FREEZE-FRACTURING IN ULTRAHIGH VACUUM AT \(-196^\circ C\)

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ABSTRACT

Conventional freeze-etching is carried out in a vacuum of \(-10^{-6}\) torr and at a specimen temperature of \(-100^\circ C\). The relatively poor topographic resolution of most freeze-etch replicas, and the lack of complementarity of morphological details in double replicas have been thought to be caused by structural distortions during fracturing, and radiation damage during replication. Both phenomena can be reduced by lowering the specimen temperature. To prevent condensation of residual gases (especially \(H_2O\)) on the fracture faces at lower specimen temperature, an improved vacuum is required. Therefore, an ultrahigh vacuum freeze-fracture apparatus has been developed which allows fracturing and Pt/C-shadowing of specimens at \(-196^\circ C\) while maintaining a vacuum of \(10^{-9}\) torr. It consists of a modified Balzers BA 350 ultrahigh vacuum (UHV) unit, equipped with an airlock which enables the input of nonhoar-frosted specimens directly into the evacuated bell jar.

A comparison of the paracrystalline plasmalemma structure in yeast cells portrayed by the conventional technique and by UHV-freeze-fracturing at \(-196^\circ C\) shows the improved topographic resolution which has been achieved with the new technique. The improvement is explained by less structural distortions during fracturing at lower temperatures. The particles of the paracrystalline regions on the P face are more regularly arranged and exhibit a craterlike substructure which corresponds with a ringlike depression in the E face. The optical diffraction patterns of these paracrystalline regions demonstrate the improvement of the structural record by showing well-defined third- and fourth-order spots.

KEY WORDS freeze-fracturing in ultrahigh vacuum - baker's yeast - membrane structure - paracrystalline arrays - very low fracturing temperature

Since the freeze-etch method was introduced (21) into biological research and developed as a routine laboratory technique (11, 12), an increasing number of scientists have applied it to an ever-growing range of problems. The main feature of the results gained by this preparation technique has been a kind of new insight into the fine structure of biological membranes (2, 3, 5, 14, 26). The introduction of the so-called "double replica technique" (6, 23, 24), allowing the replication of both halves of a cleaved specimen, revealed that freeze-etching discloses internal membrane structures which may be portrayed as elevations or depressions. The precise comparison of complementary sites in such double replicas showed, however, astonishing morphological differences (5, 6, 16). This called into question the reliability
of the structural record and led in turn to questions about possible sources of artifacts and other limitations of freeze-etching.

If these questions are to be addressed, all the physical and technical features of the method must be taken into account. Each of the preparational steps involved in the technique possesses its particular problems which must be dealt with separately. First, there are problems connected with freezing which derive from segregation phenomena in the liquid phases of cells, i.e., in the cytosol and in membranes (13, 16, 26). Second, a mechanical separation of membrane components takes place during fracturing, producing heat which facilitates plastic deformation (5, 7, 9, 16). Third, during the time between cleaving and coating there exists the danger of contaminating fracture faces with adsorbing and condensing gases (4, 8, 16, 20); an unequal distribution of the contaminant would not only hide original structures but also create artificial ones. Fourth, the fidelity of the structural record which should ideally be a copy of the specimen surface produced by coating depends on the amount of damage introduced by surface heating and on the granularity of the recording heavy metal coat (1, 15, 25). All these factors, except the segregation phenomena during freezing, could or might be responsible for the lack of complementarity in double replicas. All of them are temperature-dependent; at a lower specimen temperature, less distortion of fractured structures, less damage of radiation-heated surfaces, and less surface diffusion of condensing heavy metal atoms can be expected (15–18). The only drawback is the increased contamination at low temperatures, but this problem should be much reduced or eliminated by application of a sufficiently improved vacuum. In other words, a considerable improvement of the topographic resolution of the freeze-etch method should be attainable by freeze-fracturing under ultrahigh vacuum conditions at very low specimen temperatures.

Requirements of UHV and Freeze-Fracturing

The "improvement" of a conventional high vacuum system by increasing the pumping capacity may lead to considerable local pressure lowering, e.g., near adsorbing surfaces or shrouds and in the vacuum gauge (4, 19, 22). The main characteristic of such a system ($P \approx 10^{-7}$ torr) is, however, the inhomogeneity of its vacuum. All surfaces exposed to low pressure produce gas by desorption, and at different positions pumps with different pumping capacities are in action. As a result, a series of complicated pressure gradients is formed. In other words, the actual vacuum conditions at a given place in the system cannot be determined, except in the gauges themselves. Therefore, establishing UHV ($P \leq 10^{-8}$ torr) conditions cannot be solely a matter of increased pumping.

