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RECOMBINATION OF HUMAN ERYTHROCYTE APOPROTEIN AND LIPID

II. Visualization of Apoprotein-Lipid Bilayer Complex by Freeze-Etching

Ernst Wehrli* and Philip D. Morse III†

**Anatomisches Institut der Universität Bern and Theodor Kocher Institut, CH-3008 Bern, Switzerland*

*†Pharmakologisches Institut der Universität Bern, CH-3008 Bern, Switzerland***

Bilayers of human erythrocyte apoprotein-lipid complexes were made by dipping a mica plate through monolayers of the complex formed at the air-water interface. Stearic acid and erythrocyte lipid alone served as controls. Freeze-fracture images of the complex at high lipid surface pressures (30 dynes/cm) showed particles (average diameter, 109 Å ± 18 Å) similar to those of erythrocyte ghosts (average diameter, 102 Å ± 19 Å). Control surfaces were smooth. We conclude that part or all of the protein molecule penetrated into the lipid bilayer and that erythrocyte apoprotein-lipid complexes yield fracture faces similar to the native erythrocyte membrane.

INTRODUCTION

Lipid multilayers deposited onto a support from a monolayer have found increasing use as models for studying the properties of biological membranes. When a solid support, typically glass or mica, is dipped repeatedly through a monomolecular layer of substance spread at the air-water interface, multilayers are deposited with the polar head groups and the hydrophobic tails in alternating regions (1). Deamer and Branton (2) demonstrated that frozen multilayers of calcium stearate fractured preferentially in hydrophobic regions. When these fracture faces were replicated and viewed by electron microscopy, they appeared essentially smooth.

In contrast, freeze-fracture images of biological membranes reveal fracture faces containing a large variety of particles (Fig. 1). These particles are thought to be protein intercalated within the hydrophobic regions (3).

A variety of proteins are known to interact with lipids at the air-water interface. Changes in surface pressure induced by proteins injected beneath lipid monolayers are thought to arise from either penetration of portions of the protein into the lipid or deformation of the lipids by protein (4). If films comprised of such a protein-lipid complex were deposited onto a solid support, frozen, and fractured, the fracture faces

**Present address: Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802

Fig. 1. Freeze-etch view of erythrocyte stroma. Magnification is × 100,000. Note the presence of particles on the fracture face.

should reveal either smooth or particular surfaces. Smooth surfaces would indicate that the protein had not completely penetrated the lipid monolayer or that the protein was highly denatured when present in the complex. Particulate surfaces would indicate that protein has penetrated the lipid monolayer or that the protein deformed the lipid in some way to give the appearance of particles. We chose to study films in which total human erythrocyte apoprotein interacted with monolayers of total erythrocyte lipid because this interaction has been previously studied (5), and replicas could be compared to the biological material from which it was extracted.

MATERIALS AND METHODS

All chemicals were obtained from Merck (Darmstadt, Germany). Erythrocyte stroma was prepared from freshly collected human blood by the method of Dodge et al. (6). From the stroma, total erythrocyte apoprotein was prepared by the method of Zahler and Weibel (7) and total erythrocyte lipid was prepared by the method of Folch et al. (8). Surface pressure was measured by the Wilhelmy technique (9). The trough was constructed of solid Teflon and was separated into three compartments so that the apoprotein-lipid films could be washed free of protein if desired. The transducer was a Statham UC2 with a micro-scale accessory. The Wilhelmy plate had a perimeter of 39.6 mm. This apparatus was kept in a constant temperature room (20°C). The preparation of the apoprotein-lipid monolayers has been described previously (5). Apoprotein concentration in the subphase was 10 μg/ml. Surface pressure was maintained, at the values stated, by a movable, hand operated barrier.

