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ULTRASTRUCTURAL DEFORMATION STUDIES ON BIOLOGICAL MEMBRANES

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The ultrastructural deformation technique has been used to study various membranes, including several claimed to show subunit structure. With localized deformation fibers ca. 100–300 Å in diameter and up to 4,000 Å long were found extending across the cracks parallel to the draw direction in all membranes. Lipid extraction and proteolytic enzyme (papain) treatment of membranes has shown that the fibers are protein in nature. Deformation of membranes while still wet showed no significant change in the appearance of the fibers compared with those obtained from deformation of membranes after air drying, indicating that formation of fibers is not an artifact due to dehydration. There must be extensive interaction between the protein molecules and thus we indicate that the results do not agree with current suggestions that membranes are composed of individual protein subunits immersed in a lipid bilayer.

INTRODUCTION

Proteins and lipids are the major constituents of biological membranes. Understanding their molecular organization and interaction is required to understand the structure and function of membrane systems.

The lipids present in membranes are of three types: phospholipids, glycolipids, and cholesterol. Their chemical structure and relative number in various membranes have been well characterized due to the fact that lipids can be easily extracted from membranes and characterized with the conventional techniques of solution chemistry.

The macromolecular organization of lipids in membranes is most often interpreted as being bilayer in form on the basis of surface area experiments first reported by Gorter and Grendel (1), and, more recently, on studies of calorimetry (2, 3), electron spin labeling (4), and X-ray diffraction (5, 6). The lipids in membranes are considered to have the same arrangement as that of phospholipids dispersed in aqueous media.

More recently attention has been focused on the protein components. Although at least half of the mass of most membranes is composed of proteins, a great deal less is known about the number and size of membrane proteins than is known about membrane lipids. The major problem in this field is the limited solubility of membrane proteins and their close association with the lipids; in addition, there is the possibility that the results obtained may be an artifact of the solubilization procedure.

A number of more of less unique methods have been developed in order to study

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membrane protein composition and the location of the proteins in the membrane, e.g., sodium deodecyl sulfate-acrylamide gel electrophoresis (7–10), membrane inversion (11, 12), and membrane labeling (13–16). The results of these experiments indicate that the proteins are heterogeneous. The types and organization of the proteins in erythrocyte membranes, which are as well known as any, have recently been reviewed (17) and are nonsymmetrically located on the surfaces of the membranes.

Danielli-Davson (18, 19) and Robertson (20, 21) a number of years ago, suggested that proteins, either in a globular or in a β conformation, cover both surfaces of a central lipid bilayer. This trilamellar or unit membrane model, however, has since been found to be inadequate to account for the following experiments (1). When more than 90% of the lipids are removed from the membranes, there are no significant changes in thickness or appearance of the membranes in the electron microscope (21–24); this does not appear compatible with the idea that the protein lies solely on the surface. (2) Phosopholipase C releases 70% of the phosphorylated amines (in the polar heads of the lipids) and profoundly perturbs the fatty acid chains of the lipids, but does not show any detectable effect on the average conformation of the membrane proteins (25); this indicates that lipids are not completely covered with proteins, but rather that some of the lipids must be exposed to the membrane surfaces.

Mainly on the basis of the amphipathic properties of proteins as well as lipids, Singer et al. (25, 26) and Vanderkooi et al. (27–29) in their fluid mosaic and protein liquid-crystal models, suggested that the individual globular proteins are immersed in the lipid bilayer with the polar portions interacting with the polar head of the lipids in the aqueous phase, while the nonpolar portions are immersed in and interact with the hydrophobic region of the lipid bilayer. Similar models have been described recently by Steck (17) and Nicholson (30) for erythrocyte membranes. Both authors describe a model of more or less isolated proteins penetrating into or through the bilipid layer. The inner surfaces of the erythrocyte membranes are coated with a dense, randomly arranged layer of microfibrils of spectrin, a protein which has been described as an actomyosin system, while the carbohydrate portion of the intercalated glycoproteins are located on the outer surface.

Blais and Geil (31) utilized an ultrastructural deformation technique previously used to characterize macromolecular single crystals (32, 33) to examine the macromolecular organization of human erythrocyte ghosts. The membranes were air dried at 4°C prior to localized deformation. Uniform fibers ca. 180 Å in diameter and up to 4,000 Å long were drawn out parallel to the draw direction in the crack regions.