The essential feature of present day UHV technique is reduction of the amount of adsorbed gases at all surfaces by baking out the exposed equipment at 200°–400°C under high vacuum. This is only possible with a specially constructed vacuum apparatus designed to withstand high temperatures. The more the desorption rate is lowered by heating, the lower the pressure and the more homogeneous the vacuum that can be attained, and, at the same time, the more sensitive the system becomes to gas-producing processes. Therefore, working under UHV conditions is not only a matter of attaining UHV.

The problems of reconciling UHV and freeze-fracturing lie along these lines. Conventionally, a cold specimen or specimen holder is fixed under atmospheric conditions on a precooled stage in the vacuum chamber and exposed to a poor vacuum during pump-down. This means hoarfrosting of the specimen and its surroundings, and no possibility of backing out. Therefore, the first prerequisite of UHV-freeze-fracturing is an air-lock which enables the introduction of a nonhoarfrosted specimen directly into the ultrahigh vacuum chamber. A second problem derives from gas production by the coating procedure. In standard freeze-etching, generally no special precautions are taken to prevent pressure increase during platinum and carbon evaporation. If the same evaporators are operated under lower pressure, a drastic vacuum breakdown will take place and at least part of the expelled gases will condense on the specimen. Therefore, the second prerequisite of UHV-freeze-fracturing at very low temperatures consists of drastically reducing gas production by improving backing out and improving the cooling system of the evaporators.

Finally, the question of the vacuum required for freeze-fracture purposes at very low temperatures must be addressed. Assuming total condensation of all residual gases, the freshly cleaved specimen surface would be covered with one monomolecu-
lar layer per second under conventional high-vacuum conditions (10⁻⁶ torr). This is the same deposition rate as used for shadow-casting, and consequently heavy contamination of the specimen surface and interference with coat formation are inevitable, as has been repeatedly observed (8, 16, 20). These effects are reduced 1,000 times if UHV of 10⁻⁹ torr is applied. An interval of 10 s between cleavage and the beginning of coating would allow for only 1% of the surface to be covered by contaminants, and only one gas molecule for every 1,000 heavy metal atoms would condense during shadow-casting. In other words, under these conditions the system should be clean enough to allow a direct and undisturbed interaction between evaporated materials and the naked specimen surface. This is also a prerequisite for studies of the physicochemical properties of defined regions on the fracture faces.

MATERIALS AND METHODS

**UHV-Freeze-Fracture Apparatus**

The UHV system used in our experiments is a modified Balzers BA 350 U unit (Balzers AG, Lichtenstein) (Fig. 1). It comprises five pumps which work in series: a rotary vane pump and two oil diffusion pumps, a liquid nitrogen-cooled baffle plus Meissner trap, and a titanium sublimation pump. The main flange of the bell jar can be fitted with a gold wire or a Viton gasket. All experiments described here have been performed with Viton as gasket material. During the bake-out procedure the flange is water-cooled to ~80°C. All other flanges are sealed with gold wire. With this system, vacua in the range of 10⁻¹¹ torr can be attained. Its high pumping capacity facilitates maintenance of UHV conditions during processing. When working in the region of 10⁻⁹ torr, the partial pressure of hydrocarbons is far below 10⁻¹⁰ torr.

The lower part of the bell jar contains the UHV-compatible devices for freeze-fracturing (Figs. 2 and 3). The temperature of the liquid nitrogen-cooled specimen stage is regulated by a heater and automatically controlled via a thermocouple. Any temperature between −196°C and 0°C can be stabilized to ±0.1°C. For fracturing, a molybdenum plate is used. It is mounted on a liquid nitrogen-cooled copper block which can be advanced in the direction of the specimen stage. Above the stage two evaporators (modified Balzers electron bombardment guns) are fixed on a water-cooled support. Platinum and carbon are evaporated at a distance of 13 cm from the specimen surface.

