Proc. Nat. Acad. Sci. USA
Vol. 68, No. 9, pp. 2149–2152, September 1971

Improved Cryofixation Applicable to Freeze Etching

(spray-freezing/solute model systems/liquid nitrogen and propane)

L. BACHMANN AND W. W. SCHMITT

Institut für Technische Chemie, Technische Universität, München, Germany

Communicated by E. E. Salpeter, June 22, 1971

ABSTRACT **Freeze etching of solute model systems (e.g., glycerol or ferritin solutions) demonstrates that cryofixation can introduce serious artifacts due to the segregation of the dissolved or dispersed material from the solvent. Since, in principle, this problem can be reduced by increasing the cooling rate, a new technique has been developed which combines spray freezing with freeze etching. This spray-freeze-etching is applied by first spraying the specimen into a liquid cryomedium. The frozen droplets are then "glued" together with butylbenzene to form a regular freeze-etch specimen, while the temperature of the sample is kept at −85°C. The results obtained by spray-freeze-etching are far superior to those obtained by standard freezing. Our results, using 5% glycerol as a test specimen, are equivalent to those obtained by the high-pressure method (1). The reduction of segregation during freezing makes freeze etching a method applicable for the investigation of solute systems. Furthermore, the study of unicellular organisms or cellular fractions by freeze etching without the use of antifreeze is made possible.**

ARTIFACT PROBLEMS IN CRYOFIXATION

Since the introduction of freeze etching (2, 3), methods of adequate cryofixation for electronmicroscopic specimens have become of particular concern. In addition to offering an interesting alternative to chemical fixation for the study of cellular material, cryofixation provides practically the only method for the study of solute systems by electron microscopy.

Investigations of frozen-fluid specimens most clearly demonstrate the limits of cryofixation. The commonly available cooling rates are too low to prevent the rearrangement of solutes or particles during freezing. The well-known appearance of freeze-etched glycerol solutions is just one example. When frozen by the standard technique (by dropping a small metal specimen holder with about 1 mm^3 of solution into a liquid cryomedium) the glycerol and water separate into two different phases, i.e., compartments of water surrounded by a glycerol network (Fig. 3). For a given cooling rate, the size of the water compartments depends on the glycerol concentration and on the presence of other solute materials. Similar pictures are obtained when freeze-etching aqueous solutions of salts or sugar (4), organic solutions (e.g., benzoic acid in benzene) (5), solutions of large molecules like high polymers (dextran or polyvinylpyrrolidone) or proteins (albumin or ferritin) and even suspensions of larger particles (polystyrene latex, $\phi \leq 0.1$ μm), segregate (5). When observed by freeze-etching, all these specimens show a wide network containing the solute material in high concentration and large areas containing mainly the solvent.

Segregation can cause complex patterns, which might lead to misinterpretation of freeze-etched specimens. For instance, suspended particles (ferritin or latex) not only tend to agglomerate, but are often found arranged in a crystal-like pattern. Furthermore, when small particles (e.g., ferritin) and larger ones (polystyrene latex [$\phi = 0.3$ μm], yeast cells, etc.) are frozen together, the small particles are often observed on the surface of the larger ones, arranged in hexagonal patterns (5). The large objects merely act as mechanical obstacles against which the smaller particles are pushed by the advancing front of the growing ice crystal.

Freeze-etching techniques for biological specimens use cryoprotectants that are mainly capable of preventing one very-gross form of segregation, namely, the formation of large ice crystals that cause cell damage. On the other hand, cryoprotectants might introduce artifacts of their own, either be segregation from the aqueous solution during freezing or by their toxic effects on the biological system. In investigations of solutions or colloidal systems, cryoprotectants are of no use at all. Therefore, several attempts have been made to increase the speed of freezing, which is known to improve the quality of a cryofixed electron-microscopic specimen (3, 6–9). When the extent of partitioning of freeze-etched dilute solutions was used as a criterion*, only two methods have demonstrated a significant improvement over the standard technique. One of them is the high-pressure method. Specimens are frozen under a high hydrostatic pressure (2000 atm) with optimum heat transfer. This method makes it possible to reduce the compartment size of 5% glycerol to about 10 nm (1, 10). The necessary freezing conditions can be obtained only with an elaborate apparatus, which is not yet commercially available.

