A SIMPLE FREEZE-FRACTURE REPLICATION METHOD FOR ELECTRON MICROSCOPY

STANLEY BULLIVANT and ADELBERT AMES, 3RD

From the Mixter Laboratories for Electron Microscopy and Neurochemistry, Neurosurgical Service, Massachusetts General Hospital, Boston

ABSTRACT

A simple method to achieve results similar to the freeze-etching technique of Moor et al. (1961) is described. The frozen tissue is cut under liquid nitrogen with a razor blade outside the evaporator rather than inside with a cooled microtome. The conditions of the experiment do not favor sublimation, and it is proposed that the structure of the replica be explained by local faults in the cleavage plane which leaves structures, such as membranes, standing above the ice. Micrographs of replicas of glycerol-protected frozen small intestine of mouse prepared by the method are presented and the structural details they show are discussed. The problem of vapor-deposited contamination is discussed. It is concluded that this is a practical method for obtaining electron micrographs that are relatively free of artifact, and that further improvements may be expected from the use of rapidly frozen fresh tissue and a clean vacuum system, possibly of the ion-pumped type.

INTRODUCTION

The routine methods for cytology with the electron microscope involve chemical fixation, dehydration, impregnation with liquid plastic, hardening of the plastic with heat, cutting of thin sections, and, finally, staining prior to observation. Though the electron microscope appearance of such sections is routinely interpreted in terms of the structure of the living cell, there are good reasons to suspect that the treatment outlined above may produce significant changes in morphology.

To obtain electron micrographs which reflect more faithfully the structure of the living cell, it would be desirable to eliminate as many of the potentially artifact-producing preparation steps as possible. The ideal would be to fix tissue so that there is no movement of molecules over distances greater than the resolution limit imposed by other factors. A promising approach would be rapid freezing. This would be followed by cutting of thin sections of the cell embedded in its own ice at a temperature so low that reorganization of water and molecules could not occur. The resulting thin section might be maintained and examined at this temperature in an electron microscope with phase-contrast optics so that staining would not be required. While techniques and instruments are not available to do this at present, a suitable method of rapid freezing has been developed (Van Harreveld and Crowell, 1964), and very promising results have been obtained by impregnating tissue with gelatin solutions and then sectioning it at low temperature (Bernhard and Nancy, 1964). Freezing as a fixation method has been extensively explored in a number of ways (Gersh, 1956; Sjöstrand and Baker, 1958; Hanzon et al., 1959; Sjöstrand and Elfvin, 1964; Fernández-Morán, 1957, 1960; Bullivant, 1960, 1965; Rebhun, 1961, 1965; Van Harreveld and Crowell, 1964; Van Harreveld et al., 1965). With all these methods

the removal of water was followed by embedding in a plastic; although the preservation obtained was good, it had no better claim to reality than that of chemically fixed tissue. The main benefits from this type of work have been the confirmation, by a radically different method of fixation, of structure seen after conventional chemical fixation and the production of sections of tissue more useful for differential staining.

Another method, and the one with which this report is mainly concerned, is that which has come to be known as freeze-etching. In 1950, Hall reported a low temperature replica method for electron microscopy in which the surface ice of a frozen specimen in an evaporator was made to sublime at low temperature by the application of radiant heat. After sublimation, a replica of the surface was made by heavy metal shadowing, still at low temperature. The specimen that Hall used was a frozen suspension of silver halide particles. These particles could be seen in the replica. Steere (1957) applied the method to biological specimens, mainly virus crystals. He cut the frozen crystal with a cold scalpel prior to putting it in the evaporator and carrying out the sublimation and replication. The individual particles arranged in the crystal lattice could be seen. Steere's method differs from that to be described here insofar as the cleaved specimen was not protected from water in the atmosphere. Haggis (1961) carried the technique a step further by freezing glycerinated blood smears on thin cover slips which had been previously scored to facilitate breaking. The cover slips were inserted in the evaporator and kept at −196°C until a vacuum suitable for carrying out evaporation was attained. The temperature of the specimen was raised to −105°C and the cover slip was then snapped mechanically and a replica was made of the fractured edge of the smear. The replicas showed the outlines of cells, and it was claimed that individual hemoglobin molecules could be recognized where the fracture passed through the interior. The method which has produced the most exciting results is that devised by Moor et al. (1961). Their machine consists essentially of a low temperature ultramicrotome, set up within a high quality vacuum evaporator. Glycerinated tissue is frozen rapidly in liquid propane and mounted on a specimen stage which is maintained at −100°C. After a vacuum of 10^{-6} mm of Hg has been obtained, a series of thin slices are cut off the frozen specimen until a smooth surface is obtained. This cutting appears to be more in the nature of cleaving than of microtomy in the usual sense. After the last cut is made, the surface is left for a few seconds to allow sublimation of a layer of ice a few hundred Angstrom units in thickness. The nonaqueous components of this thin layer are thus left standing above the ice. A carbon-platinum replica, backed with carbon, is made by evaporation. After warming, the tissue is removed from the replica by chemical treatment, and the replica is mounted and examined in the electron microscope. A number of cytological studies have been made using this instrument (Moor and Mühlethaler, 1963; Moor, 1964; Moor et al., 1964; Branton and Moor, 1964). A wide variety of animal and vegetable cells have been examined. A particular advantage of the technique is that, by the nature of the cutting process, both surface views and cross-sections of organelles are seen. It must be stressed that, in all the studies done so far, glycerol has been used as a protective agent because sufficiently rapid freezing rates were not used to obtain ice crystal–free preparations without it. The objections to this have been partially circumvented, in the case of plant cells, by actually growing the plants in glycerol solutions before freezing. The image seen in the latter case represents the structure of the living glycerinated cell.

One of the main disadvantages of the Moor freeze-etching apparatus is its complexity and expense. Two simpler methods for making freeze-fractured replicas for electron microscopy will be described together with some of the results obtained by their use. The more satisfactory method differs from the original method of Moor et al., (1961) in that the fracture is made outside the evaporator with a razor blade rather than inside with a cooled microtome.

