ELECTRON MICROSCOPY OF STRUCTURAL DETAIL IN FROZEN BIOLOGICAL SPECIMENS*

By RUSSELL L. STEERE, PH.D.

(From the Virus Laboratory, University of California, Berkeley)

PLATES 11 AND 12

(Received for publication, July 23, 1956)

Cytologists are continually plagued by some uncertainty as to which of the structures they see in prepared specimens represent faithfully the structures of the living cells and which are artifacts resulting from the preparative procedures. If the specimen is to be studied at low magnification direct comparisons can sometimes be made between prepared and living materials, and artifacts which are of importance at the magnification employed can be noted. Furthermore, it is generally possible to observe specimens during preparation and determine which parts of a procedure produce artifacts. The procedure can then be modified to reduce or eliminate the undesired changes.

At magnifications greater than those that can be employed with living material it is virtually impossible to know whether the structures observed in a specimen are real or are uncontrolled artifacts. This is particularly true when the final observations are to be made with the electron microscope, for not only is the final magnification much greater, but also the observed contrast of an object is dependent upon different properties. One cannot be certain, therefore, that preparative procedures which have been accepted as satisfactory for light microscopy do not produce unrecognized artifacts of critical importance in electron microscopy. Artifacts may arise as a result of aggregation or dispersion of various components during fixation, or during the subsequent washing or embedding processes. For example, the crystalline inclusions of tobacco mosaic virus have been observed to disintegrate when cells containing them are fixed in acid solutions (3, 8, 12, 22). These crystals can be fixed under certain conditions (1, 2, 6, 21) however, though it is still unknown whether the internal structure of the crystals or only the external appearance is preserved by such chemical fixation. The micrographs presented by Brandes (6) suggest that internal structure is not well maintained within these fragile crystals when fixed with buffered OsO_4, and to the author's knowledge, no micrographs have been published which show well preserved orientation of the particles comprising such a crystal.

* This investigation was supported by grants from the National Foundation for Infantile Paralysis and the National Cancer Institute of the National Institutes of Health, United States Public Health Service.

Staining with osmium or some other heavy metal in order to increase the contrast of biological materials in the electron microscope may result in the production of unknown and uncontrolled artifacts. This staining almost certainly involves the chemical or physical attachment of the metal atoms or molecules to the surfaces of some of the particles, membranes, or other structures with which they make contact. A membrane within a cell might have a layer of the stain on each side and thus appear in thin sections as a double membrane; the actual structure could have little or no contrast with respect to the background material while the two layers of stain would appear as dark lines. Likewise surface staining could make sectioned solid objects appear to be empty sacks.

Other possible sources of artifact production during the commonly employed cytological procedures, as adapted to electron microscopy, include swelling or contraction resulting from solidification of the embedding medium, compression resulting from the removal of thin sections from the specimen block (18, 25) and the possible displacement of materials of the specimen by the embedding medium. Also, when the embedding material is removed, as it sometimes is, there is additional opportunity for displacement of the cellular contents.

In view of the likelihood that unrecognized artifacts are produced during currently employed cytological procedures it is desirable that alternate methods be developed for the preparation of biological specimens for electron microscopy. A procedure employing fundamentally different methods for the fixation of specimens and for the introduction of contrast would be of particular value.

Physical fixation by freezing might be profitably employed for if artifacts are produced by this type of fixation they should at least differ from those resulting from chemical fixation. This would be particularly so if some procedure such as freeze-drying were employed to remove the water without subsequent chemical fixation. Contrast could be enhanced by shadowing the exposed surface with a heavy metal (26–28).

It was assumed that valuable information about cytological structures might be obtained in electron microscopy by the application of a procedure incorporating surface etching by vacuum sublimation followed by low temperature shadowing and replication of the etched surface. Such a procedure would be a modification and extension of methods described by Hall (9) and Meryman and Kafig (17) and would incorporate several steps:—

1. The cells or cellular components should be frozen in a block of ice.
2. The ice should be planed away so that the material of which a micrograph is desired lies immediately beneath the planed surface of the block.
3. The cells or cellular components should next be exposed to a suitable extent by vacuum sublimation of the ice.
4. The exposed material should then be shadowed with some heavy metal to provide contrast.

5. A continuous replicating film of some stable material should be deposited on top of the shadow film.

6. The biological material should be removed from the preshadowed replica.

7. And finally, the replica should be mounted on a specimen screen in such a manner that the desired portion can be located with certainty.

The artifacts produced in specimens prepared in this manner would most probably differ from those encountered in currently employed cytological procedures. Also, there should be distinct advantages in the use of this type of procedure for the preparation of specimens which are too fragile to withstand chemical fixation and imbedding. The purpose of the present paper is to describe such a procedure that has been employed for the observation of oriented particles both *in vitro* and *in vivo*.

Instrumentation

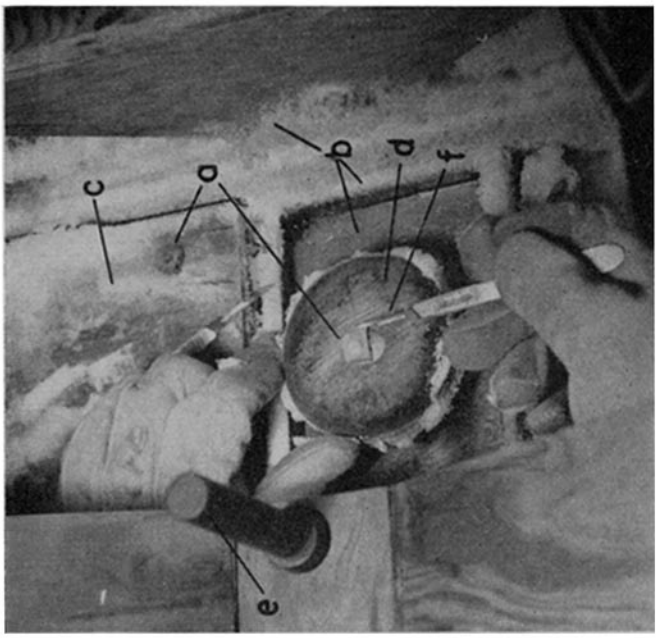
For the preparation of cytological specimens by the procedure outlined above certain specialized equipment and materials have been found desirable. They are listed and described here for convenience.

