Published November , 1976

A SIMPLE, SELECTIVE METHOD FOR FREEZE-FRACTURING SPHERICAL CELLS

H. BENJAMIN PENG and LIONEL F. JAFFE. From the Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

The conventional method of preparing single cells for freeze-fracture has involved freezing a cell pellet on a carrier. Then, the specimen is cleaved with a cold knife to expose the fracture faces. This method has several disadvantages. First, by the formation of a cell pellet, the information on the orientation of individual cells is lost and, consequently, it is difficult to determine the location of the fracture face with respect to the whole cell. Second, the cleaving process often leaves knife marks on the fracture face, and at these positions the morphological information is completely lost. Thus, the exposure of large intact fracture faces is a fortuitous event and difficult to reproduce.

Recently, several workers have reported alternative methods for freeze-fracturing cells in tissue culture or in monolayer (2, 3, 7). All these methods involve growing or plating the cells on a piece of thin metal or coverglass. A second metal piece is then applied to the top, and the whole sandwich with the cells in the middle is frozen. The top part is ripped away and this produces the fracture. Although this method works satisfactorily on flat cell layers, it has only limited use on spherical cells because the contact area between these cells and the substratum is very small.

In this paper, we present a simple and selective method to freeze-fracture large spherical cells with a diameter greater than 70 μm. We have used this method successfully to study the development of the fucoid egg (6).

MATERIALS AND METHODS

Eggs

Eggs of the monoecious alga *Pelvetia fastigiata* were used in this study. Ripe fronds of this alga were collected

Published November , 1976

for us at Pacific Grove, Calif. Fertilized eggs were obtained by the method of Jaffe and Neuscheler (4). These eggs are 100-μm spheres during the early hours of development. Shortly after fertilization, single eggs released from the oogonial sacs were collected by filtering the egg suspension through a Nitex screen (Tobler, Ernst and Traber, Inc., New York) with 130-μm openings. The eggs were then washed several times with sea water by letting them fall to the bottom of a beaker and decanting the supernate.

Filling the Nickel Screen

Washed eggs were loaded into a nickel screen (Perforated Products, Brookline, Mass.). These screens have many closely spaced, round holes. The holes are funnel shaped and eggs fit snuggly into the holes as shown in Fig. 1. The filling apparatus is schematically shown in Fig. 2. It consists of a set of Plexiglas chambers which are connected to a sink at a lower level. A screen is affixed to the opening of each chamber by a tiny bit of high-vacuum silicone grease. A gentle gravitational flow from the reservoir through the screen and the chamber to the sink is started. The flow is regulated by a capillary valve (Gilmont Instruments, Great Neck, N. Y.). A dilute suspension of eggs is then poured into the reservoir, and the cells are kept in suspension by agitating them gently with a medicine dropper. The flow gently draws the eggs into the screen's holes and holds them there. Excess eggs which sediment on the surface of the screen are "blown" off gently with a medicine dropper. The flow is turned off and the cells are left in place until they grow to the desired stage. The screens themselves are 1-cm disks with about 1,800 holes available for the cells. With this filling method, more than 90% of the holes are filled.

Until 5 h after fertilization, the eggs tend to drop off from the screen when the screen is lifted from the medium. Thus, in order to work with young eggs, we coated the filling face of the screen with a layer of poly-L-lysine (Sigma Chemical Co., St. Louis, Mo.), which is highly positively charged. A small drop of poly-L-lysine at 250 μg/ml was spread on the screen and was air-dried. This cationic film provides an excellent binding between the screen and the eggs, and virtually no eggs fell off when the screen was later manipulated. This treatment does not cause any damage to the cells as the eggs develop normally on a screen with such a coating. No cationic coating is needed to hold older eggs in the holes.