MATERIALS AND METHODS

Pieces of small intestine of mouse were protected from ice crystal formation during freezing by prior immersion in a 20% glycerol solution in Veronal acetate buffer at 4°C over a period of 20 min. Two different freeze fracture methods were experimented with.

In the more satisfactory method the fracture was made outside the evaporator. A segment of the glycerinated intestine was inserted but left protruding from the hole in the center of the heavy cylindrical, brass block shown to the right of Fig. 4. The block was placed in a plastic container and the whole immersed under liquid nitrogen which had

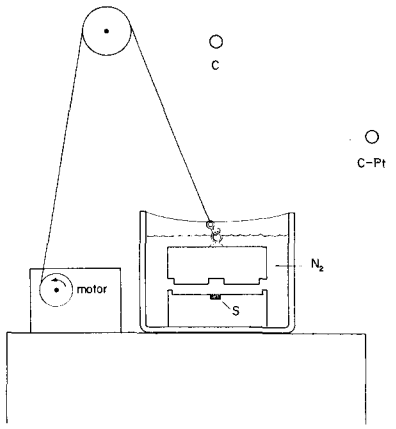


FIGURE 1 Diagram of apparatus used in first method. The lid is shown slightly raised, but in the actual experiment it is close fitting. *S*, specimen; N_2, liquid nitrogen; *C*, carbon electrodes; *C-Pt*, carbon-platinum electrodes.

been freshly poured from the storage vessel. This precaution was taken to avoid the possibility of contaminating the specimen with small ice crystals which form in liquid nitrogen when it is left standing exposed to moisture in the air. Liquid nitrogen is a poor coolant and, because of the large heat capacity of the heavy block, about 2 min were required to attain liquid nitrogen temperature (−196°C). Despite this, the replicas showed few ice crystals, and it appears that relatively slow freezing rates will suffice in the presence of glycerol. The brass lid (left of Fig. 4) was also immersed under the nitrogen and cooled. An ordinary single-edged razor blade, with no special sharpening, was gripped firmly in a pair of forceps and held under the nitrogen to cool. It was pushed across the surface of the block to cut off the top of the specimen. We placed the lid in position immediately (Fig. 3), taking care never to bring it above the nitrogen surface. The plastic container, with the block and lid remaining under nitrogen, was transferred to the evaporator and placed under

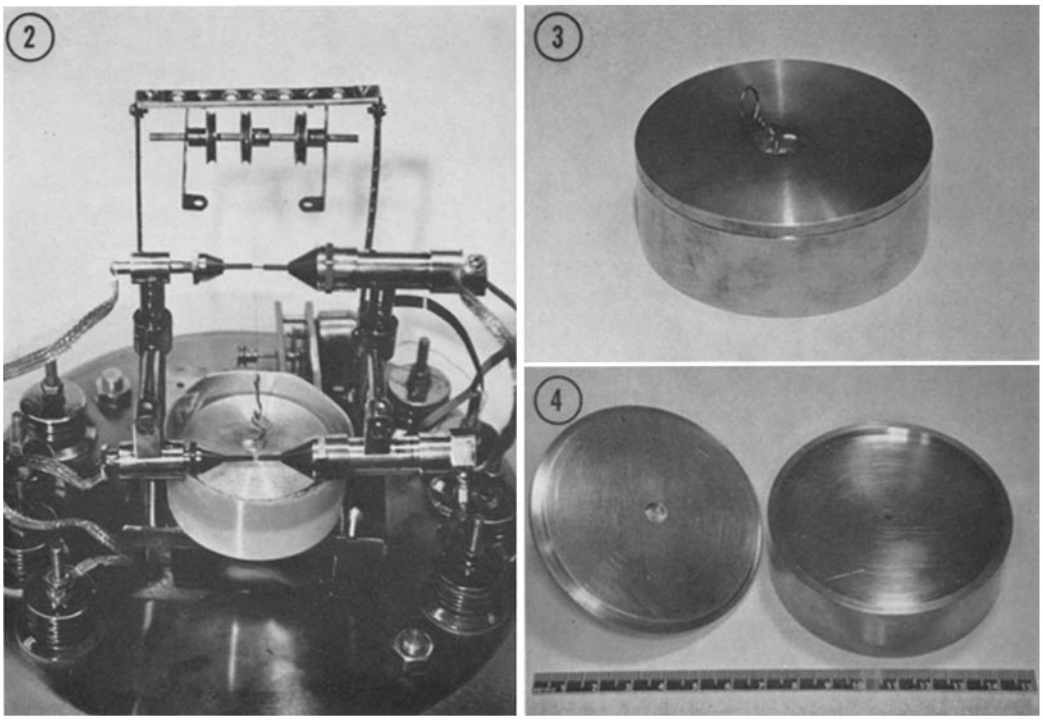


FIGURE 2 Photograph of whole apparatus for first method, set up as it would be at the beginning of an experiment, except that liquid nitrogen is not present in the plastic container.

FIGURE 3 Brass specimen block as used in first method, with lid in place.

FIGURE 4 Brass specimen block as used in first method, with lid off to show specimen hole in center of lower block.

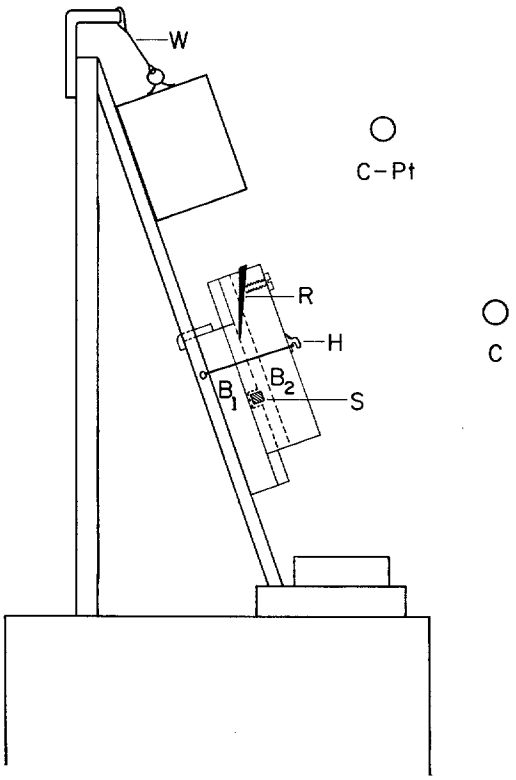


FIGURE 5 Diagram of apparatus used in second method. The specimen block B_1 is secured to the inclined plane by a thin wire passing under the hook, *H*. When the wire, *W*, is broken electrically, the heavy block falls, strikes B_2 and causes it to slide over B_1 so that the razor, *R*, fractures the specimen, *S*. *C*, carbon electrodes; *C-Pt*, carbon-platinum electrodes.