The deposition process is controlled automatically by the quartz crystal thin film monitor (situated beside the specimen stage) which is coupled to the pneumatically driven shutter. The automatic control of film thickness and the measurement of deposition rate make possible a completely reproducible coating. The airlock is attached to the lower part of the bell jar (Figs. 2-4). It consists of a chamber system which can be evacuated, a manipulator which enables the transport through the airlock towards and away from the stage, and a copper cartridge, attachable to the manipulator, which is used as
FIGURE 2 The UHV-freeze-fracture device inside the bell jar: (a) side view; (b) ground plane: (1) specimen stage; (2) specimen cartridge; (3) fracturing device; (4) quartz crystal thin film monitor; (5) shutter; (6) specimen airlock; (7) water-cooled support of the evaporators.
Figure 3 Overall view of the UHV-freeze-fracture apparatus (a, b), and the arrangement of the components inside the bell jar (c): (1) specimen stage; (2) specimen cartridge; (3) fracturing device; (4) quartz crystal thin film monitor; (5) shutter; (6) specimen airlock; (7) water-cooled support of the evaporators; (8) Pt/C-evaporator; (9) C-evaporator; (10) liquid nitrogen-cooled baffle.
vehicle for the specimens. The apparatus has been described in detail by Gross (10).

Specimen Preparation

The comparative study of methodological progress is greatly facilitated if methods of image averaging (i.e., optical diffraction) can be applied. Therefore, the plasmalemma of baker's yeast, which contains a paracrystalline structure, was chosen as test specimen.

Pressed yeast cells from a local yeast factory were suspended in distilled water at room temperature for 24 h, gently spun down, and the pelleted cells were drawn up into an injection needle. Small brass capillaries (outside diameter, 1.0 mm; inside diameter, 0.8 mm; Fig. 4) were filled by pressing out the highly concentrated cell suspension. Freezing was performed by dipping the capillaries into liquid Freon-22 at −160°C.

Airlock Procedure (Fig. 4)

Under liquid nitrogen, three capillaries are fixed in the corresponding bores of the specimen cartridge which is then screwed to the shaft of the manipulator. The cylinder attached around the end of the shaft is closed with a lid. Closure under liquid nitrogen enables transfer of cartridge and specimens to the airlock without hoarfrosting. Under continuous countercurrent of dry nitrogen gas, the cylinder is introduced into the first chamber which is then closed by fastening the manipulator flange at the front of the airlock. By advancing the shaft the cartridge is transferred to the main chamber, thereby ejecting the lid from the cylinder and pouring out the liquid nitrogen into the first chamber. The entrance to the main chamber, previously filled with dry nitrogen gas, is sealed by the shaft. After pump-down of the main chamber to $3 \times 10^{-2}$ torr, the inner gate is opened, and by further advancing of the shaft, the cartridge is inserted into the specimen stage. There, the cartridge is screwed tightly using the screwdriver provided inside the shaft. The shaft is then loosened from the cartridge, withdrawn, and the gate (which is sealed with Viton) is closed. The whole airlock procedure does not last more than 80 s.

Evacuation (Fig. 5)

Before the specimens are inserted, the bell jar is evacuated to $1 \times 10^{-9}$ torr. When the inner gate is opened during airlock procedure, the pressure rises to $-10^{-4}$ torr and, after closing, falls to $-3 \times 10^{-5}$ torr within 5 min. A vacuum of $1 \times 10^{-8}$ torr is reestablished after about 70 min. Mass spectra demonstrate restoration of UHV conditions in spite of a slight contamination with Freon-22. The specimen temperature rises from −196°C to about −140°C during the airlock procedure. Under high vacuum, the temperature is subse-
FIGURE 5 The course of specimen temperature (broken curve) and vacuum (solid curve) during evacuation: (1) beginning of the airlock procedure; (2) opening of the inner airlock gate; (3) closing of the inner airlock gate; (4-5) decontamination of the specimen stage at −100°C; (5-6) cooling of the specimen stage to −196°C; (6) starting of the titanium sublimation pump. A to E, mass spectrograms.
Fracturing

Fracturing is performed by advancing the liquid nitrogen-cooled molybdenum plate over the cartridge. The capillaries are struck one after the other at their upper end and broken at the predetermined zone (Fig. 4). The broken-off parts of the capillaries fall onto the liquid nitrogen-cooled baffle, where they are collected and prevented from thawing. During fracturing a slight pressure rise is observed, which can be shown by mass spectroscopy to be caused by water vapor. This rise lasts at most 1 s and does not exceed \(1 \times 10^{-5}\) torr.