Freeze-Fracture

At a desired stage, the cell-bearing screen was removed from the culture chamber. The cells were not prefixed or treated with cryoprotectant. A "hat" was applied to the screen either to the filling side or to the opposite side. These hats were made of thin copper, similar to those used by Collins et al. (2), except that ours were much bigger. As shown in Fig. 3, each hat has a small hole in its handle, and a fine platinum wire (0.1 mm) with a small bead at one end, formed by melting the end in a high-temperature flame, was threaded through this hole. Four small holes were also drilled through the base of the hat. These holes, when filled with ice upon freezing, enable a hat to grip the cells strongly during fracturing. The hats were sonicated in sea water to free air from the holes before each experiment. After a hat was applied to the screen, excess fluid under the screen and around the hat was removed with filter paper. The hat was then lifted up and, because the screen is very light, the surface tension of water held it to the hat. The whole sandwich was then plunged quickly into solidifying Freon 22. The frozen assemblies were stored under liquid nitrogen until ready to fracture.

To selectively obtain E and P fracture faces, we used two kinds of screens. For exoplasmic face (EF), i.e., the half of the plasma membrane facing the exoplasmic

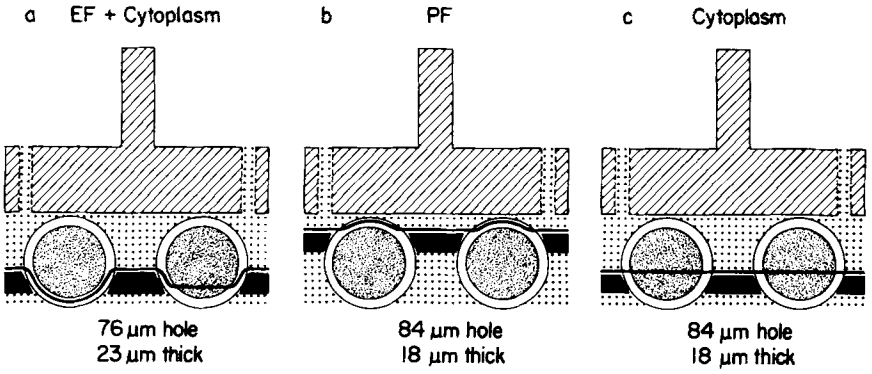


FIGURE 1 Schematic diagram of the hat-screen method. The eggs sit in the holes of a nickel screen (solid black) with the indicated dimensions. The hat is applied to either side of the screen to produce fractures which expose regions indicated above each figure. *EF*, exoplasmic face; *PF*, protoplasmic face. Ice phase (stippled) continues into the holes on the hat. Solid lines indicate the fractures. The hats are not drawn to scale.

Published November , 1976

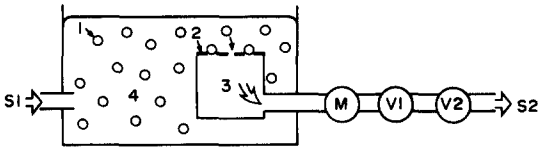


FIGURE 2 Filling chamber. A dilute egg suspension is poured into a sea water reservoir (*4*) made from a plastic petri dish. A gravitational flow (open arrows), which has been started earlier, draws the eggs (*1*) into the holes of a nickel screen (*2*) and anchors them there. The screen is fixed to the top of a Plexiglas chamber (*3*) with high-vacuum silicone grease. The flow is regulated by a three-way valve (*V1*) and a capillary valve (*V2*). A tubing manifold (*M*) allows one to fill six screens at a time. *S1*, sea water supply; *S2*, sink.

