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Biological Membrane Structure, I. The Protein Crystal Model for Membranes*

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Abstract. A geometric model for the arrangement of phospholipid and protein in biological membrane systems has been proposed. The essential principle underlying this model is that when membrane proteins polymerize, the points of contact between proteins are few, and cavities lined with predominantly nonpolar amino acids are formed. Phospholipid molecules become oriented with the fatty chains inserted into the cavities while the polar heads remain on the surface of the membrane. This orientation applies to both faces of the membrane continuum. All the lipid known to be present in membranes can be accommodated in this manner. The body of evidence supporting this model has been presented.

In 1966 Green and Perdue[1] proposed that biological membranes are two-dimensional continua of nesting lipoprotein repeating units. One of the foundation stones of the repeating unit concept is the hydrophobic bonding of phospholipid to protein. Although there is now quite general agreement about the fact of hydrophobic bonding, no satisfactory geometrical model has yet been proposed to account for it. While the repeating unit model provides a geometrical picture for the organization of lipoprotein complexes, it does not attempt to explain the relationship of the lipid and protein molecules within these complexes. The Danielli-Davson-Robertson unit membrane model,[2,3] on the other hand, is a geometrical model for the lipid-protein interactions, but this model fails to explain the observed hydrophobic bonding. Therefore, a new model was needed for this aspect of membrane structure; the purpose of this paper is to present a model which supplements rather than replaces the repeating unit model, and deals with the relationships among the individual lipid and protein molecules, rather than between organized lipoprotein complexes. The clue which led to the discovery of this new model was the examination of the literature on protein crystallographic studies. These studies have shown that there are limited points of contact among the protein molecules in crystals, and that between the points of contact are cavities which are filled with water molecules. Since the amino acid side chains which line the cavities are predominantly polar, the presence of water is energetically favorable. The water content of protein crystals in some cases exceeds 50% of the total mass of the crystal. It occurred to us that if proteins which form membranes have a relatively higher proportion of nonpolar amino acids on their surfaces, then the cavities formed by their

association to form a membrane continuum would have a more hydrophobic character and could be filled with phospholipid molecules, instead of water, as in the case of crystals of the common soluble proteins. In the present communication, this cavity concept as the basis of phospholipid-protein binding in membrane systems will be systematically developed.

A Geometric Model for the Interaction of Phospholipid and Proteins in Membranes. The model to be described should apply to all biological membranes since they all contain, if not phospholipid, at least some comparable bimodal molecule (sulfolipid, glycolipid), and about the same range of proportions of lipid and protein. It should also apply to membranes which contain neutral lipids such as cholesterol in addition to phospholipids, since a large amount of cholesterol (up to 1 mole of cholesterol per mole of phospholipid[4,5]) can be accommodated between the tails of the phospholipid molecules without significantly increasing the area per phospholipid molecule. The model, illustrated in Figure 1, consists of two layers of loosely packed globular proteins, with the crevices or interstices between the protein molecules being filled with the nonpolar tails of lipid molecules, so that the polar heads of the lipids lie at the two membrane-water interfaces. The proteins are assumed to have more or less extensive hydrophobic regions on their surfaces, permitting hydrophobic bonding with the lipid molecules. The protein molecules have limited regions of contact with each other, meaning that protein-protein interactions will be involved in giving structural stability to the membrane. The lipid molecules do not make the membrane thicker than the thickness of the double protein layer itself, since the nonpolar tails are within the bounds of the protein layers. The presence of these lipid tails in the interstices will greatly enhance the mechanical strength of the membrane; they will also greatly affect the permeability properties of the membrane.

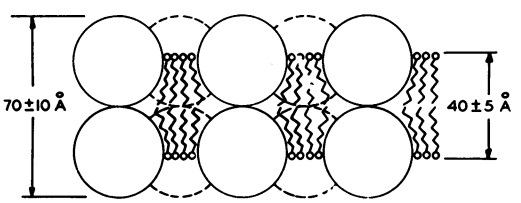

FIG. 1.—Diagrammatic cross section of a membrane, showing a double layer of protein molecules (*large circles*) with lipid bilayer regions filling the pores between them. The proteins drawn with dashed circles are understood to be in contact with the solid circle proteins behind the plane of the section.

