MEMBRANE SPLITTING IN FREEZE-ETCHING

Covalently Bound Ferritin as a Membrane Marker

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ABSTRACT

The freeze-etch technique was used to observe red blood cell ghosts labeled on both surfaces with covalently bound ferritin. Ferritin molecules were never observed on fracture faces, thus indicating that fracture does not show membrane-surface detail. Subliming away the surrounding ice did expose the ferritin on the membrane surface. These results were consistent with the concept that membranes split during the fracture process of freeze-etching.

INTRODUCTION

Face views of membranes can be observed in the electron microscope when biological samples are prepared by the freeze-etch technique. These membrane faces have been interpreted as membrane surfaces (10, 12, 13, 21) or alternatively as inner hydrophobic regions within the membrane matrix (1). At issue in these alternative interpretations is a substantial number of results which could contribute significantly to our knowledge of membrane architecture if there were a better understanding of what happens during the fracture process of freeze-etching.

Deamer and Branton (5) used radioisotopic-labeling techniques to show that the fracture process can split artificial membranes within the hydrophobic region between the methyl ends of stearic acid bilayers. We now describe experiments using a morphological label to show that fractures through frozen red blood cell membranes also occur within the membrane matrix and do not expose the morphological features of the membrane surface.

Our approach is based on etching experiments (1, 3, 15). The premise in these experiments is that if the fracture process of freeze-etching occurs within the membrane matrix, it should, nevertheless, be possible to expose the membrane surface by subliming away the surrounding ice (Fig. 1). However, the results of such an experiment are ambiguous unless the true membrane surface is labeled with a marker to differentiate it from other membrane faces. The marker must be large enough to cast a shadow resolvable by the freeze-etch technique, visible in thin sections so that its attachment can be corroborated by an independent technique, and attached to the membrane surface so that it cannot be readily broken away during the fracture process. We chose the protein ferritin because it met all of these requirements. It has a diameter of 120 A in the wet state (8), an iron dense core easily visualized in standard thin sections (8), and groups which can be covalently linked to membrane surfaces with bifunctional reagents (7).

MATERIAL AND METHODS

Ferritin (horse spleen, 6x crystallized, cadmium removed, 100 mg/ml; Pentex Inc., Kankakee, Ill., 60901) was dialyzed overnight in 10 mM Tris/HCl buffer, pH 7.5, and conjugated to the membranes of rabbit red blood cell ghosts by using the bifunctional reagent toluene—2,4—diisocyanate (TC) following

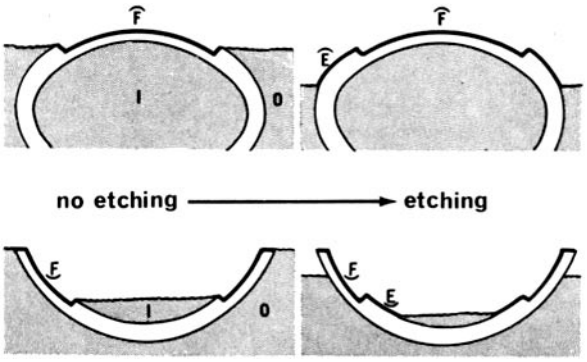

FIGURE 1 Rationale of fracturing and etching red blood cell ghosts, assuming membrane splitting. Without etching, fracture exposes convex ($\hat{F}$) and concave ($\check{F}$) fracture faces. As the ice inside (I) and outside (O) the cell is lowered by etching, convex ($\hat{E}$) and concave ($\check{E}$) etch faces are exposed.

