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Structure-Function Unitization Model of Biological Membranes

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In 1966 Green and Perdue[1,2] proposed the repeating unit model of membrane structure, the central concept of which is the postulate that membranes are built up of lipoprotein repeating units. Each such unit was assumed to be a set of proteins associated hydrophobically with phospholipid. At that time many molecular features of the membrane were unresolved. Until these molecular details were clarified further progress in developing the repeating unit model was hampered. During the past several years many of the basic molecular parameters have been satisfactorily defined both experimentally and theoretically. The predominantly lamellar or bilayer character of phospholipid in membranes has been established firmly.[3–5] The characteristic features of intrinsic (integral) membrane proteins and the distinction between intrinsic and extrinsic (peripheral) membrane proteins have been sharply drawn.[6–11] The manner in which intrinsic proteins can associate hydrophobically with bilayer phospholipid has been more realistically evaluated.[12] The models of Vanderkooi and Green[12] and of Singer and Nicolson[8] rationalize in a satisfactory fashion the above mentioned molecular parameters of membrane structure. The way was thus cleared for a re-examination of the supramolecular features of membrane structure and for a more rigorous development of the lipoprotein repeating unit concept of Green and Perdue.[1,2]

The objective of the present communication is to present a general model of biological membranes which relates structure not only to function but also to membrane biogenesis. We shall initially consider the postulates which underlie this new model as well as some of the relevant evidence for these postulates; then describe the salient features of the model; and finally demonstrate the capability of the model for rationalizing a wide variety of membrane phenomena.

I. Basic Postulates Underlying the Model

1. *Intrinsic Versus Extrinsic Membrane Proteins*

There are two kinds of proteins in membrane systems—the intrinsic proteins which are part of the membrane continuum and hydrophobically associated with phospholipid, and the extrinsic proteins

which are associated with (usually electrostatically) but are not part of the membrane continuum. The differences between the extrinsic and intrinsic classes of proteins have been considered in detail in several reviews.[6, 8, 13] In the present communication our concern will be exclusively with intrinsic membrane proteins. To avoid possible confusion we stress that "protein" implies one polypeptide chain.

2. *Bimodality of Intrinsic Membrane Proteins*

Proteins which are intrinsic to the membrane must be capable of orientation at a water-hydrocarbon interphase[7, 14] and this capability requires that the surface groups of such proteins should be predominantly polar in one sector and predominantly nonpolar in an adjoining sector. We shall refer to proteins possessing this type of distribution of surface groups as bimodal proteins. The simplest case of a bimodal protein would be a globular protein with the surface groups of one hemisphere predominantly polar and the surface groups of the other hemisphere predominantly nonpolar. The actual ratio of polar:nonpolar surface area in membrane proteins is probably variable.[7] In describing a membrane protein as bimodal, we are merely inferring that the protein is capable of stable orientation at the water–lipid interphase of the membrane continuum and that the distribution of surface groups is compatible with such orientation. Elsewhere we have considered in some detail the various molecular tactics by which bimodal distribution of surface groups can be achieved as well as the optional surface geometries which bimodal protein molecules may assume.[6, 7] The mounting experimental evidence for the bimodality of intrinsic membrane proteins has been reviewed in several recent articles.[6, 7, 9, 10, 11]

A biological membrane presents two water–lipid interphases—one at each of the two surfaces. A simple bimodal protein with polar and nonpolar hemispheres (the P-N type) can orient at only one of the interphases. A more complex bimodal protein with two polar extremities separated by a nonpolar band (the P-N-P type) can orient at the two interphases. The simple bimodal protein penetrates half way into the membrane continuum whereas the complex bimodal protein spans the membrane continuum. We shall refer to the proteins with the P-N-P type of bimodality as "through" membrane bimodal proteins.

Both phospholipid and intrinsic membrane proteins are bimodal molecules in the sense defined above. Since membranes are built up from arrays of bimodal molecules oriented at right angles to the plane of the membrane,[12] it would be expected that membranes would have a bimodal character. Indeed, the pattern for all membranes is that of a thin sheet (60–100 Å thick) with two polar surfaces separated by a nonpolar interior.

3. *The pairing of Bimodal Molecules in Membranes*

The essence of a biological membrane is the concept of paired bimodal molecules. The bilayer pattern of phospholipid arrays is an expression of this pairing principle. The pairing has its roots in the thermodynamic necessity for bimodal molecules to orient in a fashion which minimizes exposure of hydrophobic surfaces to water. When arrays of bimodal molecules are paired by apposition of hydrophobic surfaces, the resulting "membrane" with polar groups on the surface and hydrophobic groups in the interior represents the minimum free energy state and hence the most stable configurations for such arrays. The paired molecules are always oriented at right angles to the plane of the membrane. In the continuum of biological membranes, we have paired arrays both of proteins and phospholipids and as we shall discuss later, these arrays are not randomly distributed. The combination of paired arrays of protein and phospholipid leads to a more stable membrane than that composed of protein or lipid alone as evidenced by the fact that phospholipid avidly combines with lipid-free membrane proteins.[15]

Although it might appear that bimodal proteins could equally well pair with phospholipids as with other bimodal proteins, we are postulating that pairing of like with like (protein with protein and phospholipid with phospholipid) is the universal pattern in biological membranes. There are two reasons that have led us to this postulate of like pairing—first the electron microscopic evidence that the double tier structure of the membrane remains even after extraction of phospholipid[16]—and second, the evidence to be developed later that protein and phospholipids appear to form separate domains in biological membranes. We suspect that there is a much more compelling theoretical basis for like with like pairing but this has yet to be recognized. The possibility of unlike pairing of protein and phospholipid at the present stage of our knowledge cannot be excluded, but it may not be a viable possibility for reasons of stability.

