Finally, let C be the positively oriented unit circle and D be the open unit disk. Suppose that S is a subset of C of measure 2π, and that at every point $\zeta_0 \in S$ there is an arc Λ_{ζ_0} in D along which the Cauchy-type integral

$$F(z) \equiv \frac{1}{2\pi i}\int_C \frac{f(\zeta)d\zeta}{\zeta - z}, \qquad (z \in D),$$

where $f(\zeta)$ is defined and summable on C, tends to a limit $\varphi(\zeta_0)$.

THEOREM 2. *If the values of the function $\varphi(\zeta)$ ($\zeta \in S$) coincide almost everywhere on C with the values of a function that is continuous on C, then $F(z)$ is continuous on $C \cup D$.*

Proof: Because of the particular form of C, the function $F(z)$ has an angular limit at almost every point of C,[3] and hence at almost every point of S. It follows again, from the ambiguous-point theorem, that at almost every point $\zeta_0 \in S$, this angular limit has the value $\varphi(\zeta_0)$. This means that the angular limits of $F(z)$ coincide almost everywhere on C with the values of a function that is continuous on C. It is known[4] that this implies the continuity of $F(z)$ on $C \cup D$.

* Sponsored by the Mathematics Research Center, U.S. Army, Madison, Wisconsin, under contract no. DA-11-022-ORD-2059.

[1] See Golusin, G. M., *Geometrische Funktionentheorie* (Berlin, 1957), p. 379, Theorem. 1.

[2] Bagemihl, F., "Curvilinear cluster sets of arbitrary functions," these PROCEEDINGS, **41**, 379–382 (1955), Corollary 1, preceded by a conformal mapping of a region complementary to J onto the unit disk.

[3] Priwalow, I. I., *Randeigenschaften analytischer Funktionen* (Berlin, 1956), p. 137.

[4] *Ibid.*, p. 139.

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*FRACTURE FACES OF FROZEN MEMBRANES**

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The biological membrane, according to one widely accepted concept, has as its framework a bimolecular leaflet which under appropriate conditions can be seen in the electron microscope as two dark 20-Å-thick layers separated by a lighter 35-Å-thick layer.[1] Well-known theories and evidence[2–9] suggest that this structure is composed of a bimolecular leaflet of oriented lipid molecules sandwiched between two layers of protein. Though Robertson[10] has formalized these ideas as the basis of his generalized unit membrane concept, new chemical[11–13] and structural[10, 12, 14–16] evidence requires that other molecular arrangements also be considered. This has been the case in several recently proposed membrane models. Though some of these models take as their starting point the general notion of a bimolecular leaflet[17, 18] and others take as a starting point a repeating particulate subunit,[11, 14, 16] they all emphasize the possibility of dynamic interrelations between the several membrane components and explicitly deny the notion of a biological membrane which is spatially and temporally uniform.

The structural implications of these recent models are difficult to study, as there are few high-resolution techniques which can be used to examine rapidly changing forms. However, the recently improved freeze-etching technique[19, 20] should provide a direct view of membrane structure. Since this method does not involve the use of chemical fixatives or stains and the freeze-fixation employed need not kill the cells,[21] one can study membranes as they respond to a given physiological environment, rather than as they respond to a fixative environment. Furthermore, surfaces exposed in freeze-etching are three-dimensional fractures in which spatially extended areas of membranes can be examined.

Initial experiments with freeze-etching have demonstrated its applicability to a wide variety of biological specimens.[21-25] This report presents a more detailed interpretation of the fractured membrane faces exposed by this technique. Preliminary observations of freeze-etched root tip cells[24] revealed membrane faces whose morphological features could not be equated with the known features of membrane surfaces. The investigations reported here have explained this by demonstrating that what is usually considered as the true membrane surface (the interface between a membrane and any contiguous protoplasm, cell wall, or vacuolar material) is rarely seen in freeze-etched preparations. Instead, the fracture process splits the membrane and exposes an internal membrane face.

Materials and Methods.—Adventitious roots of onion sets (*Allium cepa* L., var. White Globe) were used for most of the experiments. They were grown in 20% glycerol.[24] Other experiments involved *Porphyridium cruentum* (Ag.) Naeg. or *Saccharomyces* sp. The unicellular red alga *P. cruentum* (Indiana University culture collection) was grown as described elsewhere.[26] *Saccharomyces* sp. cells (Fleischmann's yeast cakes) were separated by centrifugation from the starch used as a binder in the cakes and suspended in fresh tap water for 2 hr.