When the monolayer was ready to be deposited onto a solid support, a plate of freshly split mica was wetted, then dipped into the trough through the monolayer with a micromanipulator. No change in surface pressure was observed during this procedure. The mica was then slowly drawn up through the monolayer. This deposits the first layer with the lipid polar groups oriented toward the mica and the hydrophobic tails oriented toward the air. The meniscus was always upward during this deposition. The plate was air-dried for several minutes. Some of these plates were put aside for controls. The remaining plates were dipped rapidly back through the monolayer. The meniscus always remained downward and the area of the monolayer on the trough decreased. This showed deposition of the second layer and completed the formation of the bilayer. Only plates which satisfied these criteria were used. For control water surfaces, no lipid was present on the mica.

The plates with bilayers were then transferred in water to a large container where they were attached to a U-shaped brass holder and withdrawn from the water. The plate, with water entrained in the U of the holder, was then placed on the pre-cooled (−150°C) stage of a Balzers BA 360 freeze-etch apparatus and frozen. Under vacuum (10^{-5} torr), the mica plate was broken away from the ice with the knife assembly. The exposed surface was etched briefly (15-30 sec at −100°C), and shadowed with platinum and carbon. In Fig. 6 (a and b), the control monolayers of lipid (a) or apoprotein-lipid complex (b) were shadowed directly on the mica. The replicas were viewed with a Philips EM 200 electron microscope. Bars on the photographs represent 0.1 μ except in Fig. 2 (a, b) where the bar is 1.0 μ.

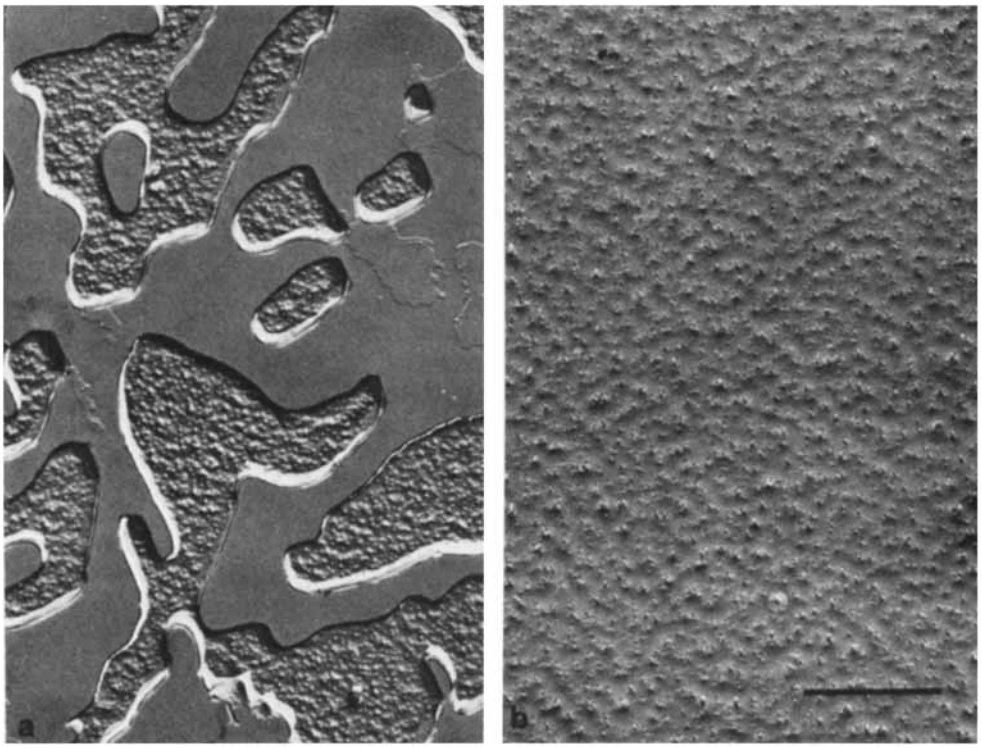

Fig. 2. (a) Frozen-etched calcium stearate multilayers × 17,500). Particles arise from contamination in the vacuum chamber. (b) Replica of frozen-etched water alone (× 17,500).