Specimen Block.—The specimen block is a brass block (Text-figs. 2 *a*, 3 *a*, and 4 *a*) $\frac{5}{32}$ inches thick and $\frac{1}{2}$ inch square with two adjacent corners rounded. The block has two holes drilled into the edge between the square corners for manipulation with curved tip forceps. The specimen block is cleaned and polished immediately before use by rubbing it with 2/0 emery paper, followed with a clean, lintless cloth.

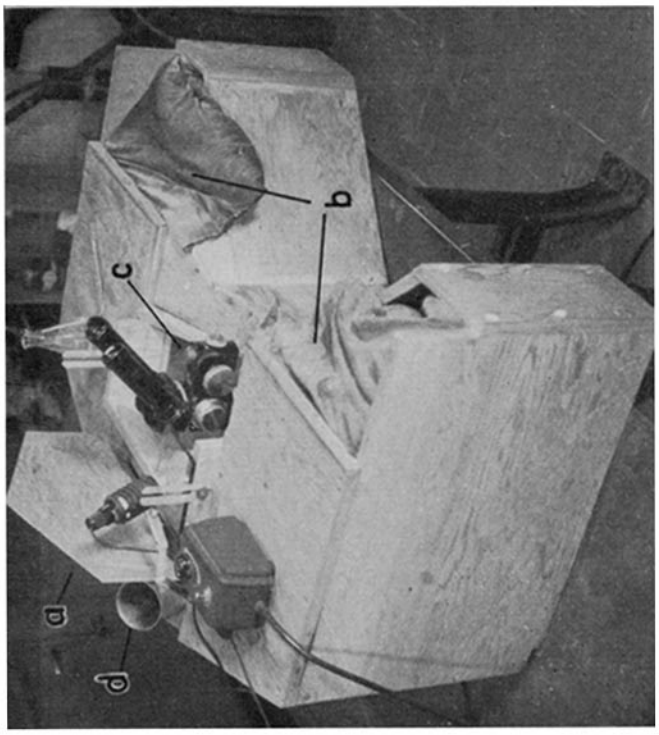
Cold Operating Box.—The insulated box (Text-figs. 1 and 2) was designed for low temperature work. There is a built-in metal container (Text-fig. 2 *b*) serving as a floor and back wall for the work area. Approximately 1 hour before the chamber is to be used this container is filled from the top back (Text-fig. 1 *a*) with a mixture of solid CO_2 and 95 per cent ethanol. A raised wooden platform, inside the box, (Text-fig. 2 *c*) serves as an arm rest and as a stand for the specimen while it is being frozen. A solid brass cylinder, the "operating block," (Text-fig. 2 *d*) rests in ethanol in a tray built into the metal container on the floor of the work area. Arm ports (Text-fig. 1 *b*) are incorporated into the box in such a position that the openings are above specimen level. This reduces the loss of cold air from around the specimen during manipulation. A Zeiss opton dissecting microscope with a lens-to-specimen working distance of 4 inches is mounted on a stand (Text-figs. 1 *c* and 2 *e*) which is provided with rack and pinion inside the box. A circular opening in the plexiglass cover permits unobstructed vision of the specimen though the microscope is outside the cold chamber. A metal tube with one end closed (Text-fig. 1 *d*) is inserted into the dry ice–alcohol mixture at the back of the cold box to be used as a container for the specimen unit (Text-fig. 3(*B*)) while it is being precooled.

Vacuum Unit.—The vacuum unit (Text-fig. 3(*A*)) is the fundamental piece of equipment for the low temperature replica procedure. It consists of a 10 inch bell jar with a 3 inch ground glass standard taper joint fused into the top (Text-fig. 3 *b*). The bell jar rests on an aluminum bed plate (Text-fig. 3 *c*) with insulated posts (Text-fig. 3 *d*) connected by wires to the jack box of a vacuum shadowing unit.[1] An ionization gauge and an air inlet are also connected through the bed plate (Text-figs. 3 *e* and 3 *f*, respectively). The diffusion pump system of the

[1] A modified shadowing unit manufactured by the Optical Film Engineering Company, Philadelphia, Pennsylvania, was used. The modification consisted of a $1\frac{1}{2}$ inch metal extension tube connected to the left side of the high vacuum system through an extra valve.