Supporting Evidence for the Model. Hydrophobic bonding of lipid to protein: The effect of salt on membrane stability and on the interaction of lipids with proteins has been studied by several laboratories.[6-10] The rationale behind these experiments is that increasing the ionic strength is known to weaken ionic bonds, so that if the interaction between protein and lipid is primarily of an electrostatic nature, addition of salt to the medium should separate the lipid from the protein. This has indeed been shown to happen in the case of the interaction of acidic or mixed phospholipid micelles with the basic protein, cytochrome *c*.[6-9] The complex which forms evidently consists of the basic cytochrome *c* molecules interacting ionically with the polar heads of the acidic phospholipids. The complex is readily extracted from an aqueous medium into

isooctane[6,7] or heptane,[8] whereas neither the phospholipid nor the cytochrome *c* is extracted independently by these solvents. The formation of the complex is strongly inhibited by as little as 0.1 M salt.[6,7] These experiments justify the assumption that salt weakens or breaks ionic protein-lipid linkages.

The cytochrome *c*-lipid binding experiments are to be contrasted with the effect of salt on membrane protein-lipid interactions. It may initially be pointed out that lipoprotein membranes are not separated into lipid and protein components by the addition of salt.[11] Lenaz *et al.*[10] have carefully studied the effect of salt on the interaction of lipid-depleted mitochondria with phospholipids. They found that just as much phospholipid would rebind to the lipid-depleted mitochondria in the presence as in the absence of 1 M NaCl; the amount rebound equaled the amount of phospholipid in intact mitochondria if a sufficiently high ratio of lipid to protein was used in the reaction mixture. These experiments clearly demonstrate that the mechanism of binding of lipid to protein is different in membranes than in the cytochrome *c*-lipid system since this binding is insensitive to salt. The logical deduction made from these experiments is that nonionic, hydrophobic forces are of primary importance for the interaction of lipids with proteins in membranes.

Location of the polar heads of phospholipid molecules in membrane systems: It is well known that cytochrome *c* is easily extracted from swollen mitochondria by 0.15 M KCl.[12,13] This shows that electrostatic forces are of primary importance in binding cytochrome *c* to the mitochondrial membrane, just as in the case described above for the interaction of cytochrome *c* with phospholipid molecules. Furthermore, the extent of binding of cytochrome *c* with purified cytochrome oxidase has been shown to be directly proportional to the phospholipid content of the cytochrome oxidase, demonstrating that phospholipid is involved in the cytochrome *c* binding.[14] (In another study by the same laboratory,[15] it was shown that the cytochrome oxidase preparation is a membranous material under the conditions that were used to assay cytochrome *c* binding.) These observations confirm the notion that the polar heads of the phospholipid molecules are located at the membrane-water interface where they are readily available for interaction with cytochrome *c*. (The alternative explanation that cytochrome *c* lies within the membrane is not consistent with its ease of salt extraction, or with its known highly polar and hydrophilic nature.)

The enzymic cleavage of membrane-bound lecithin by phospholipase C, yielding phosphoryl choline and diglyceride, also supports the interpretation that the polar heads of the phospholipids are on the membrane surface. This reaction has been performed both on human red blood cell membranes[16] and on mitochondrial membranes.[17] The same result was obtained in both cases, namely, that the water-soluble phosphoryl choline was released into solution, but the diglyceride remained membrane bound. Examination of the treated red blood cell membranes with the phase-contrast microscope,[16] and of the mitochondrial membranes with the electron microscope[17] showed that they remained intact. This result would not be expected if the phosphoryl groups were within the membrane rather than on the surface.

The protein and lipid components share the same volume: An integral fea-

ture of the present membrane model is that the nonpolar lipid tails fill the interstices between globular protein molecules. A direct line of evidence on this point comes from the electron microscopic examination of lipid-depleted membranes. In each of the three cases available (mitochondrial inner membranes,[18,19] myelin membrane,[20] and erythrocyte ghost membrane[21]), the lipid extraction did not destroy the membranous appearance of the material, nor did it significantly alter the thickness of the membrane. As usual, the interpretation of these micrographs is not unambiguous, but it would appear that the simplest explanation of them is that the lipid occupied the same layer as the protein. If the lipid had occupied a separate layer or layers, either inside or outside the protein layers, one would have expected to see a gross change in morphology.

