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Freeze-Drying Specimens for Electron Microscopy

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An improved table for freeze-drying specimens for electron microscopy has been developed. The construction of the table is described, the freeze-drying procedure employing it is outlined, and typical results of its application are presented.

The study of the structure of biomacromolecular assemblies, such as virus capsids and membrane surfaces, is often facilitated if a view can be obtained of a single surface of them. However, transmission electron micrographs of stained material do not, in general, allow surface information to be visualized without the laborious application of three-dimensional reconstruction techniques (e.g., Crowther and Klug, 1975). On the other hand, heavy metal shadowing of suitably prepared specimens provides direct access to the surface structure, since only features on the exposed surface are contrasted by the metal deposit. Simple computer methods have recently been developed which allow topographical maps to be reconstructed from micrographs of metal shadowed specimens (Smith and Kistler, 1977; Smith and Emanuilov Ivanov, 1980).

Independent observation of the two surfaces of plane-layer specimens often allows direct visualization of important aspects of their structure which would not have been seen in negative stain alone. Kistler *et al.* (1978) studied T4 polyheads using freeze-drying and shadowing and observed that transformation involved a structural change in the protein lattice in which protrusions disappeared from the inside surface of the polyheads and new protrusions appeared on their outside. Aebi *et al.* (1979) were able to show that "rectangular" T4 sheets had this morphology in negative stain because they consisted of a staggered association of two hexagonal sheets.

The value of freeze-drying as a preparation technique has prompted a search for a straightforward, reliable, and reasonably rapid method to implement it. Freeze-drying involves the rapid freezing of the specimen and the thin layer of water covering it on the grid to which it has adsorbed, followed by the sublimation of the ice in and around it. A thin layer of heavy metal is then deposited on the dehydrated specimen surface to provide contrast. Equipment to perform this operation must, first and foremost, protect the frozen specimen as it is loaded into the vacuum evaporator, and, second, protect the environment inside the evaporator so that evacuation is rapid and the possibility of contamination is obviated. Kistler *et al.* (1977) have described a table designed for use with the Balzers BAF 511 unit which meets the first of these requirements. The second requirement is met only poorly, however, since the evaporator must be opened to the air to allow the loaded table to be screwed onto the cooled stub in the bell. During this operation any clumsiness may cause loss of grids. In addition, frost can accumulate on the cooled stub and atmospheric damp can adsorb to the exposed surfaces within the evaporator, even if the unit is vigorously flushed with a dry gas, such as nitrogen or helium. The presence of this water lowers the final vacuum in the bell and reduces the efficiency of freeze-drying.

The purpose of this short note is to describe the design and construction of a

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freeze-dry table for the Balzers BAF 301 unit which satisfies the design desiderata outlined above, and to illustrate the application of the freeze-drying method to typical specimens. The improvements effected allow one to freeze-dry with consistently good results and a minimum investment of machine time.

MATERIALS AND METHODS

(a) Construction of the Freeze-Dry Table

The table (Figs. 1, 2a) is made of brass, measures 16 × 20 × 11 mm, and incorporates a locking mechanism allowing it to be clamped into the yoke of the counterflow loading device (Balzers part BB 172 050-T) which is screwed to the top of the cooling stub in the Balzers BAF 301 unit (Fig. 2b). Soft iron plugs of up to 5 mm diameter are pressed into holes drilled at each of the four corners of the top of the block. A rectangular trough, 10.5 × 15 × 0.8 mm, is milled into the top of the table. Six symmetrically placed, flat-bottomed depressions, 3.5 mm diameter × 0.2 mm deep to hold electron microscope grids, are cut into the bottom of the trough and are connected center to center in pairs by slots 1 mm wide × 4 mm long × 0.3 mm deep measured from the bottom of the trough. The front wall of the trough has a notch 1 mm wide × 0.8 mm deep cut into it to allow the trough to drain. The block is covered by an aluminum (preferable) or brass cap measuring 16 × 20 × 4 mm which has had four strong permanent magnets 5 mm in diameter fixed into holes drilled at its corner and set flush with the face of the cap.

Since the construction of a functional locking mechanism is difficult, a possible alternative is to modify a freeze-fracture table purchased from Balzers (part BB 172 075-T); the block used here was constructed from new materials.

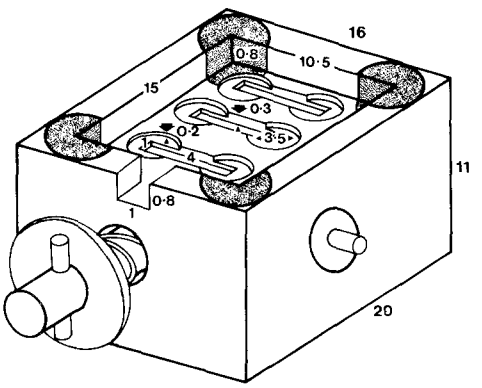

FIG. 1. A drawing of the freeze-drying table showing the recommended dimensions of the elements of its construction. Distances are in millimeters.