Coating

For platinum and carbon evaporation, modified Balzers evaporators (Balzers AG [15]) heated by electron bombardment are used. Instead of stainless steel, holders for the cathode filament and the front shield are constructed of copper, and the focusing aperture is constructed of molybdenum in order to improve heat flux to the water-cooled support. The electrodes and their holders are modified also (as indicated in Fig. 6) to reduce heat transfer to the evaporator. These features, together with outgassing of the electrodes (that is, by operating the guns near evaporation conditions for 7 min), enable maintenance of UHV during coating.

Platinum-carbon shadow-coating is performed at an angle of 45° and with a deposition rate of 1.5–2 Å/s. During the period necessary for coating with 20 Å, the vacuum is stable at a pressure of \(2 \times 10^{-9}\) torr. The mass spectra indicate only a slight increase of hydrogen partial pressure (Fig. 7).

Carbon backing is performed at an angle of 90° and with a deposition rate of 18–20 Å/s. During the period necessary for coating with 200 Å, a vacuum of \(1 \times 10^{-8}\) torr is maintained. The pressure rise is caused mainly by outbursts of hydrogen and carbon monoxide from the carbon electrode (Fig. 8). This "deterioration" of the vacuum does not matter at all because the contamination of the condensing carbon is not higher than that of the Pt-C. Assuming total condensation of all invading materials, there is only about one gas molecule among 1,000 coat atoms in both cases.

Replica and Image Processing

After coating, the specimens were withdrawn through the airlock, thawed, and the replicas were floated on 25% \(CrO_3\) in water at room temperature overnight, then washed several times in double-distilled water, and mounted on naked grids (400 mesh). The replicas were examined in a Siemens 102 electron microscope equipped with an anticontamination device and at an accelerating voltage of 100 kV. Pictures were taken on Kodak electron image plates at magnifications of 30,000 and 50,000. Magnifications were calibrated with a cross-grating (54,800 lines/inch, Balzers-Union, Lichtenstein). The negatives were used directly for producing enlarged positives or optical diffractograms. A detailed image analysis and description of applied methods will be published elsewhere.²

RESULTS

The value of any comparative study will depend greatly on the criteria used in evaluating the replicas in the microscope and in selecting representative pictures for publication. First, to ensure a reliable foundation for this report (and further studies), each experiment was repeated several times. Next, electron micrographs of all experiments were scanned by eye to find paracrystalline regions which were apparently undistorted and well shadowed. These images were then examined by optical diffraction which allows an objective analysis of all periodicities present in the images. Those images from each experiment giving the clearest optical transform and the highest-order spots were selected and compared.

Three different experimental setups were used to show the effect of vacuum improvement and temperature lowering. In the first, specimens were freeze-fractured under conventional conditions, that is, at \(-100^\circ C\) under \(1 \times 10^{-6}\) torr (Fig. 9). The most prominent structures of the yeast plasmalemma are the troughlike invaginations. On the P face, their surface seems to be quite smooth, and on the E face densely packed granules can be seen in a fir-conelike array. In the flat areas of the P face between the invaginations, patches of particles in a hexagonal array are visible. The particles have a diameter of \(100 \ Å\) and a center-to-center spacing of \(165 \ Å\). On the E face no equivalently prominent structure can be found; all that can be observed are patches of the same size showing a slightly different structure than their surroundings. In other words, in two cases positive structures (granules and particles) are found on one half of the membrane and no corresponding depressions on the opposite half.

In a second experimental setup, the specimen temperature was kept at \(-100^\circ C\) but the vacuum improved to \(10^{-9}\) torr (Fig. 10). The main features of the structural record are the same as under high vacuum. The granules on invaginations (E face) and the particles in the patches (P face) seem to be less prominent, whereas the regions where the

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depressions could be expected again show little detail.