The through membrane bimodal protein may be looked upon as a fusion of two paired bimodal proteins of the P-N type and hence as an extension of the pairing principle. As we shall discuss later on, the through membrane bimodal protein appears to be specialized for immunochemical processes and may be an exception rather than the rule for intrinsic membrane proteins.

High resolution electron microscopy has established that membranes in cross section consistently show two tiers of staining centers[17] and these two tiers can be equated with the pairing of protein and lipid bimodal molecules in the membrane continuum. Electron microscopic examination of freeze fractured membranes has led to the now widely accepted interpretation[18–20] that the 60–100 Å thick membrane

sheet can be split down the hydrophobic middle into two sheets of half the thickness.

4. *Lipoprotein Repeating Structures as the Units of Membrane Construction and Function*

Biological membranes generally can be depolymerized to lipoprotein repeating units which spontaneously can coalesce to generate vesicular membranes when the depolymerizing reagent is removed[21–25] (see Fig. 1). These lipoprotein repeating units have been found to correspond to multimeric sets of proteins (complexes) associated hydrophobically with a complement of phospholipid—the units being stabil-

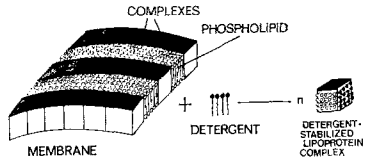

Figure 1. Depolymerization of membranes into lipoprotein repeating units.

ized by the detergents used for depolymerization of the membrane. We may consider the lipoprotein repeating units as unitized elements of the membrane stabilized by detergents and capable of generating *de novo* vesicular membranes. The ultimate structural unit of a membrane is thus a multimeric set of proteins (complex) with its complement of associated phospholipid. When the multimeric complex is further depolymerized into its component proteins, then at that point the structural identity of the complex is lost perhaps irreversibly.

A large number of functionally defined complexes have been isolated from a wide variety of membranes as lipoprotein particles stabilized by detergent and whenever tested, they have been shown to be capable of generating membranes *de novo* upon removal of the stabilizing detergent. We may conclude that the lipoprotein repeating units which generate membranes *de novo* are in fact functionally defined complexes with their associated complement of phospholipid. That is to say, the units of membrane construction are also the units of membrane function. Included among the complexes thus identified are the complexes of the mitochondrial electron transfer chain,[22,23] the electron transfer complexes of the chloroplast thylakoid membranes,[27,28] the Na^+-K^+ ATPase of the plasma membrane,[29,30] the Ca^{2+}-ATPase of the sarcoplasmic reticulum,[31] the electron transfer complexes of the bacterial electron transfer chain,[32–34] and the two complexes which collectively catalyze TPNH-dependent hydroxylations.[35,36] Since all membranes must be built up of complexes as judged by the capacity of membranes to undergo depolymerization to membrane-forming

lipoprotein units, then complexes clearly must be the units for all categories of membrane function and not only for the category of enzymic catalysis. Such functions would include active transport, transprotonation, facilitated transport, photoreception, nerve transmission, etc. While at present we are still unaware of the full scope of membrane functions, our postulate is that whatever the function, the unit of its expression will be a complex (see item 1 of addendum).

In view of the wide assortment of functions which membranes can subserve additional to that of catalysis, statements frequently made that certain membranes like myelin are inert and devoid of function can only be described as inaccurate.[37] Every membrane fulfills some category of function, be it catalysis of transport, electrical insulation, transmission of a perturbation, or response to hormonal triggering, etc. Myelin, it must be remembered, arises from a loop of the plasma membrane of a nerve cell and it would hardly be expected that a plasma membrane would be devoid of some function.

We are proposing that membranes generally are constructed from lipoprotein complexes and this proposal is based on the above mentioned evidence that all membranes that have been tested have been found without exception to have the capability for depolymerization to lipoprotein repeating units. In turn the depolymerized lipoprotein repeating units have been found to have the capability for *de novo* membrane formation. There is a strong impression in the literature that the rod outer segment membrane would not conform to the pattern of lipoprotein repeating units and that in this membrane the unit of both structure and function is a single molecule of rhodopsin. In a later section of the present article, we shall be considering the problem of rhodopsin in some detail. For now it is sufficient to point out that the available experimental data are insufficient to rule out the possibility that rhodopsin exists in membranes as part of a complex as required by the present model.

5. *Complexes as Informational Sets of Protein Molecules*

A fundamental distinction has to be made between the individual bimodal proteins and multimeric sets of bimodal proteins (complexes). The distinction is not simply the difference between the parts and the whole. The complex has an ordered three dimensional structure[38] and it is this unique order not achievable by self-assembly that is the essence of the membrane dilemma. The corollary of the unique position of the complex is that the component proteins of the complex are never the unit of structure, function or biogenesis.

The link between membrane and hereditary process may well be the complex (see Fig. 2). Since the complex is most likely assembled on the ribosome presumably by a polycistronic message,[39] and since only the lipoprotein particles derived from complexes can give rise to

membranes *de novo*, it may be stated that DNA is the ultimate determinant of membrane structure. How the complex is assembled and when and where the complement of phospholipid is added to the complex are still unanswered questions. We are aware from the work of Pollak[40] that the reticulosomes are paracrystalline structures which generate membranes when supplemented with phospholipid—a token that reticulosomes are arrays of complexes still unassociated with phospholipid.