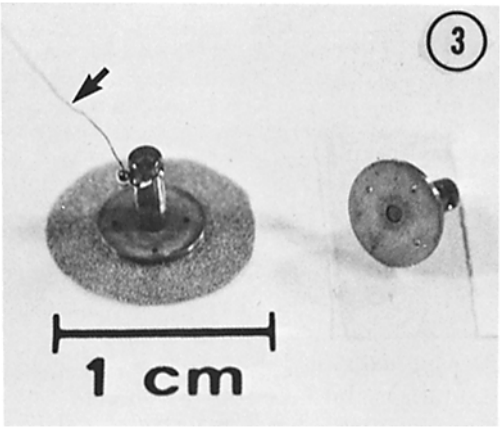


FIGURE 3 The hat and screen assembly. Note the holes on the hat and the pulling wire (arrow).

space, screens with a thickness of 23 μm and a hole diameter of 76 μm were used, and the hat was applied to the screen's filling face, i.e., the face with larger cell protuberances (Fig. 1*a*). For protoplasmic face (PF), i.e., the half of the membrane facing the cytoplasm (according to the new nomenclature [1]), screens with a thickness of 18 μm and a hole diameter of 84 μm were used, and the hat was applied to the antifilling face, i.e., the face with smaller cell protuberances (Fig. 1*b*). The eggs of about 100-μm diameter protrude 6% farther from the antifilling face of a screen with larger holes. This increase provides the necessary clearance for the exposure of PF from the face with smaller cell protuberances. To obtain extensive fractures through the cytoplasm, the hat was applied to the filling faces of screens with larger holes (Fig. 1*c*).

To fracture, the hat-screen assembly was transferred to the standard 4-specimen stage of a Balzers BA 360 M freeze-etch device. The screen was held down with a specially made clamping cap. The platinum wire on the hat was fastened to the knife holder. The specimen was fractured by pulling the hat off the screen with the rotary movement of the knife, which had been cooled down before this step. The fracturing was done at a stage temperature of −130°C and under a vacuum better than 2×10^{-6} torr. The fracture face of the screen was immediately replicated with platinum and carbon.

The replica was freed as follows: the screen was thawed and floated on 2.5 M NaOH. The solution was heated up gently to 80°C in a water bath for 1–2 h. Then, the screen was cleaned with distilled water. After this step, the replica could usually be floated on 70% sulfuric acid by slowly introducing the screen into acid at a shallow angle. The screen was taken out immediately after the replica was freed. We routinely obtain large replicas (especially EF ones) with intact areas up to 5-mm wide.

After acid digestion, a replica is cleaned with distilled water and picked up on a grid. To avoid shattering the replica during transfer between solutions, we carried the replica on a piece of nickel screen and lowered it gently onto the surface of a new solution. This procedure avoided the initial shock due to concentration difference, which easily shatters replicas.

With screens heavily coated with poly-L-lysine, it was normally impossible to free the replica from the screen. Such a screen, after extensive NaOH digestion, was floated on 70% sulfuric acid for a brief period of time (5 to 10 min). Then, it was rinsed with distilled water and dried. This procedure was normally enough to yield clean replicas. Disks, 3 mm in diameter, at the region occupied by the replica were punched out of the screen with a clean grid punch. Since the replicas of the cell fractures lay right over the screen holes, these screen disks provided excellent grids for carrying the replicas. The disks were sometimes coated with formvar to strengthen the replica. All replicas were examined with a Philips EM300 electron microscope. The shadowing direction for all electron micrographs, except Fig. 4 and Fig. 7, run roughly from the bottom of the picture to the top.

RESULTS

Fig. 4 shows a representative fracture obtained with the method shown in Fig. 1*a*; i.e., the hat was applied to a 76-μm hole screen on the side with larger cell protuberances. Nearly every cell underneath the hat was fractured and replicated. This yields hundreds of single-cell replicas of both the plasma membrane and the cytoplasm, located exactly in the holes as shown in Fig. 4. Fig. 5 shows the E face of a small portion of the plasma membrane obtained with this method. If screens with 84-μm holes were used and the hat was applied to the side with smaller cell protuberances as shown in Fig. 1*b*, then large areas of P face were exposed (Fig. 6); if these same screens were