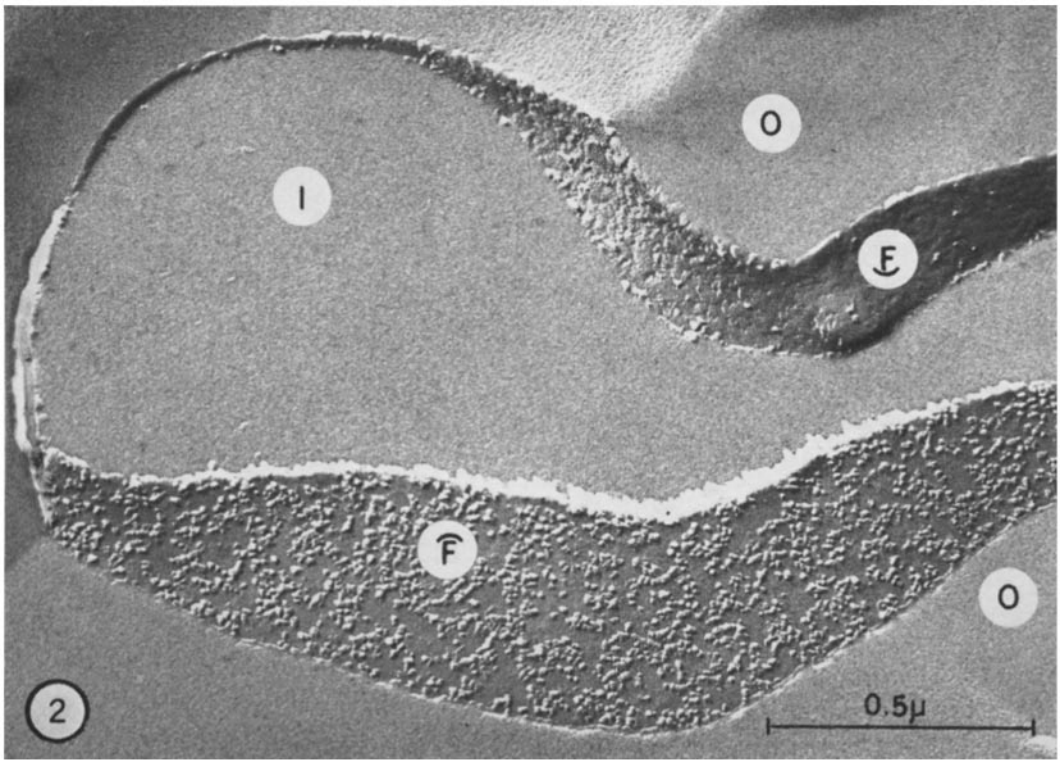

FIGURE 2 Fracture faces of a nonetched control cell. Symbols as in Fig. 1. × 70,000.

the method of Gyenes and Sehon (7). TC was chosen since it produces exclusively covalently linked conjugates (18). Red blood cell ghosts were prepared by the method of Kepner and Macey (9). The reaction mixture contained: 10–20 mg of ferritin, 2 ml of 10 mM Tris/HCl buffer pH 7.50,[1] 0.2 ml of a 1:1 suspension of freshly prepared rabbit red blood cell ghosts washed four times prior to use, and 0.2 ml of a solution containing 20 μl of TC dissolved in 7.5 ml of dioxane. Control mixtures contained no TC or dioxane and 10 times as much ferritin to provide a more rigorous test of the washing procedure. After 10–15 min, the initial pH of the reaction mixture

[1] Similar results were obtained in another series of experiments in which phosphate buffer 7.5 mM, pH 7.5, was used during the entire experimental procedure. The ghosts in these experiments were obtained by lysis in 7.5 mM phosphate buffer, pH 7.5 (6), and the ferritin solution was dialyzed overnight against the same buffer.

(6.2–6.5) was raised to 8.3 with 0.1 M sodium borate. Reaction then continued for an additional 20 min. All reactions were performed at room temperature, and the suspensions were continuously stirred during the entire period. At the end of the reaction time, the mixture was placed in ice-cold centrifuge tubes which were filled with the cold buffer solution and centrifuged for 30 min at 4°C, 20,000 rpm in the SS-34 head of a Sorvall centrifuge (48,200 *g*). The supernatant was removed, and the cells were washed by resuspending and pelleting five times in 12 ml of 10 mM Tris/HCl buffer, pH 7.5. Special care was taken