A complex represents a set of intrinsic membrane proteins in a defined and invariant sequence with stable noncovalent links that maintain the sequential order. Given the order imposed by the

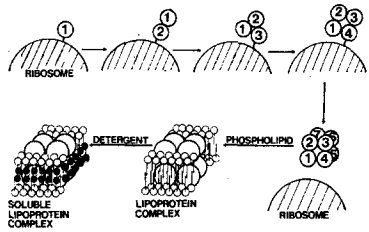

Figure 2. Biogenesis of complexes.

hereditary synthetic process, the complex will spontaneously rearrange when exposed to phospholipid to generate an element of a membrane which can associate with other such elements to generate a vesicular membrane *de novo*. The sequence of the intrinsic proteins in a complex may stand in the same relation to the three dimensional form of the complex as does the sequence of amino acids in a polypeptide chain to the final conformation of folded protein. It may be the order of associated proteins in a complex that eventually determines the way in which this set of proteins in presence of phospholipid will fold and rearrange to form an element of a membrane.

Implicit in the notion of the ribosome-dependent assembly of complexes is the postulated incapacity of the individual intrinsic proteins to form the complex by self-assembly. There have been many attempts to demonstrate self-assembly of membrane complexes from the proteins of the depolymerized complex. However, all but one have been unsuccessful.[41–43] Racker and his associates attempted to reconstitute Complex III from its component proteins and reported some limited success.[44] However other workers have raised serious doubts whether any reconstitution of Complex III was in fact achieved.[45] Green and Hechter[38] have already considered the reasons why self-assembly of membrane complexes from the component proteins would be an unlikely process.

Although the functionally unique membrane complex cannot be spontaneously self-assembled, nonetheless intrinsic membrane proteins can generate spontaneously what may be described as nonsense

complexes, and these like the physiological complexes can interact with phospholipid to form membranes *de novo*. The capacity for forming nonsense complexes appears to be inherent in all intrinsic membrane proteins other than glycoproteins. The nonsense complex may consist of one polypeptide species or a mixture thereof.

6. *Cooperativity in Membranes and Protein Domains*

A considerable set of membrane phenomena can be rationalized satisfactorily only in terms of the cooperativity of membranes. Cooperativity appears to involve protein–protein interactions, the effects of which radiate throughout a membrane. It has been calculated that when a red blood cell membrane is exposed to 80 molecules of growth hormone, the change in fluorescence of tryptophane residues in the membrane is of a magnitude that would require perturbation of all the proteins in the membrane.[48] The absorption of one quantum of light by rhodopsin can trigger a nerve impulse.[49] This means that the excitation of one molecule of rhodopsin by a single photon can trigger a perturbation of the rod outer segment membrane that can radiate from the point of excitation to the point of junction of the rod outer segment membrane with a sensory membrane where an impulse travelling to the optic center of the brain can be triggered. A few molecules of acetyl choline can trigger precisely that kind of radiating perturbation in a nerve membrane (a wave of discharge of the nerve potential and a wave of ion movements).[50] When mitochondria are energized by electron transfer, the cristae can undergo a major change in configuration which bespeaks a significant conformational change in each of the repeating structures in the membrane.[51–53] All these clear examples of membrane cooperativity argue for a structure of the membrane that will allow of rapid transmission of perturbations. The principle of protein domains provides a basis for the rationalization of cooperativity in membranes.

From the wide spread occurrence of crystallinity in membranes,[6, 54, 55, 56] it has been deduced that proteins and phospholipids form separate domains.[7] In other words, both protein and lipid are in separate continua within the membrane and there is alternation of the respective domains (see Fig. 3). We may define the protein domain as the domain in which complexes are lined up one behind the other in a continuum with noncovalent links for keeping the complexes in tight associations. Each such protein domain would be one complex wide and would extend through the thickness of the membrane. If we accept this simplistic interpretation of membrane structure, it would follow that in a plane normal to the surface of a membrane, each complex is surrounded on two sides by phospholipid (the phospholipid domains) and on the other two sides by complexes (the protein domain). The depolymerization of membranes to lipoprotein repeating units induced

by detergents would be satisfactorily rationalized in terms of this domain hypothesis. The detergent would weaken the interactions between complexes and the membrane would fall apart into individual complexes with their complement of phospholipid. In order for such a lipoprotein unit to be stable in an aqueous medium, the phospholipid arrays would have to reorient through an angle of 90°, and the two hydrophobic faces of the complex exposed by depolymerization, would have to be covered by molecules of the bimodal detergent.

In formulating the hypothesis that protein domains provide structural bases for membrane cooperativity, we have assumed that com-

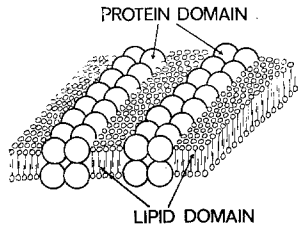

Figure 3. Domains of protein and phospholipid in membranes. The geometry of protein domains and the relation of protein to lipid domains are highly variable from membrane to membrane. The simplistic pattern shown in this figure merely illustrates the domain principle.

plexes penetrate the thickness of a membrane. In other words, each complex has a double tier structure like that of the membrane continuum.

7. *The Cavity-Channel Principle in the Construction of Complexes*

Thus far we have been vague about the macromolecular structure of a complex. How many proteins are there in a complex and how are these arranged? Are all complexes built up of the same number of proteins? We are in no position to provide final answers to any of these questions. There is sufficient analytical data to specify the probable subunit compositions of only two complexes of the mitochondrial electron transfer chain. Complex III has a molecular weight of about 200,000 and it contains 7–8 proteins.[57, 58] Complex IV has a molecular weight of about 100,000[59] and the average molecular weight of the monomers (12,000)[60] would argue for 8 protein molecules in the complex. At least for these two complexes, an octet pattern would appear to be the closest approximation to the probable number of protein molecules. Assuming 8 molecules of protein per complex, the dimensions of the two respective complexes would accommodate a double tier cuboidal pattern with four bimodal proteins on one tier (each set of four being in a square pattern). The hydrophobic faces of the paired bimodal proteins would be apposed to maximize thermodynamic stability.