When low temperature (−196°C) was applied in addition to UHV, the situation altered drastically, as seen in Fig. 11. The granular structures become much more clearly visible, more details are evident, and their arrays are more regular. The complementary structures on the opposite site can now be seen with about the same distinctness. Most prominent is the substructure of the particles in the patches. They exhibit a craterlike shape on the P face with corresponding ringlike depressions in the E face. The array of this structure is now so perfect that it can be called paracrystalline. The diffraction patterns deriving from conventional and UHV-low temperature experiments (Fig. 12) yield information about the average repeating features in the paracrystalline regions. The improvement of the structural record achieved with the new technique is indicated by the appearance of well-defined third- and fourth-order spots of the reciprocal lattice in the optical transform of both halves of the membrane, and diffuse diffraction spots not extending beyond the second order are found after conventional preparation. The occurrence of higher-order spots does not prove conclusively that the record includes finer details because higher-order spots could also be caused by sharper portrayal of the large units. The existence of smaller features can be demonstrated, however, by image processing, i.e. removal of the aperiodic noise component by Fourier filtration and reconstruction of the filtered data. A description of these methods and results will be published elsewhere.

DISCUSSION
The insignificant difference between applying high vacuum or UHV at −100°C, on the one hand, and the great improvement achieved by additionally lowering the specimen temperature, on the other hand, indicates that the structural record is primarily temperature-dependent. A close inspection of the pictures does not reveal remarkable differences in the granularity of the shadow-cast. Therefore, reduced surface diffusion cannot be held responsible for better resolution. The observation of reduced order in paracrystalline structures of "high-temperature" specimens can be explained by the action of mechanical forces during fracturing. Cleaving involves an energy input for the separation of components that are originally bound. The breaking of bonds produces heat which elevates the temperature in the cleavage plane. If the specimen is cleaved at −100°C, the temperature might rise so much that membrane lipids are softened, enabling lateral dislocation of protein complexes (particles) and lipid molecules. This may lead to disorder in the arrangement of particles and to closing of pits in the lipid matrix of the opposite half of the fractured membrane. A loss of structural information in the
Figure 7  Vacuum and deposition rate during Pt/C-shadowing.
Figure 9 Yeast plasmalemma, freeze-fractured at -100°C under $1 \times 10^{-6}$ torr. Between the invaginations the PF (a) shows patches of hexagonally arranged particles (one is encircled). Between the structures corresponding to the invaginations no equivalently prominent structure can be found on the EF (b). Arrows indicate direction of shadowing. $\times 170,000$. 

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FIGURE 10  Yeast plasmalemma, freeze-fractured at $-100^\circ$C under $1 \times 10^{-9}$ torr (UHV). The main features of the structural record are the same as under high vacuum (Fig. 9). Arrows indicate direction of shadowing. $\times 170,000$. 
Figure 11 Yeast plasmalemma, freeze-fractured at −196°C under 1 × 10−9 torr (UHV). (a) On the PF the particles in the paracrystalline patches are much more clearly visible with more details and in a more regular array. (b) Their negatives on the EF are shown with a comparable distinctness. One of the patches is encircled. Arrows indicate direction of shadowing. × 170,000.
The paracrystalline regions of both fracture faces of the yeast plasmalemma after conventional freeze-fracturing (a, d) and UHV-low temperature replication (b, c, e, f) are shown at higher magnification (× 300,000). a* to f* represent the corresponding optical diffractograms. The improvement of the structural record by the new technique is indicated by the appearance of well-defined third- and fourth-order spots in the diffractograms of both fracture faces. Arrows indicate direction of shadowing. Bars, 500 Å.
lipid matrix could also be explained by surface heating during coating, but particle dislocation would be much less probable. Therefore, we can conclude that the improvement is achieved primarily by fracturing at lower temperature. UHV conditions are necessary only to keep the fracture faces clean. Low-temperature surfaces become contaminated, either by bad vacuum or by fracturing outside the vacuum chamber. Water, which is the main contaminant, condenses according to the physicochemical properties of the exposed surfaces, that is, crystallites may be produced which look like membrane particles. This phenomenon has been repeatedly observed (8, 16, 20) and will be described quantitatively elsewhere. But this condensation on selected surface areas indicates also that “specific” decoration is possible at very low temperatures. It enables the use of “contamination” as a label for particular physicochemically defined regions on the fracture faces. Therefore, if membrane structure is to be portrayed at highest resolution, or certain surface sites marked with crystallites of suitable materials, UHV is an indispensable prerequisite.