In this initial study, the deformation of a number of possible model systems was examined. In particular, we note that neither solvent cast films of both the lipids extracted from erythrocyte membranes by a modification of the Rose-Oklander (41) technique or cholesteryl myristate, nor films of the soluble protein extracted by the technique of Lenard and Singer (34) or lysozyme cast from water yielded fibers. The lipids as well as the proteins seemed merely to "flow" into the cracks in the carbon film or break up into unconnected islands as a possible model for membranes containing preformed fibrils, e.g., the spectrin in erythrocyte membranes, a fibrillar suspension of poly (γ-benzyl-L-glutamate) was dried down. When the randomly arrayed fibrils were drawn across the cracks in the carbon film, the result was a network of criss-crossing, overlapping fibrils.

Several amorphous (brittle and rubbery) polymer films were also drawn, but failed to produce isolated fibrils. In fact, the only materials in which a similar deformation process has been observed to date are single crystals of macromolecules in which the long-chain molecules are oriented and folded essentially perpendicular to the 100-Å thick lamellae (33).

The fact that air-dried samples were used in this experiment created concern about the effect of the dehydration, i.e. whether or not the fibers formed during deformation were the result of denaturation or other molecular rearrangement accompanying dehydration, or were characteristic of the molecular arrangement in the hydrated state. In this paper we discuss the results of a limited further study of the ultrastructural deformation of the membranes. In particular we consider (1) the effect of hydration, (2) the nature of the fibers, and (3) an extension to other membrane systems including several which have been shown to have a subunit structure. In addition to localized deformation as used for the erythrocyte ghosts, uniform deformation up to 100% was used in order to reveal more detail concerning the deformation process.

The subunit structure claimed for a number of membranes is based on electron micrograph studies. The relationship of these subunits to the structure of the membrane is not clear; in some cases they may only be enzymes or other proteins systems on the surface, whereas in other cases they may have a structural function. Several of these membranes, as described below, were chosen for use in this study.

Chromatophores of Rhodospirillum rubrum have been shown to have hexagonal subunits of ca. 90 Å diameter when they are negatively stained with phosphotungstic acid (35) or positively stained with uranyl acetate-lead citrate (36). These subunits, termed quantasomes, were considered to be the photosynthesis units of this bacterium. Micrographs of shadowed chloroplast membranes of spinach and morning glory were interpreted as showing 90-Å subunits which aggregated together to form the quantasome structure of these membranes. These larger quantasomes are considered to be the photosynthesis unit machinery of higher green plants (37, 38). Micrographs of plasma membranes of rat liver have been interpreted as showing globular knobs 50–60 Å in diameter and 70–80 Å hexagonal subunits for samples negatively stained with phosphotungstic acid at room temperature and 37°C, respectively (39). In addition, rat liver nuclear membranes and rat kidney plasma membranes were used.

An examination was conducted of the effect on erythrocyte membrane deformation properties of storage time similar to that utilized in blood banks. Although holes were found to develop in membranes from cells stored in acid citrate dextrose (ACD) at 4°C over a period of 7 wk, no change was observed in the deformation characteristics. Fibers similar to those observed in fresh samples were observed for 2-, 3- and 7-week old samples.

EXPERIMENTAL PROCEDURES

(A) Membrane Systems

Standard techniques were used for the preparation of the various membranes. Human erythrocyte ghosts were prepared by the method of Dodge (40) following the procedure for maximum removal of hemoglobin. The ghosts following preparation were either colorless or very faintly pink. When desired, lipids were extracted using isopropanol-chloroform according to a method adapted from Rose and Oklander (41).

Chloroplast membranes of spinach were isolated following the method of Park (37, 42), while the chloroplast membranes of morning glory (collected from the wild) were prepared in a similar manner, except that the leaves initially were homogenized in a medium of 0.5 M sucrose and 0.1 KPO_4 buffer at pH = 7.4.

The photosynthetic bacteria Rhodospirillum rubrum were obtained from Dr. R. S. Gage, Department of Physics, University of Guelph, Ontario, Canada. The bacteria were cultivated in the liquid medium described by Ormered and Gest (43) under anerobic conditions at 37°C using illumination conditions similar to those used by Gage (36). The chromatophores were isolated by the method of Cohen-Bazire and Kumisaiva (44).