Elsewhere Green and Brucker[7] have considered the concept of a central cavity in the interior of a complex with channels leading to this cavity from the exterior phase. The cavity as well as the channels could be either polar, nonpolar or some blend thereof. It is to be noted that polar cavities and polar channels are not incompatible with the bimodality of the intrinsic proteins but for such a cavity and such channels to be stable, the association of polar (or nonpolar) patches in the nesting proteins of the complex has to be precise. Otherwise the energy price would be excessive.

The central cavity and the associated channels of complexes provide a device whereby polar molecules can move from one side of the membrane to the other without "seeing" the hydrocarbon phase or the phospholipids (see Fig. 4), and a device whereby the substrates of a chemical reaction catalyzed by a complex can be insulated from the

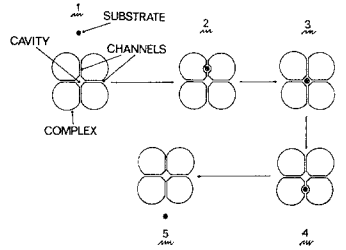

Figure 4. Cavity-channel principle of membrane complexes.

aqueous phase. Entry into the central cavity has to be conformationally mediated, and a high degree of selectivity has to be exercised in respect to the molecules capable of such conformationally controlled entry into and exit out of the central cavity.

The concept of internal cavities and channels opens the door to a new way of looking at complexes. A complex is no longer viewed merely as a structureless collection of proteins sitting in the water-lipid interphase but is regarded as an elaborate system of internal cavities and channels endowed with the capability of carrying out specific enzymic and transport functions. The geometric shape of internal cavities and channels need not be static but may be highly dynamic due to the precisely programmed conformational maneuvers accompanying the catalytic and transport functions of a complex. The polarity of the walls of the internal cavities and channels may also vary with time in such a way that the electrostatic microenvironments created within cavities and channels are regulated by conformational changes of the component proteins. In view of the fact that complexes are the most probable species to harbor active sites of membrane catalysis and transport, it is logical to anticipate that complexes possess highly

ordered internal structures that are determined genetically to fulfill specific functions.

The notion of internal cavities or clefts within single proteins such as cytochrome *c*,[61] myoglobin,[62] and lysozyme[63] is well established. M. Perutz was one of the first to emphasize the fundamental significance of internal cavities in enzymes in general.[64] There is thus a continuity between individual proteins and complexes of proteins with respect to the cavity concept.

II. Structure-Function Unitization Model

We are proposing a model of biological membrane based on the principle that the unit of structure, function and biogenesis is one and the same and that this unit (the complex and its associated complement of phospholipid) has universal validity for biological membranes. It was E. Korn who first recognized the inevitability of this unitary principle.[86] But until the complex as a membrane entity was systematically explored, the identification of the complex as the unit of structure, function and biogenesis was not immediately obvious.

It has not been easy to find a convenient name for designating the model. We are suggesting that the model be described as the structure-function unitization model (or the unitization model for short). We have picked only one of the central ideas of the model as the basis of the name but a more complete name would of course be too unwieldy for general use.

It may be appropriate at this point to recapitulate the basic concepts which are crucial for the structure-function unitization model, although these concepts have already been considered in the previous section (1) Intrinsic proteins are bimodal; (2) sets of intrinsic proteins form complexes which are the units of both structure and function; (3) membranes are composites of interdigitating protein and lipid domains; (4) protein domains are arrays of associated complexes; (5) complexes with associated phospholipid are the units of membrane biogenesis; (6) the protein domain provides the structural basis for membrane cooperativity; (7) membrane complexes are ribosome-assembled informational supramolecules which cannot be self-assembled from the component bimodal proteins; and (8) complexes possess precisely structured internal cavities and channels that provide protected microenvironments for most membrane-centered physical and chemical processes.

One could schematize the purely structural aspects of the model by means of a simple geometric diagram such as is shown in Fig. 3. But it should be emphasized that no one diagram can cover adequately the structural nuances of the model no less the functional and biogenetic. There are many crystalline states[6,54–56,65] which membranes can

exhibit—an indication of the variable way in which the protein and phospholipid domains can be arranged in different membranes. If we take into consideration the possible variation in the subunit structure of complexes (e.g. octet versus sextet) and the possible variation in the geometric pattern of the protein and lipid domains, then the inadequacy of a single geometric representation of the structural features of the model becomes obvious.

In specifying that complexes are the repeating units of the protein domain, we are not excluding the possibility that some intrinsic proteins may occur singly and that such proteins may be mobile in the phospholipid domain (see Fig. 5). Marchesi[66] has shown by sequence analysis and other evidence that an intrinsic glycoprotein of the red blood corpuscle plasma membrane is a through membrane bimodal protein. Capaldi[67] has further demonstrated that this glycoprotein unlike the other intrinsic proteins of the red blood cell membrane cannot be cross

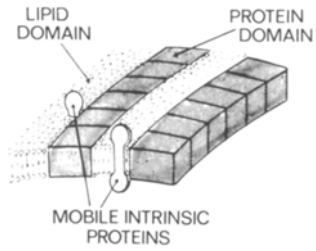

Figure 5. Glycoproteins as mobile species in membranes.

linked to other proteins by glutaraldehyde—a token that this protein is not part of a fixed set of proteins. In view of an unusual feature of the glycoprotein, namely a helical hydrophobic sector some 10 Å thick which connects the two polar regions of the molecule (see Fig. 5), it could be inferred that the glycoprotein would be capable of translational freedom in the phospholipid phase of the membrane. The narrow helical section would make it possible for the glycoprotein to serve as a mobile intrinsic protein in the membrane, and as a mobile component, the glycoprotein would not be subject to the translational restraints of the intrinsic proteins which are components of membrane complexes.