In preparation for freeze-etching, onion roots were cut in half lengthwise. A 1–2-mm piece of the half tip was placed in a thin syrup of gum arabic dissolved in 20% glycerol, transferred to a 3-mm copper disk, and then rapidly frozen in liquid Freon 22 (chlorodifluoromethane). Gum arabic helped to cement the frozen root tip to the copper disk but had no observable effect on cell ultrastructure. The *Porphyridium* and *Saccharomyces* cells were collected by centrifugation. Small droplets of the cell pellet were placed on copper disks and frozen in Freon 22.

The frozen specimens were freeze-etched (Fig. 1) as described by Moore *et al.*[20, 22] In some experiments the amount of etching applied to the fractured surface was varied by manipulating either the etching time or the etching temperature, or both. "Normal etching" was accomplished by leaving the freshly fractured specimens *in vacuo* (less than 2×10^{-6} torr) for 1–5 min at −100°C. "No etching" was accomplished by keeping the temperature of freshly fractured surfaces below −165°C and by replicating the surfaces as rapidly as possible (less than 10 sec) after they were fractured. "Deep etching" was accomplished by leaving the freshly fractured surfaces *in vacuo* for 10 min at −95°C.

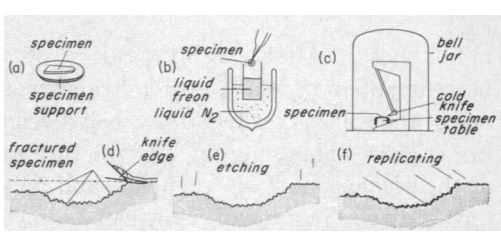
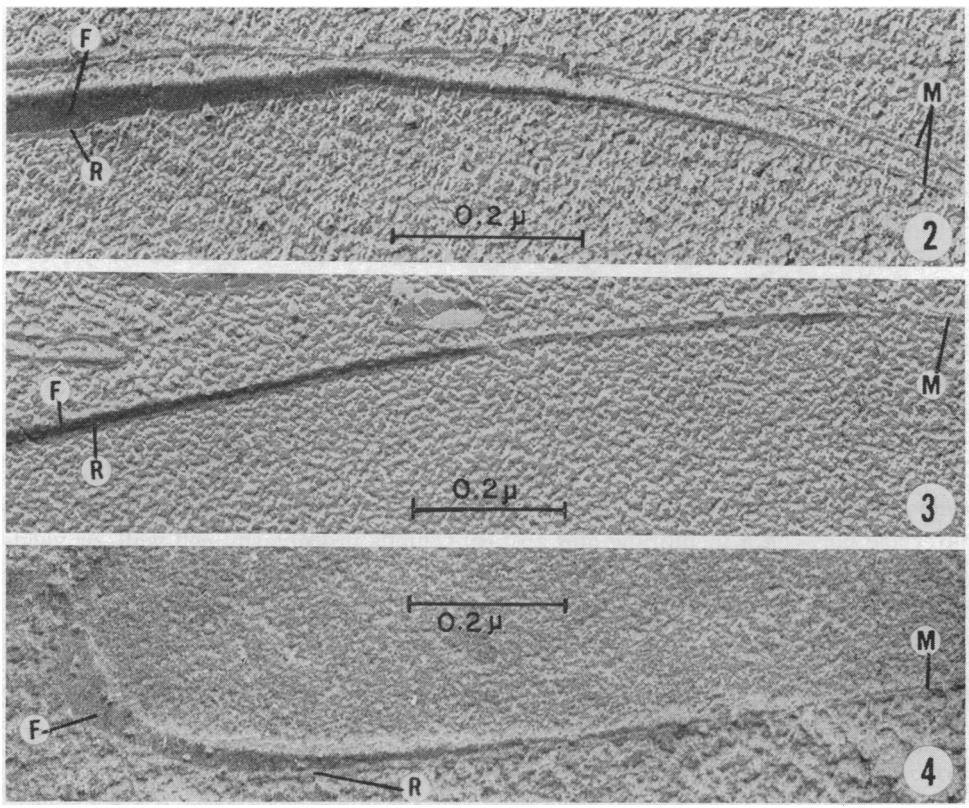


FIG. 1.—Freeze-etching. (*a*) The fresh specimen was placed on a Cu disk, (*b*) rapidly frozen in liquid Freon 22, and (*c*) placed in the precooled freeze-etching vacuum chamber. (*d*) The frozen specimen was fractured with a microtome knife at −185°C, and in some cases (*e*) the freshly fractured surface was etched. (*f*) The surface was shadowed and replicated with Pt and C.