Rat kidney plasma membranes (RKPM), prepared by the Fitzpatrick method (45), were supplied courtesy of Dr. J. E. Zull, Biology Department, Case Western Reserve University, Cleveland, Ohio. Rat liver plasma membranes (RLPM) were supplied by courtesy of Dr. N. Wong (46). The membranes were prepared using a method described in his Ph. D. thesis. Rat liver nuclear membranes (RLNM) were supplied by courtesy of Dr. R. T. Cook, Institute of Pathology, Case Western Reserve University.

It is noted that great care in purifying and characterizing each of the membrane preparations was not taken. However, although some of the preparations may have been contaminated with other types of membranes, or the membranes themselves may not have been, and probably were not free of cell coats (e.g., spectrin for the erythrocyte membranes), the generality of the results speaks for the generality of the conclusions.

(B) Ultrastructure Deformation Technique

The ultrastructure deformation technique involves deformation of individual membranes on a substrate, followed by shadow casting (31). Uniform deformation up to 100% can be obtained using Mylar substrates. The Mylar deforms uniformly along the draw direction, contracting normal to this direction. Localized deformation up to much higher degrees can be achieved by first coating the Mylar with evaporated carbon; upon deforming the Mylar the carbon film breaks at about 1-μ intervals. The deformation procedures are as follows: 1 mil Dupont Mylar film was cut into ca. 2-cm wide and 6-cm long pieces and mounted in a sample stretcher and, if desired, put in the evaporator to be coated with carbon. A few drops of membrane suspension were deposited on the Mylar or carbon-coated Mylar, allowed to air dry at room temperature (unless otherwise specified), and stretched the desired amount. The whole assembly was then returned to the evaporator for platinum-carbon shadowing at about 45°, and coated with another carbon layer normal to the surface of the Mylar. Aqueous polyacrylic acid (PAA) from B. F. Goodrich Chemical Company, diluted to 10% with distilled water, was used to peel off the replica. The PAA was removed by floating the replica on water for about 4 hr, and the replica was then picked up with copper grids for the electron microscopy observation.

Deformation of wet samples was accomplished as follows: A few drops of membrane suspension were allowed to remain in contact with the Mylar carbon substrate for ca. 1 day in a water-saturated atmosphere at about 4°C and then deformed at 4°C. The membranes were still immersed in the suspension medium used in the last step of the preparation process, i.e. pH 7.4 and phosphate buffered, during the deformation process, some of them apparently sedimenting and adhering sufficiently while standing to permit deformation. The specimens were then air dried prior to shadow casting. Although some membranes would dry down across the cracks during the air drying, it was easy to select those which had deformed.

RESULTS AND DISCUSSION

Micrographs of a drawn human erythrocyte ghost (HEG) and, for comparison purposes, a polyethylene (PE) single crystal are shown in Fig. 1. Fibers ca. 100–200 Å in diameter are observed parallel to the draw direction and spanning the cracks in both specimens. Blais and Geil (31) in their initial work pointed out that no other model system for a membrane that they tried showed this behavior, that only polymer single crystals, such as PE, showed to that of deformation behavior HEG (i.e., individual, parallel fibrils). PE single crystals consist of 100-Å thick lamellae with lateral dimensions on the order of micrometers. The long-chain molecules are oriented essentially normal to the lamellar surfaces and are folded back and forth on themselves with the 100-Å thickness (fold period) (33). A number of polypeptides, such as polyglutamic acid, polyalanine, and polylysine (47, 48), and even DNA (49), have been shown to crystallize in a similar fashion. The physical cohesion of the crystal is a result not only of Van der Waal's bonds and, in some polymers, hydrogen bonds between the adjacent parallel segments of the same and neighboring molecules, but also of the covalent bonds involved in the folds on the surfaces. Cohesion of neighboring molecules during deformation is a result, presumably, of the extensive length over which the Van der Waal's bonds act between neighboring chains.