the electrode assembly (Ladd Research Industries, Inc., Burlington, Vermont). The hook from a crane made from Erector set parts was placed in the loop on top of the lid (Fig. 2). The bell jar was placed in position and a vacuum of 1×10^{-5} mm of Hg obtained in about 10 min. During this pumping, the liquid nitrogen in the container boiled vigorously at first and then solidified so that at this point the temperature of the block and the specimen was in the region of −210°C. The frozen nitrogen sublimed and the temperature of the block rose. Water frost on the outside of the plastic container also sublimed. There was no frost on the block and lid or on the inside of the container, since these had been protected by the covering of nitrogen. Once vacuum was achieved, the carbon platinum pellet and the carbon rod were outgassed by passage of a low current to heat them to a cherry-red color. The carbon electrode was normal to the specimen surface, and the carbon platinum electrode was at 45° to it (Fig. 1). A small 1.5 v motor with suitable gearing (purchased from a hobby shop) actuated the crane and lifted the brass lid. As soon as the specimen surface was exposed to the carbon platinum pellet, the latter was flash-evaporated by passage of a high current. Immediately following this, a coat of carbon was evaporated. When air was admitted to the evaporator, there was transient condensation on the block which indicated that the temperature was still below −183°C, the boiling point of oxygen. The tissue was removed from the replica with hot concentrated nitric acid, washed in one change of clean nitric, and then transferred to distilled water. These steps were carried out with the aid of a fine platinum loop. The replicas were picked up from the water on uncoated 400-mesh grids and examined in a Siemens Elmiskop I.

The second method was an attempt to duplicate more nearly the Moor technique with less expensive equipment. Because of factors which will be described in the Discussion, it did not make replicas as satisfactory as those made with the first method described above. It will be described both because it was a step in the evolution of the more successful method and because with some modification it may also work well. The specimen was frozen by immersion under liquid nitrogen as before, but this time in a hole in a brass block B_1 (Figs. 5 and 7) which was dovetailed with another block B_2 containing a razor blade so mounted that, when the block B_2 slid over the block B_1, the specimen was cleaved. The whole assembly, B_1 and B_2 complete with specimen, was cooled with the two sliding parts staggered with respect to one another. When it reached liquid nitrogen temperature, it was transferred to the evaporator and mounted on an inclined plane with guide slots made from Erector set parts. Sliding of the top block was prevented by a piece of thin copper wire secured to the inclined plane. When a vacuum of 10^{-5} mm of Hg was attained and the electrodes had been outgassed, a third heavy block was released from the top of the incline by passing a current through and breaking the thin wire (*W*). This block fell until it struck B_2, to which it transferred some part of its momentum, breaking the restraining wire and causing the specimen to be cleaved. Carbon-platinum and carbon were evaporated and air was admitted. It is estimated that the temperature never rose above −170°C until after the replica was made. The situation immediately after removal of the bell jar is shown in Fig. 6. The replica was treated as before. The main disadvantage of the technique as described is that frost forms on the assembly during transfer into the evaporator. This will be discussed later.

RESULTS

Examples of electron micrographs, all obtained by the first method, are shown in Figs. 8 to 13. The

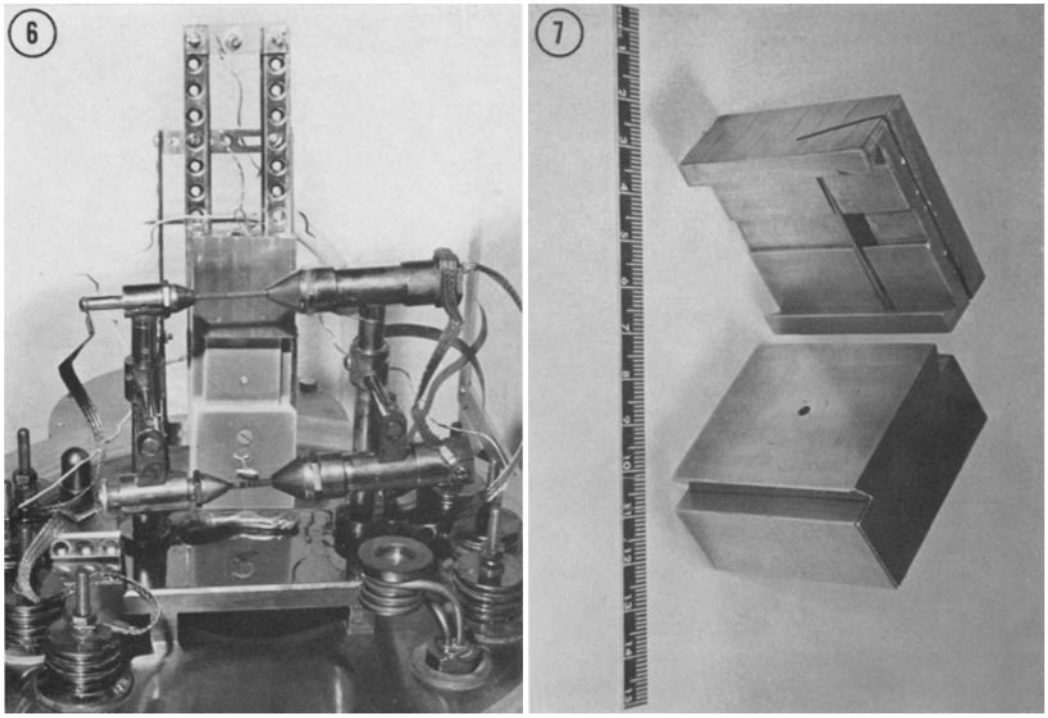


FIGURE 6 Photograph of whole apparatus for second method, shown at the end of an experiment. The fractured specimen can be seen in the center of the middle block.