All micrographs have been printed so that shadows appear as light areas and have been mounted for publication with shadows extending from bottom to top.

Results.—Basic observations: A consistent feature in all freeze-etched preparations is the presence of a small ridge (Figs. 2–4) at the base of most exposed mem-



FIGS. 2–4.—FIG. 2.: Endoplasmic reticulum in onion root tip. FIG. 3: Vacuolar membrane face in onion root tip; view from inside the vacuole. FIG. 4: Vacuolar membrane face in onion root tip; view from outside the vacuole. In Figs. 2, 3, and 4, the fractures are tangent to the membrane surfaces on the left and almost perpendicular to the membrane surfaces on the right. The small ridge (*R*) at the base of an exposed membrane face (*F*) on the left is continuous with one of two ridges which forms the typical freeze-etch image of a single membrane (*M*) on the right.

brane faces. After careful scrutiny of a large number of photomicrographs, it became apparent that the small ridge was in fact continuous with and identical to one of the ridges that had previously been assumed to represent part of a unit membrane.[22, 24] This same type of fracture was observed in freeze-etched preparations of the plasma, nuclear, vacuolar, and dictyosomal membranes. Figure 5 is an interpretative diagram of what can be seen in the micrographs of Figures 2–4. It implies that during fracturing, membranes are split to expose either one or the other

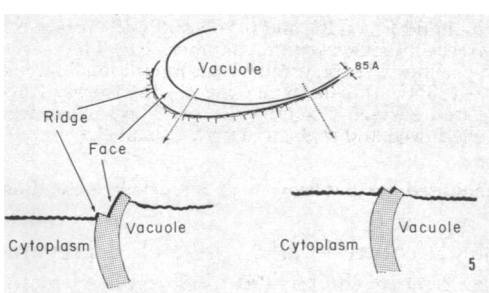


FIG. 5.—*Top*, a representation of Fig. 4. *Bottom*, diagrams of imaginary sections perpendicular to the plane of the page through the fractured tissue along the *dashed arrows*. These diagrams assume arrival of shadow-replica material from upper left, and show why fracture of an inclined *ca.* 75-Å-wide single membrane frequently produces the freeze-etch image seen in Figs. 2–4.

of two nonetchable inner faces. Since the diagram is intended as a generalized scheme, it also implies that freeze-etching will show neither the true membrane surfaces nor the surfaces of any materials contiguous to membranes. These implications have been verified by the experiments and observations described below.

Etching variations: Two sets of onion root tips were freeze-etched. In order to control the amount of water sublimed from the fractured surface, one set received no etching, whereas the other set received normal etching. Figure 6 shows cells which received no etching, and Figure 7 shows cells which received normal etching. Comparison reveals a striking similarity in the over-all appearance of the membrane faces in spite of clear differences in the textural appearance of the rest of the protoplasm. Whereas etching had little apparent effect on the membrane faces, it gave the protoplasmic matrix a distinct pebbled appearance. In other words, water was sublimed from the fractured cytoplasmic matrix, karyoplasm, and vacuole, but not from any of the faces along which the fractures followed membrane contours. This suggested the prediction, diagrammed in Figure 8, that deep etching would reveal more of the true membrane surface than had normal etching. Since etching removes primarily water and not other cell constituents, the effects of deep etching could best be seen in yeast cells which had been suspended in plain tap water for 2 hr. Figure 9 is the result of such an experiment and shows that with deep etching a greater portion of the true membrane surface is exposed. As anticipated, this is particularly noticeable at the edge of vacuoles, because of the high water content, and consequent etchability, of vacuolar fluid.