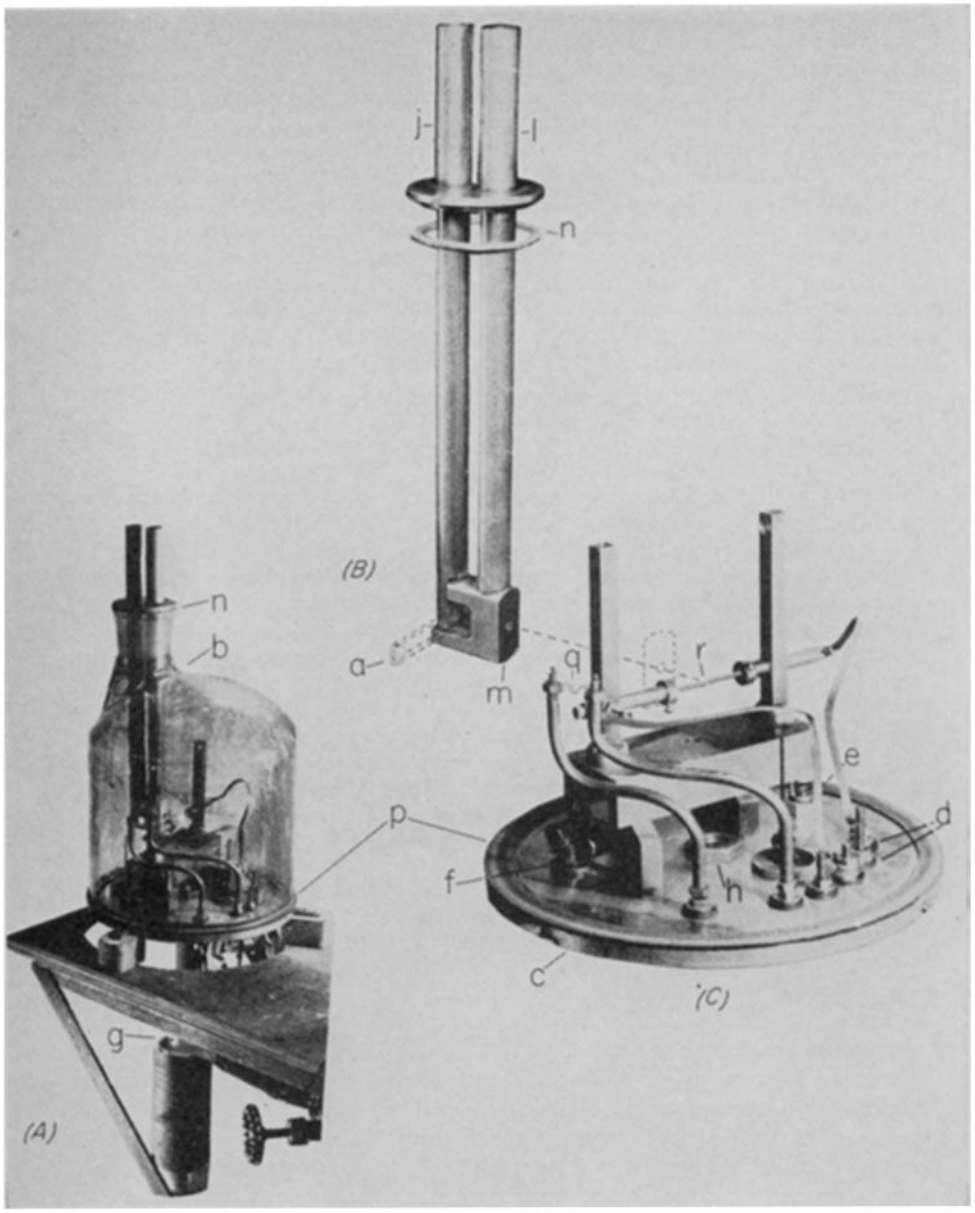


TEXT-FIG. 2



TEXT-FIG. 1

TEXT-FIG. 1. Cold operating box. Outside view with microscope mounted in position.
TEXT-FIG. 2. Cold operating box. Looking inside with plexiglass cover removed.



TEXT-FIG. 3. Vacuum unit. Assembled unit left. Exploded view at right.

vacuum shadowing unit is connected through a glass cold trap (Text-fig. 3 *g*) to a ½ inch (inside diameter) tube mounted in the center of the bed plate (Text-fig. 3 *h*). The specimen unit (Text-fig. 3(*B*)) is inserted into the standard taper at the top of the bell jar. It consists of two ⅞ inch O. D. brass tubes which have been soldered in place in a brass plate with 4 inches of each tube extending above the plate and approximately 12 inches below. One of these tubes, the specimen tube (Text-fig. 3 *j*), is cut away at the lower end for a distance of 1½ inches. This cutaway end is closed with a brass plate. The other tube serving the purpose of a cold trap (Text-fig. 3 *l*) has a brass block attached to its lower end. This block has been cut so that it forms a box around three sides of the specimen surface. A tapered hole in the face of this box opposite the specimen (Text-fig. 3 *m*) permits deposition of a carbon film on the specimen. Rubber gaskets are employed to provide airtight seals between the specimen unit and the top surface of the standard taper (Text-fig. 3 *n*) and between the bell jar and the bed plate (Text-fig. 3 *p*).

A coiled loop filament is attached to one set of posts (Text-fig. 3 *q*) and is used for the evaporation of a chromium shadow film. To a second set of posts is attached a carbon evaporating unit[2] (Text-fig. 3 *r*) for the deposition of the carbon films (4, 5).

Ammonium Acetate Solution.—An ammonium acetate solution containing 150 gm. of salt per liter was employed to fasten the specimen block to the operating block and later to the specimen tube. This solution freezes at approximately −60°C. but does not fracture and permit the specimen block to become loosened when chilled to −190°C.

Scalpel and Blade.—A scalpel blade attached to a metal handle (Text-fig. 2 *f*) is used for planing the ice. The blade is carefully sharpened and polished with 4/0 emery paper by pushing and pulling the blade across the paper so that the cutting edge is always parallel to the direction of the polishing strokes. The few rough spots which remain on the cutting edge are sufficiently distant from each other that smooth cuts (scratch-free as determined by microscopic examination at 80 ×) can be made over most of the surface of a specimen.

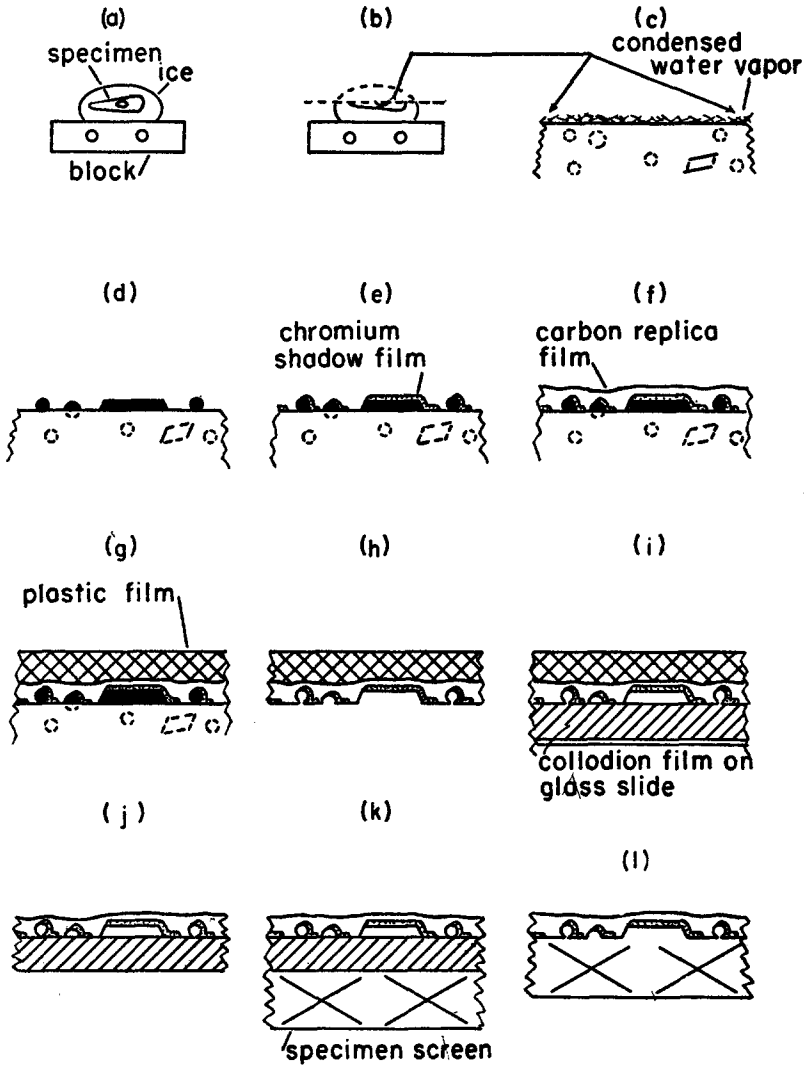
Methacrylate.—A polymerized mixture of 75 per cent butyl- and 25 per cent methyl-methacrylate dissolved in 1,1-dichloroethane is prepared for use in stabilizing the carbon replica film during early stages of manipulation. This preparation is a viscous liquid at −40°C., and the solvent is volatile at sub-freezing temperatures. These properties permit the use of the mixture at −15°C. at which temperature evaporation of the solvent is complete at atmospheric pressure within an hour of application.