FIGURE 7 Photograph showing specimen block and sliding block with razor as used in second method.

replicas show intracellular detail and may be compared with chemical fixation and thin sectioning (see Trier and Rubin, 1965, for example) and with freeze-etching of similar material (Moor, 1964; Moor et al., 1964). Membrane structures are most obvious, but other structures, such as the fibrillar terminal net below the microvilli, are also shown (Fig. 8). In common with the work of Moor, the structures seen tend to confirm previous work with chemical fixation. Despite the rather crude method of cutting, large areas of the replica appear flat, although the combination of two- and three-dimensional appearances referred to already is obvious. For example, some mitochondria have been left entirely in the surface, some have been sectioned to reveal internal detail, and some have been scooped out (Figs. 8 to 10, and 12). Combinations of these processes are found. Good internal detail of mitochondria is revealed in Fig. 10, and the cristae are seen to contain an appreciable space within them. The Golgi region is shown, in Fig. 11, to have the classic appearance of a stack of flattened envelopes, and this impression is heightened by the fact that a surface view of one of the sacs is shown. The fine granular projections, seen on many membranes and cut surfaces, will be described in more detail below. The mosaic pattern seen on the surface of some membranes (Fig. 13) may represent ribosomes. Fig. 12 shows the nuclear membrane to advantage. The nucleoplasm has been scooped out. The double-layered envelope with pores is seen in cross-section and also in surface view as seen from the interior of the nucleus. The difference in size of the nuclear pores in the inner and outer membranes is very noticeable, those in the outer membrane being much larger. A section through the nucleoplasm is shown in Fig. 13. The surface is finely granular, but in certain areas, corresponding to the distribution of the heterochromatin in glutaraldehyde-fixed material, there is a coarser 250 A granulation.

In addition to the mosaic pattern on some membranes and the coarser granularity following

the distribution of heterochromatin in the nucleus, the replicas show a fine pock-marked appearance. In the best replicas, obtained with the first method, this fine granularity does not interfere too much with the image. In replicas made with the second type of apparatus, and also in those made with an earlier version of the first, in which a smaller block with a poorly fitting lid was used, the fine granularity was much more marked, and in some cases obliterated the other structures. It is interesting that this granulation appeared preferentially on certain biological structures, the surfaces and the edges of membranes in particular, rather than on plain ice surfaces such as the lumen.

DISCUSSION

Advantages of the Method

As mentioned in the Introduction, there are reasons to suspect that the routine methods of preparing tissues for electron microscopy may introduce structural changes. Schultz and Karlsson (1965) have demonstrated that the usual fixatives do not immobilize membranous components sufficiently to prevent their subsequent movement, owing, in their study, to changes in osmolarity.

Two aspects of the preparative procedure would appear to be particularly suspect: (*a*) the change from aqueous medium to alcohol and then to liquid plastic may be associated with changes in the volumes of membrane-limited compartments, since the membranes probably continue to act as differential barriers to the solvents; and (*b*) replacing the aqueous medium, with its high dielectric constant and appreciable ionic strength, by the less polar alcohol would increase the electrostatic forces existing between fixed charges in the tissue. If, for example, we consider two closely adjacent membranes bearing charges of the same sign, the repulsion between them would be increased and they might be pushed farther apart. Effects such as these might well alter intercellular and interorganelle distances as well as the shapes and sizes of organelles and the intermolecular distances and configurations of macromolecules.

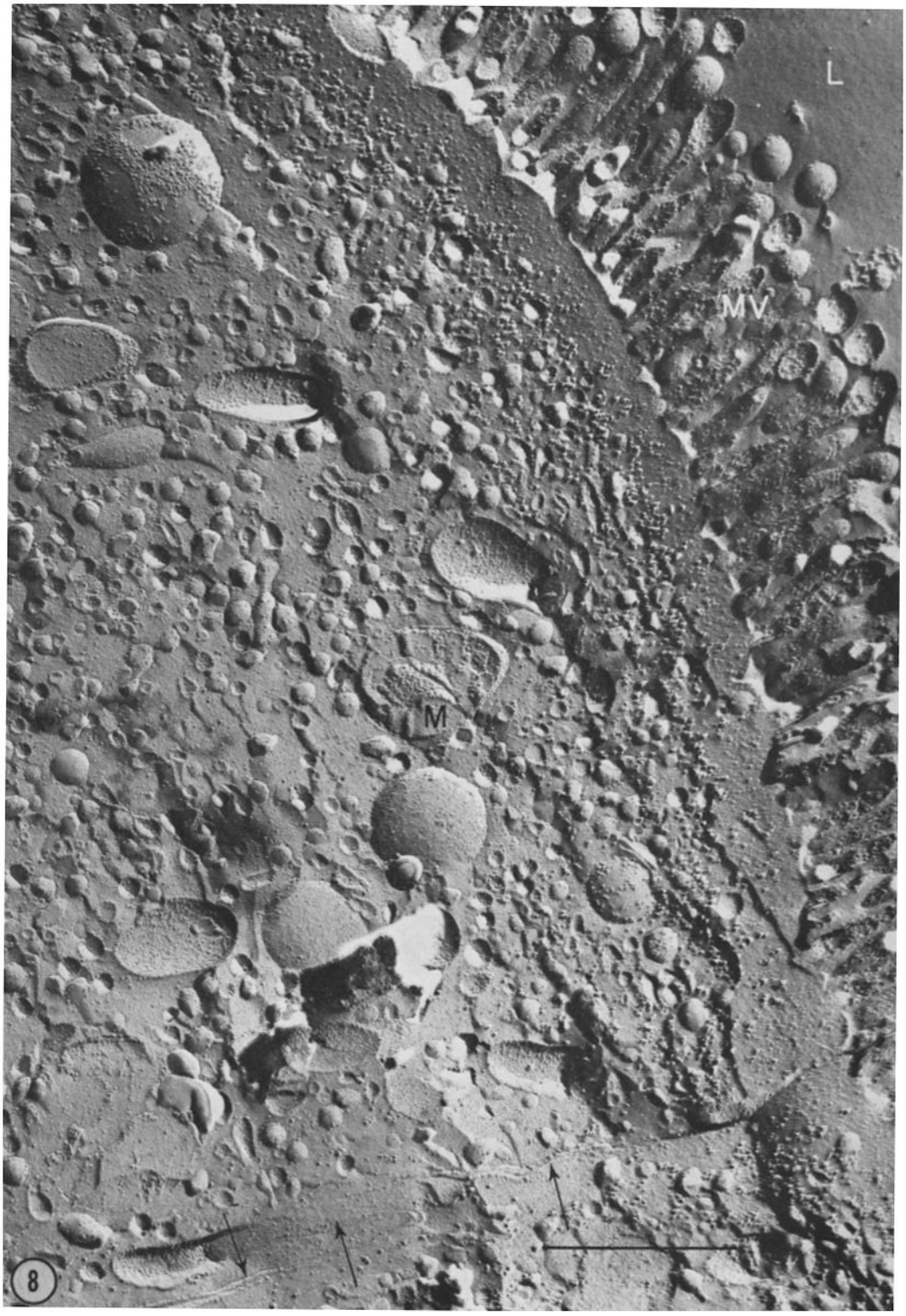
Moor's method of freeze-etching would appear to have eliminated most preparation-induced changes. The advantage of the present modification of Moor's method is that it is both inexpensive and simple. The time from freezing of the specimen to examination of the replica in the electron microscope is about 30 min. Meryman (1957) has shown that migratory recrystallization of ice takes place at temperatures as low as −96°C and it is thought that this process may occur to some extent until the temperature is reduced below −120°C. The present method uses temperatures below −183°C in contrast to the −100°C at the time of shadowing in the Moor apparatus. This avoidance of migratory recrystallization is not of such importance with the present work on glycerinated tissue but can be expected to assume importance when work is done with rapidly frozen fresh tissue.