Surface features: If freeze-etching splits membranes so as to reveal inner faces rather than the true surface, structural features known to exist on membrane surfaces should not be visible on membrane faces exposed in freeze-etching. Ribosomal particles, frequently associated with the surface of the endoplasmic reticulum in chemically fixed preparations, cannot be seen on the endoplasmic reticulum faces revealed in freeze-etching. Small particles, averaging *ca.* 85 Å in diameter, are

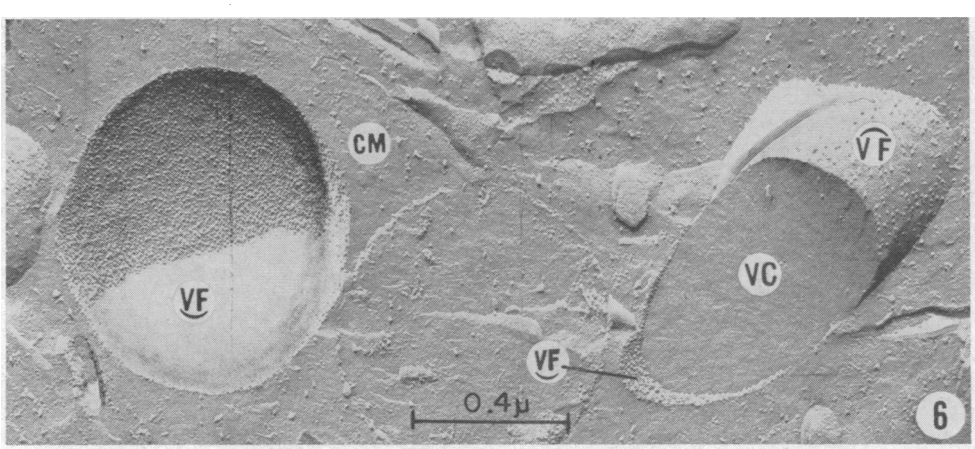


FIG. 6.—Part of an onion root tip cell with no etching. With the exception of a few small protuberances, the surface of the cytoplasmic matrix (*CM*) and vacuolar contents (*VC*) is relatively smooth. Both concave faces (*VF*) and convex faces (*VF*) of vacuolar membranes have been exposed. The vacuole in the upper right has been partially fractured and shows that the concave and convex vacuolar membrane faces had been apposed before fracturing. See Fig. 7.