Experimental Procedure

Freezing of Specimen.—A small drop of water (2 mm. in diameter) containing the material to be studied is placed in the center of the upper surface of the specimen block (Text-fig. 4 *a*). If it is desirable to orient the material such manipulation is accomplished at this stage. The block, resting on a glass slide, is then placed on the wooden platform in the cold chamber (Text-fig. 2 *c*) and allowed to cool by radiation and freeze. Microscopic observation of the specimen during this period suggests that the water becomes supercooled; the freezing generally occurs after a slight disturbance and appears to take place instantaneously. The rapid solidification results in the formation of a block of clear ice in contrast to the opaque ice that forms when the specimen is frozen by conduction, as occurs when the warm specimen block is mounted directly on the prechilled operating block. After the drop of water freezes the specimen block is transferred to the top of the operating block (Text-fig. 2 *d*) and made fast with a drop of the ammonium acetate solution. The microscope is then mounted over the specimen and brought into focus.

[2] Supplied by the Optical Film Engineering Co.

Creating a Planed Surface.—Thin layers of ice are removed with the sharpened scalpel blade and are discarded. When the material of which a replica is desired remains immediately below the surface (Text-fig. 4 *b*) a final layer of extreme thinness



TEXT-FIG. 4. Diagrammatic presentation of low temperature replica procedure outlined in text.

is very carefully removed and the operating block rapidly withdrawn from the cold box. Curved tip forceps are inserted into the two holes provided in the specimen block and a downward force is applied to pry it loose from the operating block. It is immediately frozen to the inside face of the precooled specimen tube (Text-fig. 3 *j*)

with a drop of the ammonium acetate solution. The specimen unit is then lowered into the bell jar and liquid nitrogen is poured into the specimen tube, into the cold trap tube, and into a dewar flask into which the cold trap beneath the bell jar has been inserted. The valve to the vacuum pumps is opened and a high vacuum obtained while the specimen and both traps are maintained at liquid nitrogen temperature.

Etching by Vacuum Sublimation.—When a vacuum of about 10^{-3} mm. of Hg is obtained the liquid nitrogen is removed from the specimen tube and this slowly becomes warmer, reaching a temperature of −95° C. in about 15 minutes. At a temperature of approximately −92° C. the water vapor (Text-fig. 4 *c*) that had condensed during transfer as a white film on the surface of the specimen disappears. The specimen is then allowed to etch for approximately 30 seconds, a time generally sufficient for etching to proceed to a depth of 100 to 300 A (Text-fig. 4 *d*). Liquid nitrogen is then poured immediately into the specimen tube to prevent further etching. The two cold traps are kept full of liquid N_2 throughout the run to condense all vapor liberated from the specimen and the specimen tube and to minimize back-condensation when the specimen surface is returned to liquid nitrogen temperature.

Shadowing and Replication.—The etched specimen is shadowed with chromium at about a 2-to-1 angle (Text-fig. 4 *e*). The carbon source, approximately 2 inches directly in front of the ice block, is then heated at 30 amps until approximately 0.1 mg. of carbon evaporates from the pointed electrode. A portion of this carbon is deposited as a continuous replica film (4, 5) on top of the shadow film and serves to support it during later manipulations (Text-fig. 4 *f*).

The valve to the vacuum pumps is closed and nitrogen is removed from both the specimen tube and the cold trap tube. Then the air inlet is opened. The specimen unit is withdrawn from the bell jar and the specimen block pried from it and placed in the freezing compartment of a refrigerator maintained at −15° C. The layer of frost which condenses on the upper surface of the carbon film during transfer sublimes within 2 hours, then a thick solution of the dissolved methacrylate (at room temperature) is dropped on the upper surface of the replica film and allowed to flow over the edge of the block. It is allowed at least an hour to dry and solidify (Text-fig. 4 *g*) before removal from the refrigerator.

Removal of Biological Residue.—After the plastic film has dried the specimen block is removed from the cold chamber and that portion of the plastic containing the desired specimen is cut out and transferred, specimen side down, to the surface of a saturated solution of sodium hydroxide in a small container. This is placed in a larger container of boiling water for 5 minutes, removed, and allowed to cool to room temperature. The specimen film is then washed in several changes of distilled water to remove denatured protein and salts (Text-fig. 4 *h*). The clean replica film is placed chromium (shadow film) side down in a small drop of water on a collodion coated microscope slide and dried under the heat of a 100 watt lamp held 3 inches above the specimen. The heat of the lamp during the drying period helps to soften the thick plastic film so that the chromium shadow film makes good contact with the collodion (Text-fig. 4 *i*). When all the water has evaporated the slide is placed in benzene to dissolve the thick plastic film. The collodion film is then cut along the edges of the slide, moistened with the breath, and the slide, held almost horizontal, is slowly lowered into a dish of clean distilled water. The thin collodion film floats from the

slide and remains on the surface of the water (Text-fig. 4 *j*). A plastic ring (13 mm. outside diameter, 9 mm. inside diameter, and 1½ mm. thick) is coated with the adhesive from cellulose tape (dissolved in benzene) and is placed on the collodion film so that it encircles the specimen. A 3 inch piece of ½ inch wide cellulose tape is held sticky side down with an end between thumb and forefinger of each hand. It is carefully lowered until it makes contact with the collodion film everywhere except where the ring keeps the film away. Then it is withdrawn one end first and the collodion film is allowed to dry before the final transfer is made.

