic problem than the other, is not discussed in this paper. To overcome the damaging effect of surface tension during drying, sections were lyophilized or embedded in thin films of several materials before drying. As mentioned before, these procedures have not yet been successful. Alternatively, filling of voids in the sections with substances of low density was considered to be effective. Negative staining with a 0.2% sucrose-0.5% PTA solution, in fact, gave rise to remarkably well-defined surface structures of bacteria (Figs. 2 a-c) or erythrocytes, but the formation of pits by the evaporation of sucrose from thickly deposited areas (Fig. 2 a) reduced its value as a general procedure. Nonetheless, this experiment clearly indicates the value of negative staining for preserving the three-dimensional organization of frozen sections.

#### Positive Staining Vs. Negative Staining

Bernhard and Viron (2) and Iglesias et al. (3) demonstrated well-preserved structures of rat liver after the double staining with UA and Pb, and, in the present study, similar results were obtained in various tissues. The reduction of the section thickness from 800-1,000 to 400-600 Å, however, did not improve the morphological definition, contrary to expectation. One cause may be an increased susceptibility of the thinner sections to surface tension but another is probably an increased sensitivity to the chemical effects of the staining solutions.

OsO<sub>4</sub> staining, with or without additional staining with UA and Pb, results in tissue morphology similar to that seen in conventional section, with membranes of a positive density. The delineation of the membranes, however, is not so clear as in conventional sections and the trilamellar structure of a unit membrane is clearly seen only when the membrane is rigidly supported by cross-linked proteins, as in such cases as the plasma membrane of the erythrocyte (Fig. 11). The degree of structural disarray is, in fact,

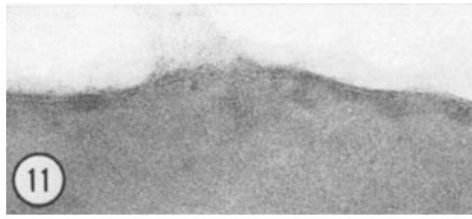


FIGURE 11 Part of a human erythrocyte in a clot, stained triply with  $\text{OsO}_4$ , UA, and Pb. The trilamellar structure of the plasma membrane is clearly seen.  $\times 100,000$ .

generally greater with  $\text{OsO}_4$  staining than without, suggesting that  $\text{OsO}_4$  staining may be more damaging than UA or Pb staining to the preservation of the structural organization of the frozen section.

Although the chemical and structural effects of the positive staining are not exactly known, it is important to distinguish between the problems of structural preservation and of how best to delineate the structures. Positive staining is required to recognize certain structures such as the trilamellar structure of the membrane but the morphological delineation is seen to be generally better with negative staining than with positive staining, partly, perhaps, because of a protective effect of the negative staining against surface tension during drying.

Negative staining, too, however, requires careful evaluation. Both PTA and UA stain sections more or less positively, depending on the time from the application of the staining material to complete drying. In fact, at low magnification, "negatively" stained frozen sections are often quite similar to positively stained conventional sections in general appearance (Figs. 3-7). Nonetheless, one major effect of negative staining may be to darken hydrophilic regions but not hydrophobic areas, as Nicolson (11) suggested in his evaluation of the negatively stained images of chloroplasts embedded in BSA. In addition, however, if there are open spaces within a section, the negative stain will generate profiles of any elevated structures by filling the voids. This effect can occur even when the elevated structures are hydrophilic and will vary depending on the staining intensity. For instance, ribosomes can be observed in some of negatively stained sections of spinach leaf (Fig. 8 c) but not in others (Fig. 8 d). Similarly, peripheral doublets of the axoneme of sperm or spermatid

tail are sometimes hard to recognize with PTA negative staining (Fig. 10 a) but are clearly visible on other occasions (Fig. 10 b).