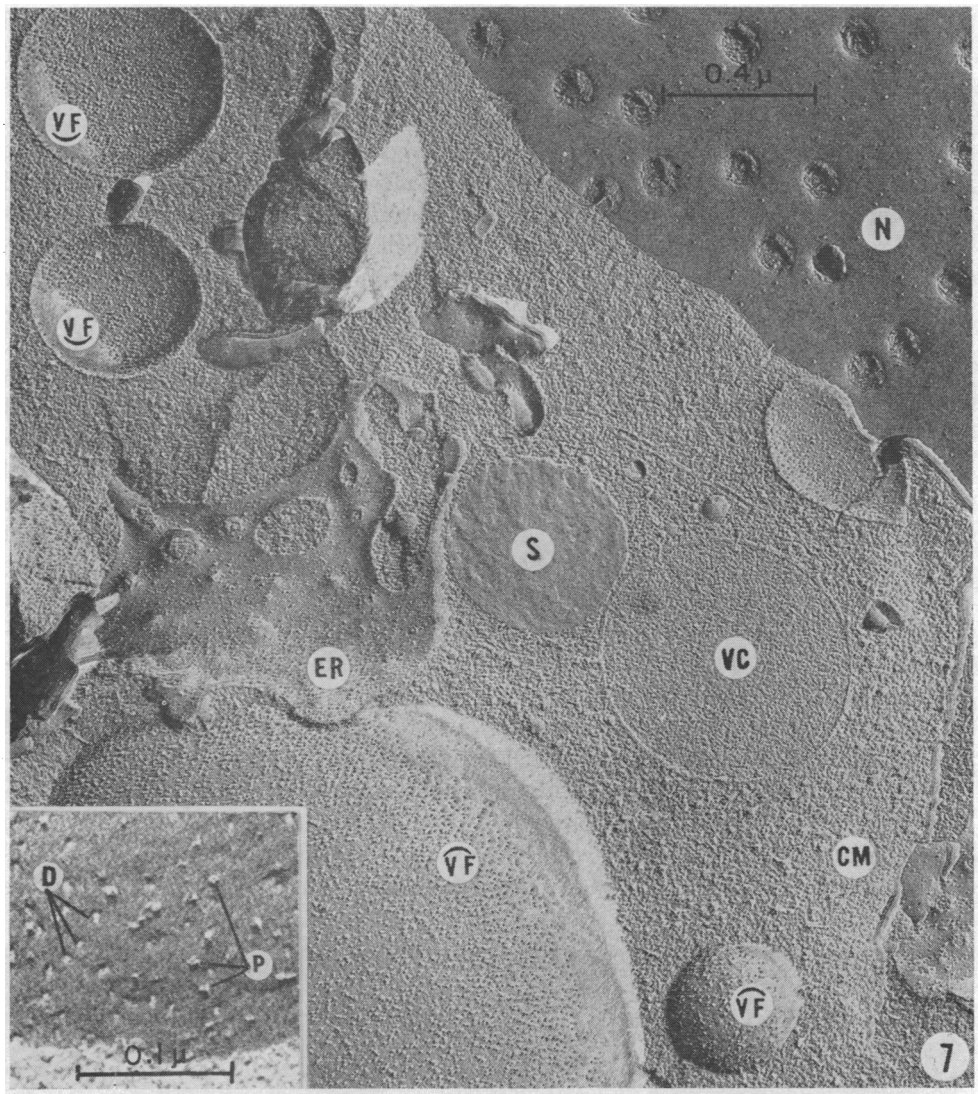


FIG. 7.—Part of an onion root tip cell with normal etching. Surfaces of the cytoplasmic matrix (*CM*) and vacuolar contents (*VC*) are pebbled as the result of etching, but vacuolar membrane faces (*VF*) are similar to those in Fig. 6, indicating that they are nonetchable. Note the smooth, nonetchable appearance of lipoidal material in the spherosomes (*S*) and compare with the smooth portions of membrane faces, including nuclear membrane (*N*) and endoplasmic reticulum (*ER*). Compare with Fig. 6. *Inset* shows a typical vacuolar membrane face and associated small particles (*P*), as well as depressions (*D*) where some of these particles were pulled off during the fracture process.

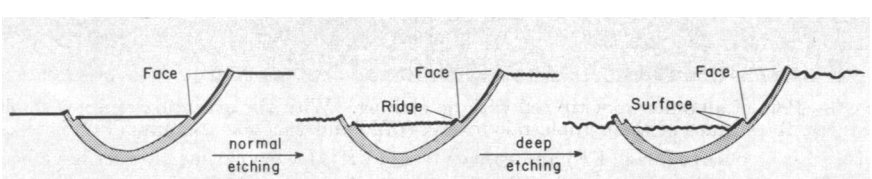


FIG. 8.—Interpretation of the etching procedure.

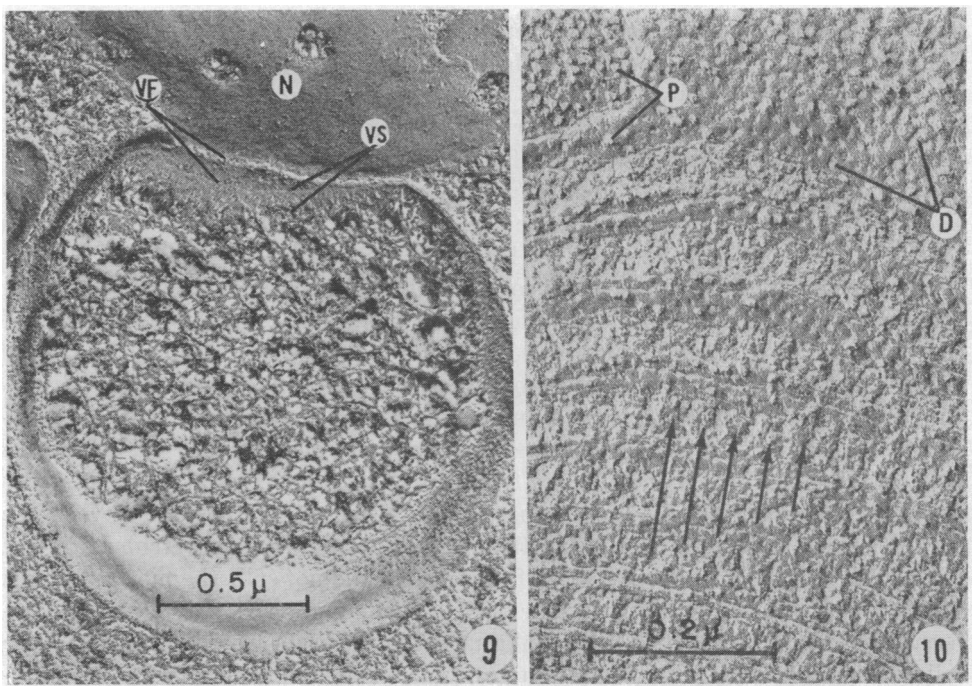


FIG. 9.—Deep etching in a yeast cell. Nuclear (*N*) and vacuolar membrane faces (*VF*) are similar to those seen in normally etched preparations (Fig. 7), but, as predicted in Fig. 8, a portion of the true vacuolar membrane surface (*VS*) has been exposed by the deep etching. Cf. with Fig. 3.