For the final transfer a notched specimen screen is used. The notch serves in the electron microscope as a reference point. The specimen is centered over the screen and the desired portion is positioned near the notch and mounted in the manner described by Williams and Kallman (25). The screen supporting the replica film on collodion is transferred to a piece of filter paper and exposed briefly to acetone vapors to make the collodion adhere tightly to the specimen and screen (Text-fig. 4 *k*) or else flooded with acetone to remove the collodion film entirely (Text-fig. 4 *l*).

RESULTS

Specimens which have been successfully prepared by the frozen replica method to date include crystals of purified tobacco ringspot virus in 0.01 M phosphate buffer at pH 7, crystals of purified turnip yellow mosaic virus in ⅓ saturated $(NH_4)_2SO_4$, crystals of squash mosaic virus in 0.01 M phosphate buffer at pH 7, and tobacco mosaic virus crystals within the living hair cells of infected Turkish tobacco plants. These crystals were employed during the development of the procedure because they are composed of particles having known dimensions and arranged in some regular pattern. Unstable crystals were selected because it was thought that a procedure which could reveal the arrangement of individual particles inside such crystals would be suitable for the study of many unstable cytological structures.

Crystals of purified virus were used initially because it was assumed, and correctly so, that this material would be easier to remove from the replica film than cellular debris. When satisfactory micrographs of these were obtained the procedure was employed to study the intracellular crystals of tobacco mosaic virus. The cell walls were difficult to remove but it was finally discovered that they could be torn from the film with a pair of forceps before the replica was placed in the NaOH solution. The replica of the cell walls was generally removed with them, but the remainder of the replica was essentially undisturbed by this action.

The crystals of purified tobacco ringspot virus (Fig. 1) and squash mosaic virus (Fig. 2) were sufficiently large that they could be observed during the planing procedure. Consequently it is known that these micrographs show planes through crystals rather than outer faces exposed by etching away ice from around the crystals. It should be noted that the particles are beautifully oriented with hexagonal packing. In a few places the predominant layer has

been gouged out and particles of the next lower layer are visible (Fig. 1 *A*). In other places scattered particles are left on the surface (Fig. 1 *B*). The planing of the squash mosaic virus crystal was less well accomplished but produced interesting results: Several successive layers of the crystal were exposed, each of which exhibits well defined hexagonal packing (Fig. 2).

The crystals of turnip yellow mosaic virus were much smaller than those of tobacco ringspot virus and squash mosaic virus and could not be seen as individual crystals during preparation. A suspension was used, therefore, which contained so many crystals that it would have been almost impossible to find an area of the final replica of any considerable size that would not include part of a crystal. It is uncertain, however, whether a particular micrograph shows an external surface of a crystal or a plane through it. The micrograph (Fig. 3) shows a suggestion of hexagonal packing, but orientation in all these crystals was far less pronounced than in crystals of tobacco ringspot or squash mosaic. It is interesting to note the uniform knobs on the turnip yellow mosaic particles (arrow Fig. 3). These were not present in micrographs of crystals of the other viruses nor in micrographs of turnip yellow mosaic virus frozen dried from distilled water (7). It is not known whether they resulted from the presence of $(NH_4)_2SO_4$ in the crystal suspension or represent actual surface structure of the particles which has been preserved by the new method of preparation but their uniformity of size and occurrence would suggest that they reflect fine surface structure.

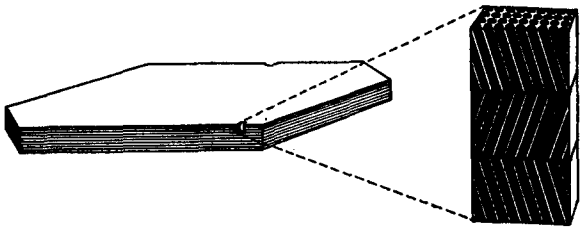
Typical hexagonal crystals of tobacco mosaic virus were located within hair cells of infected Turkish tobacco plants. A hair containing a suitable crystal was cut from a strip of epidermis and manipulated in a drop of water on the brass specimen block so that the crystal was oriented with its axis parallel to the face of the block (hexagonal surface of crystal perpendicular to face of specimen block). The specimen was frozen and the ice planed to a depth that would insure transection of the crystal. Figure 5 shows a micrograph of the replica of a portion of such a crystal. Note the herringbone pattern representing the arrangement of particles in adjacent layers. The length of the individual virus rods in these crystals determined with Dow polystyrene latex, lot 580 G, as an internal reference, is very close to 300 mμ. A replica of a tobacco mosaic virus crystal in which the particles were cut transversely (Fig. 4) shows that the particles within a layer are arranged in a square net.

DISCUSSION

Previously published electron micrographs (10, 11, 13–16, 20, 29, 30) have shown the orientation of protein and virus particles on the surface of crystals. Others (19) show oriented particles of animal viruses within the nuclei of infected cells. Now we are able to see, in oriented array, individual particles with diameters of less than 30 mμ *within* virus crystals. Furthermore, we can

see details of the internal structure of crystals of tobacco mosaic virus within cells which have not been exposed to chemical fixation.

The arrangement of particles (assumed to be virus particles) within the crystalline inclusion bodies of epidermal cells of plants infected with tobacco mosaic virus was considered in some detail by Wilkins and coworkers (24). They concluded that these crystals are built up of thin layers lying parallel to the hexagonal face of the crystal and that within each layer the individual virus rods are aligned parallel to each other, but with an orientation not quite parallel to that of the virus rods in adjacent layers. Steere and Williams (23) have since demonstrated that the crystals are indeed composed of virus particles. Now we are able to see the orientation of the particles within adjacent layers of such a crystal and find a herringbone pattern, similar to that which



TEXT-FIG. 5. Diagrammatic view of crystal of tobacco mosaic virus reconstructed from data obtained.

Wilkins and coworkers (24) had concluded. Though micrographs have been obtained of several crystals which show non-parallel orientation of particles of adjacent layers, we cannot be positive that all tobacco mosaic virus crystals are oriented in this fashion. In fact one micrograph has shown parallel orientation of particles of adjacent layers. This specimen was overetched, however, and it is possible that there was some rearrangement of the particles during preparation. It is probable that both types of crystal are formed in nature.

