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An Alternative Model For Molecular Organization in Biological Membranes

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Numerous models have been proposed for the molecular organization of lipids and proteins within biological membranes. Each model has its own merits and is consistent with at least some of the evidence derived from one or more membrane species, but no general structure applicable to all membranes is yet available. In fact such a structure may not exist. It is possible, however, that there are specific associations between lipid and protein molecules which do contribute to the structure of most biological membranes.

Several reviews of membrane organization at the molecular level have recently been published.[1–4] It is not within the scope of the present article to further weigh the evidence for various proposed structures. It is intended, rather, to suggest an alternative model for lipid-protein associations in membranes, which to the author's knowledge has not been previously emphasized. The model is consistent with several lines of evidence which in past studies have been used to support a variety of apparently contradictory hypothetical structures. It also suggests several definitive experimental tests.

Since the model is specifically derived for lipid-protein complexes within membranes, it would be useful to briefly outline structural features of current models of this interaction.

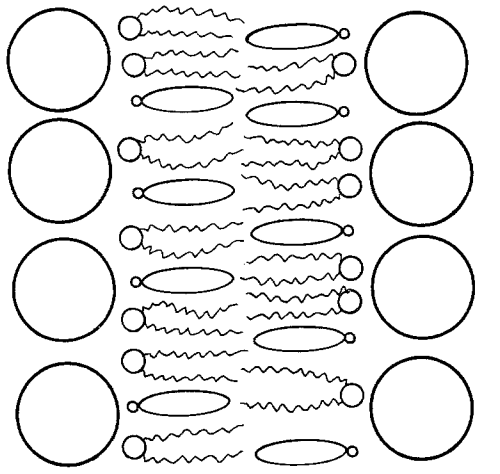
Protein-Lipid-Protein Membrane Models

Protein-lipid-protein (PLP) models are represented by the familiar structures proposed by Davson and Danielli[5,6] and Robertson[7] in which a bimolecular layer of lipid is sandwiched between two layers of protein (Fig. 1). The hydrocarbon tails of the lipid are directed inward, and in modern variations a certain amount of the protein may extend into or through the interior lipid phase.[2,8] The major forces by which lipid and protein associate in PLP structures would be electrostatic in nature, involving interaction between phospholipid head groups and charged protein groups.[2,9–11]

Lipid-Protein-Lipid Membrane Models

A great deal of interest has recently been focused on measurements of optical parameters in membrane protein. Optical rotatory dispersion (ORD), circular dichroism (CD) and infrared spectra have provided evidence that the major portion of membrane protein is α helical.[12–17] Certain anomalies in the spectra from various membranes were also found. For instance, ORD and CD spectra tended to be red shifted and were of remarkably low amplitude. These results led several groups to suggest that at least the α helical portion of membrane protein was in a nonpolar environment such as would

be provided by lipid hydrocarbon chains.[13, 15, 16, 18, 19] Thus, in the lipid-protein-lipid (LPL) model it is envisaged that lipid is still present in two layers but that membrane protein is arranged as a hydrophobically bound core between the lipid layers (Fig. 2) with the lipid chains directed into or around the bound protein. The major forces between lipid and protein in LPL structures would be London–van der Waals dispersion forces and hydrophobic interactions. Major contributions of hydrophobic interactions to membrane stabilization have also been proposed by previous investigators on the basis of other lines of evidence.[20–23]



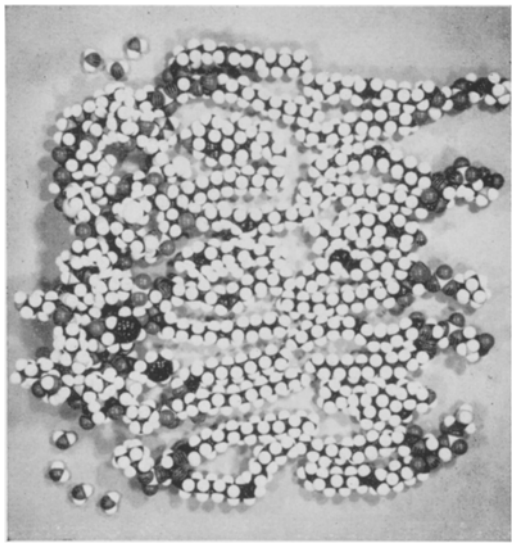


Figure 1. PLP membrane model. In Figs. 1–4, phospholipids, cholesterol, protein and water are shown both diagramatically and as molecular models. In the diagrams, phospholipid is represented by two chains attached to a head group, and cholesterol by an ellipse with a smaller head group. Proteins are shown as large circles. In Fig. 1, a polypeptide chain is shown interacting electrostatically with one side of a lipid bilayer. Water molecules (above and below the polypeptide chain) provide scale.