Cooperativity within the membrane may be operative at two levels—within the proteins of a complex and between complexes, i.e., at the intra- and inter-complex levels. The degrees of freedom which a bimodal protein can manifest in a membrane are highly reduced. In fact, we may say that one of the biological functions of a membrane is to diminish the translational degree of freedom of intrinsic proteins in the membrane continuum so that the protein–protein interactions can be

more effectively controlled. This is not to say that protein and phospholipid domains may not fluctuate translationally in limited local regions but this fluctuation may operate within the restraint that the relative positions of proteins and phospholipids do not change appreciably. It is the domains which can fluctuate translationally, not the individual proteins nor phospholipids separately. Enzymology in the protein domain of a membrane is the new frame of reference that has to be considered. The proteins in a catalytically active membrane complex are subjected to a highly specialized and controlled environment. As alluded to above, each complex is exposed to three environments simultaneously, namely other complexes, bilayer phospholipid and a water phase.

Given the high degree of variability among membranes, is the unitization model sufficiently flexible to account satisfactorily for the full range of structural variation? Can one structural module (i.e., the complex) rationalize the properties of membranes so different as the inner mitochondrial membrane and myelin? There are multiple devices by which membrane properties can be modulated without changing the basic constructional pattern. Cholesterol and glycolipids may respectively affect both the permeability of membranes and the ratio of protein to lipid required for maximum stability of the membrane.[68,69] The sialic acid groups on intrinsic glycoproteins may augment by a large factor the charge density of the membrane surface[70] and this modulation of the charge density may have profound effects on various membrane parameters. In a large number of membranes, headpiece-stalk projections are attached at very regular intervals to one side of the membrane[71] and thus contribute to the asymmetry of biological membranes. These are only a few of the chemical devices for modulation of membrane properties. Therefore, we see no difficulty, in principle, of rationalizing the full gamut of membrane variation within the framework of the structure-function unitization model.

It is important to recognize that the structure-function unitization model of the membrane has an analogy in the unitization of structure and function among polymeric soluble enzymes; a polymeric enzyme is the unit of structure, function and biogenesis. The unitization model of the membrane thus has deep roots in biological precedents.

The thesis has been developed by Green and Goldberger that all living cells operate within the framework of a universal set of principles and that all the fundamental processes and structures fall within the framework of these universals.[72] The universals include the principles of heredity and energy transduction, and the fundamental metabolic processes. The principles of membrane structure and function undoubtedly would fall within the framework of biological universals if for no other reason than that the membrane is the very essence of living systems.

This principle of universality has enormous tactical advantages in the sense that it provides assurance that underneath the bewildering variety of specific membrane properties there is always a set of properties which is independent of the source or specialization of the membrane. It is the description of this set of universal attributes of membrane systems to which the present model is addressed. Moreover, in reaching decisions in a field with an abundance of soft and often times ambiguous data, the principle of universality may be invaluable as a guide to the separation of the wheat from the chaff.

The structure-function unitization model which we are proposing has many similarities to Changeaux's model of membrane structure.[73] The approach of Changeaux has been prophetic of the new direction which the membrane field is now pursuing. The unitization model of the membrane can readily accommodate the essential elements of the Changeaux model.

Finally, a few comments are in order about the tactical approach which we have followed in the development of the unitization model. While the model is indeed steeped in an extensive substratum of experimental evidence it goes beyond available experiment. The *a priori* approach has thus played a key role in the genesis of the model. The ultimate test of a model is neither the logic of nor the experimental justification for its genesis but rather the extent to which it rationalizes the major membrane phenomena, introduces order and clarity in the field, and predicts new relationships. The crucial test of the model, therefore, lies in this fitting of theory and experiment.

III. Applications of the Model

In this section, we shall be considering a selected set of membrane phenomena which demonstrate the versatility and scope of the structure-function unitization model.

Crystalline cytochrome oxidase. Purified preparations of cytochrome oxidase show a highly characteristic herring bone crystalline character when examined by negative staining with uranium acetate, in positively stained thin sections, or in thin section without any stain.[65] There are two quite different aspects of this crystallinity that are worthy of note.

The crystalline pattern of cytochrome oxidase is observed when the oxidase is in the oxidized state (nonenergized). When the oxidase is reduced and thereby energized, the crystalline pattern disappears. Vanderkooi *et al.*[65] have shown that in negatively stained preparations the individual complexes are visualized, and each complex is oriented in a precise herring bone pattern with respect to its neighboring complexes. When the complex is reduced, this regular orientation of complexes disappears and the orientation appears to be randomized. Several important conclusions may be drawn from these observations. The

geometry of the protein-lipid domains is not constant. The domains can fluctuate from a highly regular pattern to a random pattern. This fluctuation of pattern must mean that the angle of packing of complexes in the protein domain is variable. Note that the dimensions of the individual complex do not change appreciably during the oxidized to reduced transition.[54] Reduction of the complex among other things may lead to charge separation (separation of electrons and protons) and conformational strain.[74] As a result of one or both of these perturbing influences, the individual complexes in their reduced form may assume new orientations relative to their neighbor complexes.