The fact that the ghosts showed similar deformation characteristics as PE single crystals, suggests that some form of interaction in the membrane is also necessary. As shown below, related research has strongly suggested that the fibers in the ghosts are predominently composed of proteins with the low molecular weight lipids contributing little or nothing to the formation of the fibers. However, the current models of membrane stucture, as pointed out above, suggest that the proteins are present either individually, as more or less spherical particles, or are aggregated into mosaic blocks immersed in the lipid bilayer. In particular in many models there is apparently presumed to be little or no interaction between neighboring particles or blocks, permitting a high degree of fluidity. In other models of HEG in particular, spectrin has been suggested to form a skeletal support with which at least some of the intramembranal proteins are associated (17). If, according to these models fibers could form at all, one would expect that the proteins would be observed as units in or on the fibers. However, in the deformation study of HEG, no sign of any subunit or nonuniformity in the fibers was observed. In addition, we emphasize that the observations are not consistent with a fibril structure being present in or on (e.g., spectrin) the membranes prior to deformation; in such a case the fibrils would be expected to cross each other when drawn across the crack, as observed for the model system consisting of poly (γ-benzyl-L-glutamate) fibrils (31). These experimental observations, confirming those of Blais and Geil (31), suggest that there must be extensive interactions between the individual protein molecules in the ghosts in the air-dried state. We note, however, that the interaction need not be the same as in the folded chain polymer crystal; e.g., entanglement or interpenetration of neighboring coiled protein molecules would be sufficient. A similar mechanism of fibril formation has been suggested for amorphous polymers undergoing crazing (50, 51), i.e. cracks are formed spanned by numerous, parallel fibrils. In addition, the fibrils formed in the ghosts appear to be restricted in length 4,000 to 5,000 Å, whereas in crystals of most, but not all, polymers the fibrils can apparently be of indefinite length. While this length could possibly be related to the protein length, the fibrils themselves, on the basis of their

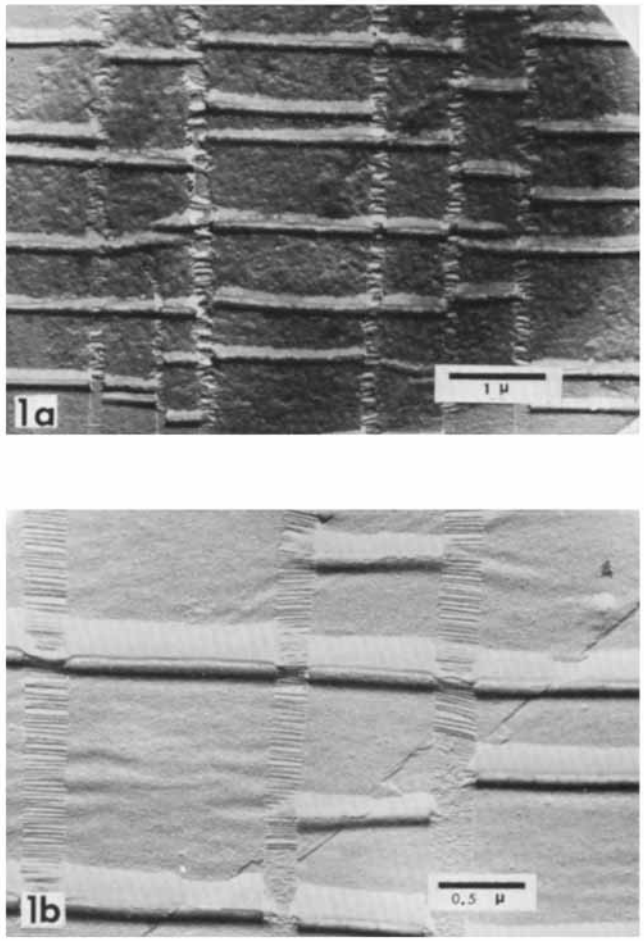

Fig. 1. Electron micrographs of (a) human erythrocyte ghosts, and (b) polyethylene single crystals drawn on carbon-coated Mylar.

diameter, must contain a large number of molecules, requiring the interaction hypothesized above. At this time it is not known if the molecules are fully straightened out in the fibrils or retain some of the coil or other conformation originally present; in the polymer fibrils a substantial number of folds are retained.

The same deformation technique was applied to chromatophores of Rhodospirillum rubrum (35, 36) and rat liver plasma membranes (39) in which we observed the hexagonal arrangement of subunits in negatively stained samples. The deformed membranes are shown in Fig. 2. Smooth fibers were found to pull out parallel to the draw direction similar to those observed for ghosts. In these samples also there is no sign of any subunits in the fibers.