Particulate Membrane Models

A third membrane model is the concept of particulate or sub-unit membranes.[24–27] This concept does not place the lipid in layers but rather considers that membranes are composed of lipoprotein sub-units which may be dispersed and reconstituted into membranes by specific techniques. In a sense, the particulate model is a variation of the LPL concept, since much of the protein is considered to be in the interior of the membrane with hydrophobic interactions stabilizing the structure.

An Alternative Model For Lipid-Protein Associations

A major difficulty in the PLP concept arises when forces binding proteins to the lipid bilayers are considered. As noted previously, the obvious forces would be electrostatic in nature. However, many membrane proteins do not behave as though this were a simple charge interaction. A major portion of the protein is in fact strongly bound to the membrane and can be readily solubilized only by rather drastic measures such as use of detergents.

Strong lipid-protein interactions are provided by LPL membrane models in which protein is bound hydrophobically to the interior structure of the membrane, but it is difficult to imagine how the α helices of a large protein molecule might be distributed among the hydrocarbon chains of lipid

molecules. Furthermore, the LPL model implies that a large portion of the protein surface is composed of aliphatic amino acid side chains in order to provide hydrophobic interactions with lipid chains. A requirement for hydrophobic surfaces complicates mechanisms by which cells may synthesize membrane proteins in aqueous environments.

In order to avoid the necessity for hydrophobic protein surfaces, but still provide strong binding of protein to lipid, the following model is proposed:

(1) It is generally assumed that both hydrocarbon chains of phospholipids are directed inward in membranes, since this is the most acceptable conformation for lipids in bulk phase and in lipid bilayer membranes. However, the presence of protein in lipoprotein membranes allows an alternative conformation. The present model proposes that membrane lipid is arranged in a bilayer, but that only one of the hydrocarbon chains is necessarily directed inward. The other chain may be directed outward into a protein layer (Fig. 3). Since a lipid molecule forms a bridge between surface protein and the membrane interior, we shall hereafter refer to this structure as the lipid bridge model.

(2) The outwardly directed chain interacts hydrophobically with a protein molecule which has one or more binding sites for hydrocarbon chains. An important model of this interaction would be the serum albumin-fatty acid binding site which strongly binds fatty acids through hydrophobic interactions with their hydrocarbon chains.[28,29] A single protein molecule may have more than one chain binding site. For instance, Ji and Benson[23] have reported that chloroplast lamellar protein binds up to 36 hydrocarbon chains per mole, presumably by hydrophobic interactions, whereas serum albumin has six high energy hydrophobic binding sites per mole for fatty acid chains.[29]

(3) The inwardly projecting tails are shown as interdigitating in the center of the membrane. This is proposed in order to provide an area per phospholipid

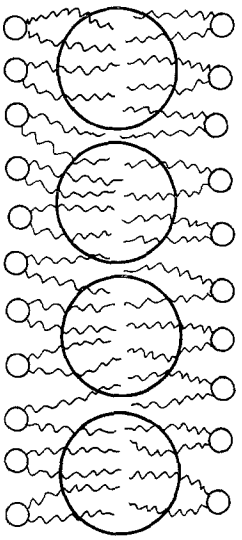


Figure 2. LPL membrane model. In this model, protein is hydrophobically bound to the non-polar interior of a membrane. The diagram is highly simplified and is intended only to illustrate a hydrophobic bonding mode. The polypeptide chain in the photograph (between the lipid layers) contains 50 amino acids. A typical membrane protein might have 5–10 times this number of amino acids and would occupy a correspondingly larger volume than shown here.