Published November , 1976

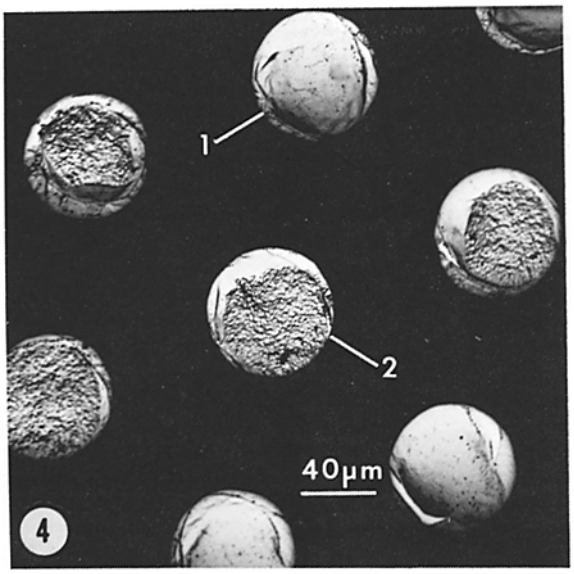


FIGURE 4 Electron micrograph showing replicas in the screen of several fractured cells. Large, intact replicas of plasma membrane (*1*) and cytoplasm (*2*) can be easily obtained. Solid black region is the screen. × 250.

used but the hat applied to the opposite side, i.e., the side with larger cell protuberances (Fig. 1*c*), extensive cytoplasmic fracture faces were invariably obtained. An example is shown in Fig. 7 and detailed in Fig. 8. Since the size of the fracture face is physically determined by the size of the holes, intact single cell replicas, either of the plasma membrane or of the cytoplasm, with a diameter of 70–80 μm can be easily obtained with this method.

DISCUSSION

In this paper we have demonstrated a simple method for freeze-fracturing large spherical cells. This method has the following distinctive advantages over the conventional method (5). (*a*) Superb freezing rates can be achieved because the nickel screen is very thin and the cells touch the freezing agent directly. This enables us to achieve excellent ultrastructural preservation of these marine eggs without prefixation and without using a cryo-protectant. (*b*) Selective exposure of either PF or EF of the plasma membrane or of the cytoplasm can be achieved simply by varying the hole size of the screen and by applying the hat to either side of the screen. (*c*) Large pieces of intact replica can be routinely obtained. (*d*) The information on the position of the fracture with respect to the whole cell is constantly preserved. For cells whose polarity can be defined while they are on the screen, e.g., *Pelvetia* eggs in this study, this method provides an easy means to obtain fractures at a desired orientation and location.