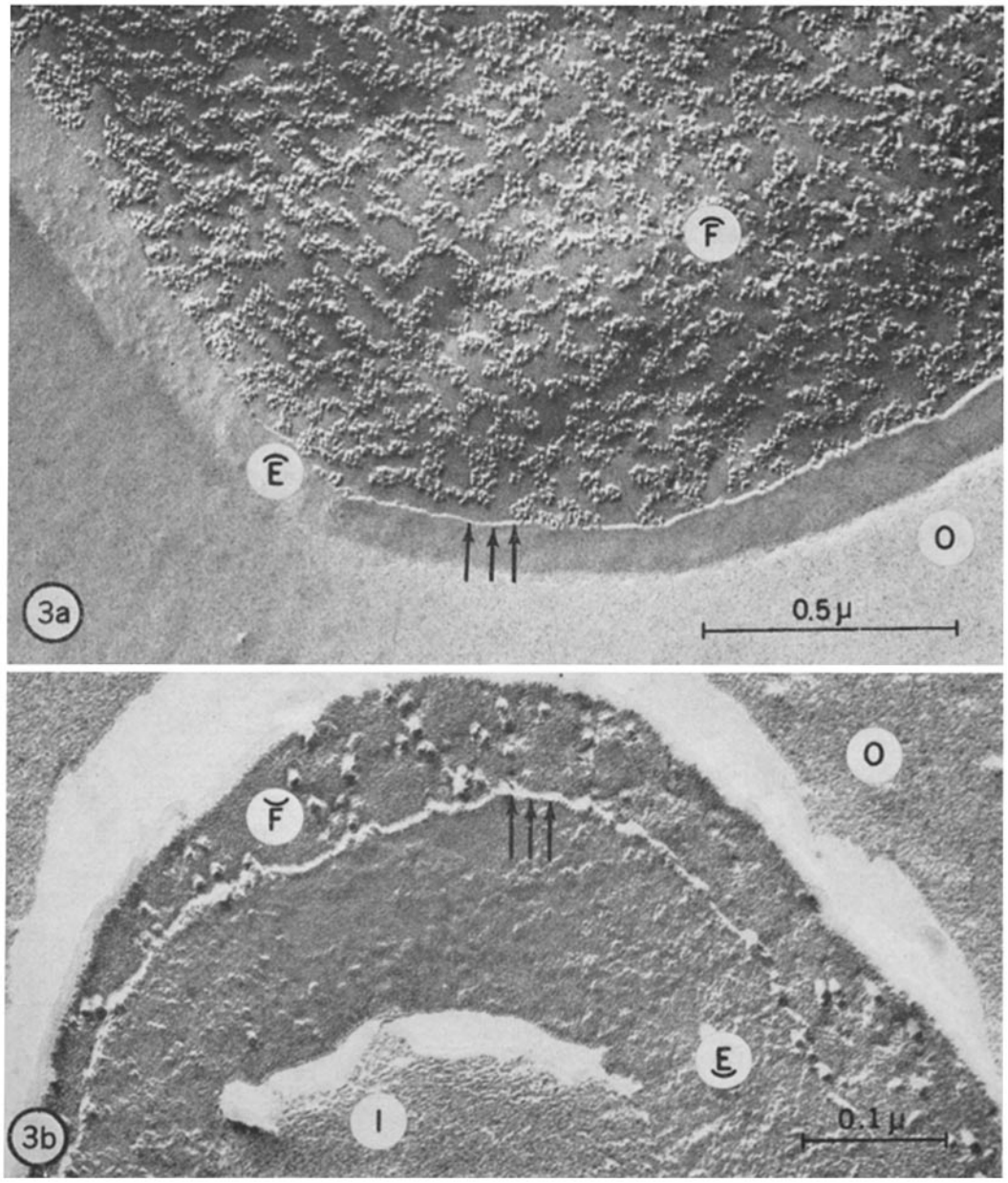

FIGURE 3 Membrane faces in etched, control cells. A ridge (triple arrow) separates the fracture faces from the etched faces. No ferritin is seen on any membrane faces. Symbols as in Fig. 1. Fig. 3 *a*, Convex fracture. × 70,000; Fig. 3 *b*, Concave fracture. × 200,000.

during resuspension to stir the pellet until it had been thoroughly dispersed. Preliminary experiments with fixed cells which could be washed in distilled water showed that the presence of 10 mM buffer was no obstacle to etching experiments.