#### *Certain Fine Structural Aspects*

If frozen sections are smoothly sectioned and the problems of staining and surface tension are overcome, there is no a priori reason that frozen sections should contain less information than conventional sections. On the contrary, frozen sections should be free from the artifacts which may occur during the process of conventional embedding and

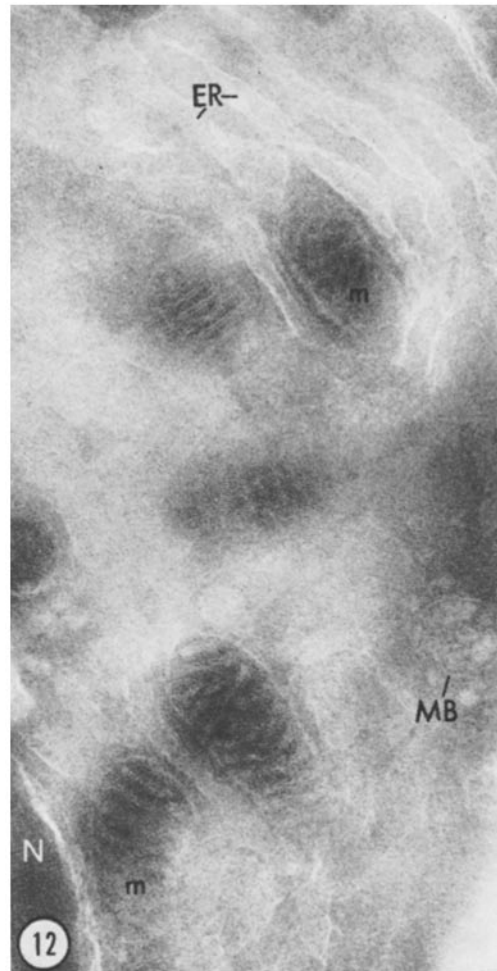


FIGURE 12 Part of a plasma cell in mouse spleen. Membranes of endoplasmic reticulum (ER), mitochondria (m), a multivesicular body (MB), and vesicles inside the body are all seen in negative contrast. N, nucleus.  $\times 40,000$ .

ultramicrotomy. Indeed, in our experience, negatively stained, frozen sections of many tissues show morphological detail which is comparable to or, sometimes, even greater than that seen in conventional sections. Although it is not the primary purpose of this paper to describe these structural features in detail, some observations are described here.

In negatively stained sections of animal cells (Figs. 9, 12), plant cells (Figs. 8 *a-d*), or bacteria (Figs. 2 *a-c*), all membrane structures are seen in negative contrast, perhaps as a result of their hydrophobic nature. The width of such negatively stained membranes appears to match the overall width of the trilamellar structure of the unit

membrane (e.g., Fig. 2 *b*). Despite the very different appearance of plasma membrane in a negatively stained frozen section from one in a positively stained conventional section, the myelin sheath in a negatively stained frozen section shows the familiar pattern of alternating dense and faint lines, similar to those seen in conventional sections both in periodicity and in morphology (Figs. 9 *a, b*). This resemblance, however, only holds at low or medium magnification. At high magnification, the "white" or low density lines are found to be of significantly greater width in frozen sections than in conventional sections: about 50 Å, both in rat (Fig. 13 *a*) and rabbit sciatic nerve, compared to 20 Å. This dimension coin-