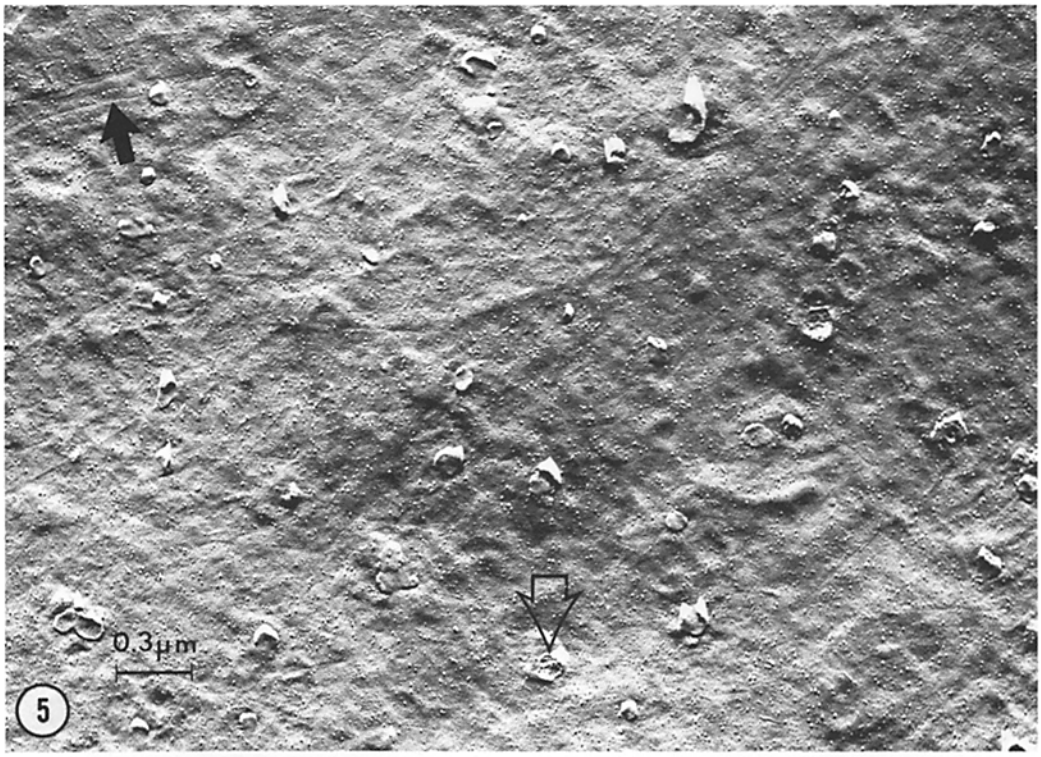
Our method of using a metal "hat" as the top part of the sandwich closely resembles the methods used by Collins et al. (2), Pfenninger et al. (7), and Fisher (3). These workers have used the sandwich method to freeze-fracture cells in tissue culture or in a monolayer. Our present method further advances its application to spherical cells or spherical cell masses. Since the screens are obtainable in a variety of hole sizes, this method can be easily applied to cells with a wide size range.

Pelvetia embryos have a rigid cell wall as thick as 1 μm outside their plasma membranes. Our results indicate that such a cell coating presents no difficulty in obtaining fractures through the plasma membrane or the cytoplasm. On the other hand, this method is equally applicable to unfertilized or newly fertilized eggs which have naked cell membranes or only a very thin wall. These two conditions should cover almost all plant or animal cells with a spherical shape.

SUMMARY

A simple and selective method for freeze-fracturing spherical cells is described. The cells are loaded into the holes of a thin nickel screen. A