A droplet of the material was mounted on a 3 mm cardboard disk and quickly frozen in Freon 22 (E. I. du Pont de Nemours & Co., Wilmington, Del.) partially solidifed by cooling with liquid nitrogen. No glycerol or other cryoprotective agent was used.

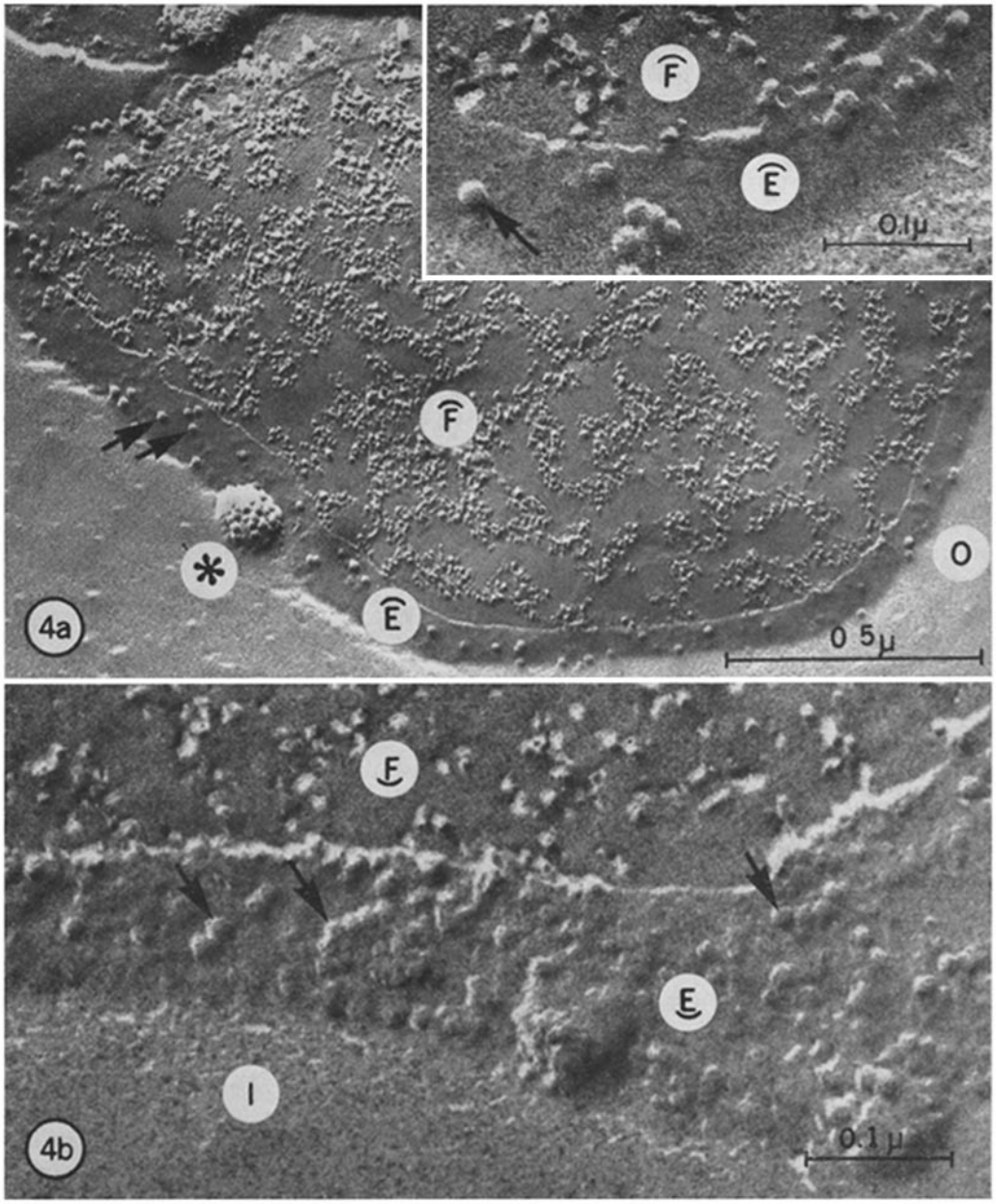

FIGURE 4 Membrane faces in etched, ferritin-conjugated cell. Ferritin molecules (arrows) are associated with the etched faces ($\hat{E}$ and $\underset{\smile}{E}$), but not visible on the fracture faces ($\hat{F}$ and $\underset{\smile}{F}$). Compare with controls in Fig. 3 and thin sections in Fig. 5. The globular structure (*) also seen in Fig. 5 *a*) does not seem to be a pure ferritin aggregate but may be membranous material coated by ferritin molecules. Symbols as in Fig. 1. Fig. 4 *a*, Convex fracture. × 70,000; *inset*, × 200,000; Fig. 4 *b*, Concave fracture. × 200,000.

Freeze-etching was carried out in a Balzers apparatus as previously described (1, 12), with 1 min of etching at −100°C or no etching at −115°C. Some of the ferritin-conjugated membranes were fixed in 1% glutaraldehyde for 1 hr at room temperature prior to freezing.

Samples for thin sectioning were fixed in glutaraldehyde (1% in 7.5 mM phosphate buffer pH 7.5 for 1 hr at room temperature then overnight at 4°C), embedded in 1% agar, postfixed for 1 hr in osmium tetroxide (1% in 0.1 M phosphate buffer pH 7.4 at room temperature), stained overnight with uranyl nitrate (1% at 4°C), embedded in Epon 812, sectioned in a Porter-Blum MT-2 ultramicrotome, and poststained for 15 min in Reynolds' lead citrate. Replicas and sections were examined in a Siemens Elmiskop 1, direct magnification ranging from 20,000 to 50,000. All micrographs are mounted with shadow direction from bottom to top. Shadows are white.

RESULTS AND DISCUSSION

The surfaces exposed during the freeze-etch process were those predicted by the scheme represented in Fig. 1. When the specimen was not etched after fracture, the only surfaces exposed were those in Fig. 2. We refer to these as fracture faces. As with most biological membranes, the