To overcome this difficulty, a spray-freezing method was developed, which can be applied to freeze-etching.

Since this "spray-freeze-etch" method has previously been described only in a short note (11), the details of the procedure are given in this paper.

THE TECHNIQUE OF SPRAY-FREEZE-ETCHING

Principles

The approach of the spray-freeze-etch technique is to increase the cooling rate by injection of very small droplets of the

* It is recommended that different techniques should be evaluated by the use of simple model systems, such as glycerol solutions. Segregation patterns of solutions react very sensitively to changes in freezing conditions and are much easier to reproduce and interpret than freeze-etched cells.

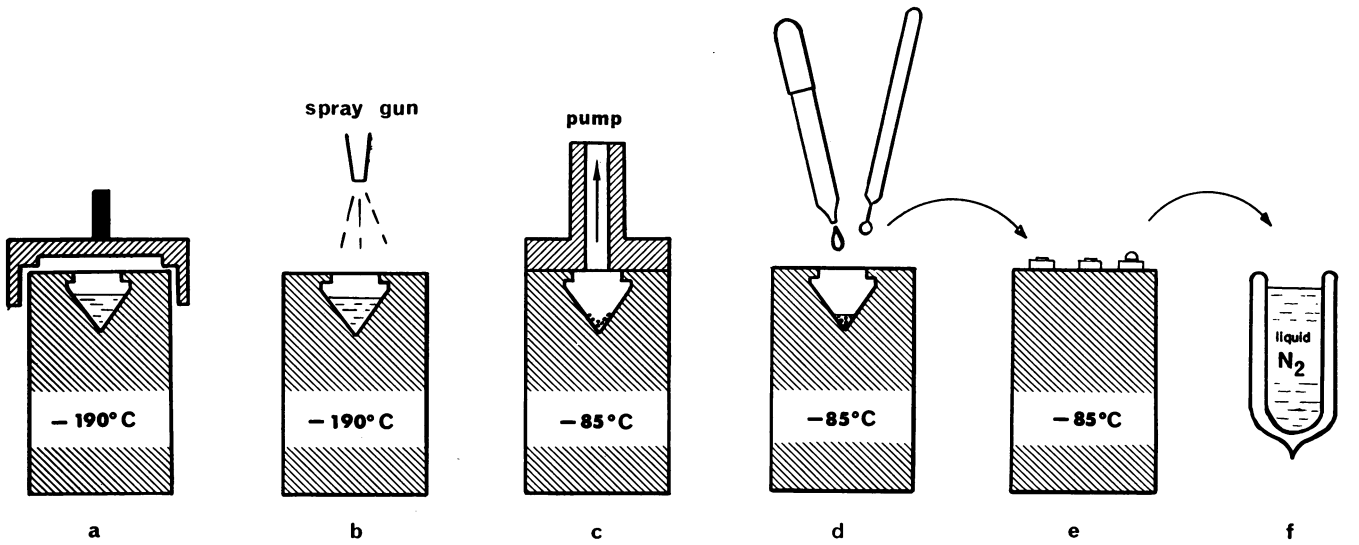

FIG. 1. Scheme of specimen preparation. (*a*) The cryomedium (liquid propane) is in a cavity ("cryochamber", about 10 mm in diameter) in a copper block cooled with liquid nitrogen. The condensation of moisture is prevented by the use of a metal lid. (*b*) The specimen is sprayed into the cryomedium. The ice layer, which thereby forms on the flat top of the block, should not fall into the cryochamber and has to be removed. (*c*) After transfer of the block into a cryostat inside a glove box, the propane is evaporated under reduced pressure at −85°C. (*d–f*) A drop of cooled butylbenzene is added to the cryochamber and mixed with the frozen specimen droplets. A drop of this mixture is then transferred with a platinum loop onto cold Balzers specimen mounts. These are then dropped into liquid nitrogen. All tools must be kept at −85°C or colder.

specimen into a cryomedium, thus reducing the probe volume, as compared to the standard method, by a factor of about 10^6. A similar principle has already been applied with good results for freeze drying (12). However, for freeze etching, the problem is how to handle minute specimen particles (diameter of the order of 10 μm) for further preparation. This was accomplished by embedding the frozen particles in an inert medium with a melting point only slightly above the usual cleaving temperature in freeze etching (i.e., $T_m >$ −100°C) and a vapor pressure comparable to, or lower than, that of water. After mixture of the frozen specimen particles with the binding medium at low temperature (to prevent recrystallization), a drop of the mixture is transferred to a cooled Balzers specimen mount, solidified in liquid nitrogen, and further processed like regular freeze-etching specimens. In this way, the freezing of the sample and the mounting on specimen carriers become two independent procedures, which can no longer interfere with each other.