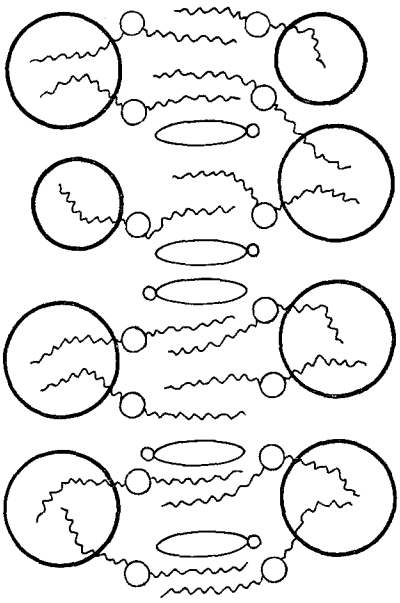
molecule consistent with those calculated for erythrocyte membranes.[30,31] Evidence that interdigitation may occur in lipid–protein model systems has been provided by X-ray diffraction data.[32] However, interdigitation is by no means a requirement of the model. There is no present evidence which compels us to specify an area per hydrocarbon chain within the membrane.[3,30] Cholesterol, when present, would also be arrayed in the inner layer of lipid chains. It should be noted here that all the lipid chains in a membrane need not be in the lipid bridge configuration. Those not interacting with surface protein sites would presumably have both chains directed inward.

(4) A variant of the lipid bridge model is shown in Fig. 4. In this structure, only a monolayer of lipid is required and both tails of the phospholipid would interact hydrophobically with protein on either side of the membrane. A model similar to that shown in Fig. 4 has also been suggested by Hybl and Dorset.[33] These investigators found that derivatives of 11-bromoundecanoic acid had an extended chain configuration in crystals, and proposed that monolayers of phospholipid in the extended chain configuration might occur in some cell membranes. Cholesterol would be present as a bilayer intercalating with the chains of the phospholipid.

The general model described in 1–4 above resolves the problem outlined earlier. Membrane protein could have strong hydrophobic associations with membrane lipids, in accordance with ORD and CD data, but would not necessarily be in the membrane interior. There are a number of other features to the model:

(1) It provides a rationale for the presence of two fatty acid chains per phospholipid. One interacts hydrophobically with a binding site on a protein molecule and the other binds the complex to the rest of the membrane.

(2) It is known that single phospholipid



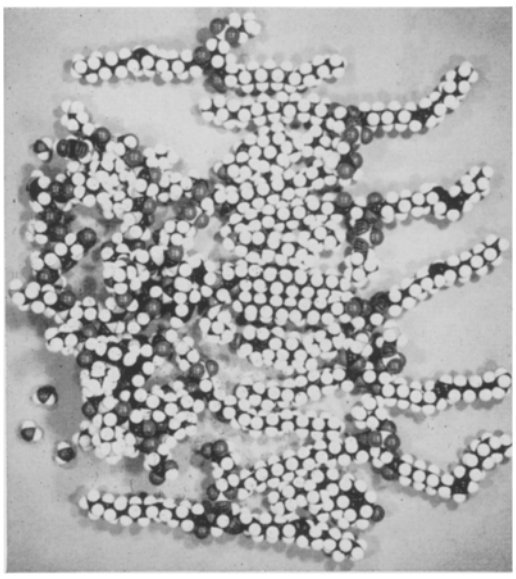


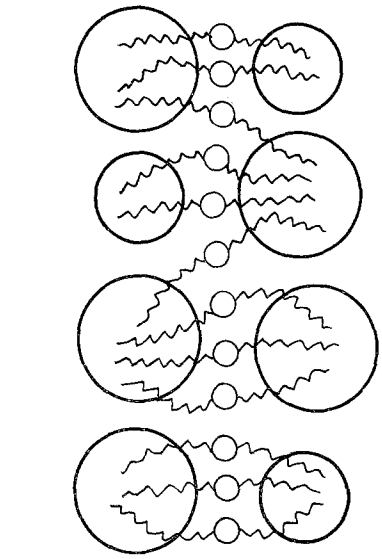
Figure 3. Alternative lipid–protein association. In this structure, lipids are present as a bilayer, but each phospholipid has one chain directed into the non-polar membrane interior and the other chain interacting with a hydrophobic bonding site on membrane protein. Cholesterol is distributed among the inner hydrocarbon chains.

molecules typically have both unsaturated and saturated fatty acid chains.[34,35] These may function differentially as described above. For instance, saturated chains might reside in the inner non-polar layer, since this would maximize van der Waals interactions. Unsaturated chains would then interact with protein binding sites. Placement of unsaturated chains in the protein is also supported by evidence from the serum albumin–fatty acid model (Alec Keith, unpublished observations. See also ref. 29). In general, saturated fatty acids are less strongly bound than unsaturated acids. Palmitic acid is an exception to this rule.[29]