The Mechanism of Freeze-Fracture Replication

In the first method, the temperature of the cleaved surface at the onset of shadowing was below −183°C and the chamber pressure was 1×10^{-5} mm of Hg. The vapor pressure of water over ice at the temperature of the specimen is very low and presumably no appreciable sublimation occurred. Hall (1950) calculates that even at −130°C the rate of sublimation in a perfect vacuum would only be 0.01 A sec^{-1}. There are three possibilities to account for the detail seen in the replicas: (*a*) The ice layer was somehow lost under the liquid nitrogen after cleaving. This was

FIGS. 8 to 13 show replicas of cells from glycerinated, frozen-fractured mucosa of the small intestine of the mouse. They were all prepared by the first method (see text). Magnification is indicated by a micron mark on each plate.

FIGURE 8 Intestinal absorptive cell showing microvilli (*MV*) projecting into the lumen, *L*. Cut fibrils of the terminal net are seen subjacent to the microvilli. The mitochondrion, *M*, is sectioned to show internal detail. Small vesicles fill the cytoplasm. Plasma membranes forming a cell junction are indicated by arrows. Shadowing from lower left. × 30,000.


L
MV
M
8

proved not to be the case by the results obtained with the second method, in which the cleaving was done in the vacuum immediately prior to shadowing. The replicas had the same appearance although there was more of the fine pock-marking referred to previously. (*b*) Heat from the evaporation caused local warming of the surface to the region of −90°C, where sublimation would occur. Since the carbon-platinum electrode was 10 cm from the specimen and the flash evaporation was rapid, this seems unlikely although it cannot be ruled out entirely. (*c*) Sublimation did not occur and the structure was revealed by faults in the cleavage plane occurring, for example, at a membrane where there were local differences in the constitution of the ice. This would have somewhat the same result as sublimation, for it would leave certain structures standing above the ice surface. In the experiments reported here, this seems the most likely explanation, and hence the process is referred to as freeze-fracture replication, rather than freeze-etching. Since the replicas produced by the freeze-fracture method resemble so closely those produced by the Moor method, it is possible that, in this latter method also, faults in the cleavage plane are more important than sublimation in reflecting fine structure. It would be of interest to add varying degrees of sublimation to the fractured surface to see if this added to the information obtained.

It should be remembered that we are dealing with a replica which has in it the shapes and dimensions of the original structures, but none of their other properties. The method gives structural information only, and the thin sectioning method has to be used to make density measurements or derive cytochemical information.