Spray freezing

A copper cylinder containing a cavity as indicated in Fig. 1 is cooled with liquid nitrogen. To prevent condensation of moisture, the top is covered with a metal lid. The cryomedium, (e.g., liquid propane) is condensed in the cavity† and the suspension or solution to be investigated is sprayed in short pulses into it. The spray gun used is a commercial retouching air brush (Grapho^R) with a nozzle opening of 0.1 mm. The gun was operated with compressed air at 0.5–1 atm pressure at a distance between 5 and 10 cm. A minimum of about 0.2 ml of the specimen suspension is required. It is important that most of the droplets be injected into the cryomedium rather forcefully, otherwise they might freeze in the cold-gas layer before they reach the liquid propane. Such slowly injected droplets show the characteristic segregated appearance of slowly frozen specimens. The shape of the cryochamber prevents the liquid cryomedium from being blown out by the spray. An ice layer forms on the top of the copper cylinder during the spraying of the specimen. This has to be removed so that no part of it falls into the cryomedium. The removal of the ice layer is simplified if a copper plate with a hole slightly smaller than the opening of the "cryochamber" is placed on top of the copper cylinder during spraying. After spraying, this plate is removed and the cylinder is again covered with the cooled lid and placed into a cryostat, inside a glove box with good thermal insulation. The cryostat is kept at −85°C. We no longer recommend a simple dry-ice–methanol bath as previously used (11), because the condensation of CO_2 on liquid-nitrogen-cooled surfaces or tools may be annoying. Unsatisfactory heat-transfer occasionally caused recrystallization by annealing.

Mounting of the specimen

All subsequent steps are performed in the glove box with all tools also kept at −85°C or colder. The cover lid over the cryochamber is replaced by a cylinder that has a flared-gasket surface on one end and is connected to a rotary pump on the other end. The propane is evaporated off within 1 min (vapor pressure about 100 Torr at −80°C). The frozen specimen in the cavity of the copper block is now free of the cryomedium. A cooled drop of a hydrophobic medium is then added and thoroughly mixed with the specimen. This "embedding" medium must not penetrate the frozen specimen at all but serve merely as a glue to keep the isolated droplets together for further processing. We used *n*-butylbenzene, which is easily cleaved at −100°C; its vapor pressure

† Although the melting point of pure propane is −190°C, commercial fuel propane usually does not solidify at liquid-nitrogen temperature (−196°C). However, it should be remembered that at liquid-nitrogen temperature, oxygen might condense together with the propane to form a potentially explosive mixture (14). This hazard can be avoided if the temperature of the cryochamber is kept slightly above −190°C.