Hayashi *et al.*[65a] have found that a variety of conditions can abolish crystallinity in the oxidized form of cytochrome oxidase, namely high pH (>8·3) and high ionic strength (e.g. 1·5 M NaCl and 0·7 M NaBr). These observations suggest that the orientation of complexes within the protein domain is highly sensitive to environmental factors and only in a narrow range of conditions will complexes orient in the characteristic crystalline pattern.

The crystallinity of cytochrome oxidase is three dimensional as shown by the identical herring bone pattern in thin section.[65] In negatively stained preparations (surface view of dried preparations), the crystallinity is a reflection of the orderly orientation of complexes and the orderly alternation of protein and lipid domains. In positively stained, thin sectioned preparations (cross sectional view of dehydrated preparations), the same interpretation could be made regarding the arrangement of proteins and phospholipids in the crystal lattice.

Based on these observations, we may conclude that the double tier of globular particles is clearly visualizable in thin sectioned crystalline preparations and that these particles are seen even without external staining. Hence, the problem of staining is eliminated. Presumably by virtue of the perfect alignment of particles in a crystalline array, the individual protein particles have been visualized directly. The dimensions of the particles are consistent with the known mass of the proteins in cytochrome oxidase. Thus in the case of the crystalline cytochrome oxidase membrane, the component proteins in the two tiers of the membrane appear to be arranged in a highly regular and precise herring bone pattern.

Viral membrane formation. What is known about the genesis of viral membranes may be summarized by two observations, first that the membrane is pinched off from the host cell membrane, and second, that the protein composition of this pinched off membrane is different from the protein composition of the host membrane.[75–78] This means that viral proteins are introduced into the cell membrane and then pinched off by the developing virus. The point we would like to make is that the final structure of viral membranes suggests that repeating structures with projecting headpiece-stalks are introduced into the cell membrane.

These structures are induced by the interaction of viral nucleic acid with the hereditary apparatus of the cell. The conclusion that the viral membranes contain repeat structures follows from the fact that the viral membrane shows highly regular center to center distance between the projecting elements.[79] We infer therefore that viral membrane formation involves the introduction of sets of complexes with associated headpiece-stalk projections into an already existing cell membrane and the subsequent pinching off of this packaged set of complexes by the viral nucleic acid as it leaves the cell with formation of a viral membrane. Since viruses are formed in practically every type of cell, it would follow that the formation of the host cell membrane in all these different types of virus-susceptible cells depends upon the principle of complexes as the building blocks of membranes. This argument is based on the postulate that viral membrane formation is an exact counterpart of cell membrane formation. That is to say, if the viral membrane arises from complexes containing virus-induced intrinsic proteins, then the cell membrane which is used as the vehicle for viral membrane formation must arise in the same way from preformed complexes.

Viral coat formation. Virus particles such as the tobacco mosaic virus (TMV) do not have a lipoprotein membrane but rather a protein envelope which is one molecule thick.[80] In the biogenesis of this protein envelope, Klug has shown that the starting point for assembly is a set of double discs.[81] Each disc corresponds to a multimeric ring of proteins and the discs always come in pairs. The paired proteins in the apposed discs may stand in the same relation to one another as the pairing of bimodal proteins in a membrane. We might draw an analogy between the biogenesis of the viral coat and the biogenesis of membranes. The double disc of viral proteins may be considered as the equivalent of a membrane complex. The paired discs associate with one another to form the protein continuum of the viral coat. The double tier structure thus applies both to the viral discs and to the complexes. We are predicting that the pairing of viral proteins in the paired disc is a reflection of the bimodality of these proteins although this bimodality may not be as clear-cut as that of intrinsic membrane proteins as judged by the water solubility of the viral coat proteins. However, the tendency of viral coat proteins to form polymeric associations is an indication of partial hydrophobic character.[81] Perhaps the most outstanding difference between the paired viral discs and the intrinsic membrane complex is that the former but not the latter can be self-assembled.[82] But this difference may reflect the greater water solubility of the viral coat proteins—a property which allows for the extensive experimentation required for self-assembly.

Induction of intrinsic enzymes in membranes. Reagents such as barbiturates can induce enzymes in microsomal membranes which are intrinsic to

the membrane.[83] It is important to emphasize the point that the enzymes which we are considering in the present context are not membrane-associated (extrinsic) but integral to the membrane (intrinsic). The mixed function oxidases are among the intrinsic membrane systems that have been found to be inducible and these oxidases are unambiguously membrane-forming complexes.[84] The theory of membranes which underlies our model requires that the induction of enzymes involve the induction of sets of protein (complexes) which are at the same time the building blocks of membranes. Insofar as these induced intrinsic enzymes have been studied from the standpoint of their structure, it would appear that the results to date are fully consistent with this prediction. Intrinsic induced enzymes are invariably enzyme complexes with the capability for *de novo* membrane formation.[85]

Crosslinking of intrinsic proteins by glutaraldehyde. Since intrinsic membrane proteins come in sets or complexes, it would be predictable that reagents such as glutaraldehyde which can cross link tightly associated proteins should completely alter the molecular weight pattern of the monomeric protein components of membrane complexes. All the monomeric species should disappear and a new set of dimeric, trimeric, etc. species should appear. Capaldi[87] has indeed verified this prediction for the behavior of the proteins in a membrane generated by purified cytochrome oxidase. The monomeric protein species largely disappeared upon glutaraldehyde treatment and a new set of polymeric protein species appeared. This dramatic demonstration of the cross linking of all proteins within the cytochrome oxidase membrane establishes the capability of the glutaraldehyde technique for "measuring" the distances separating intrinsic proteins in a membrane. Cross linking of two proteins by glutaraldehyde would require that the two proteins should be no more than 5 Å apart and probably considerably less than 5 Å. The same technique applied to the proteins of a submitochondrial particle (ETP) which is essentially an inner membrane preparation also yielded the same results as for the cytochrome oxidase membrane, namely disappearance of the monomeric protein species. Thus, in a membrane which is known to contain only complexes, the glutaraldehyde technique verifies the tight association of intrinsic proteins within the membrane implicit in the concept of complexes.