Structural Details Shown in the Replicas

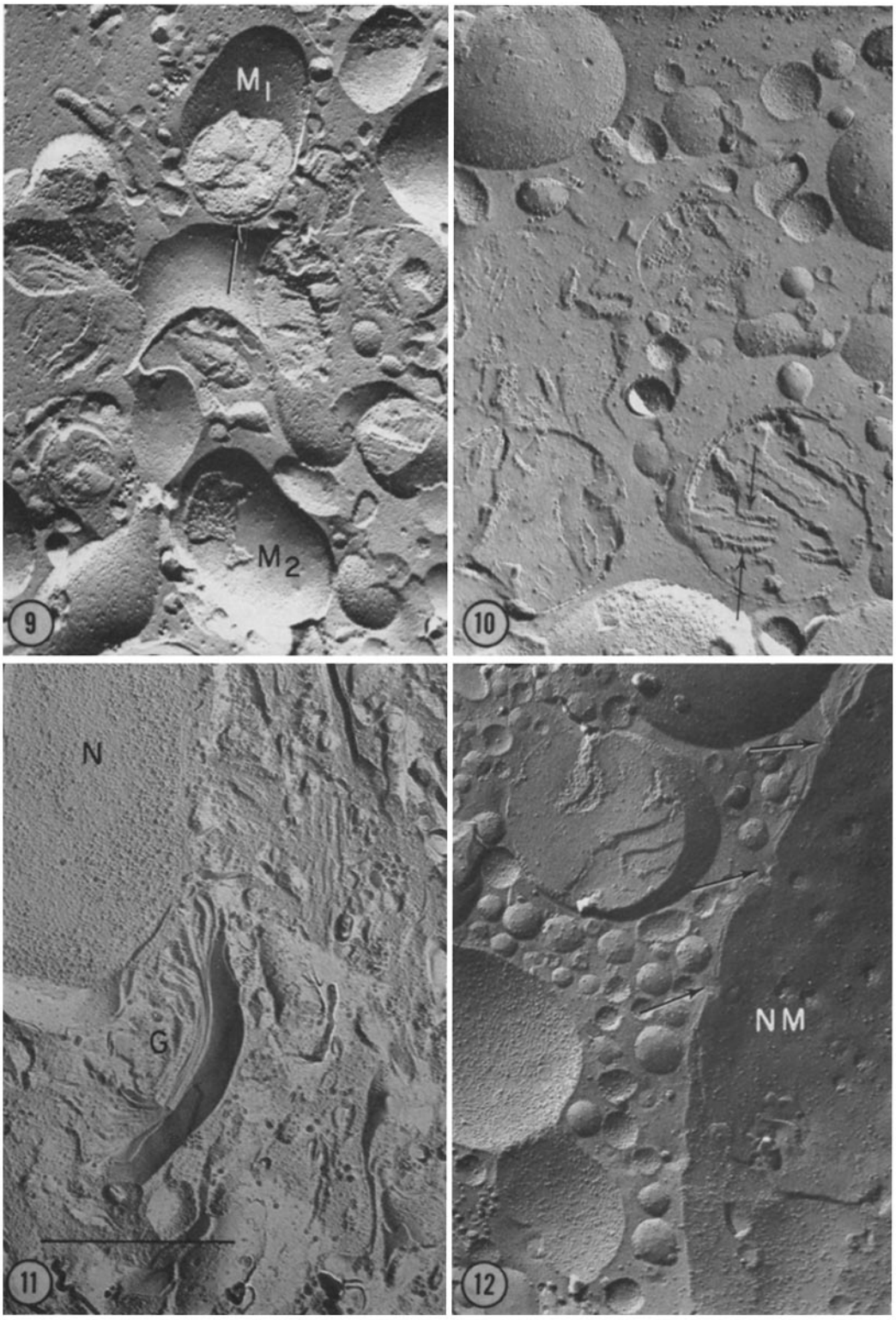
According to the conventional methods of preparation, the space within the mitochondrial cristae may be either open or closed. When it is closed, the two membranes give the appearance of a tight junction. An open space is shown in the cristae after osmium tetroxide or glutaraldehyde and osmium tetroxide, but a closed space is shown after glutaraldehyde alone, freeze-drying (Sjöstrand and Elfvin, 1964), freeze-substitution of frozen glycerinated (Bullivant, 1964; Rebhun, 1965) or rapidly frozen fresh tissue (Malhotra and Van Harreveld, 1965). An open space was shown on replicas of frozen glycerinated tissue both by the work of Moor and his associates and also by this present work. Assuming that replication gives a more faithful image than the freeze-substitution because the temperature of the tissue is never raised above −183°C until shadowing is completed, then we can state that glycerinated mitochondria really do have a space within the cristae. Until the experiment of following very rapid freezing of fresh tissue with freeze-fracture replication has been done, we cannot be sure of the true size of the space in living nonglycerinated mitochondria. If replication gives a more faithful image than substitution, we must assume, in the

FIGURE 9 Portion of cytoplasm of intestinal absorptive cell. The mitochondrion M_1 protruded above the ice surface and was also truncated to reveal internal structure. Inner and outer membranes are indicated by the arrow. The mitochondrion M_2 also protruded but was not sectioned. A portion of its outer membrane was removed. Shadowing from upper center. × 30,000.

FIGURE 10 Portion of cytoplasm of intestinal absorptive cell. Mitochondrial cristae with well defined internal space are shown by arrows. Shadowing from lower left. × 30,000.

FIGURE 11 Intestinal absorptive cell showing nucleus, *N*, and Golgi region, *G*. Sacs of Golgi apparatus are shown in both cross-section and surface view. Shadowing from left center. × 30,000.

FIGURE 12 Intestinal absorptive cell in which the nucleoplasm has been scooped out. The nuclear membrane has been sectioned to reveal its double nature in cross-section with pores (arrows). Pores are also seen as holes in the surface of the inner nuclear membrane (*NM*) and as larger holes in the outer membrane (lower right). Shadowing from lower right. × 30,000.


M1
M2
9
10
N
G
11
NM
12

case of Malhotra and Van Harreveld, that the space was originally open in living tissue and was closed by the substitution or embedding processes.

The second point, which will be discussed very briefly, is the structure of nucleoplasm, particularly as shown in Fig. 13. The distribution of the more coarsely dotted material follows that of heterochromatin in glutaraldehyde-fixed and freeze-substituted material (Bullivant, 1965). As will be discussed, this may be contamination or may be real, but it does have some underlying structural basis. The dots are about 250 A in diameter, and it is tempting to speculate that they may be the cut ends of nucleoprotein fibers. Using the Kleinschmidt spread cell method, DuPraw (1965) has shown that the basic fibers of nucleoprotein have a diameter ranging around 250 A.