FIG. 10.—Freeze-etched *P. cruentum*, normal etching. Note the regular array of large particles (*arrows*) between the chloroplast membranes, but the absence of these particles on exposed membrane faces. Only smaller particles (*P*) and depressions (*D*) are on the exposed faces.

seen in varying numbers on all of the membrane faces in onion root tip cells (Fig. 7). Similar particles have been reported in other freeze-etch studies.[21-24] As previously suggested,[24] these small particles cannot be equated with ribosomes because they are too small and are found on membranes (including Golgi membranes) which are known from fixed and sectioned preparations to be devoid of ribosomes.[27]

Photomicrographs of *Porphyridium cruentum*[26] show that in these cells 320-Å particles are attached in extremely orderly arrays on the outer surfaces of the chloroplast membranes. Our own sections of chemically fixed *P. cruentum* confirmed these findings. The extraordinary regularity and large size of these particles suggested their use as markers of the outer membrane surface. Figure 10 illustrates the appearance of the plastid membranes in freeze-etched *P. cruentum*. Although the 320-Å particles are seen between the chloroplast membranes, neither these particles nor any depressions out of which they might have been fractured are evident on the tangentially fractured membranes; only smaller, randomly distributed particles and depressions similar to those found in other plant chloroplasts[28] are visible on these fractured faces. These observations confirm the proposition that freeze-etching does not normally expose the true outer membrane surface and indicate that freeze-etching does expose a hitherto unseen inner membrane face.

Discussion.—Three lines of evidence suggest that during freeze-etching, membranes are split in half, revealing either of two internal membrane faces. The first

evidence for this type of fracture was encountered when it became possible to follow the contours of a single membrane which had been fractured almost normally in one part and tangentially in another part. In such preparations (Figs. 2–4) it became clear that the *ca.* 85-Å-thick rim representing a normally fractured membrane was an image formed by the confluence of two ridges, one bordering the base, the other forming the top, of the tangentially fractured portion. It appears unlikely that either of these ridges alone can be considered the entire membrane, as this would reduce the thickness of the biological membrane to less than 40 Å. For similar reasons these confluent ridges cannot be mere eutectic mixtures or organized, but nonmembrane, cytoplasmic components, as this would reduce the dimension of the biological membrane to that of a Euclidean plane.

A second line of evidence arises from the fact that in vitrified cells all of the fracture planes which follow membrane surfaces are nonetchable. Figure 6 shows convex faces over the top of vacuoles and concave faces out of which vacuoles have been removed. Comparison of these faces with analogous faces in Figure 7 shows that all exposed membrane faces appear to be identical whether or not they are etched. Such a result indicates that the fracture process in freeze-etching exposes two nonetchable membrane faces. If the fracture did not split the membrane but separated it from contiguous vacuolar or cytoplasmic material, only one of the exposed faces would be that of the membrane while the other would be that of some cell material such as protoplasmic matrix or vacuolar fluid. If this were the situation, at most one of the two exposed faces—that of the membrane—would be nonetchable. The other face would be that of protoplasmic matrix, vacuolar fluid, etc., and therefore would be etchable (cf. Fig. 6 with Fig. 7).

The third line of evidence rests on the observation that freeze-etched membrane faces do not show the structural features associated with the true membrane surfaces. The absence of the 320-Å particles on freeze-etched *P. cruentum* chloroplast membrane faces, in spite of the demonstrable presence of these particles in chemically fixed and in freeze-etched material, is graphic evidence that the membrane faces exposed after normal freeze-etching are not true membrane surfaces. A completely analogous situation has been observed in attempts to view the outer cell wall surface of yeast and bacterial cells. The outer wall surface of these unicells is rarely exposed by the fracture process but it can be exposed by deep etching.