RESULTS

Calcium stearate multilayers were prepared at 17 dynes/cm as a control (Fig. 2a) and closely resembled those shown by Deamer and Branton (2). Particulate structures on the smooth lipid surfaces were due to contamination in the bell jar (10) and could not be completely eliminated. The regions of ice covered with lipid could easily be distinguished from the exposed ice surface (Fig. 2b). Bilayers of total erythrocyte lipid prepared at a surface pressure of 30 dynes/cm showed no particulate contamination on the exposed lipid surface (Fig. 3).

When human erythrocyte apoprotein was injected under monolayers of human erythrocyte lipid at 2 dynes/cm, the surface pressure rose 18 dynes/cm. The apoprotein-lipid monolayer was maintained at 21 dynes/cm while the bilayers were formed. A large number of particles were observed in plates prepared under these conditions (Fig. 4). A patch effect was occasionally seen in some areas. When the lipid film was first spread to an initial surface pressure of 30 dynes/cm, protein penetrated to a much lesser extent (surface pressure increase of 4 dynes/cm). This was reflected by a decreased number of particles observed (Fig. 5). The average particle size was 109 Å ± 18Å, which compared favorably with the average particle size in the erythrocyte membrane (102 Å ± 19 Å) (Fig. 1). Surface replicas of apoprotein-lipid monolayers prepared under similar conditions showed a highly irregular surface (Fig. 6a). Surface replicas of monolayers containing only erythrocyte lipid were smooth (Fig. 6b).

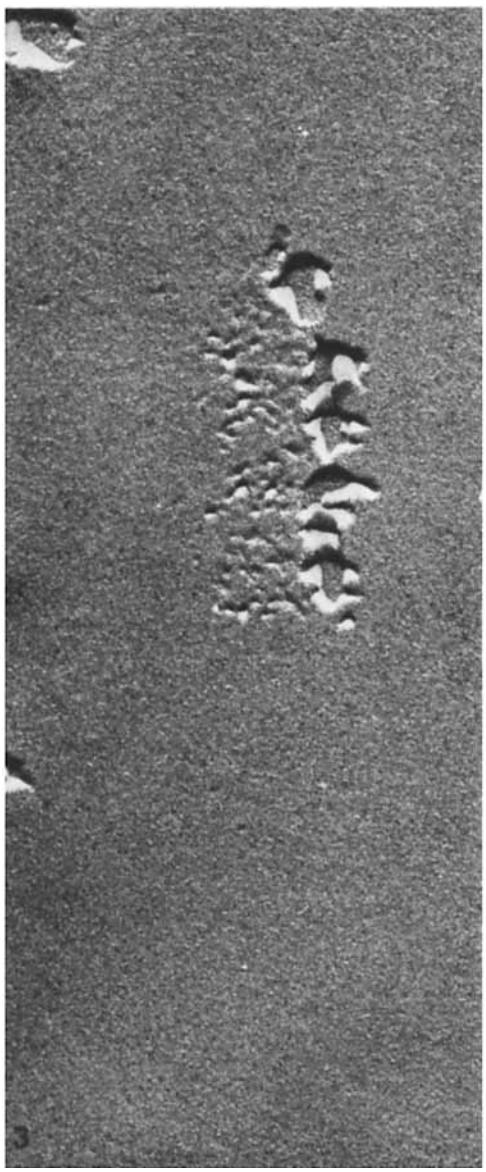

Fig. 3. Total human erythrocyte lipid bilayer after freeze-etching (× 100,000). Note the complete absence of particles.

Fig. 4. Bilayers of human erythrocyte apoprotein-lipid complex after freeze-etching (× 100,000). The bilayer was formed by injecting apoprotein under an erythrocyte lipid monolayer at 2 dynes/cm and depositing two successive layers on a mica plate. The mica plate with one of the layers was split away and the layer on the ice was replicated.