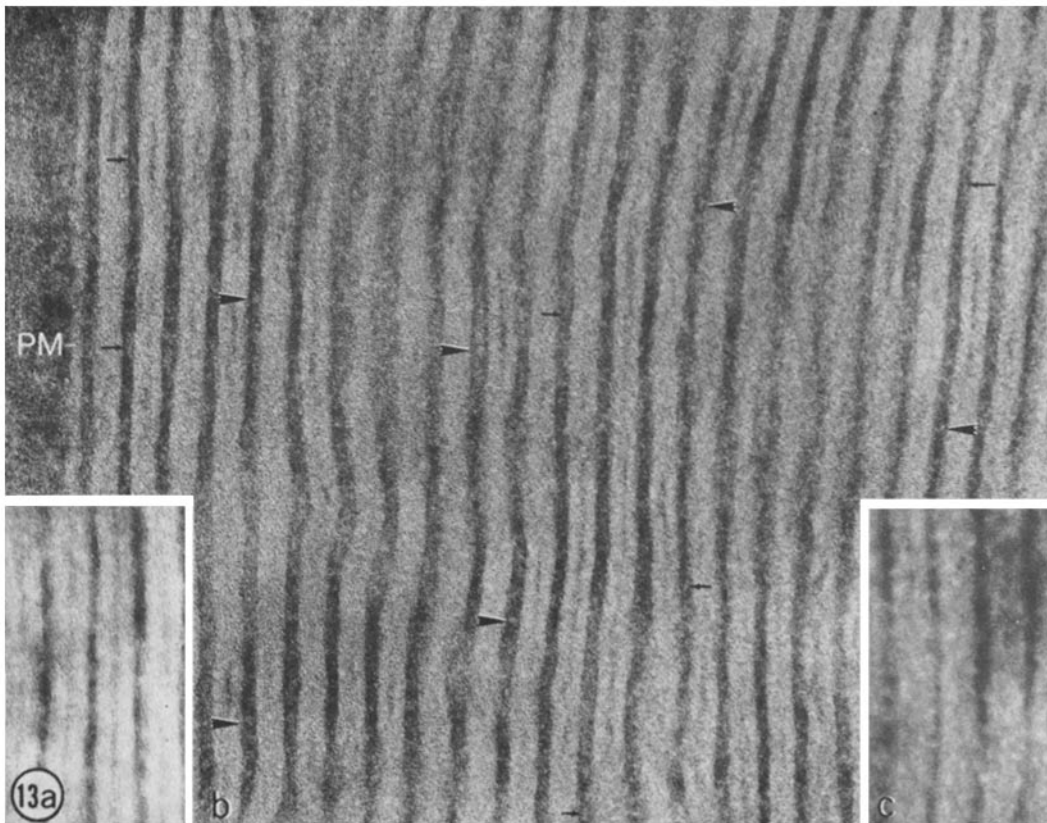


FIGURE 13 Parts of myelin sheaths of rat sciatic nerve, sectioned in the same manner as described in the explanation of Fig. 9. In (*a*), the major or dark line-to-dark line periodicity is about 120 Å, a value similar to the periodicity seen in conventional section, but the width of white lines is about 50 Å which is much greater than that of white lines in positively stained conventional section, about 20 Å. In (*b*), the dark lines or spaces are widened but the faint lines are not. Minute, globular (arrowheads) and filamentous (small arrows) structures are clearly visible in the widened dark spaces. In (*c*) of a very thin section, each of the white lines is vaguely discerned and appears to be composed of 30–50 Å granules in negative contrast. *PM*, plasma membrane. (*a*),  $\times 500,000$ ; (*b*),  $\times 360,000$ ; (*c*),  $\times 500,000$ .