(b) The Freeze-Drying Procedure

The freeze-dry table and cap were cleaned in acetone and then ethanol using a water-bath sonicator, the manipulator arm was attached to the table, and both table and cap were placed in a 60°C oven to dry. Once they were hot they were transferred to the liquid nitrogen bath. The washing and heating steps insured that the table was clean and dry and that water vapor would not condense onto it as it was placed into the liquid nitrogen. The liquid nitrogen bath was made of styrofoam and the table and manipulator lay in it with the liquid nitrogen level no lower than halfway up the side of the table. The sides of the bath should extend about 20 mm above the liquid nitrogen level to trap a layer of cold dry nitrogen above the table. This dry nitrogen should be steadily replenished by the gentle boiling of the liquid nitrogen in the bath. Air movements above the bath should be avoided, particularly in humid atmosphere, since this causes "snow" to precipitate onto the table.

Freeze-drying was performed essentially by following the modification of Kistler *et al.* (1977) of the method described by Nermut *et al.* (1972). Specimens were fixed (usually 1%, final w/v, paraformaldehyde) and suspended in distilled water. Grids to be used were 300-mesh copper, covered with thin parlodion film stabilized by relatively thick layer (>200 A) of evaporated carbon. Grids were glow discharged before use. The specimen was absorbed to the support film from a drop of the suspension placed on a grid. The drop was withdrawn gently using filter paper, and the adsorbed specimen was washed thoroughly by touching it to a number of drops of distilled water. The washed grid, carrying a small drop of water from the last washing drop, was then poised over the liquid nitrogen bath, touched against a piece of filter paper to withdraw the excess water, and immediately plunged into the liquid nitrogen or into a small receptacle containing Freon 22 at its melting point (−155°C). Once frozen it was put into one of the depressions in the table. The loaded table was covered by the precooled cap which lightly attached itself to the table by the magnets.

The Balzers unit was prepared for operation and evacuated to 10^{-5} Torr. Stub cooling was initiated about 20 min before freezing the last specimen. As soon as the stub reached −150°C the unit was vented with dry nitrogen, the manipulator arm was used to load the table onto the cooled stub through the small access port against the nitrogen stream, and the unit was pumped once again. Loading took no longer than 20 sec. A pressure of better than 3×10^{-5} Torr was generally obtained after about 2 min of pumping and cooling of the knife arm was then initiated. Once the knife reached −150°C it was used to knock the cap

from the table. The stub was then warmed to, and maintained at, −35°C for 90 min to allow the ice in and around the specimen to sublime away. Bell pressure was usually better than 2×10^{-6} Torr at the end of the sublimation step. The stub was cooled to −150°C once again, the specimens were shadowed with platinum from an electron gun at an elevation angle of 30°C, and the metal deposit was stabilized with a thin carbon layer, as described by Kistler *et al.* (1977). The stub was warmed to 40°C, the bell vented with dry nitrogen, and the table removed from the unit.

RESULTS AND DISCUSSION

The essential elements of successful freeze-drying have been described in detail by Williams (1952). These include the rapid freezing of the specimen, sublimation at low temperature, and protection of the dehydrated sample from atmospheric moisture after freeze-drying. Nermut *et al.* (1972) developed a freeze-drying table (Balzers part BB 176 294-T) which allowed an optimal sublimation environment to be established about the specimen and permitted the shadowing to be done without breaking the vacuum. Kistler *et al.* (1977) manufactured an improved table consisting of a copper cylinder with a shallow trough milled in its top and a cap to cover it secured with a wire clip. This provided protection to the grids on loading so that they were kept frozen and isolated from frost due to atmospheric moisture.

The table described here (Figs. 1, 2a) is essentially a refinement of the one developed by Kistler *et al.* (1977). The provision of individual recesses for each grid keeps them in place. The slots permit forcep tips to slip under the grids so that they can be removed easily without damage. The drain allows the trough to empty itself of liquid nitrogen as it is being loaded; small quantities of liquid nitrogen retained in the trough can explosively vaporize when pumping begins throwing the grids from their recesses. The magnets provide a light but sufficiently secure attachment of the cap to the block to allow mechanical clips to be dispensed with.