(3) The model readily accounts for electron microscopic images obtained from sectioned membranes. If saturated fatty acid chains were directed inward, as suggested above, and unsaturated chains bound to protein layers, osmium would preferentially react with the unsaturated chain and produce the trilaminar structure found in a number of membranes. No migration of stained material would be required.[36,38] On the other hand, in membranes with high concentrations of unsaturated fatty acids, such as chloroplast grana, osmium would stain the membrane interior also and trilaminar structure would be less apparent. This in fact has been noted in a number of studies.[38–40]

(4) It is not necessary that membrane proteins have hydrophobic surfaces, as required in LPL models where protein is surrounded by hydrocarbon chains. Only hydrophobic binding sites are necessary, as in the serum albumin–fatty acid model. Membrane proteins could thus be synthesized by the cell in essentially soluble form. They would become "insoluble" only after associating with membrane lipid chains.

The lipid bridge model is not intended to account for all lipoprotein interactions in membranes. It seems necessary that some protein extend through the membrane,



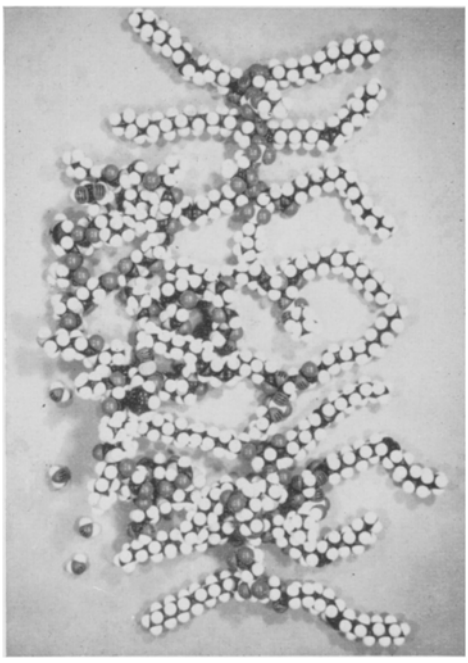


Figure 4. Alternative lipid–protein association. This is a variation of the structure shown in Fig. 3 and could occur in membranes with low lipid/protein ratios. Only a monolayer of lipid is required, and both lipid tails interact hydrophobically with protein. Cholesterol would not readily fit into such a structure.

particularly enzymes involved in transport processes. Furthermore, several membrane proteins such as cytochrome *c* and the various coupling factors have only loose associations with membranes. These proteins behave as though their interactions were electrostatic in nature. The present model does provide a possible lipoprotein structure for proteins which are arranged on membranes but need not extend through it.

There are a number of experimental tests which would determine whether the lipid bridge structure plays a major role in lipid–protein associations within membranes:

(1) Most membrane proteins should be potentially soluble in aqueous solutions, once the hydrophobic associations with membranes were broken. There is some initial evidence pertinent to this point. Ruby and Mazia[41] have found that extended dialysis of erythrocyte membranes against water does finally solubilize the protein moiety.

(2) X-ray diffraction data should show nonpolar regions in membranes to be less than 30 Å thick, if lipid chains are indeed interdigitated as drawn in Fig. 3. Recent studies by Worthington and Blaurock[42] have provided evidence that the apolar region of myelin is less than 20 Å thick, and it was suggested by these investigators that bilayers of lipid in the usual sense could not exist in myelin membranes.

(3) Differential thermal analysis comparing quantitative heat capacities of membranes and membrane lipid offers a third test. Steim *et al.*[43] have shown that transition temperatures of hydrocarbon chains in *Mycoplasma* and *E. coli* membranes are similar to transition temperatures of lipids derived from those membranes. Since not all of the lipid chains would be directed inward in the proposed model, it would be expected that heat capacity of lipids in membranes would be lower than the same lipids in bulk phase. In initial experiments, it was found that heat capacity of *Mycoplasma* membrane is in fact approximately 25% lower than the heat capacity of the extracted lipids. This was attributed to possible interaction between membrane protein and lipid and could be accounted for by the model proposed here.