The orientation of rods in a square net as seen end-on (Fig. 4) would permit the presence of considerable water around each particle. This is in agreement with previous observations (23, 24) that only 30 to 40 per cent of the volume of the tobacco mosaic virus crystal can be accounted for as dry virus.

A diagrammatic representation of a crystal of tobacco mosaic virus is presented in Text-fig. 5. Here the herringbone arrangement is shown and the relationship of the layers to the intact crystal is demonstrated.

Not only do the micrographs of these crystal replicas show the internal organization of the various crystals, but they permit one to measure the particle spacing in hydrated crystals which were prevented from shrinking during preparation by the solid block of ice in which they were embedded. Unfortunately, there must be some expansion during freezing which would make these

values a bit too large. It is of interest to note that the spacing in both the tobacco ringspot virus and the turnip yellow mosaic virus crystals allows for a maximum diameter of 25 mμ for each particle. For squash mosaic virus the maximum possible diameter is 21 mμ.

It is clear that this low temperature replica procedure is capable of preserving considerable detail in virus crystals formed either *in vitro* or *in vivo*. What then are the advantages and disadvantages of the procedure?

The advantages are several:

1. Fixation is by rapid freezing. This differs from ordinary chemical procedures and will certainly be better for some specimens.
2. The specimen is kept fixed until the etched surface is replicated. Particles which are only partially exposed by the etching procedure are held firmly in place in the ice.
3. Contrast is enhanced by shadowing a surface exposed by differential sublimation. Artifacts due to differential fixation, differential staining, or surface staining are avoided.
4. The planing removes layers of material from above the specimen surface; but the specimen itself is not removed from the block by a sectioning procedure. Therefore no artifacts are formed as a result of compression of the specimen during removal from the block.
5. Structures that are too fragile to be fixed, embedded, and sectioned may be prepared by this procedure.
6. Cellular components that are not fixed by commonly used reagents or do not stain well with heavy metals might be revealed by this procedure.

Disadvantages of the low temperature procedure fall into two groups: (1) those that are inherent in the physical principles involved and (2) technical difficulties that can probably be solved by additional work.

The inherent difficulties include:

1. Small particles that do not remain firmly held in the ice are free to be displaced by ambient physical forces. They may recede with the surface of the ice or become attached to the surface of other particles; or they may be removed from the specimen surface entirely in the vaporization stream.
2. The chemical nature of the objects seen in the replica cannot be determined.
3. Some displacement of materials may occur during the freezing of the specimen regardless of the freezing procedure employed.
4. Structures surrounded by too much salt or other non-volatile substances may not be exposed sufficiently well by vacuum sublimation to be revealed in preshadowed replicas.

Technical difficulties are:

1. The procedure is rather time-consuming and cumbersome, requiring two days for the preparation and microscopy of not more than six specimens.
2. Serial sections are not obtainable by current methods.

3. Etching is not uniform over the surface of a single specimen.

4. The length of time required to etch different kinds of specimens to the same depth is not constant, and hence it is difficult to obtain uniform results without a preliminary trial and error process.

5. The planing procedure is apt to disrupt the specimen surface in several ways: (*a*) If too much pressure is applied to the knife, or if the knife or specimen is not kept sufficiently cold, the surface of the specimen might be caused to melt locally. (*b*) Fragments of ice and frozen specimen may be left on the planed surface. (*c*) The cutting edge of the blade with which the specimen is planed must be free of rough regions if a smooth, unscratched surface is to be obtained.

6. The level to which the specimen must be cut to obtain a picture of the desired material is very critical and often difficult to determine during the planing procedure.

Regardless of the difficulties of the low temperature replica procedure, the results which have been obtained with fragile but highly oriented virus crystals are encouraging and suggest that it will be a useful procedure for the study of many cytological problems. It should be stressed, however, that this method is not proposed as a replacement for other cytological techniques, but merely as an additional or complementary method for studying cytological detail.

When greater precision has been developed in the use of this procedure it is expected that the various steps of chemical fixation and embedding can be examined in an effort to find out more precisely what effect they have on biological materials.

SUMMARY

A procedure is described whereby preshadowed replicas can be obtained from frozen biological specimens which have been cut and then etched by sublimation of the ice from their surfaces. Electron micrographs showing details of the internal structure of plant virus crystals are presented to demonstrate the values of the procedure. Crystals of purified tobacco ringspot virus and squash mosaic virus and some portions of turnip yellow mosaic virus crystals have been shown to exhibit hexagonal packing. Sections through *in situ* crystals of tobacco mosaic virus show the rods to be parallel within each layer and arranged in a square net as viewed end on. Individual rods in each layer of the latter measure 300 mμ in length and are somewhat tilted with respect to the rods of adjacent layers. This results in the formation of a herringbone appearance when a crystal is cut perpendicular to its hexagonal face.

It is suggested that the procedure outlined here might well serve to supplement other procedures for the preparation of many cytological specimens for electron microscopy.

It is with pleasure and gratitude that I acknowledge my indebtedness to Professor Robley C. Williams for continuous encouragement and cooperation during the development of this procedure. I am grateful also to Mr. K. A. Miller for his excellent technical assistance in the preparation of the photographs for this paper.