Figure 3 shows the result of uniform deformation (75%) of the rat liver plasma membranes. The membrane is seen to break up along shear lines (i.e. at 45° to the draw

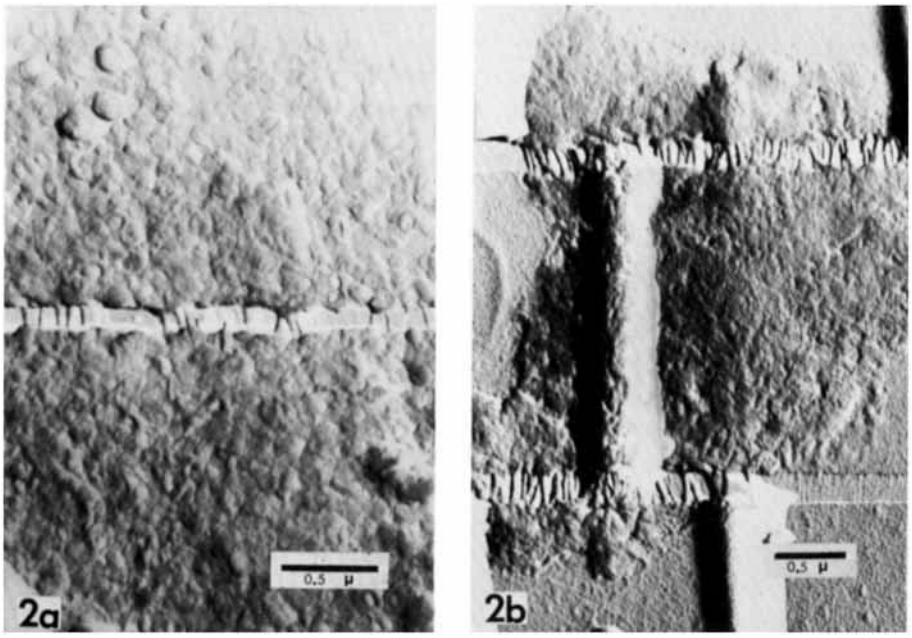

Fig. 2. Electron micrographs of (a) chromatophores of Rhodospirilum rubrum, and (b) rat liver plasma membrane down on carbon-coated Mylar.

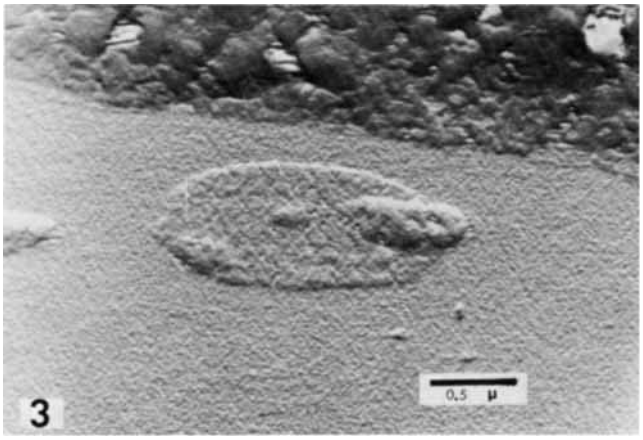

Fig. 3. Electron micrograph of rat liver plasma membranes uniformly drawn 75% on Mylar.

direction) into rectangular blocks 200 to 700 Å in size. This is considerably larger than the observed "subunit" size (∿75Å) in negatively stained samples (39). In the larger cracks, particularly in regions containing several superimposed membranes, fibers can be seen drawn out across the cracks. The blocks are also considerably larger when the

membranes are superimposed. Thus, although one might interpret the observed blocks as subunits of the membrane, we suggest instead that they are limiting size regions which can undergo plastic deformation, assuming a uniform strain in the substrate.