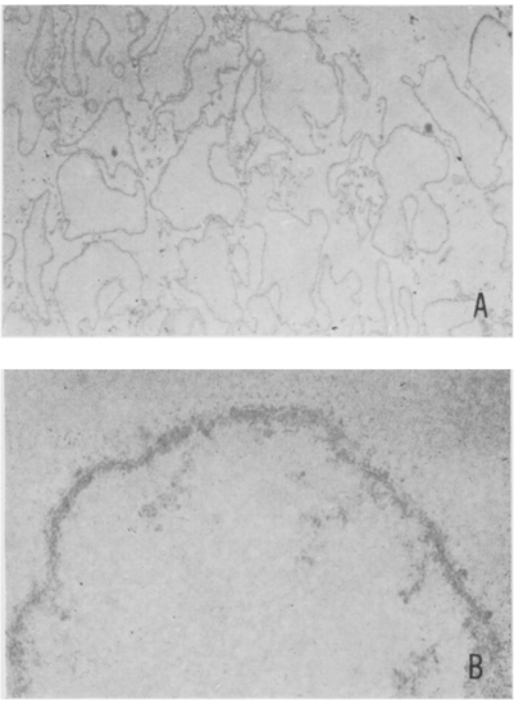


Figure 5. Glutaraldehyde-fixed beef erythrocyte ghosts. Trilaminar structures may be observed in portions of the membrane under high magnification. The membranes vary from 100 to 200 Å in thickness. Some of the apparent thickness is probably due to residual hemoglobin which has been allowed to remain with the ghosts. (A) 5,700×, (B) 120,000×.

(4) The last test involves the appearance of fracture planes in frozen-etched membranes. Initial studies have been completed and the pertinent results will be outlined here. Details of these experiments will be reported elsewhere.[44]

Membrane proteins involved in lipid bridge interactions should have binding sites for hydrocarbon chains. Such proteins would be normally bound to membranes through

the lipid bridge, but single-chained amphipathic molecules would be expected to solubilize them by competing for the binding sites on the protein. Terry *et al.*[45] for instance, have shown that sodium dodecyl sulfate completely displaces lipid from membrane protein, and lysolecithin readily solubilizes membranous systems such as myelin.[46] It follows that if it were possible to fix the proteins so that they could not be released from the membrane, single-chained lipids would still compete for hydrophobic binding sites and displace double chained lipids, but the membrane structure would remain intact.

If such a replacement could be carried out, any membrane properties which depended on continuous lipid apolar phases would be dramatically altered in the lipid bridge model, since the two chains of a lipid molecule are in different environments. This immediately distinguishes the lipid bridge model from unit and LPL models, since in the latter both chains are in the same environments, and replacement with single-chained species would not effect the general hydrophobic interactions within membranes.

One property dependent on extensive apolar phases in membranes is the fracture plane which occurs during freeze-fracture and freeze-etch electron microscopy. Branton[47] first proposed that this fracture passes along nonpolar planes in membranes, and abundant evidence is now available which strongly supports this concept.[1,48,49] We will therefore assume here that the presence of fracture planes indicates extensive nonpolar regions in membranes. Absence of fracture planes would then indicate the absence of such regions. If the lipid bridge model is correct, it would be expected that single-chained lipids would readily replace double-chained lipids from fixed membrane protein by competing for hydrophobic binding sites. It is further seen that extensive nonpolar regions would then be lost, since only a single tail is present on the replacing molecule. Freeze-fracture planes should therefore be absent in such membranes. This clearly distinguishes the proposed structure from other models. Simple replacement of double-chained lipids by single-chained lipids would not alter the general nonpolar interactions within unit or LPL models, and fracture planes should therefore be present.