BIBLIOGRAPHY

1. Bald, J. G., The development of amoeboid inclusion bodies of tobacco mosaic virus, *Australian J. Sc. Research, Series B*, 1948, **1,** 458.
2. Bald, J. G., Additional methods for fixing and staining viruses in infected plant tissues, *Am. J. Bot.*, 1949, **36,** 335.
3. Beale, Helen P., Relation of Stanley's crystalline tobacco virus protein to intracellular crystalline deposits, *Contrib. Boyce Thompson Inst.*, 1937, **8,** 413.
4. Bradley, D. E., Evaporated carbon films for use in electron microscopy, *Brit. J. Appl. Physics*, 1954, **5,** 65.
5. Bradley, D. E., An evaporated carbon replica technique for use with the electron microscope and its application to the study of photographic grains, *Brit. J. Appl. Physics*, 1954, **5,** 96.
6. Brandes, J., Über das Aussehen und die Verteilung das Tabakmosaikvirus im Blattgewebe. Elecktromikroskopische untersuchungen an ultradünnen Mikrotomschnitten, *Phytopath. Z.*, 1956, **26,** 93.
7. Cosentino, V., Paigen, K., and Steere, R. L., Electron microscopy of turnip yellow mosaic virus and the associated abnormal protein, *Virology*, 1956, **2,** 139.
8. Goldstein, Bessie, A cytological study of the leaves and growing points of healthy and mosaic diseased tobacco plants, *Bull. Torrey Bot. Club*, 1926, **53,** 499.
9. Hall, C. E., Low temperature replica method for electron microscopy, *J. Appl. Physics*, 1950, **21,** 61.
10. Hall, C. E., Electron microscopy of crystalline edestin, *J. Biol. Chem.*, 1950, **185,** 45.
11. Hall, C. E., Introduction to Electron Microscopy, New York, McGraw-Hill, Inc., 1953, 348 and 382.
12. Iwanowski, D., Über die Mosaikkrankheit der Tabakspflanze, *Z. Pflanzenkrankh.*, 1903, **13,** 1.
13. Labaw, Louis W., and Wyckoff, R. W. G., Molecular arrangement in crystals of the southern bean mosaic virus protein, *Nature*, 1955, **176,** 455.
14. Labaw, L. W., and Wyckoff, R. W. G., Crystal structure of turnip yellows virus protein, *Science*, 1956, **123,** 849.
15. Markham, R., Smith, K. M., and Wyckoff, R. W. G., Molecular arrangement in tobacco necrosis virus crystals, *Nature*, 1948, **161,** 760.
16. Mattern, C. F., and duBuy, H. G., Purification and crystallization of coxsackie virus, *Science*, 1956, **123,** 1037.
17. Meryman, H. T., and Kafig, E., The study of frozen specimens. Ice crystals and ice crystal growth by electron microscopy, *Naval Med. Research Inst., Research Rep., Project NM 000 018.01.09*, 1955, **13,** 529.
18. Morgan, C., Bergold, G. H., and Rose, H. M., Use of serial sections to delineate the structure of *Porthetria dispar* virus in the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 23.
19. Morgan, C., Howe, C., Rose, H. M., and Moore, D. H., Structure and development of viruses observed in the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 351.

20. Price, W. C., and Wyckoff, R. W. G., Electron micrographs of molecules on the face of a crystal, *Nature*, 1946, **157**, 764.
21. Rubio Huertos, M., Rapid extraction of intact crystalline inclusions from the cells of plants infected with tobacco mosaic virus, *Nature*, 1954, **174**, 313.
22. Sheffield, Francis M. L., Micrurgical studies on virus-infected plants, *Proc. Roy. Soc. London, Series B.*, 1939, **126**, 529.
23. Steere, Russell L., and Williams, R. C., Identification of crystalline inclusion bodies extracted intact from plant cells infected with tobacco mosaic virus, *Am. J. Bot.*, 1953, **40**, 81.
24. Wilkins, M. H. F., Stokes, A. R., Seeds, W. E., and Oster, G., Tobacco mosaic virus crystals and three dimensional microscopic vision, *Nature*, 1950, **166**, 127.
25. Williams, R. C., and Kallman, Frances, Interpretations of electron micrographs of single and serial sections, *J. Biophysic. and Biochem. Cytol.*, 1955, **1**, 301.
26. Williams, R. C., and Wyckoff, R. W. G., The thickness of electron microscopic objects, *J. Appl. Physics*, 1944, **15**, 712.
27. Williams, R. C., and Wyckoff, R. W. G., Electron shadow micrography of virus particles, *Proc. Soc. Exp. Biol. and Med.*, 1945, **58**, 265.
28. Williams, R. C., and Wyckoff, R. W. G., Electron shadow micrography of the tobacco mosaic virus protein, *Science*, 1945, **101**, 594.
29. Wyckoff, R. W. G., The electron microscopy of macromolecular crystals, *Acta Cryst.*, 1948, **1**, 292.
30. Wyckoff, R. W. G., Electron Microscopy: Technique and Applications, New York, Interscience Publishers, Inc., 1949, Chapter 10, page 221.