The plasma membrane of the red blood corpuscle contains both extrinsic (40%) and intrinsic proteins (60%). The mass ratio of extrinsic to intrinsic proteins (1:1·5) has been determined by Capaldi.[87] A vesicular membrane can be generated by the intrinsic protein fraction of the red blood corpuscle membrane. In such a membrane stripped of extrinsic proteins, the glycoproteins account for 5% of the total mass. It would be anticipated that all the intrinsic proteins except for the glycoproteins should show crosslinking with glutaraldehyde by virtue

of being components of complexes whereas the glycoproteins would be unaffected since these are mobile species which can move freely through the phospholipid domain. Capaldi has fully confirmed this prediction.[87] The glycoproteins but none of the other intrinsic proteins were unaffected by glutaraldehyde in respect to molecular weight. In other words, components of complexes were crosslinked whereas mobile intrinsic protein species were not crosslinked by glutaraldehyde.

Paracrystalline structures in spaces between membranes. In mitochondria paracrystalline arrays (parallel bars) have been observed in the intracristal space linking outer and inner membranes or linking two neighboring and apposed cristal membranes.[88] These arrays have a periodicity of about 100–115 Å. Similar paracrystalline structures with periodicities in the same range have been reported in the synaptic gap between nerve membranes and in the spaces between apposed cell membranes.[89, 90] The periodicity of the paracrystalline structures is identical with the periodicity of protein domains in membranes (90–120 Å between the centers of two domains). We, therefore, interpret the intermembrane paracrystalline structures in terms of soluble proteins in the aqueous space separating two membranes undergoing polymerization in periodic bands. These bands of polymerized protein may connect protein domains in one membrane to protein domains in the apposed membrane. It is to be noted that the membranes must be very close before these cross bridges are formed. This very proximity may trigger the polymerization required for the proteins to form cross bridges between the two membranes. The paracrystalline appearance of these cross bridges may be no more than a reflection of the exact periodicity of protein domains in a membrane—a periodicity established by the evidence from crystalline membranes.

Cell walls of bacteria. Bacterial cells of the gram negative class have basically two enveloping membranes—the so-called cell wall and the conventional cell membrane.[91] The two membranes are separated but are possibly linked through a rigid network system known as the peptidoglycan layer.[104] Let us consider how the cell wall membrane may be looked upon as a variation on the theme of biological membranes. At high resolution the cell wall membrane shows a "double tier" character (paired stain centers) as does the cell membrane.[104] Moreover, the cell wall membrane can be depolymerized with appropriate detergents into lipoprotein repeating structures which can reform the cell wall membrane when the detergent has been removed by suitable means.[92] These two observations suggest that the cell wall membrane is built up on the same constructional pattern as the cell membrane, and that complexes and associated lipid are the units of membrane construction.

The cell wall membrane differs from the cell membrane in two important respects—first in respect to its mechanical rigidity and

second in respect to the presence in the membrane continuum of a third bimodal molecule, namely lipopolysaccharide. Let us consider each of these two novel features in turn. The rigidity of the cell wall membrane is in large measure referable to the peptidoglycan support structure.[91a] Protein projections extend periodically from the peptidoglycan network to appropriate sites on the cell wall membrane and these cross connections apparently increase to a very high degree the mechanical stability of the cell wall membrane. Procedures which readily rupture ordinary membranes such as ultrasonic irradiation usually have no effect on the cell wall membrane. But when the peptidoglycan support structure is disrupted by appropriate means, the cell wall membrane loses its mechanical stability and behaves like ordinary membranes such as the cell membrane.

Cell wall membranes contain about equal parts of two lipids, namely phospholipid and lipopolysaccharide. The ratio of protein:total lipid (phospholipid plus lipopolysaccharide) is about 1:1. Lipopolysaccharides generally can generate vesicular membranes *de novo*.[94] The thickness of such membranes (about 100 Å) would argue that the membranes arise by the pairing of lipopolysaccharide monomers in the same fashion as phospholipids formed paired arrays in the generation of liposomal membranes. In view of the very different dimensions of phospholipid versus lipopolysaccharide molecules (25 Å versus about 50 Å) it would be a reasonable prediction that there are three domains in cell wall membranes—protein, lipopolysaccharide and phospholipid—probably arranged in the order protein–lipid–protein–lipopolysaccharide.

Capitalize Endotoxins of gram negative bacterial cells have all been identified with the lipopolysaccharides of the cell wall membrane.[95] These endotoxins are all capable of *de novo* membrane formation and of fusing with cell membranes in the sense that the endotoxin becomes incorporated into the membrane.[96] The interaction of certain cell membranes with endotoxin can lead in many instances to the complete loss of membrane function.[97]

The stability of membranes as a function of interactions between complexes. Implicit in the unitization model of the membrane is the notion that the protein domains are composed of associated complexes in such a way that each complex in the continuum is linked to two other complexes in one direction and to phospholipid on both sides in the other direction (see Fig. 3). The stability of the links between complexes obviously must affect the stability of the membrane and weakening of the links must lead to the fragmentation of membranes into smaller and smaller vesicles and eventually into lipoprotein particles. There are several instances of precisely this kind of transition. Tzagoloff *et al.*[98] showed that inner membrane submitochondrial particles (ETP) became depolymerized to quasi-soluble lipoproteins by adjusting the

pH of the particle suspension to about 9·0 and then sonicating in absence of added salts. A substantial proportion of the particles were no longer sedimentable after this treatment. Electron microscopic examination showed that the membranes had been depolymerized to lipoprotein particles of the dimensions of single complexes. When salt was added back to the lipoprotein dispersion or the pH was readjusted to neutrality, the dispersed lipoprotein units regenerated vesicular membranes *de novo*. The experiments of Tzagoloff *et al.* thus demonstrated that when complex–complex interactions were weakened by increasing the charge repulsion between complexes (alkalinization) and by decreasing the screening effect of salt (low ionic strength), then membranous vesicles of the inner mitochondrial membrane underwent fragmentation to quasi-soluble lipoprotein units.