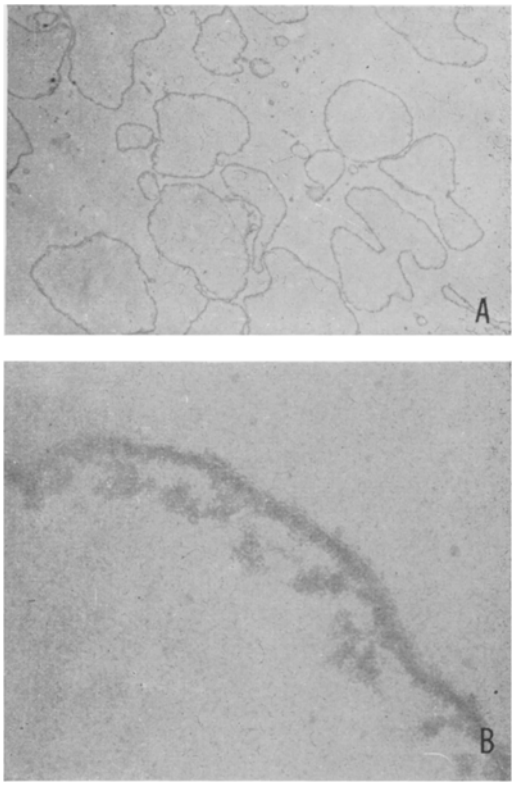


Figure 6. Glutaraldehyde-fixed beef erythrocyte ghosts. The same preparation as in Fig. 5 was incubated 1 h with lysolecithin (2·5 mgs/mg protein) prior to fixation in osmium tetroxide. Trilaminar structures are still apparent in portions of the membranes. (A) 5,700×, (B) 120,000×.

These points were tested by using glutaraldehyde to fix and stabilize the position of protein in a membranous system. Glutaraldehyde is a five carbon dialdehyde which

cross-links amine groups in biological polymers.[50] However, it does not fix lipids to membranes, with the possible exception of phosphatidyl ethanolamine[51] nor does it affect freeze-etch images of membranes.[48] Lysolecithin was chosen as a single-chained lipid, since it is readily soluble in water and has a polar head group similar to those of natural membrane lipids (lecithin and sphingomyelin). The rationale of the experiment was therefore to fix membranes with glutaraldehyde and expose the fixed membranes to solutions of lysolecithin. The membranes were then examined by sectioning and freeze-etch electron microscopy and lipid composition was estimated by analytical and thin layer chromatographic techniques.

Lipid replacement was carried out on bovine erythrocyte membranes. Ghosts were prepared by the procedure of Dodge *et al.*[52] and fixed in 2·0% glutaraldehyde, pH 7·0, for 1 h at 20°C. A small amount of hemoglobin was purposely left in the ghosts, since it was found that residual hemoglobin helped stabilize the fixed ghosts during later washing procedures.

The fixed ghosts were then exposed to several concentrations of lysolecithin for one hour at 20°C and washed twice in 10 mM Tricine buffer. Lipids of unfixed and fixed ghosts, and lysolecithin treated ghosts were extracted with chloroform:methanol 2:1 and chromatographed on thin layer silica gel G plates. Phosphate and cholesterol were measured on each sample.