The question may be raised whether the proteins in a membrane may touch each other, as postulated, and still allow enough room between them for the large amount of lipid known to be present in membranes. The membranes in which we are interested have protein to lipid ratios ranging from 0.8 to 4.0 by weight,[22] or, in other words, from 20 to 55% lipids by weight. On a volume/volume basis, these figures become 27–64% lipid, assuming a density of 1.3 for the protein and 0.9 for the lipid. This range includes all the membranes, except myelin, for which data are given in reference 22. The question raised may be answered in the affirmative, based on information gained from the study of protein crystals. Protein crystals characteristically contain vast amounts of solvent of crystallization which fill the spaces between the protein molecules. For example, the lysozyme,[23] myoglobin,[24] and ferricytochrome c[25] crystals contain 33.5, 40, and 55% solvent of crystallization respectively. (Since the density of the solvent of crystallization is approximately equal to the mean protein density in these cases, percentages by weight and volume are nearly equivalent.) These data demonstrate the feasibility of constructing protein lattices stabilized by interprotein interactions, while at the same time maintaining adequate space between the molecules to include a large amount of solvent. We therefore conclude that sufficient space can also exist between protein molecules in membranes to accommodate all of the lipid, since the range of lipid percentages found in membranes nearly coincides with the range of percentage of solvent found in crystals. This conclusion was also confirmed by the construction of membrane models in which the proteins were represented as uniform spheres, and were packed in various regular two-dimensional lattices.

The hydrophobic nature of membrane proteins: If the interactions between lipids and membrane proteins are hydrophobic, then the membrane proteins must display nonpolar, or at least nonionic, regions on their surfaces where this kind of interaction can occur. For all of the soluble proteins on which crystal data are available,[24–27] the central cores of the molecules are made up primarily of amino acids with hydrophobic or at least nonionic side chains, while virtually all of the polar side chains are on or near the surface. There is a partial exception to this rule in the case of ferricytochrome c,[28] where two nonpolar "pseudochannels" extend from the nonpolar core to the surface. All of these proteins are readily soluble in aqueous media, and in their crystals the interstitial spaces are filled with aqueous solution. The essence of what we are now proposing is

that a fundamental difference between membrane proteins and soluble proteins is the proportion of nonpolar amino acids on their surfaces, with the proportion being significantly greater for the membrane proteins. This is consistent with the low solubility of the majority of membrane proteins in aqueous media in the absence of lipid or detergent. We do not mean to propose that the ionic side chains become buried inside of the protein, since that would require the stripping off their hydration shells which is energetically very unfavorable.[29] Rather, we are suggesting that a larger-than-normal proportion of nonpolar side chains are on the surface in addition to the polar ones, perhaps in a manner similar to, but greater than, that found for cytochrome *c*.[28]

In a membrane, the most highly polar part of the protein surface probably lies at the membrane-water interface. It is inevitable however that some of the ionic groups will also be on the sides of the protein facing the interior of the membrane; we assume that these will retain their hydration shells within the membrane. Direct experimental evidence bearing on the presence and amount of water in membranes is sparse, and this problem should be examined more carefully, perhaps by differential thermal analysis and thermogravimetric techniques.[30] The presence of some water within the membranes with which we are concerned is assured, however, by the mere fact that they are permeable to water.

The globular nature of membrane proteins: We assume that membrane proteins in general have compact, spheroidal, globular shapes, and are not extended fibrous structures. Consequently, lipid molecules cannot penetrate individual protein molecules, except perhaps in special cases. The fact that the proteins of many membranes (e.g., mitochondrial, chloroplast, retinal rod) act as enzymes supports this view, since the vast majority of enzymes for which the shape is known are globular, and a globular form evidently is necessary to retain the integrity of the active site of an enzyme.

Electron microscopy of membranes has in some instances given definite evidence for the presence of globular material in membranes. Crane and Hall[31] have published micrographs of mitochondrial membranes showing 50-Å globules which they interpret to be proteins. Similar globules are also evident in the micrographs of Cunningham *et al.*[19] that show mitochondria from which the lipid had been extracted with acetone. We have recently obtained some excellent micrographs of mitochondrial cristae which show regular double layers of 30 to 35-Å particles making up the membranes. The main point to be noted for present purposes is that the general form of the material is spheroidal and not fibrous.