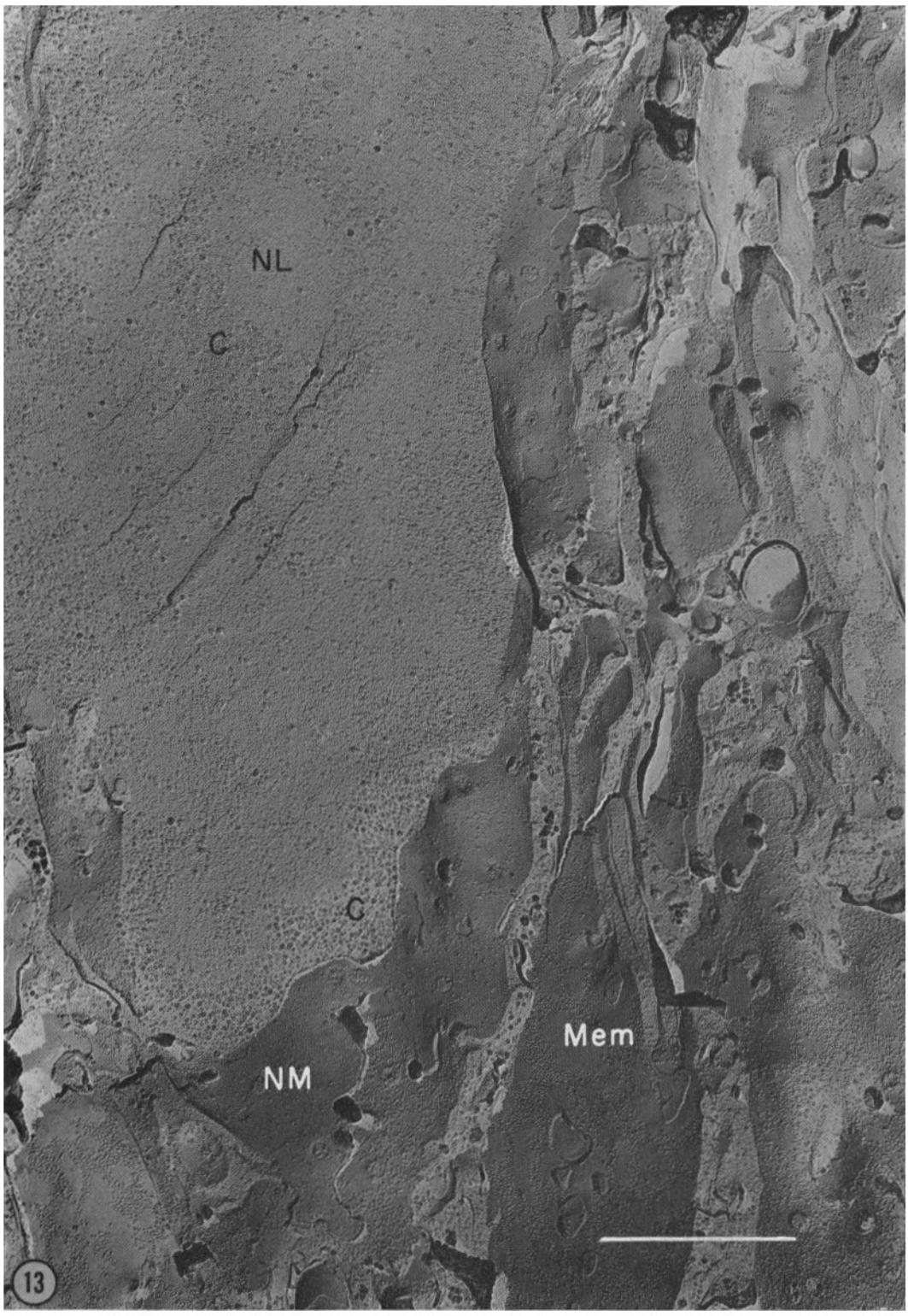
The basis for the fine pock-marking or granulation which appeared on the replicas has not been completely established. This fine granulation is distinct from the coarser granulation of the heterochromatin regions and the mosaic pattern on the membranes. Both these latter may be real, representing nucleoprotein in the first case and ribosomes in the second but, until contamination as a source of this granulation can be discounted, we cannot be certain. It does seem certain that the finer granulation or pock-marking represents contamination. It is more generally distributed. Replicas produced by the Moor method also show a similar fine granulation.

Specimens prepared by the first method, but with a smaller diameter specimen block and lid being used, showed more of the fine granulation, and it was thought that this was due to contamination occurring either during the liquid nitrogen stage or making its way between the block and the lid in the vacuum. Later the larger block and lid were made. This was machined so that the lid was a very close fit, on the theory that the flow of contaminant molecules at low pressure is extremely dependent on the diameter of the channel through which they flow (approximately to the fourth power). The results presented were obtained with this arrangement. The second type of machine, in which the fracture occurs in the vacuum, was built, and the replicas showed much fine granulation. It may be possible that with the first type of experiment the shielding due to the lid was so good that at the moment of exposure the surface of the specimen was as clean as if freshly cleaved in the vacuum. A small amount of contamination then occurred in the short interval before shadowing. The evaporator used was not a special one, but the one routinely used for making carbon films (a Kinney PW 400 with nitrogen trap). An improvement can be expected from a cleaner vacuum system. In the second method, a lot of frost was unavoidably picked up during transfer to the evaporator. This was in contrast to the first method where the only frost was on the outside of the plastic container, and as this was at a much higher temperature than the specimen block the frost thus sublimed away. (The second method could be made to work by having a means of cooling the block while in the evaporator so that one could wait until all the frost had gone before cutting. The advantage of the first method is that the block is so large and has such heat capacity that it does not warm appreciably during the experiment and no external cooling is required). From the way in which fine granulation has been reduced throughout the development of the method, by the application of remedies which would physically be expected to reduce contamination, it does seem that at least some of the granular appearance is due to the deposition of contaminant vapor, largely water. It is anticipated that the use of a cleaner vacuum system, possibly an ion-pumped type, will resolve the problem of contamination.

CONCLUSIONS

The technique described produces good replicas of the fractured surfaces of frozen cells. It is simple to perform and the equipment required

FIGURE 13 Nucleus and cytoplasm of cell thought to be a Paneth cell. Chromatin areas (*C*) appear coarsely granulated and are distributed around the periphery of the nucleus and around the nucleolus (*NL*). Pores can be seen in the nuclear membrane (*NM*). Some membranes (*Mem*) show a granulated mosaic appearance. It is not certain if the granules are ribosomes (see text). Shadowing from upper left. × 30,000.


NL
C
C
Mem
NM
13

consists only of a vacuum evaporator and some modestly priced homemade apparatus. Interesting features shown in glycerinated frozen cells, prepared by the method, are the presence of a space within mitochondrial cristae, larger pores in the outer nuclear membrane than in the inner, and a mosaic of smooth and granulated areas on the surface of some membranes. Further improvement in the quality and fidelity of the electron micrographs may be expected with the reduction of contamination by the use of cleaner vacuum systems and the use of rapidly frozen rather than glycerol-protected frozen tissue. It seems reasonable to expect that resolution at a macromolecular level may be achieved by this technique, with the added confidence that the molecules occupy the position they had in life.