The most important improvements in operation come from the adaption of the freeze-dry block to the counterflow loading device. The slip-on mount allows the smooth, rapid attachment of the block to the cooling stub with minimal possibility of disturbing the grids. Since the block is loaded by lifting it from the liquid nitrogen bath and inserting it into the bell against a stream of dry nitrogen, frosting on the block, which could reduce its thermal contact with the cooled yoke, is largely prevented. The dry nitrogen with which the bell is flushed, can be pumped out to high vacuum (10^{-5} Torr) in 3 min whereas if the bell is opened to the atmosphere it can take over an hour to reach this pressure. Runs are therefore done in a much shorter time. A further saving in time can be had by

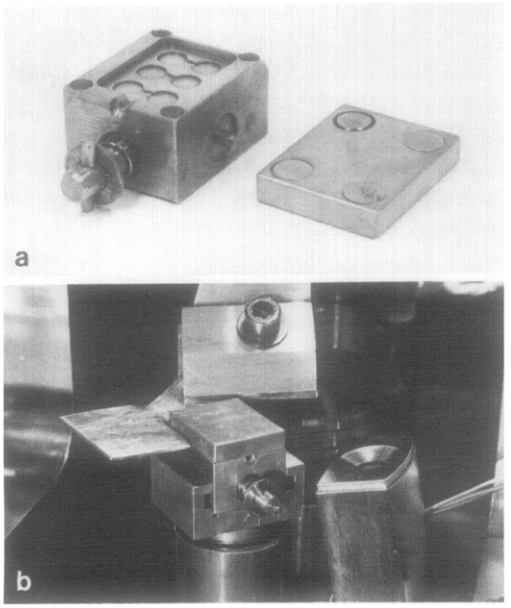

FIG. 2. (a) A photograph of the freeze-drying table and the cap used to cover it showing the magnets set into the cap. Not all the refinements in the design shown in Fig. 1 have been incorporated in this table. (b) The table is shown mounted in the yoke on the cooling stub in the BAF 301 unit. The knife arm is behind the table and holds the shutter. The cap is knocked from the table by bringing the cooled knife forward where it remains to act as a cold trap as the ice is sublimed. Rotating the knife to the right from the position in which it is seen here causes the shutter to cover the table during the initial stage of metal evaporation. Rotation further to the right places it over the thin film monitor which it protects when carbon is deposited from above.

reducing sublimation time; 90 min is longer than necessary to freeze-dry many specimens and so it is worthwhile investigating the use of shorter times if experimental demands warrant it.

The exposed specimen surface is not in good thermal contact with the cooled table below it, and is consequently susceptible to local heating during metal evaporation. Such heating may favor disruption of specimen surface structure and facilitate metal ion migration on the surface, neither of which is desirable. To help minimize such effects the specimen table is cooled to −150°C prior to metal shadowing and, following Kistler *et al.* (1977), a shutter (see Fig. 2b) is used to keep the specimen isolated from the radiant heat of the electron gun until metal evaporation has stabilized.

A potentially serious problem is the presence of the eutectic network (Kistler and Kellenberger, 1977), which can partially ob-