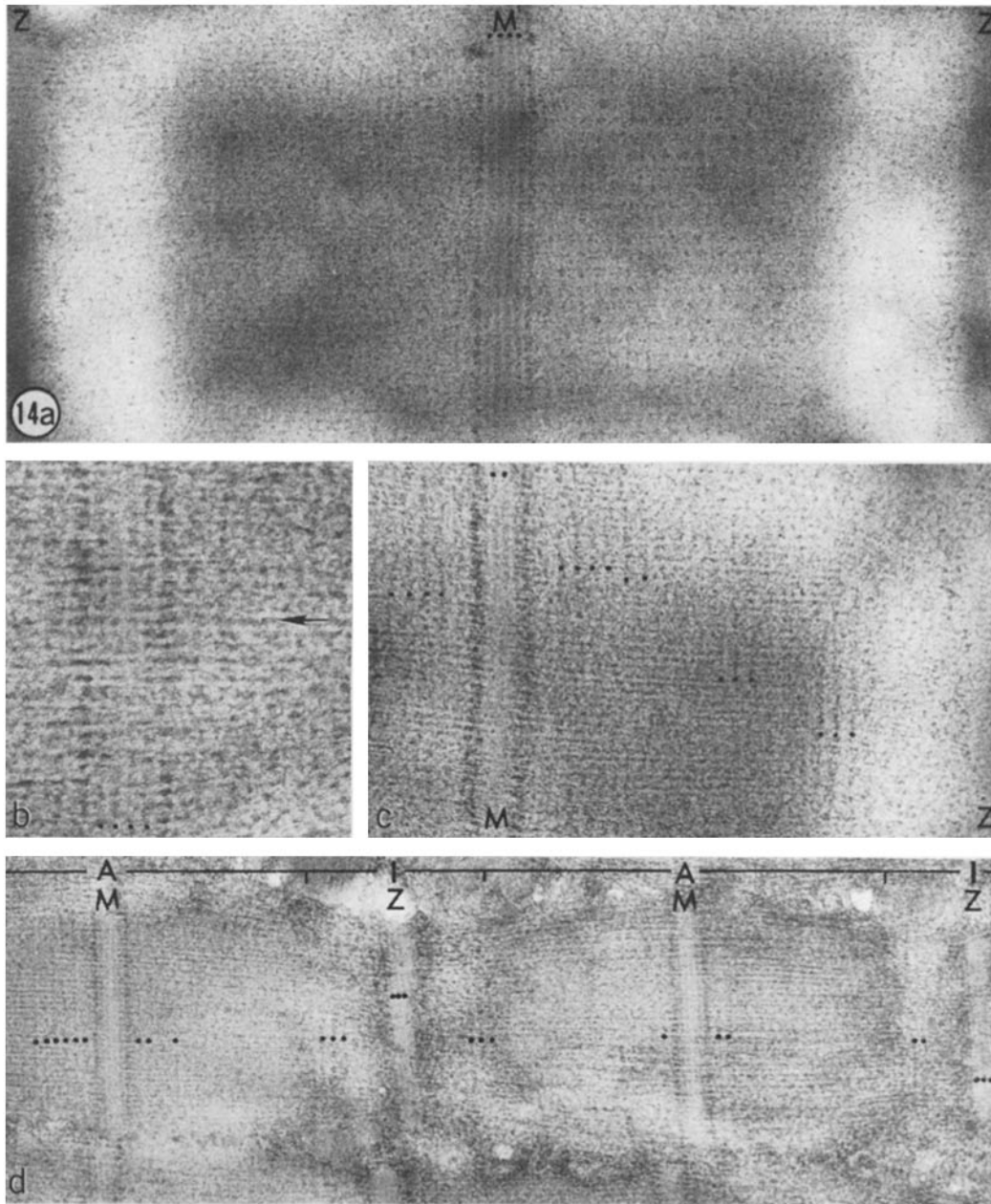


FIGURE 14 Contracted rat skeletal muscle, in sections stained negatively with 0.5% UA. A sarcomere from Z to Z lines, with an M line with two outer dense and four inner less dense substriations, is shown in (a). In (b), each of the six substriations of the M line is seen to be a series of "dots." A region of negative stain can be followed in the direction of an arrow and is seen to become a dot as it passes each of the six substriations. In (c) of the same magnification as that of (a), the M to Z distance is seen to be about the same as that in (a). Yet, the number of the inner substriations in the M line is only two (two black spots) at the top of the figure and none at the bottom. In (c) and (d), delicate substriations in the A and I bands are marked with black spots. In (d), three substriations are seen in the Z lines, as indicated by black spots. (a),  $\times 65,000$ ; (b),  $\times 100,000$ ; (c),  $\times 65,000$ ; (d),  $\times 37,000$ .

cides with the value revealed by the X-ray analysis of electron density profiles of rabbit myelin and interpreted as representing the thickness of the lipid bilayers (12).

Schmitt et al. (13) revealed, in their classical X-ray study, that the periodicity of fresh myelin was about 180 Å in dog and cat spinal roots. The periodicity recognized in the present study is variable: a 120 Å periodicity in rat sciatic nerve (Fig. 13 *a*), observed after 1 h fixation with 2% glutaraldehyde, and a 150–170 Å periodicity in rabbit sciatic nerve (Fig. 9 *b*), seen after 3 h fixation with 5% glutaraldehyde. Whether this variation is due to the difference in species, in the fixation conditions, or in the degree of postsectioning artifact is unknown at present. How the cross-linking of protein matrices with glutaraldehyde affects the dimensions of different structures requires further analysis.