Though at first it may appear surprising that membranes should split in two, consideration of the same types of evidence which led Danielli and Harvey[4] to postulate the presence of proteins on membrane surfaces leads to the prediction that fractures in vitrified cells might not occur along the true membrane surface. Danielli and Harvey postulated the existence of protein layers on membrane surfaces to account for the lower interfacial tension observed at surfaces of living cells. Though recent evidence[11, 12] suggests that in some membrane systems lipids with highly polar groups may assume the emulsifying roles which Danielli attributed to proteins, any adsorbed emulsifier would not only reduce interfacial tension, but would also provide the membrane interface with mechanical stability.[29] A macromolecular interface such as the surface of a membrane can interact with the surrounding aqueous phase by various types of polar bonds, and must be encased in a thin layer of bound water molecules whose properties merge, gradually, with those of the bulk phase.[18] As a result, it appears unlikely that the surface of a membrane with low

interfacial tension vis-à-vis any contiguous protoplasmic material would present a sharp discontinuity of the sort which would make it uniquely liable to mechanical rupture while in the vitrified state.

Small particles, averaging *ca.* 85 Å in diameter, are seen on many freeze-etch membrane faces (see figures here and in refs. 21–25). One important difficulty presently under study, and one which should serve to emphasize the tentative nature of all freeze-etch interpretations so far proposed, is the absence of an adequate number of depressions (Figs. 7 and 11) into which these particles can be fitted. Although the nature of these small particles is currently being investigated, the tentative hypothesis adopted is that these substructures represent units within which membrane components have assumed globular or micellar configurations (Fig. 11). Such particles may represent lipoprotein associations analogous, perhaps, to those which have been reported in mitochondria,[11, 15] plastids,[12, 16] and other membranes.[14] According to this interpretation, the smooth regions between the 85-Å particles would represent regions in which the membrane components exist as an extended bilayer. The appearance and nonetchability of these smooth faces is similar to that of lipid material seen in spherosomes (Fig. 7). Thus, these smooth regions appear to be free of water and may be lipid faces.

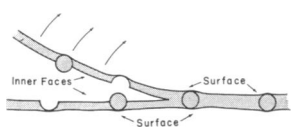


FIG. 11.—Interpretation of the fracture process. The inner membrane faces are seen in normal freeze-etching. Either particles, or depressions out of which the particles have been fractured, are seen (see *inset*, Fig. 7).

Comparative studies show that the number and manner in which the small particles are associated within a given membrane is a function of the type of cell organelle examined.[22, 24] For example, the freeze-etch results shown here indicate that the nuclear membrane in onion root tips (Fig. 7) appears to exist primarily as an extended bilayer, whereas similar freeze-etching studies of chloroplast membranes[28] show that they are composed almost entirely of the globular substructures. The function of these particles in different membranes as well as the environmental factors which modify their configuration is under study.

Summary.—Fracture planes within frozen cell membranes have been examined by freeze-etching. The frozen membrane is fractured so as to expose inner membrane faces. Examination of these faces suggests that the biological membrane is organized in part as an extended bilayer and in part as globular subunits. The relative proportion of the membrane which exists in either of these organizational modes varies among the different cell organelles.

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*THE HILL REACTION OF CHLOROPLASTS ISOLATED FROM GLUTARALDEHYDE-FIXED SPINACH LEAVES**

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In the course of studying effects of various hydrolytic enzymes on chloroplast fine structure, we found it useful to isolate chloroplasts from leaves previously fixed in neutral 6 per cent glutaraldehyde.[1] Chloroplasts isolated in this way were not only morphologically indistinguishable from *in vivo* chloroplasts, as viewed by light microscopy or by electron microscopy on freeze-etched preparations, but also retained the optical rotatory dispersion and absorption spectra of unfixed chloroplasts. These initial observations indicated that the environment of chlorophyll was not greatly changed during glutaraldehyde fixation, and encouraged us to look for photochemical activity in these chloroplasts. In this paper we show that chloroplasts isolated from glutaraldehyde-fixed leaves perform both the ferricyanide Hill reaction, as assayed manometrically by O_2 evolution, and indophenol reduction. The Hill reaction of these fixed chloroplasts is sensitive to reagents such as DCMU, methylamine, and phosphorylation cofactors, and proceeds with an efficiency 25 per cent that of unfixed chloroplasts. The Hill reaction capacity of the fixed chloroplasts is almost unchanged after storage periods of many weeks. These results indicate that quantum conversion and electron transport in photosynthesis are explainable in terms of a rigid protein framework with lipid dispersed through it. Conformational changes as such are apparently not necessary for quantum conversion, O_2 evolution, and electron transport.