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REFERENCES

1. BACHMANN, L., R. ABERMANN, and H. P. ZINSHEIM. 1969. Hochauflösende Gefrierätzung. Histochemie. 20:133–142.
2. BRANTON, D. 1969. Membrane structure. Annu. Rev. Plant Physiol. 20:209–238.
3. BRANTON, D. 1971. Freeze-etching studies of membrane structure. Phil. Trans. Roy. Soc. London, Ser. B. 261:133–138.
4. BULLIVANT, S., and A. AMES. 1966. A simple freeze-etch replication method for electron microscopy. J. Cell Biol. 29:435–447.
5. BULLIVANT, S. 1974. Membranes. Freeze-etching techniques applied to biological membranes. Phil. Trans. Roy. Soc. London, Ser. B. 268:5–14.
6. CHALCROFT, J. P., and S. BULLIVANT. 1970. An interpretation of liver cell membrane and junction structure based on observation of freeze-etch replicas of both sides of the fracture. J. Cell Biol. 47:49–60.
7. CLARK, A. W., and D. BRANTON. 1968. Fracture faces in frozen outer segments from the guinea pig retina. Z. Zellforsch. Mikros. Anat. 91:586–603.
8. DEAMER, D. W., R. LEONARD, A. TARDEI, and D. BRANTON. 1970. Lamellar and hexagonal lipid faces visualized by freeze-etching. Biochim. Biophys. Acta. 219:47–60.
9. DUNLOP, W. F., and A. W. ROBARDS. 1972. Some artifacts of the freeze-etching technique. J. Ultrastruct. Res. 40:391–400.
10. GROSS, H. 1977. Gefrierätzung im Ultrahochvakuum (UHV) bei −196°C. Diss. 5581, ETH-Zürich, Switzerland.
11. MOOR, H., K. MUEHLETHALER, H. WALDNER, and A. FREY-WEYSSLING. 1961. A new freezing ultramicrotome. J. Biophys. Biochem. Cytol. 10:1–13.
12. MOOR, H., and K. MUEHLETHALER. 1963. Fine structure in frozen-etched yeast cells. J. Cell Biol. 17:609–628.
13. MOOR, H. 1964. Die Gefrier-Fixation lebender Zellen und ihre Anwendung in der Elektronenmikroskopie. Z. Zellforsch. Mikros. Anat. 62:546–580.
14. MOOR, H. 1969. Beitrag der Gefrierätzmethode zur Aufklärung von Struktur und Funktion der Biomembranen. Ber. Disch. Bot. Ges. 82:385–396.
15. MOOR, H. 1970. High resolution shadow casting by the use of an electron gun. Proc. Int. Congr. Electron Micros., 7th. 1:413–414.
16. MOOR, H. 1971. Recent progress in the freeze-etching technique. Phil. Trans. Roy. Soc. London, Ser. B. 261:121–131.
17. SLEYTR, U. B. 1974. Freeze-fracturing at liquid helium temperature for freeze-etching. Proc. Int. Congr. Electron Micros., 8th. 2:30–31.
18. SLEYTR, U. B., and W. UMBRATH. 1974. A simple device for obtaining complementary fracture planes at liquid helium temperature in the freeze-etching technique. J. Microsc. (Paris). 101:187–195.
19. SLEYTR, U. B., and W. UMBRATH. 1976. Freeze-etching: technical developments and general interpretation problems. Proc. Eur. Reg. Conf. Electron Micros., 6th. 2:50–55.
20. STAHELIN, L. A., and W. S. BERTAUD. 1971. Temperature and contamination dependent freeze-etch images of frozen water and glycerol solutions. J. Ultrastruct. Res. 37:146–168.
21. STEER, R. L. 1957. Electron microscopy of structural detail in frozen biological specimens. J. Biophys. Biochem. Cytol. 3:45–60.
22. STEER, R. L. 1969. Freeze-etching simplified. Cryobiology. 5:306–323.
23. STEER, R. L., and M. MOSELEY. 1969. New dimensions in freeze-etching. Ann. Proc. Electron Microsc. Soc. Am., 12th. 202–203.
24. WEBER, E., K. MUEHLETHALER, and H. MOOR. 1970. Membrane structure as seen with a double replica method for freeze-fracturing. Exp. Cell Res.
25. ZINGSHEIM, H. P., R. ABERMANN, and L. BACHMANN. 1970. Shadow casting and heat damage. Proc. Int. Congr. Electron Micros., 7th. 1:411-412.

26. ZINGSHEIM, H. P. 1972. Membrane structure and electron microscopy. The significance of physical problems and techniques. (Freeze-etching). Biochim. Biophys. Acta. 265:339-366.