Micrographs of deformed chloroplast membranes of morning glory and spinach are shown in Fig. 4. Again fibers were pulled out over the crack regions. For the morning glory membranes, the fibers vary in diameter from 100 to 300 Å both between and within the fibrils. The fibers from the spinach membranes are considerably more uniform in diameter. The chloroplast membranes of spinach and morning glory have both been shown to have a surface structure due to the presence of particles called quantasomes. These quantasomes contain the photosynthetic apparatus of the membranes and are composed predominantly of proteins. Figure 5 shows a micrograph of a shadowed sample of an undeformed morning glory chloroplast membrane in which a "subunit" structure, presumed due to the presence of these quantasomes on the external surface, can be seen. The bumps on the morning glory chloroplast fibers, we suggest, are from the surface-associated material which was pulled out along with the fibers. In our spinach samples, no subunits were seen on the surface of the membranes; this may be due to the observation of Park (37, 38) that the structures depend on the photoperiod during growth of the green plant and, in particular, are only seen on the inner surface of the membrane. Although, for these samples we cannot rule out the presence of structural subunit particles, the formation of fibers requires that there be physical cohesion between the proteins both within a given subunit and in neighboring subunits: they cannot be merely immersed as highly mobile units in a lipid bilayer at the time the membranes are deformed (i.e. following dehydration).

A chloroplast membrane of morning glory that was uniformly deformed is shown in Fig. 6. Again, shear lines separating blocks of material are seen at ca. 45° angles to the draw direction. The presence of the quantasomes, however, results in considerable surface texture on the 100 Å size scale. The irregularity of packing of the quantasomes both before and after deformation makes it impossible to determine if plastic deformation has occurred within the blocks or whether they act as rigid bodies. Thus, it is not possible to determine if the quantasomes contribute to the structural integrity of the membrane or are merely carried along as a surface feature.

The results of deformation of rat kidney plasma membranes and rat nuclear membranes are shown in Fig. 7. Fibers ca. 200 Å in diameter are observed. Although some irregularity was seen in a few micrographs of the kidney plasma membranes, the fibers na here are seen to be essentially smooth, similar to those seen in the erythrocyte ghosts. e

As pointed out above, the ultrastructural deformation technique has been applied to a number of different types of membranes. In all cases, fibers were found to be pulled out parallel to the draw direction. These experimental results strongly suggest that this deformation behavior is a universal property of biological membranes, containing typical levels of protein (protein-poor membranes, such as myelin, were not examined; however, similar features are expected, since we attribute the cohesion of the membranes in vivo to the same macromolecular interaction postulated above). However, consideration must be given to the effect of dehydration; it is highly possible that there is a rearrangement of the molecular organization during drying prior to the deformation. The only way to solve this problem is to stretch the membranes while they are still wet for comparison with the

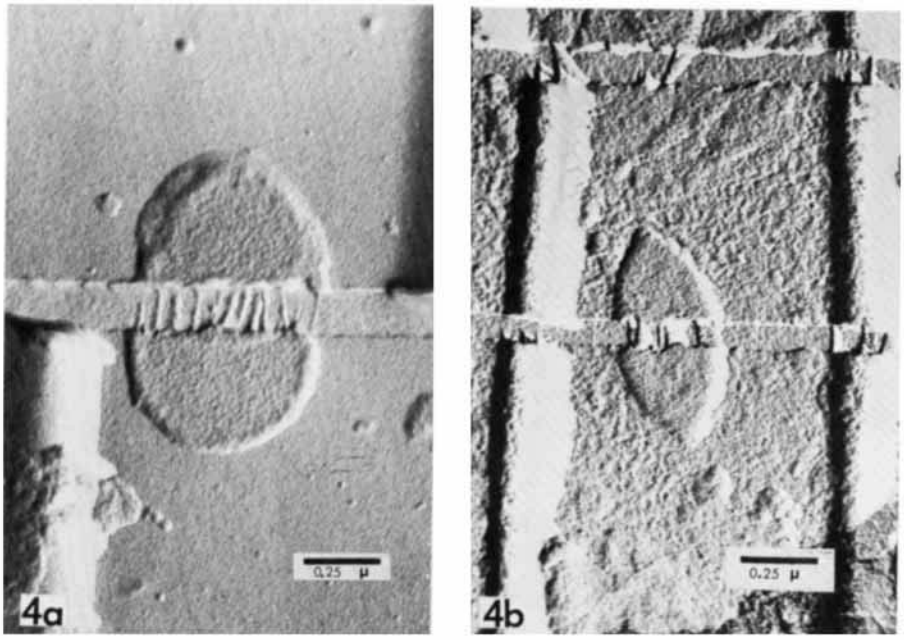

Fig. 4. Electron micrographs of (a) morning glory chloroplast membrane, and (b) spinach chloroplast membrane drawn on carbon-coated Mylar.