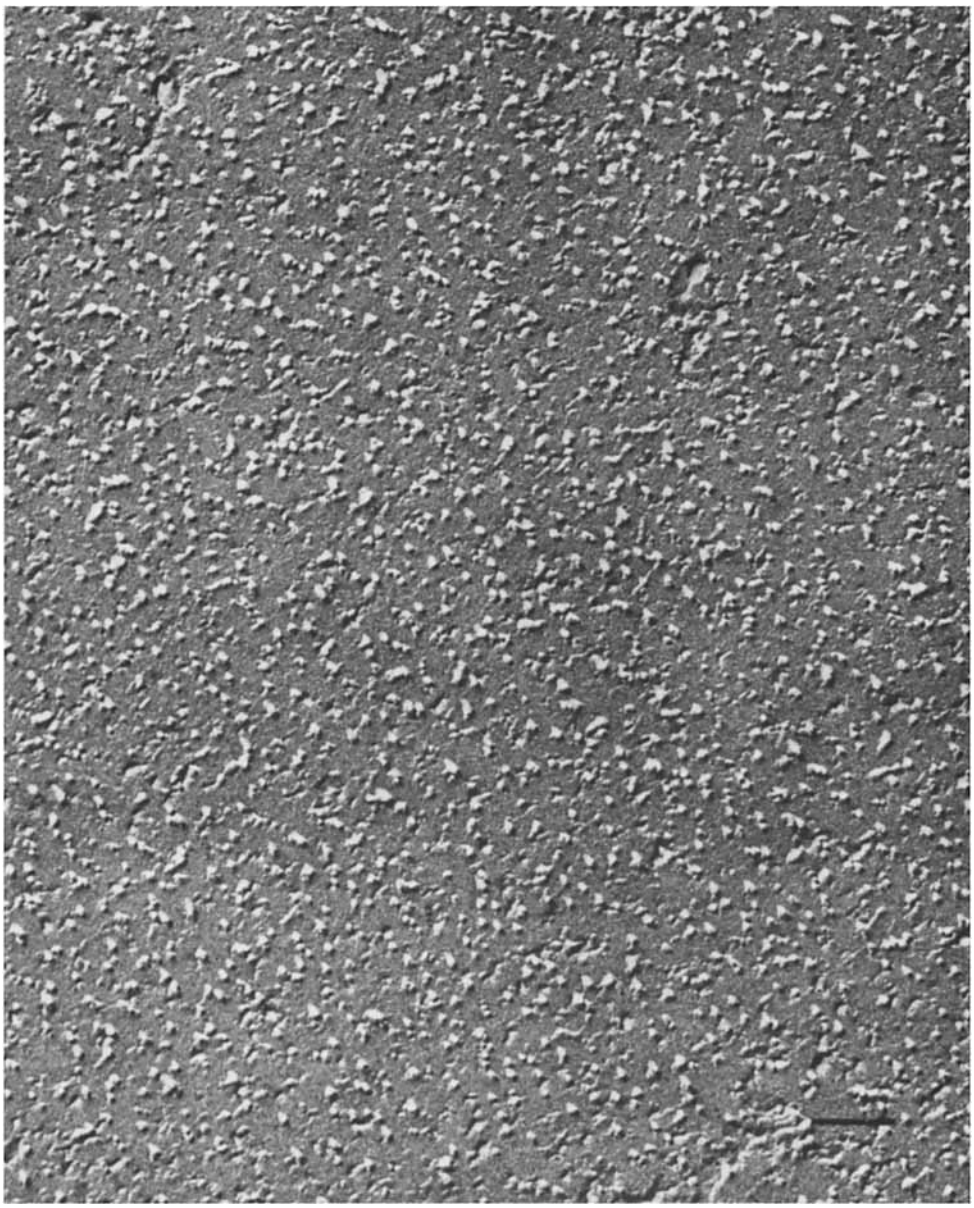

Fig. 5. Bilayers of human erythrocyte aproprotein-lipid complex after freeze-etching (× 100,000). This bilayer was formed by injecting apoprotein under an erythrocyte lipid monolayer at 30 dynes/cm and was otherwise treated as in Fig. 4. Fewer particles are seen here than in Fig. 4.