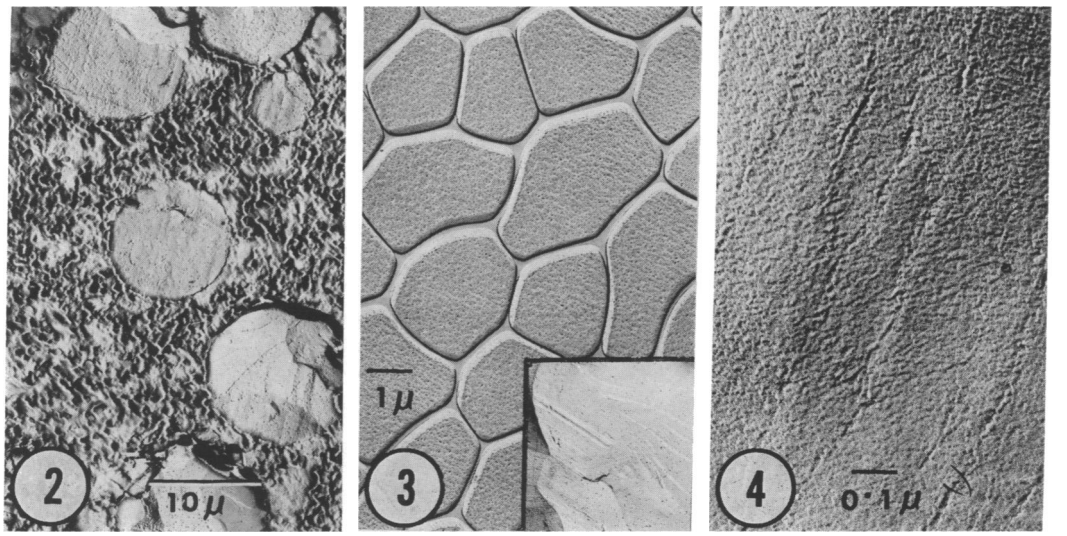

FIG. 2. Spray-freeze-etched sample. The specimen droplets (5% glycerol) are embedded in (but not penetrated by) a matrix of butylbenzene "glue". Magnification: ×1,500.

FIG. 3. 5% glycerol–water solution, freeze etched after standard freezing. The ice compartments are up to several μm in size. Insert: same solution prepared by spray-freeze-etching; the compartments are far too small to be visible at this magnification. Magnification: ×5,700.

FIG. 4. 5% glycerol–water solution, spray-freeze-etched. At high magnification, a granularity due to some segregation becomes visible (compare with Fig. 7 e–h in Riehle, 1968). Magnification: × 57,000

is lower than that of water (melting point −88°C, boiling point 183°C). A drop of this mixture is then transferred with a liquid-nitrogen-cooled wire loop onto Balzers specimen carriers, which are also kept at −85°C. Then the specimens are dropped into liquid nitrogen. It should be emphasized that the procedure given has nothing to do with freeze substitution.

Freeze etching and processing of the replicas

Freeze cleaving and etching was done in a Balzers BA 360 M at −100°C. After replication, the spray-frozen samples have to be immersed in acetone to dissolve the butylbenzene. The replicas, which then float free in acetone, were transferred onto water with a pipette, where they spread on the surface. Insoluble organic material was removed as usual by floatation of the replicas on 70% sulfuric acid.

Variations in technique

Two variations have been tried. In the first, a small quantity of the suspension is sprayed onto a liquid-nitrogen-cooled metal block to form a thin frozen layer. The block surface containing the specimen is then submerged in the liquid nitrogen, where the frozen layer is scraped off. Specimen bits and nitrogen are then poured into the cryochamber. After evaporation of the nitrogen, butylbenzene is added and freeze-etch specimens are prepared as described above. In the second

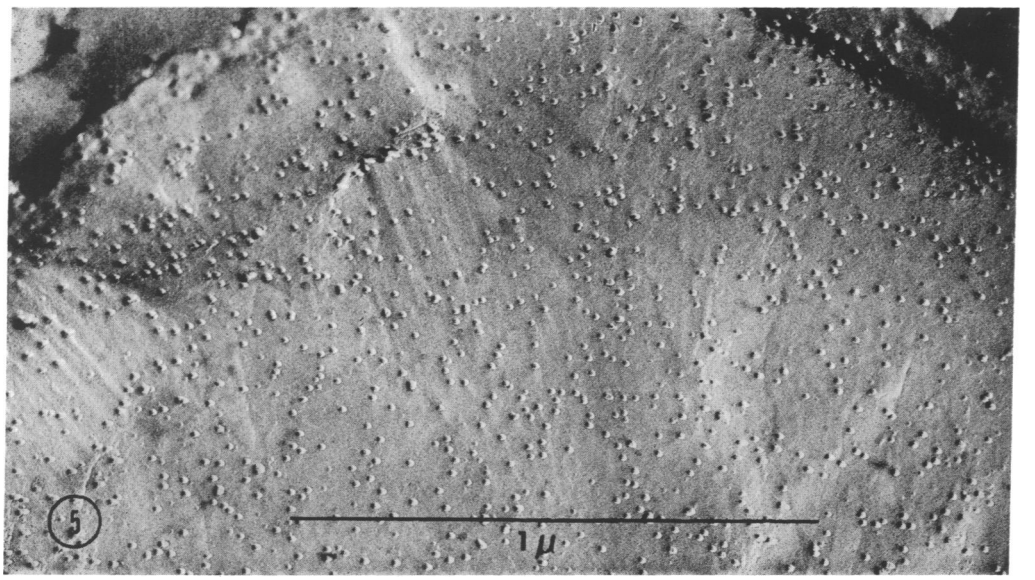

FIG. 5. Spray-freeze-etched ferritin solution. Concentration dependence studies have established that the particles are indeed ferritin. The border of the droplet is visible at the upper-left corner. Magnification: ×70,000.

method, the fluid sample is sprayed directly onto liquid-nitrogen-cooled specimen carriers. Two apertures are used, so that only the central area of the specimen support is hit by the spray. The frozen sample gradually forms a little mound suitable for freeze etching. No further manipulation is necessary. The spraying is interrupted several times to make sure that the surface of the growing specimen is always kept cold enough.