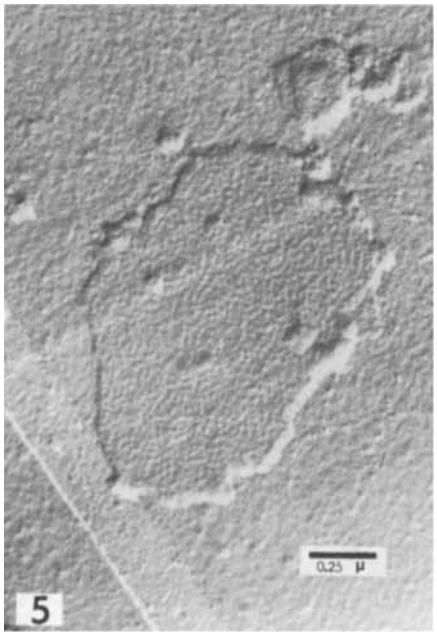

Fig. 5. Electron micrograph of morning glory chloroplast membrane.

Fig. 6. Electron micrograph of morning glory chloroplast membrane uniformly drawn 75% on Mylar.

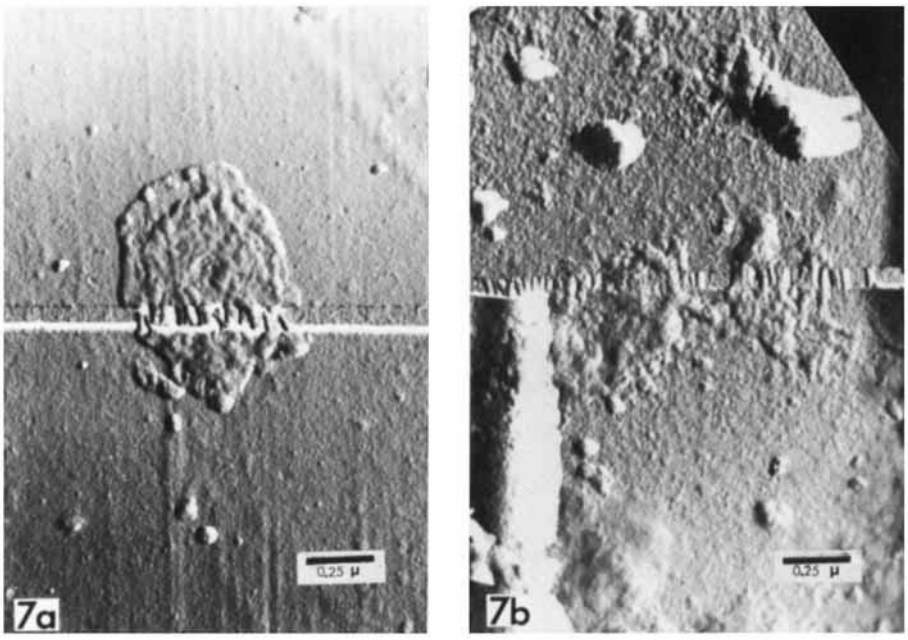

Fig. 7. Electron micrographs of (1) rat kidney plasma membrane, and (b) rat liver nuclear membranes drawn on carbon-coated Mylar.

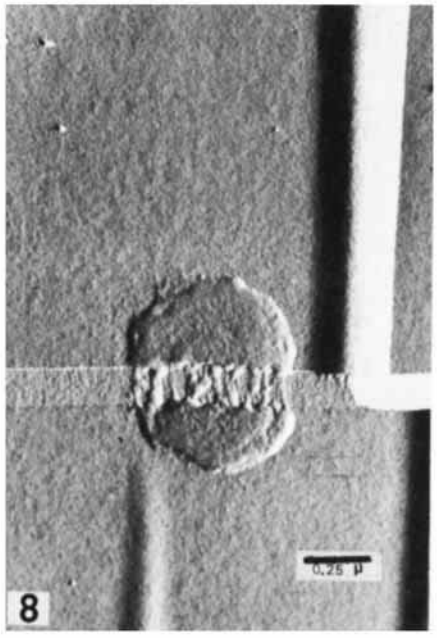

Fig. 8. Electron micrograph of morning glory chloroplast membrane drawn while wet on carbon-coated Mylar.