We would like to thank Dr. Ronald S. Weinstein for helpful discussion and suggestions. We are indebted to Mr. Richard Hebert for assistance with the experiments, to Mr. John Fields for machining some of the parts and preparing the illustrations, and to Mrs. Marilyn Schaefer for typing the manuscript.

This work was supported by grants GM 11427-03, CA-07368-02, and 5-K3-MH-3769-05 from the National Institutes of Health.

Received for publication 27 January 1966.

Note Added in Proof: An improvement of the first method has recently been made. A three tier block is now used. The lower holds the cleaved specimen. The middle has two $\frac{3}{32}$ in. diameter holes drilled in it so that both carbon-platinum shadowing and carbon coating can be done without removal of this block. The top block is the lid and is removed as before. The advantages are: (*a*) reduction of contamination as the specimen is only exposed to the atmosphere of the evaporator through the small solid angle represented by the holes, and (*b*) an increase of resolution brought about by the aperturing effect of the holes.

REFERENCES

BERNHARD, W., and NANCY, M-T., 1964, Coupes a congélation ultrafines de tissu inclus dans la gélatine, *J. Micr.*, **3,** 579.

BRANTON, D., and MOOR, H., 1964, Fine structure in freeze-etched *Allium cepa* L. root tips, *J. Ultrastruct. Research*, **11,** 401.

BULLIVANT, S., 1960, The staining of thin sections of mouse pancreas prepared by the Fernández-Morán helium II freeze-substitution method, *J. Biophysic. and Biochem. Cytol.*, **8,** 639.

BULLIVANT, S., 1964, Observations on the structure of mitochondrial membranes, *J. Cell Biol.*, **23,** 16A(abstract).

BULLIVANT, S., 1965, Freeze-substitution and supporting techniques, *Lab. Inv.*, **14,** 1178.

DUPRAW, E. J., 1965, Macromolecular organization of nuclei and chromosomes: a folded fibre model based on whole-mount electron microscopy, *Nature*, **206,** 338.

FERNÁNDEZ-MORÁN, H., 1957, Electron microscopy of nervous tissue, *in* Metabolism of the Nervous System, (D. Richter, editor), London, Pergamon Press, Inc., 1957, 1.

FERNÁNDEZ-MORÁN, H., 1960, Low temperature preparation techniques for electron microscopy of biological specimens based on rapid freezing with liquid helium II, *Ann. New York Acad. Sc.*, **85,** 689.

GERSH, I., 1956, The preparation of frozen-dried tissue for electron microscopy, *J. Biophysic. and Biochem. Cytol.*, **2** suppl., 37.

HAGGIS, G. M., 1961, Electron microscope replicas from the surface of a fracture through frozen cells, *J. Biophysic. and Biochem. Cytol.*, **9,** 841.

HALL, C. E., 1950, A low temperature replica method for electron microscopy, *J. Appl. Physics*, **21,** 61.

HANZON, V., HERMODSSON, L. H., and TOSCHI, G., 1959, Ultrastructural organization of cytoplasmic nucleoprotein in the exocrine pancreas cells, *J. Ultrastruct. Research*, **3,** 216.

MALHOTRA, S. K., and VAN HARREVELD, A., 1965, Some structural features of mitochondria in tissues prepared by freeze substitution, *J. Ultrastruct. Research*, **12,** 473.

MERYMAN, H. T., 1957, Physical limitations of the rapid freezing methods, *Proc. Roy. Soc. London, Series B*, **147,** 452.

MOOR, H., 1964, Die Gefrier-Fixation Lebender Zellen und ihre Anwedung in der Elektronenmikroskopie, *Z. Zellforsch. u. Mikr. Anat.*, **62,** 546.

MOOR, H., MÜHLETHALER, K., WALDNER, M., and FREY-WYSSLING, A., 1961, A new freezing-ultra microtome, *J. Biophysic. and Biochem. Cytol.*, **10,** 1.

MOOR, H., and MÜHLETHALER, K., 1963, Fine structure in frozen-etched yeast cells, *J. Cell Biol.*, **17,** 609.

MOOR, H., RUSKA, C., and RUSKA, H., 1964, Elektronenmikroskopische Darstellung tierischer Zellen mit der Gefrierätztechnik, *Z. Zellforsch. u. Mikr. Anat.*, **62,** 581.

REBHUN, L. I., 1961, Applications of freeze-substitution to electron microscope studies of invertebrate oocytes, *J. Biophysic. and Biochem. Cytol.*, **9,** 785.

REBHUN, L. I., 1965, Freeze substitution: fine struc-

ture as a function of water concentration in cells, *Fed. Proc.*, **24,** suppl. 15, S-217.

SCHULTZ, R. L., and KARLSSON, U., 1965, Fixation of the central nervous system for electron microscopy by aldehyde perfusion. 2. Effect of osmolarity, pH of perfusate and fixative concentration, *J. Ultrastruct. Research*, **12,** 187.

SJÖSTRAND, F. S., and BAKER, R. F., 1958, Fixation by freeze-drying for electron microscopy of tissue cells, *J. Ultrastruct. Research*, **1,** 239.

SJÖSTRAND, F. S., and ELFVIN, L-G., 1964, The granular structure of mitochondrial membranes and of cytomembranes as demonstrated in frozen-dried tissue, *J. Ultrastruct. Research*, **10,** 263.

STEERE, R. L., 1957, Electron microscopy of structural detail in frozen biological specimens, *J. Biophysic. and Biochem. Cytol.*, **3,** 45.

TRIER, J. S., and RUBIN, C. E., 1965, Electron microscopy of the small intestine: a review, *Gastroenterology*, **49,** 574.

VAN HARREVELD, A., and CROWELL, J., 1964, Electron microscopy after rapid freezing on a metal surface and substitution fixation, *Anat. Rec.*, **149,** 381.

VAN HARREVELD, A., CROWELL, J., and MALHOTRA, S. K., 1965, A study of extracellular space in central nervous tissue by freeze-substitution, *J. Cell Biol.*, **25,** 117.