The dense lines of myelin sheath in frozen sections vary in width (compare Figs. 13 *a* and 13 *b*). This is probably due to the expanding force of surface tension exerted on the section, as it spreads on the sucrose droplet, and suggests an interesting aspect of myelin structure. The dense lines which are in physical continuity with the Schwann cell cytoplasm appear to expand readily, whereas the faint lines which are structurally continuous with the extracellular space, or glycocalyx, do not.

Minute structures, which are not definitely recognized in the dense lines of a tightly layered myelin sheath (Fig. 13 *a*), become clearly visible when these spaces are expanded (Fig. 13 *b*). Their shapes vary from globular to fibrillar and the widths vary from less than 20 Å to 40 Å. In very thin or densely stained sections, white lines show a 30–50 Å granularity (Fig. 13 *c*), in agreement with past studies (2, 14). The significance of such a granularity, however, is unclear and the possibility of artifact cannot be excluded at present.

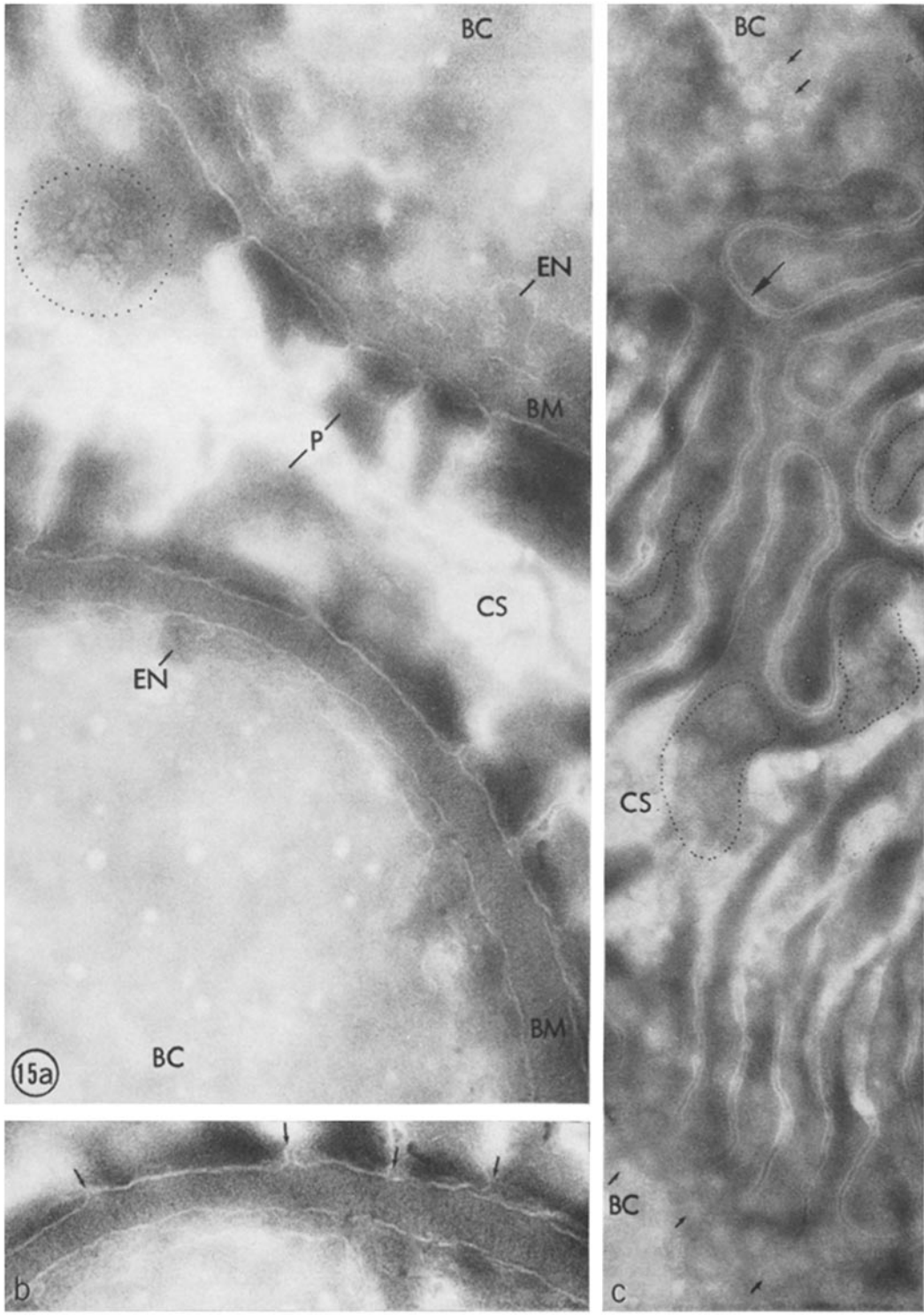
The M line of contracted skeletal muscle fibers in frozen sections often shows cross-striated substructures (Fig. 14 *a*) which are each a series of "dots" when observed at high magnification (Fig. 14 *b*). The number of striations varies from four, excluding two outer dense striations (Figs. 14 *a, b*), to none. This variation is sometimes seen within a single M line (Fig. 14 *c*). Whether such a variation is due to a slight difference in the degree of contraction or a local tilting of the M line or other causes is unknown at present. Delicate substriations