EXPLANATION OF PLATES

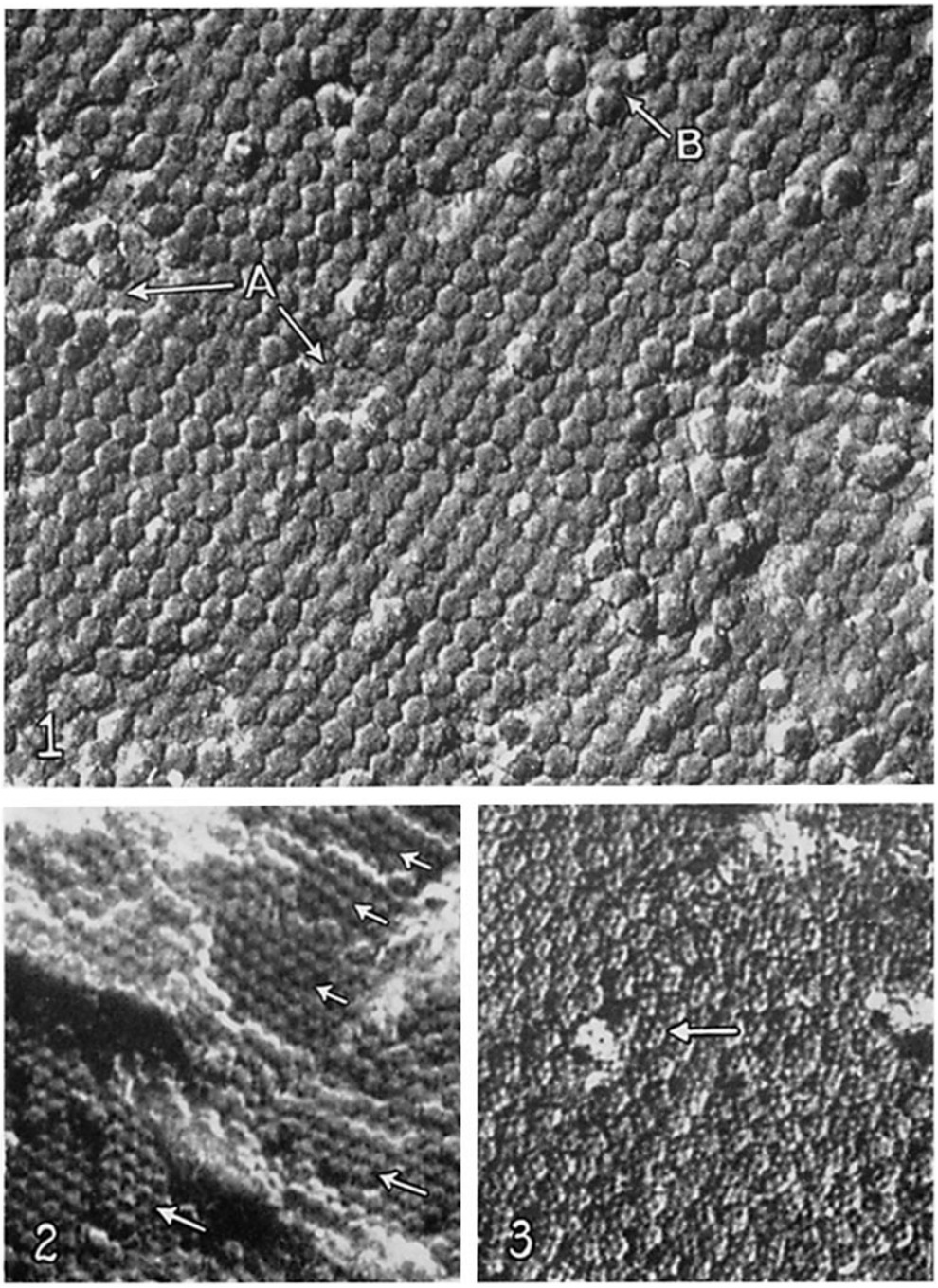
PLATE 11

Electron micrographs of specimens prepared by the low temperature replica procedure.

FIG. 1. Plane through a crystal of purified tobacco ringspot virus in 0.01 M phosphate buffer. Note the hexagonal outline of individual particles and the hexagonal packing within the crystal. Note also the gouged out depressions in specimen (*A*) and the particles deposited on the surface (*B*). × 165,000.

FIG. 2. Section through part of a crystal of purified squash mosaic virus in 0.01 M phosphate buffer. Note the several levels of the crystal exposed by rough planing of the surface (arrows). Each level exhibits hexagonal packing but the individual particles are not distinctly hexagonal as are those of tobacco ringspot virus. × 165,000.

FIG. 3. Replica of a portion of a crystal of purified turnip yellow mosaic virus in 30 per cent saturated $(NH_4)_2SO_4$. Note the uniform knobs (arrow) on each particle. Due to the small size of the crystals employed for this picture it is not known whether this micrograph represents a plane through part of a crystal or an outer surface of a crystal. × 165,000.

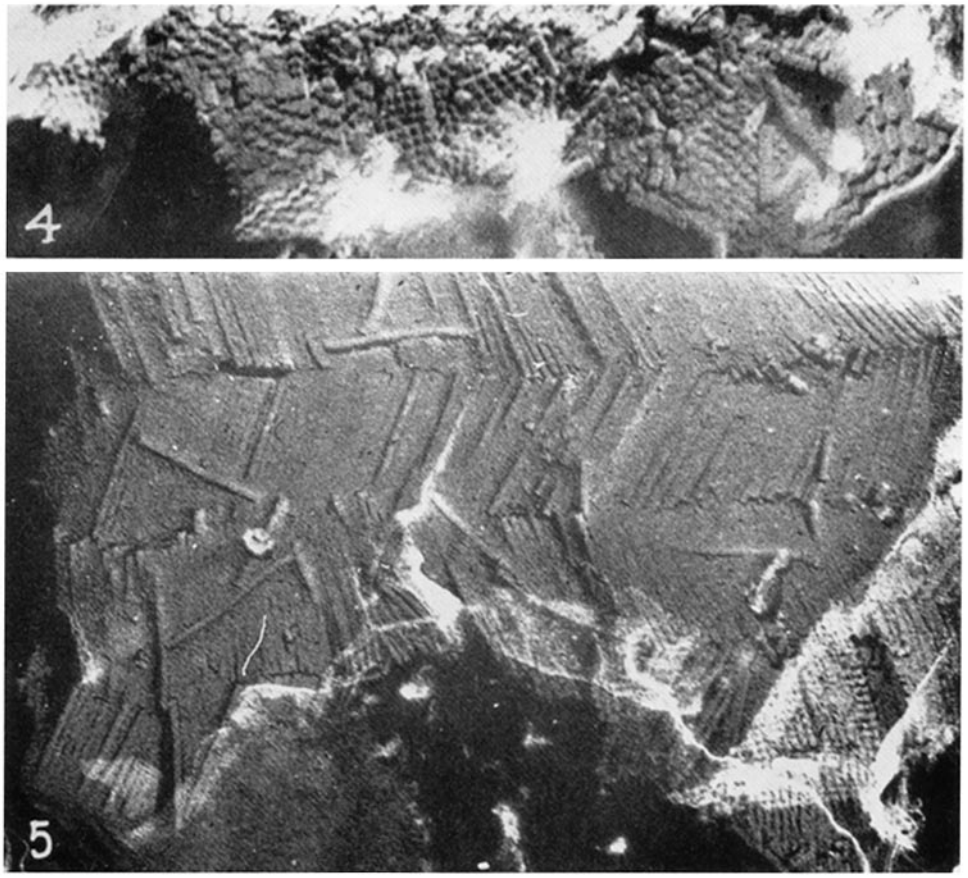


(Steere: Electron microscopy and structural detail)

PLATE 12

FIG. 4. Portion of a typical hexagonal crystal of tobacco mosaic virus within a hair cell of an infected Turkish tobacco plant. Crystal cut parallel to hexagonal face. Note the square net formed by the transversely cut particles. × 65,000.

FIG. 5. Portion of a typical hexagonal crystal of tobacco mosaic virus within a hair cell of an infected Turkish tobacco plant. Crystal cut perpendicular to hexagonal face. Note herringbone pattern due to the parallel arrangement of particles within each layer and the angle between particles of adjacent layers. Individual particles measure approximately 300 mμ in length. × 65,000.



(Steere: Electron microscopy and structural detail)