DISCUSSION

Erythrocyte apoprotein extracted by 2-chloroethanol may be highly denatured. Nonetheless, apoprotein and total erythrocyte lipids do interact strongly (5) and this suggests that at least a portion of the apoprotein penetrates into the hydrophobic region of the lipid monolayer. Proteins probably penetrate lipid monolayers by unfolding and inserting portions of their side chains into the lipid. However, most of the protein molecule still resides in the aqueous region below the lipid (12). When a single lipid monolayer containing partially penetrated apoprotein is picked up on a solid support

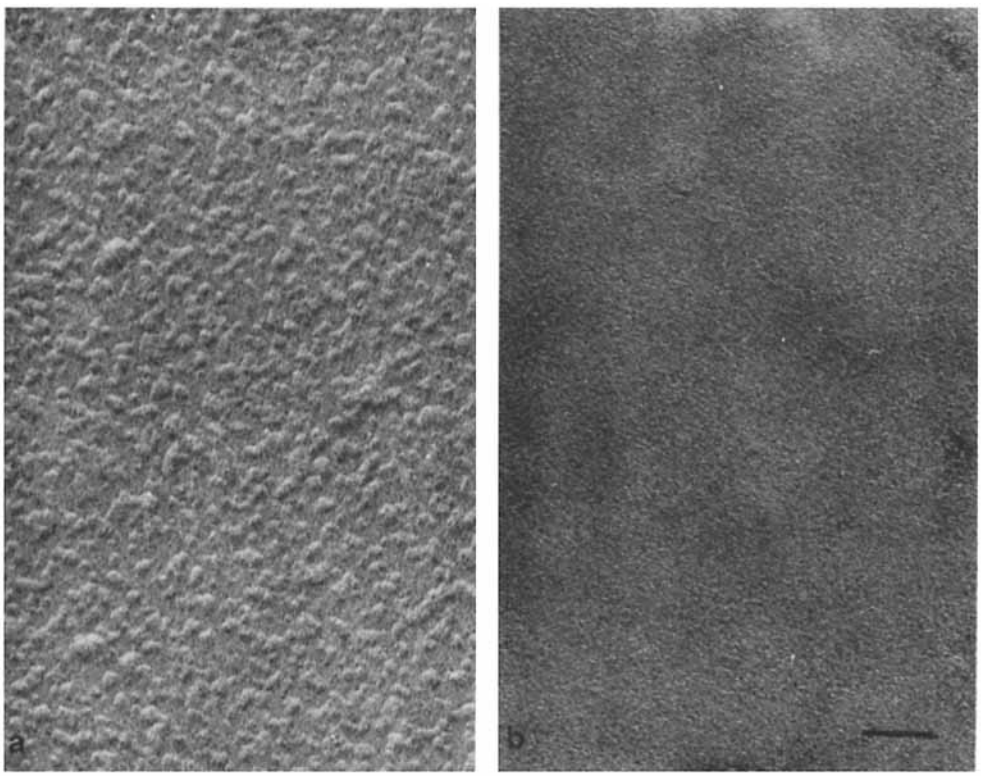

Fig. 6. (a) Surface replica of a monolayer of apoprotein-lipid complex (× 100,000). Only one layer of the complex was deposited onto the mica and its surface replicated. Initial surface pressure of the erythrocyte lipid was 30 dynes/cm. The bumps are thought to represent protein attached to and absorbed beneath the lipid monolayer. (b) Surface replica of a monolayer of erythrocyte lipid alone deposited onto mica at 30 dynes/cm (× 100,000).

and dried, the protein molecules beneath the lipid distort the monolayer surface and form the diffuse bumps seen in Fig. 6a. (Fig. 7a).