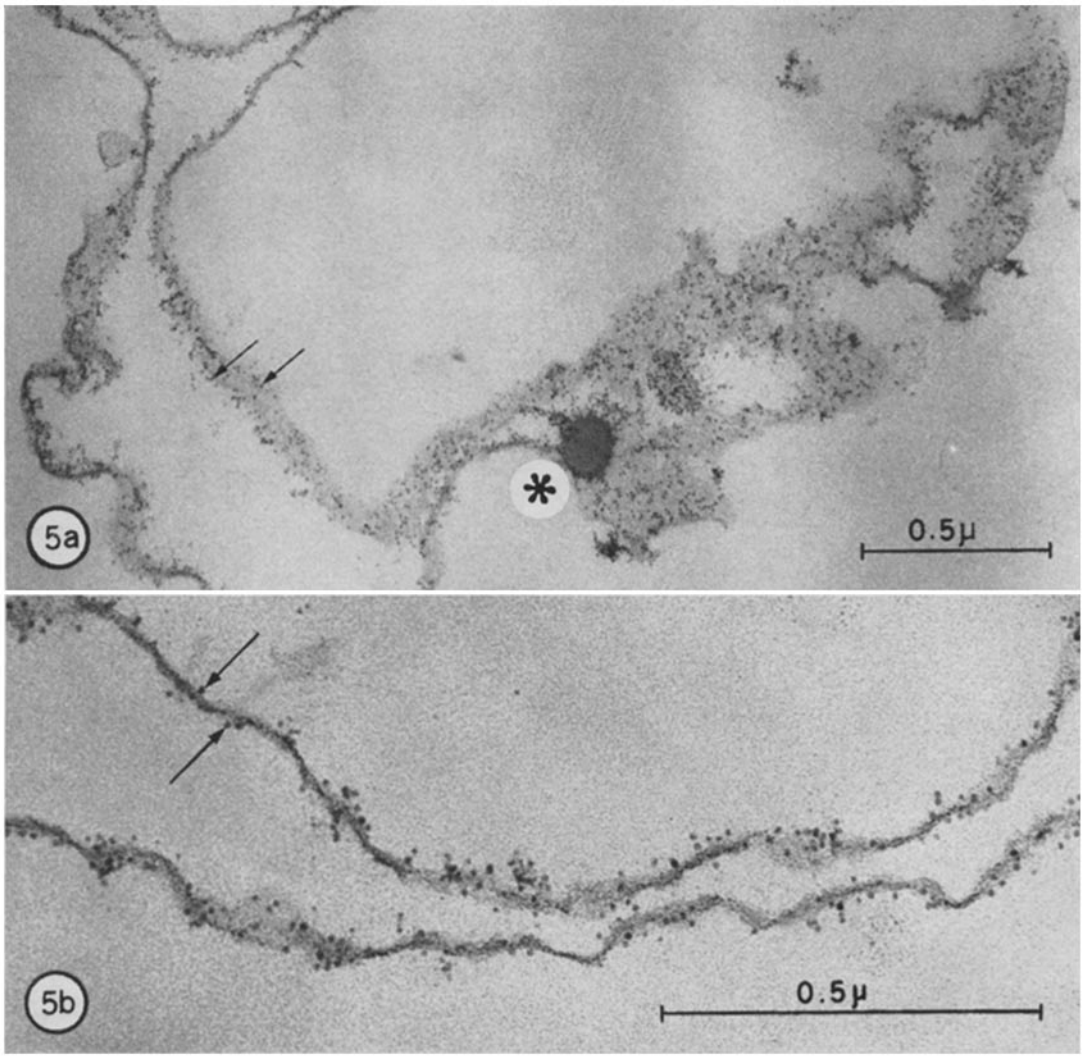

FIGURE 5 Sections of red blood cell ghosts conjugated with ferritin. Ferritin (arrows) is associated with both surfaces of the membrane. Fig. 5 *a*, × 50,000; Fig. 5 *b*, × 100,000.

fracture faces showed numerous particles which, in the red blood cell, average 85 A in diameter (2). One face ($\widehat{F}$) showed a much greater number of membrane particles than the other face ($\underset{\frown}{F}$). When fracture was followed by etching, a new surface was exposed next to each fracture face (Fig. 3). This face did not present the problems of interpretation raised by the fracture faces as it must, a priori, represent either the true surface of the membrane or this surface covered by a thin, nonsublimable layer of the medium in which the cells were frozen.

Fig. 3 shows red blood cell ghosts which were exposed to the control medium containing ferritin, but no conjugating agent. No ferritin molecules were seen on any of the fracture or etch faces. Thin sections of the same material (not illustrated) showed no membrane-associated ferritin. Thus, in the absence of conjugating conditions no ferritin was attached to the red blood cell ghost. When TC-conjugating agent was present, ferritin could be seen on both etched faces (Fig. 4) but never on the fracture faces which were comparable to the controls. Thin sections of TC-conjugated material (Fig. 5) demonstrated the success of the conjugation procedure and showed the association of ferritin molecules with both membrane surfaces.

The absence of ferritin molecules on all fracture faces and their presence on the etched faces is consistent with the hypothesis that the membranes are split along an inner face during freeze-etching. Our observations are not consistent with suggestions that fracture exposes detail on membrane surfaces, because ferritin molecules covalently bound to the surfaces could be observed only on the etched faces of red blood cell ghosts.

Several surface fracture schemes have been proposed. Some (10, 13, 21) assume that fractures proceed along either the outside or inside membrane surface so that the membrane itself is always included under the replica (Fig. 6 *a*). Others (12) assume that the fracture proceeds along the outer membrane surface only for some membrane types (Fig. 6 *b*) and along the inner membrane surface only for other membrane types (Fig. 6 *c*). However, no surface fracture scheme can predict our observations which show that none of the fracture faces represent the surface morphology of the red blood cell ghost. Plaque formation, which Staehelin (19) attributed to alternate surface fracture and membrane splitting (Fig. 6 *d*), was never seen under our freeze-etch conditions.