Specimens were prepared for electron

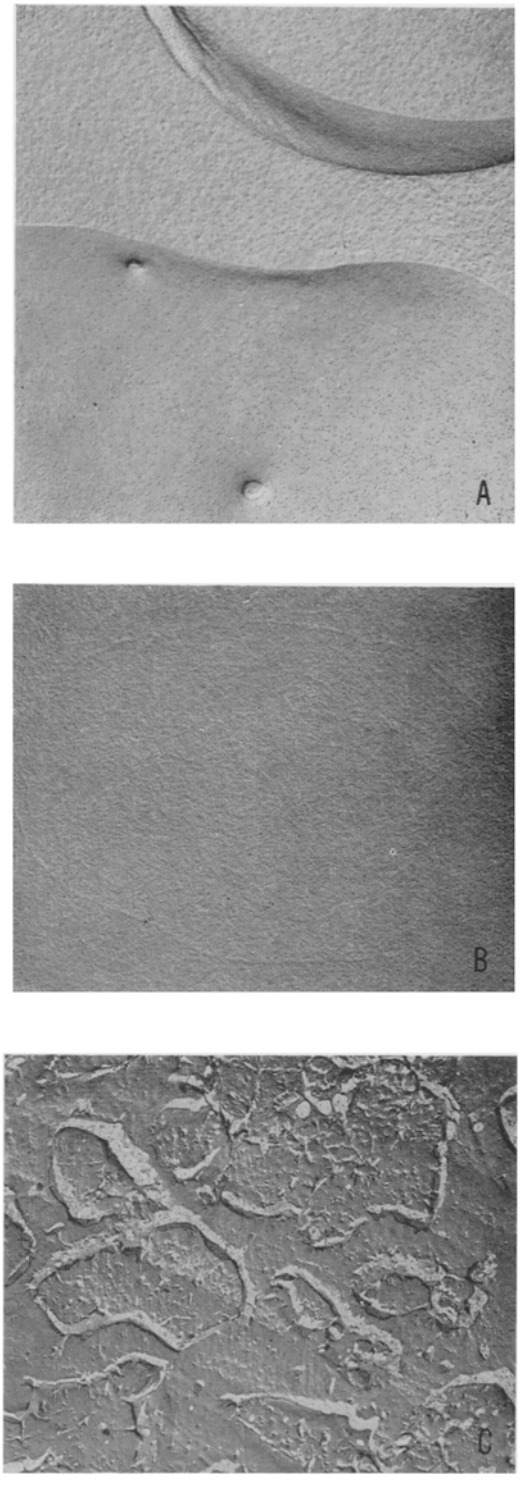


Figure 7. Frozen-etched beef erythrocyte ghosts. (A) Glutaraldehyde-fixed ghosts frozen in 2 M sucrose. Fracture planes are abundant. Particles are distributed on all fracture faces. 35,000×, 1-min etching. (B) Glutaraldehyde-fixed ghosts frozen in 2 M sucrose following lysolecithin incubation. Fracture planes are absent, and only cross-fractured membranes can be found. 20,000×, 1-min etching. (C) Same as (B), but frozen in 10 mM Tricine buffer. Deep etching has occurred, revealing membrane surfaces. Fracture planes are absent. 5,700×, 1-min etching.

microscopic examination by fixation and staining in 1% osmium tetroxide, followed by dehydration in acetone and embedding in Epon 812 resin. Sections were post-stained in uranyl acetate and lead citrate. Freeze-etching was carried out as described in previous studies.[1]

The results of these experiments may be summarized as follows:

(1) Unfixed ghosts were highly labile to the concentrations of lysolecithin used in the replacement studies. However, glutaraldehyde fixation prevented solution of ghost membranes, and examination of ghost suspensions by phase contrast microscopy disclosed little difference between fixed ghosts before and after lysolecithin treatment. Even high concentrations (5 mM) of detergents such as Triton X-100 and sodium dodecyl sulfate did not disrupt the ghost membranes.

(2) Thin-layer chromatography of lipids extracted from unfixed ghosts showed the major lipid constituents to be sphingomyelin, phosphatidyl ethanolamine, phosphatidyl serine and cholesterol, in agreement with previous data.[53] However, phosphatidyl ethanolamine and phosphatidyl serine were not extractable after glutaraldehyde fixation, confirming the finding of Gigg and Payne[51] that phosphatidyl ethanolamine cannot be extracted from glutaraldehyde-fixed tissues. Since phosphatidyl ethanolamine and serine compose about 48% of bovine erythrocyte phospholipids[53] it is assumed that these lipids are not available for replacement in the fixed membranes.

(3) Exposure to lysolecithin (2·0 mgs/10^9 ghosts) caused nearly total displacement of sphingomyelin from ghost membranes. Approximately 75% of the cholesterol was also displaced. Lysolecithin was strongly bound to the ghosts and was not lost during two washes in Tricine buffer.

(4) Comparable cross sections of control and lysolecithin treated membranes are shown in Figs. 5 and 6. Trilaminar structures could be found in all membranes, and in general little difference was seen when control and lysolecithin treated membranes were compared.

(5) Frozen-etched membranes are shown in Figure 7. Fracture planes were abundant in both unfixed and fixed membranes. This confirms earlier reports[48] that glutaraldehyde fixation does not appreciably affect fracture planes. No marked differences in fracture surfaces could be seen when fixed and unfixed material was compared.

(6) Fracture planes were totally absent from lysolecithin treated membranes. Only faint cross-sections of membranes could be found (Fig. 7B). If preparations were deep-etched (Fig. 7C) the usual membrane surfaces were readily apparent.

These studies have established that a single-chained phospholipid can displace double-chained species from glutaraldehyde-fixed membranes. Much of the cholesterol is also lost during this process. The appearance of sectioned membranes is not markedly altered by lysolecithin treatment. However, the fracture planes of frozen-etched membranes are totally absent. These results are consistent with and predictable from a lipid bridge conformation in plasma membranes. They do not differentiate between the model proposed here and the structure suggested by Hybl and Dorset.[33]

An alternative explanation in terms of the PLP model is that lysolecithin displaces double-chained lipids from fixed membranes but does not in turn form a bilayer with extensive non-polar regions. This possibility cannot be discounted in interpreting the present results. However, the results are not readily understood in terms of the LPL membrane model. Replacement of double-chained lipids with single-chained lipids

would not be expected to markedly alter hydrophobic interactions in the LPL model.

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