Published November , 1976



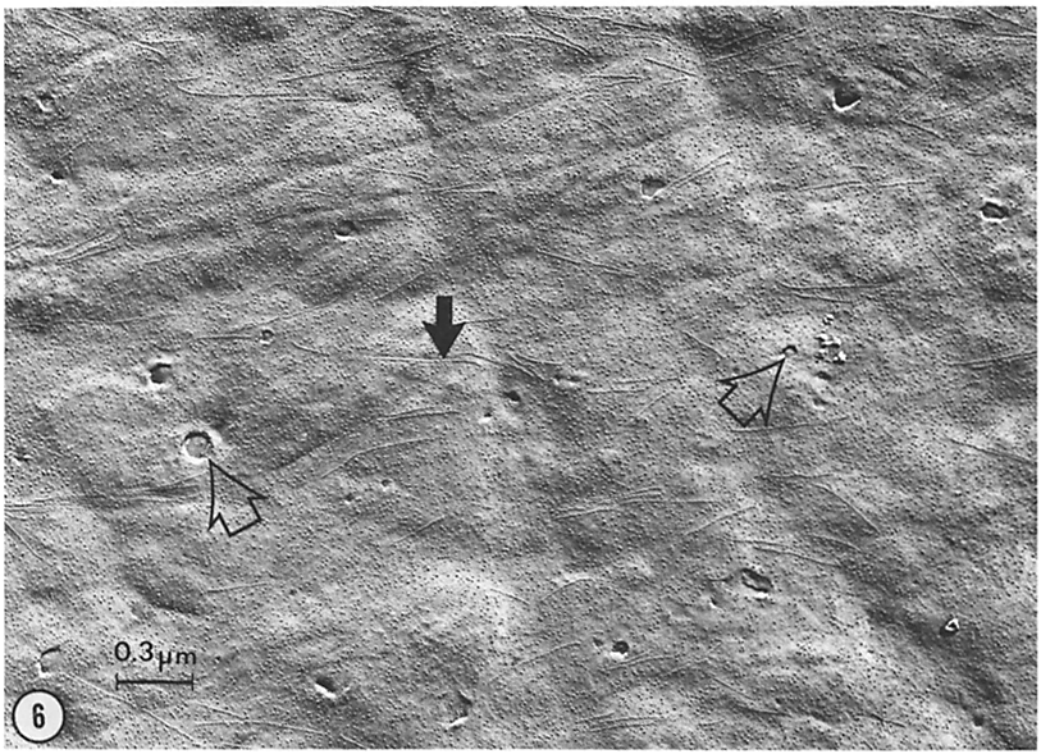


FIGURE 5 E face of the plasma membrane obtained with the method shown in Fig. 1*a* (the cell on the left side). Complementary depressions of particle strings (solid arrow) can be seen. The structure pointed out by open arrow is the opening of a secretory vesicle. Compare with the P-face images in Fig. 6. × 34,000.

FIGURE 6 P face of the plasma membrane obtained with the method shown in Fig. 1*b*. Particle strings (solid arrow) and openings of secretory vesicles (open arrows) are conspicuous membrane structures in addition to isolated 10-nm membrane particles. × 36,000.