Measurements of intact membranes obtained by optical rotatory dispersion and circular dichroism have indicated the presence of an appreciable degree of α-helix content.[16,32–35] It is not necessary for a protein to have a high α-helix content in order to be globular (e.g., α-chymotrypsin[36] is spheroidal but contains very little α-helix), but the presence of a large amount of α-helix rules out the possibility that membrane proteins exist primarily in an open, β-type conformation. A compact structure for membrane proteins is thus indicated by these experiments.

Protein-protein interactions in membranes: It appears to be a general rule for the structure of protein crystals that the individual molecules touch their neighbors at only a limited number of points. It has been reported that there are no more than 4–6 limited regions of contact of each protein molecule with its neighbors in the lysozome,[26] myoglobin,[24] ferricytochrome *c*,[25] and oxyhemoglobin[27] crystals; the majority of these contacts are between polar parts of the proteins. These limited contacts are adequate to give the crystals their structure and to partially account for their stability. The interstitial solvent is also partially responsible for the stability of these crystals, as noted by the instability which results if the solvent is removed.

The significance of this information derived from crystal data for membrane structure is that the membrane proteins need only touch each other at a few points in order to appreciably affect the structure and stability of the membrane. The membranous appearance of lipid-depleted membranes[18–21] is evidence for the existence and importance of protein-protein contacts. The extracted membranes are much weaker than the intact membranes, which we presume to be an effect brought about by the substitution of organic solvent molecules (i.e., the extracting or fixing medium) for the lipid molecules in the interprotein spaces.

Conclusions. In this paper we have presented a model to account for the interaction of phospholipid and protein in membranes and have presented evidence to support it. We have not by any means exhausted all that might be said about it, nor have we mentioned many of the membrane properties which are explained by it. The freeze-etching technique of electron microscopists shows globular material when membranes are fractured; this would be the expected result for a membrane of two protein layers with bilayer lipid in the crevices. The electrical conductivity of some membranes is similar to that of lipid bilayers; this would be expected, since the membrane can be thought of as a sparse bilayer with a nonpolar interior and a polar exterior. Many membranes are permeable to water and other polar molecules; the existence of some polar groups on the protein surfaces within membranes, together with their hydration shells, can account for this permeability. The lipid in membranes has physical properties similar to lipid in bilayers; this results because of the similar orientation and freedom of motion of the lipid molecules in the membrane as in the bilayer. The membrane shows trilaminar structure, just as a bilayer does; this would be expected, since both have an essentially nonpolar core and polar surface. Although membranes and bilayers are similar in several respects, there are many different kinds of membranes with diverse chemical and physical properties; this diversity cannot be explained on the basis of lipid bilayer properties alone, but the present membrane model is sufficiently versatile to do so. We hope to discuss some of these topics in greater detail in future papers.

Note added in proof: As a result of a study of the available x-ray diffraction data on the retinal rod outer segment disk membranes (namely, Blasie, J. K., and C. R. Worthington, *J. Mol. Biol.*, **39**, 417 (1969)), the essentially static picture of a membrane presented in this paper has been modified toward a more dynamic two-dimensional liquid crystal model, in which the proteins and lipids are both in thermal motion in the plane of the membrane (Vanderkooi, G., and M. Sundaralingam, these PROCEEDINGS, in press). A consequence of this modification is that the lipid bilayer, on a time-average, forms a continuum, and the protein-protein interactions are made and broken in a fluctuating, liquid-like manner.

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* An earlier, preliminary version of the membrane model contained in this paper was presented at the Coral Gables Conference on the Physical Principles of Biological Membranes; Green, D. E., and G. Vanderkooi, in *Physical Principles of Biological Membranes*, ed. F. Snell, J. Wolken, G. Iverson, and J. Lam (New York: Gordon and Breach Science Publishers, Inc., 1970), pp. 287–304. The model presented here is an elaboration and extension of the earlier version, with several important changes in emphasis and interpretation.

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