With glycerol–water solutions, both variations give results similar to that of the spray-freeze–etch technique described above, but they seem to be more difficult to perform. The first variation is of advantage when very little sample is available. The second one can be used for freeze-etching nonaqueous solutions in cases where neither a suitable cryomedium nor a "glue" can be found.

RESULTS

By the spray–freeze–etch method, the specimen consists of dispersed droplets embedded in a matrix of butylbenzene (Fig. 2). Only the content of these droplets will be referred to as the specimen.

Solutions of 5% glycerol in water look very similar when spray-freeze-etched or when frozen by the high-pressure method, although the freezing conditions are quite different (Fig. 4). The large ice compartments that are formed by the standard technique (Fig. 3) are not present (Fig. 3, *insert*). Segregation merely manifests itself by a granularity of the order of 5–15 nm, seen only at higher magnification. Preferred orientation of these small compartments, as described by Riehle (1), also occurs frequently (11).

Solutions of ferritin, dextran, albumin, etc., which become heavily segregated by standard freezing, show dispersed individual molecules when sprayed frozen (Fig. 5). Luckily, at the same time, the surface of spray-freeze-etched water appears considerably smoother than in specimens frozen by the standard technique. With the latter procedure, pure water usually appears loaded with particles, bumps, or "warts" of still-unknown origin (4, 13, 11).

Further studies are needed to test other cryomedia and to make sure that the currently used "embedding temperature" of −85°C is low enough to prevent noticeable recrystallization during the time needed for the preparation of the specimen. However, the results already demonstrate that spray freezing can be combined with freeze etching in a way that opens new possibilities for the electron microscopic investigation of solute systems. The successful application of this procedure to the study of unicellular organisms (i.e., Chlorella, Euglena, and spermatozoa) without the use of antifreeze will be described elsewhere.

The work was supported in part, by the Deutsche Forschungsgemeinschaft, the Balzers AG, Balzers, Liechtenstein and grant GM 10422 from the National Institutes of Health. We thank Dr. M. M. Salpeter (Cornell University) for many helpful discussions.

1. Riehle, U., *Chem. Ing. Techn.*, **40**, 213 (1968).
2. Steere, R. L., *J. Biophys. Biochem. Cytol.*, **3**, 45 (1957).
3. Moor, H., and K. Muehlethaler, *J. Cell Biol.*, **17**, 609 (1963).
4. Moor, H., K. Muehlethaler, H. Waldner, and A. Frey-Wyssling, *J. Biophys. Biochem. Cytol.*, **10**, 1 (1961).
5. Schmitt, W. W., H. P. Zingsheim, L. Bachmann, *Proc. 7 Int. Congr. Electron Microscopy (Grenoble)*, I, 455 (1970).
6. Stephenson, J. L., *J. Biophys. Biochem. Cytol.*, **2**, Suppl., 45 (1956).
7. Fernández-Morán, H., *Ann. N.Y. Acad. Sci.*, **85**, 689 (1960).
8. Moor, H., *Z. Zellforsch. Mikrosk. Anat.*, **62**, 546 (1964).
9. Bullivant, S., *Lab. Invest.*, **14**, 1178 (1965).
10. Moor, H., and U. Riehle, *Proc. 4th Eur. Reg. Conf. Electron Microscopy (Rome)*, II, 33 (1968).
11. Bachmann, L., and W. W. Schmitt, *Naturwissenschaften*, **58**, 217 (1971).
12. Williams, R. C., *Exp. Cell Res.*, **4**, 188 (1953).
13. Davy, J. G., and D. Branton, *Science*, **168**, 1216 (1970).
14. Stephenson, J. L., *Nature*, **174**, 235 (1954).