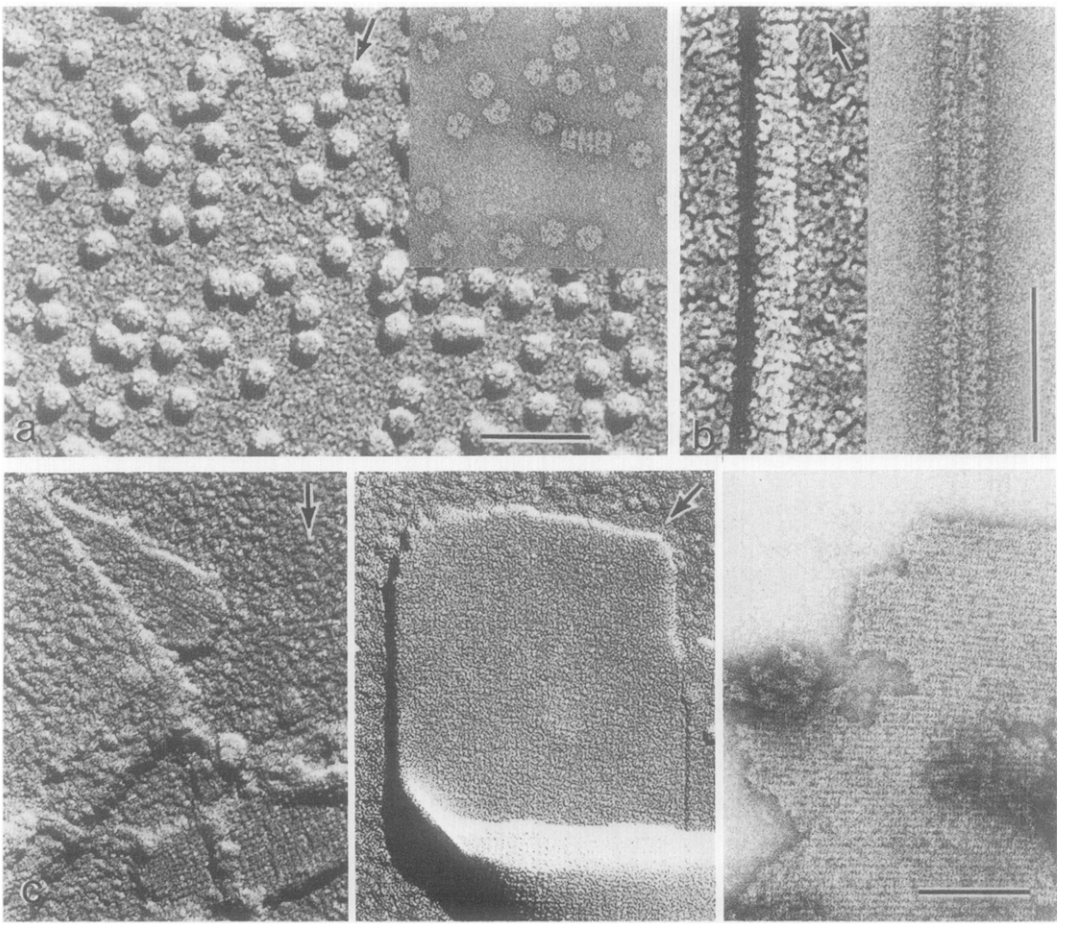

FIG. 3. (a) A field of freeze-dried and shadowed glutamine sythetase particles with a negatively stained area shown as an inset for comparison. (a) Freeze-dried and shadowed (left) and negatively stained (right) Sendai virus nucleocapsids after high-salt treatment and fixation. The nucleocapsid nucleoprotein helix has a pitch of 50 Å and the strand can be seen as it winds in a left-hand fashion in the micrograph to the left. (c) (Left) An actin cylinder showing the difference in structure between the "smooth" outside of the cylinder with its "rough" interior. (Middle) A "rectangular" sheet with rough surfaces on both sides. (Right) A negatively stained actin sheet. Light areas in the shadowed specimens have accumulated metal, dark areas are in shadow; arrows indicate the direction of metal deposit. Bars indicate 500 Å in (a) and (b) and 1000 Å in (c). Negative staining was with 1% uranyl sulphate in (a) and (b) and 1% uranyl acetate in (c).

scure the support film and much reduce the usable area on it. As noted elsewhere (e.g., Williams, 1952; Kistler and Kellenberger, 1977) the extent and composition of the eutectic depends on the solute composition and quantity of water left on the grid when it is frozen. Eutectic has been reduced to negligible amounts by drawing off as much water as possible from the grid by touching it to filter paper just prior to freezing it. This procedure leaves an extremely thin film of water on a hydrophilic grid which can be seen if the grid is illuminated from behind and viewed against a dark background. Such a film dries in about 2–3 sec, which is more than enough time to freeze it.

Representative examples of the results obtained with various specimens are presented in Fig. 3. In Fig. 3a a field of freeze-dried shadowed glutamine synthetase molecules is shown with an area of negatively stained molecules in the inset for comparison (see e.g., Frank *et al.*, 1978). Figure 3b shows a stretch of freeze-dried and shadowed Sendai virus nucleocapsid after high-salt treatment (Heggeness *et al.* 1980) in comparison with a negatively stained specimen. The freeze-dried specimen allows the direct visualization of the left-handed single-start nucleoprotein helix for the first time. Figure 3c (left) shows the broken end of a cylinder made of *Acanthamoeba* (non-muscle) actin crystalline sheet which has a "rough" inside and a "smooth" outside (U. Aebi, G. Isenberg, T. D. Pollard, and P. R. Smith, manuscript in preparation). The middle panel shows a folded piece of "rectangular" sheet made from two actin sheets back to back with their "rough" sides outward. The right panel shows the actin sheet negatively stained; the fact that it has a "smooth" and a "rough" side cannot be deduced from this image alone.

The freeze-drying table described above has incorporated the two elements which are important for the successful application of this procedure irrespective of the evaporator employed to actually sublime away the ice and to shadow the specimen with heavy metal. These are first, the protection of the frozen specimens from the atmosphere during transfer into the evaporator, and second, the protection of the environment within the evaporator during specimen insertion. The table we have constructed is very simple, and can be made in most modestly equipped machine shops. The introduction of its use in our laboratory has allowed freeze-drying to become a routine procedure which is being applied to an ever widening range of specimens.

I am particularly grateful to Mr. Lou Senden for his skillful construction of the freeze-dry table.

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