Published November , 1976

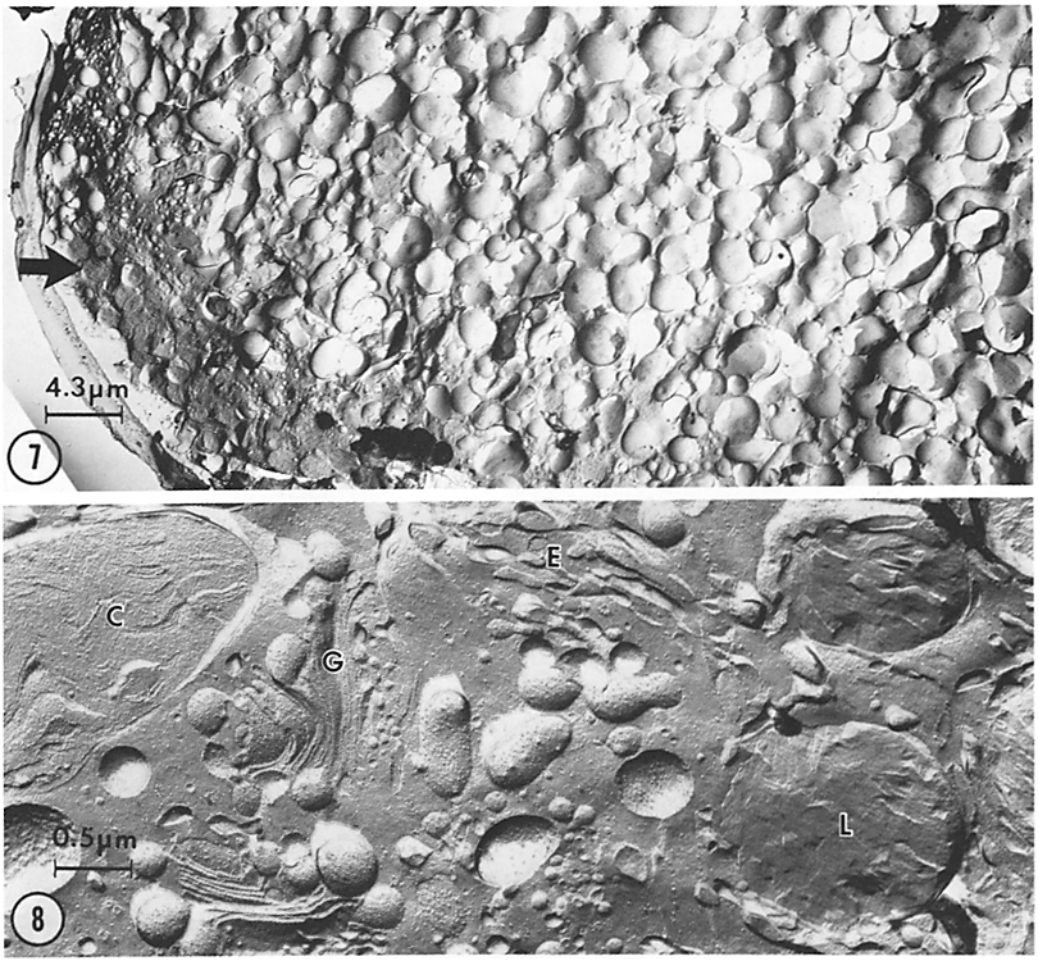


FIGURE 7 A fracture through the cytoplasm obtained with the method shown in Fig. 1*c*. The cytoplasm was stratified by centrifugation before the experiment. The centripetal pole is to the left. Numerous lipid bodies (arrow) are aggregated at this pole. It is interesting to note that all the lipid bodies are cross fractured. × 2,300.

FIGURE 8 The details of a region at the centripetal end of the cell as shown in Fig. 7. *C*, chloroplast, cross fractured; *E*, cisternae of the endoplasmic reticulum; *G*, Golgi apparatus; *L*, lipid bodies. × 22,000.

metal hat is applied to the cell monolayer and the whole assembly, hat-cells-screen, is frozen and then fractured by ripping the hat off. The fractured face on the screen is replicated. By varying the size of the screen holes and by applying the hat to either side of the screen, this method can selectively expose the E face (or the outer half of the plasma membrane), the P face (or the inner half of the plasma membrane), or the cytoplasm of the cells. It also provides a means to produce fractures at a preselected area on the cell, if the cells can be loaded onto the screen in an oriented fashion.

We thank Mr. T. Rand Collins, Mrs. Caroline Schooley, and Dr. Kenneth R. Robinson for technical advice during the course of this work. We also thank Dr. Peter Satir, Dr. Birgit Satir, and Dr. Rudolf Schinz for valuable comments.

This work was supported by a National Institutes of Health biophysics training grant to H.B.P. and a National Science Foundation research grant to L.F.J.

This paper is from the Ph.D. Thesis of H.B.P.

Received for publication 26 March 1976, and in revised form 8 July 1976.

REFERENCES

1. BRANTON, D., S. BULLIVANT, N. B. GILULA, M. J. KARNOVSKY, H. MOOR, K. MÜHLETHALER, D. H. NORTHCOTE, L. PACKER, B. SATIR, P. SATIR, V. SPETH, L. A. STAEHLIN, R. L. STEERE, and R. S. WEINSTEIN. 1975. Freeze-etching nomenclature. *Science (Wash. D. C.)*. **190**:54–56.
2. COLLINS, T. R., J. C. BARTHOLOMEW, and M. CALVIN. 1975. A simple method for freeze-fracture of monolayer cultures. *J. Cell Biol.* **67**:904–911.
3. FISHER, K. A. 1975. "Half" membrane enrichment: verification by electron microscopy. *Science (Wash. D. C.)*. **190**:983–985.
4. JAFFE, L. F. and W. NEUSCHELER. 1969. On the mutual polarization of nearby pairs of fucaceous eggs. *Dev. Biol.* **19**:549–565.
5. MOOR, H., and K. MÜHLETHALER. 1963. Fine structure of frozen-etched yeast cells. *J. Cell Biol.* **17**:609–628.
6. PENG, H. B. 1976. Polarization of the fucoid egg: galvanotropism and ultrastructure. Ph.D. Thesis. Purdue University, West Lafayette, Indiana.
7. PFENNINGER, K. H., and E. R. RINDERER. 1975. Methods for the freeze-fracture of nerve tissue cultures and cell monolayers. *J. Cell Biol.* **65**:15–28.