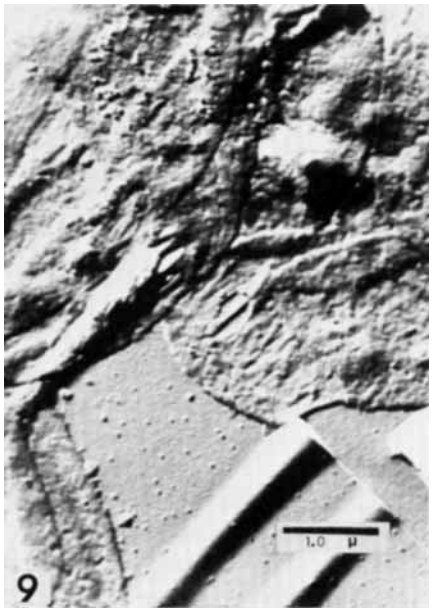

Fig. 9. Electron micrograph of human erythrocyte ghosts with lipids extracted drawn on carbon-coated Mylar.

dry ones. Figure 8 shows the results of deforming, at 4°C, membranes of morning glory while they were immersed in the suspension medium. No significant changes was observed as compared to the air-dried membranes; fibrils are drawn out across the cracks. Although the quantasome structure is not seen on the membrane surface, the bumpiness of the fibers is similar to that shown in Fig. 4.

Several experiments suggest that the fibers are predominantly composed of the membrane's structural proteins: (1) in the initial work of Blais and Geil (31), limited attempts were made to determine the nature of the fibers. As they showed, when the lipids are extracted from HEG before stretching, as shown in Fig. 9, fibers are still observed. Although the individual fibers are of about the same diameter as in the normal ghosts, they are many more per unit crack length. This is probably due to the larger amount of protein per unit area. As they observed also, the membranes shrink upon lipid extraction; we did not, however, observe holes in the membranes as they did. The shrinkage suggests that structural changes have occurred within the membranes. The fact that fibers, or aggregates thereof, are still observed indicates, however, that they are composed of interacting protein molecules. This is also suggested by their observations that similarly stretched cast thin films of the extracted lipid, chloresteryl myristate, the soluble protein from the ghosts, and a globular protein (lysozyme) did not show any evidence of fiber formation; failure occurred at the edge of the cracks in the carbon film with the lipid apparently flowing into the crack. (2) N. Wong (46) investigated* the effect of papain (supplied by Sigma Chemical) on the ability of membranes to form fibers during deformation. Rat liver plasma membranes, after drying down on the Mylar-carbon substrate, were treated with a papain suspension overnight, allowed to air dry, and then stretched. No fibers were observed, the membrane itself becoming smooth. In addition, when the membranes were treated in suspension with papain for various periods of time, 15, 30, and 45 min, air dried, and then subjected to stretching, it was shown that the number of fibers decreased as the treatment time increased, eventually disappearing. Although, to our knowledge, the papain was not tested for phospholipase contamination which could alter the lipid structure and properties, the above results and those with the model systems all lead to the conclusion that the fibers are composed primarily of proteins; lipid can be drawn along with the fibers in the normal membranes but does not contribute to the fibers' integrity.

As pointed out above, the results of our ultrastructural deformation studies on various membranes do not agree with current models which suggest that the proteins in membranes, wet or dry, form individual aggregates surrounded by lipids. The formation of fibers in the membranes subjected to deformation strongly suggests that there must be considerable intermolecular interaction and cohesion between the protein molecules, either by covalent bonds, interpenetration, or entanglement. This interaction must be between the proteins in the membrane and cannot be simply a result of their association with, for instance, a network of spectrin fibrils; a network of bumpy fibrils extending across the cracks would then be observed. However, it is possible that the spectrin or similar structural proteins participates in the interaction and cohesion between the membrane proteins. The protein molecules in the membranes uncoil or unfold to cooperatively

*Working with J. Zull of the Department of Biology, Case Western Reserve University.

form fibers as the membranes are deformed. In general no evidence of a subunit structure within the membranes is seen, although, in some cases (e.g., MGM) enzymes or other macromolecular systems attached to the surface may be drawn along with the fibers. In addition, no evidence was found for a change in the deformation characteristics of red cells as a function of storage time.

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