It may be objected that surface fracture does occur, but that ferritin is not seen on the fracture faces because it is split away, probably with some smaller protein molecules to which it is bound. This objection seems to be an inherent limitation to labeling experiments with macromolecules. However, the conjugating agent (TC) acts as an inter- and intraprotein cross-linker (22). In our experiments, it probably formed interconnecting covalent bonds between the subunits of the ferritin molecules and the membrane proteins, thus in-

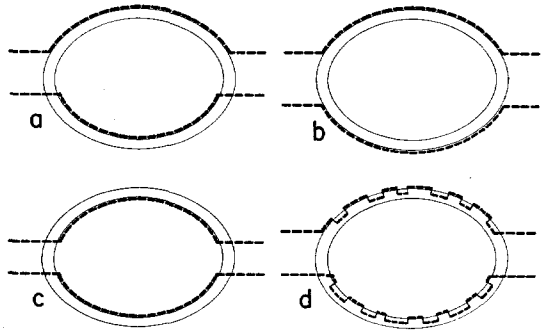

FIGURE 6 Hypothetical fracture schemes: Fig. 6 *a*, Both membrane surfaces provide a fracture plane. Fracture exposes one or the other of these membrane surfaces, but never the previously apposed ice. Fig. 6 *b* and *c*, A unique membrane surface—either the outside surface (*b*) or the inside surface (*c*) depending upon membrane type—provides the fracture plane. Fracture exposes either the membrane surface or the previously apposed ice. Fig. 6 *d*, Both membrane surfaces as well as a plane within the membrane matrix are fracture faces. Fracture exposes any of these faces, but never the previously apposed ice.

creasing ferritin-membrane as well as intramembrane and intraferritin protein linkages. Furthermore, even in cases in which TC conjugation was followed by glutaraldehyde fixation to provide additional cross-linkage (not illustrated), the results were identical, i.e. no ferritin was seen on the fracture faces.

Although our experiments show that the fracture process does not expose the surface morphology of red blood cell membranes, these results cannot demonstrate the exact location of the fracture plane. Cleavage between methyl end-groups has been demonstrated in a stearic acid bilayer (5), but caution must be exercised in extrapolating these results to the red blood cell ghost whose structure is more complex and, at the molecular level, largely unknown. However, reviews of the available evidence (2, 11, 20) suggest that also in the red blood cell membrane a natural cleavage plane could be formed by the juncture of methyl end-groups in a lipid bilayer. Here, the prevalent intermolecular forces would be of the London–van der Waals type (about two orders of magnitude smaller than the other intermolecular forces relevant in the polar regions of the membrane) and, because of their end-to-end interaction, largely of a noncooperative nature (17).

The fracture plane in the red blood cell ghost is interrupted by particles associated with both halves of the membrane. A greater number of particles is always associated with the fracture face closest to the inside of the cell (Fig. 2), but the particles on one fracture face are not perfectly matched by corresponding depressions on the other. Any fracture through a solid object should produce matching faces unless there is material loss or plastic deformation (4). A more detailed analysis of the fracture process will be required to explain the imperfect match of particles and depressions.

Indeed, the function and composition of the particles remain to be established. Indirect evidence suggests that they represent some[2] of the membrane protein (2). Because our fracture experiments with ferritin-labeled membranes show that the particle-containing faces are within the membrane matrix, our observations are most easily reconciled with a bilayer membrane model in which the lipid components are locally intercalated with protein differentially associated with each half of the membrane.

CONCLUSIONS

(*a*) During the freeze-etch process, the red blood cell ghost membrane is fractured along its inner matrix. The plane of fracture may be formed by the methyl end-groups of fatty acid chains in a lipid bilayer membrane with localized protein intercalations. (*b*) Detail on the membrane surface can be visualized on the etch faces that are exposed after sublimation of the surrounding medium. (*c*) Etching provides a method for observing macromolecules or large molecular aggregates on membrane surfaces. This should be useful in localizing antigens, specific proteins, and the sites of enzymatic activity or viral penetration.

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[2] A simple calculation shows that the membrane particles can represent only part of the total membrane protein. (32% if we assume: spherical shape; density 1.3; 4,200 membrane particles/μ^2; average diameter 85 A (2); 4.15 $\times$ 10^{-13} g total lipid per cell (14); protein lipid ratio 60:40 (11); surface area 110 μ^2 (16).

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