are often observed all over the other areas, including the Z line (Figs. 14 *c, d*). Substriations observed over the A band quite probably represent orderly arrangements of cross bridges between the thick and the thin filaments (17) but the significance of similar substriations in the I band is unclear. The definition of these substriations is comparable to or probably even better than that seen in conventional section (15, 16), and suggests that the highly crystalline structure of skeletal muscle may be better preserved in frozen sections than in conventional sections.

The structure of the renal glomerulus is excellently preserved in frozen sections (Figs. 3, 15), although the visualization of certain structures with negative staining is more difficult than that with positive staining used in conventional ultramicrotomy. For instance, round vesicles, 400–1,000 Å in diameter, which are similar to protein absorption droplets (18) in morphology are seen, often in clusters, in pedicels (Figs. 15 *a, c*). A feature which is not as clearly defined in a conventional section, however, is that the glomerular basement membrane shows striations which seem to be composed of coarse fibrillar elements arranged approximately perpendicular to the surfaces of the membrane (Figs. 15 *a, b*). The striations become less clear near the surfaces. The amorphous material near the surface of the epithelial side of the basement membrane is seen to be continuous with the material which occupies the narrow intrapedicel spaces (Figs. 15 *a, c*).

In conclusion, although more remains to be done to define the optimal parameters of fixation, freezing, surface tension, and staining, the technique of ultracryotomy is already in a state to be considered as an alternative to conventional embedding and ultramicrotomy for ultrastructural studies. It is a particularly promising method of specimen preparation for the application of cytochemical and immunocytochemical procedures, since it is likely to allow greater retention of antigenic or enzymatic activities of macromolecules than most other methods. Such studies are under way in our laboratory.

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FIGURE 15 Rat renal glomeruli. In (a), two blood capillaries (BC), endothelial cells (EN), basement membranes (BM), pedicels (P), and capsular spaces (CS) are well defined. In (b), which is a high contrast print of a part of (a), the basement membrane shows cross striations which seem to be composed of coarse fibrillar elements. The striations become obscure near both surfaces of the membrane and the amorphous material near the surface of the epithelial side is seen to be in continuity with the material in the narrow interpedicel spaces (arrows). In (c), tangentially sectioned pedicels show an interlocking pattern and the continual presence of a material in the narrow, interpedicel spaces is seen (large arrow). Tangentially sectioned fenestrations of endothelial cells show round profiles (small arrows). In (a) and (c), small round vesicles are seen in clusters, as marked with dotted lines. (a) and (b),  $\times 40,000$ ; (c),  $\times 25,000$ .