In contrast, frozen-etched bilayers of lipid and apoprotein reveal fracture faces which show particles that are sharp and well defined. One explanation of this result is that during formation of the bilayer, protein molecules which were formerly only partially penetrated into the monolayer become completely incorporated into the interior of the bilayer (Fig. 7b). Protein molecules simply absorbed to the outside of the bilayer would be surrounded by water (ice) and would not distort the surface as in Fig. 6a. We cannot exclude the possibility that penetration of the apoprotein into the monolayer may cause structural changes in the lipid which would produce the particulate images. This requires further study.

When an excess of protein, as is the case in this study, is injected beneath the lipid monolayer, at least a monolayer of protein is present to interact with the lipids (11, 12). However, the amount of apoprotein penetrating the monolayer is never this great since the lipids themselves will always occupy space on the surface. Thus, the number of particles observed does not correspond to a complete monolayer of apoprotein.

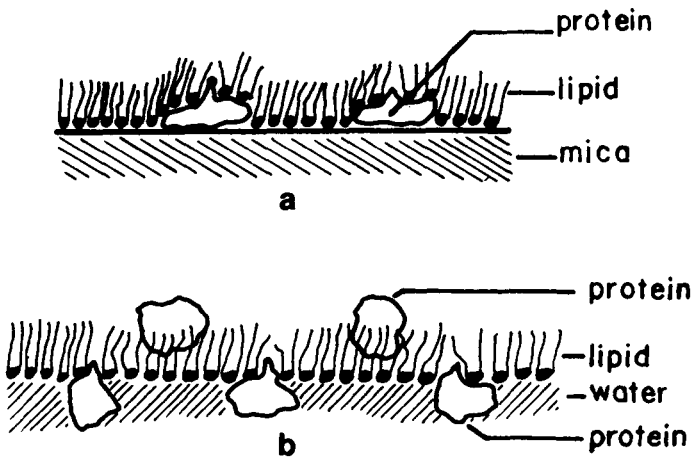

Fig. 7. (a) Diagrammatic representation of Fig. 6a. The presence of apoprotein attached by penetration to the lipid monolayer is accentuated by drying the apoprotein lipid complex onto a flat mica surface. (b) Diagrammatic representation of Fig. 5. Apoprotein present in the bilayer is visualized by replication, but apoprotein beneath it does not deform the surface.

The results further illustrate that erythrocyte apoprotein-lipid complexes in monolayers yield fracture faces similar to the native erythrocyte. Solubilization of the erythrocyte membrane destroys all previous protein-protein and protein-lipid relationships; thus, the apoprotein used to prepare the monolayers and bilayers was totally randomized. However, the penetration and unfolding of apoprotein at the air-water interface does not seem to be a physically irreversible process. This is supported by the similar particles size in the artificial and intact erythrocyte membranes.

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REFERENCES

1. Blodgett, K. B., J. Amer. Chem. Soc. 57:1007 (1935).
2. Deamer, D. W., and Branton, D., Science 158:655 (1967).
3. Branton, D., and Deamer, D. W., "Membrane Structure," Springer-Verlag, Wien and New York (1972).
4. Kimelberg, H. K., and Papahadjopoulos, D., Biochim. Biophys. Acta 233:805 (1971).
5. Morse, P. D., II, J. Supramol. Struct. 2:63 (1974).
6. Dodge, J. T., Mitchell, C., and Hanahan, D. J., Arch. Biochem. Biophys. 100:119 (1963).
7. Zahler, P., and Weibel, E. R., Biochim. Biophys. Acta 219:320 (1970).
8. Folch, J., Lees, M., and Sloan-Stanley, G. H., J. Biol. Chem. 226:497 (1957).
9. Wilhelmy, L., Ann. Phys. Chem. 114:177 (1863).
10. Deamer, D. W., Leonard, R., Tardieu, A., and Branton, D., Biochim. Biophys. Acta 219:47 (1970).
11. Quinn, P. J., and Dawson, R. M. C., Biochem. J. 113:791 (1969).
12. Quinn, P. J., and Dawson, R. M. C., Biochem. J. 115:65 (1969).