The plasma membrane of the red blood corpuscle can become completely solubilized when exposed to EDTA for extended periods.[99] This observation of J. Reynolds was followed up by R. Capaldi[100] who showed that membranous vesicles of the ghost membrane can be depolymerized to quasi-soluble lipoprotein particles during such exposure and that these lipoprotein particles will generate vesicular membranes *de novo* when Mg^{2+} is added back to the medium and EDTA is eliminated. Here again EDTA by tying up Mg^{2+} or Ca^{2+} exerts two effects: (a) increases the repulsion between complexes by virtue of unscreening the charge repulsive action of sialic acid (in the complexes of the ghost membrane, glycoproteins with high sialic acid content play a dominant role and divalent metals are required to neutralize the charge of the sialic acid); and (b) decreases the interaction between complexes possibly by eliminating metallochelate links. In the *halobacteria*, Brown[101] has shown that membranes which are stable in 5 M salt media depolymerize to quasi-soluble lipoproteins when the molarity of the medium is decreased below 1–2 M. The intrinsic proteins of the complexes of the *halobacteria* have the properties of polycarboxylic acids.[101] High salt is required to reduce the charge repulsion between neighboring complexes; dilution of salt then weakens the interaction between complexes to the point that the membrane depolymerizes reversibly to quasi-soluble lipoprotein particles.

Membrane fluidity. The thermal transition points of the hydrocarbon chains of phospholipids in membranes nearly match those of the hydrocarbon chains of phospholipids in micellar dispersions.[5] Is this near identity of transition points compatible with the interdigitation of protein and lipid domains? There is no reason why the phospholipids in the lipid domain of membranes should behave any differently than phospholipids in dispersions. In both cases the phospholipids are in bilayer arrays, and the interactions between the nonpolar groups of proteins and the fatty chains of phospholipids are no different from the nonpolar interactions between one phospholipid and another.[116]

A second question is why the nature of the fatty acid chains in the phospholipid of membranes should have a profound influence on processes such as active transport or bacterial growth.[102] This correlation poses no special difficulty for a model of membrane structure based on the principle of complexes, and of protein and lipid domains. Integral

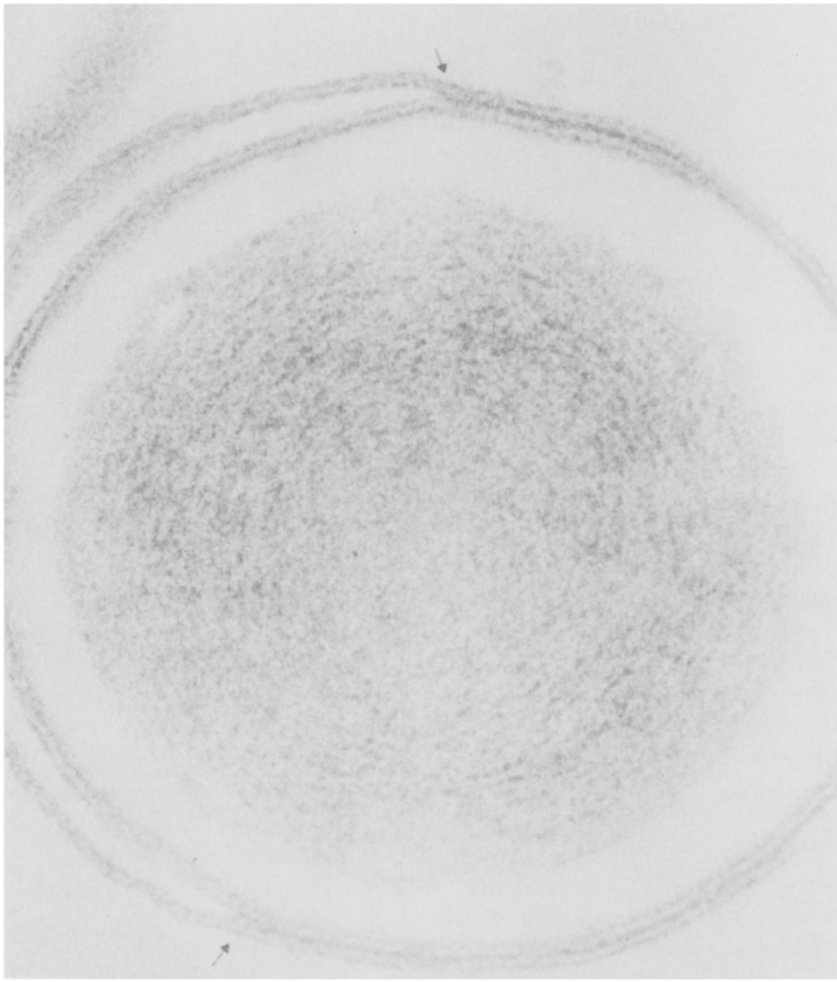

Figure 6. Fusion of two phospholipid membranous sheets to form a hybrid triple tiered structure. Osmium stained thin section. The electron micrograph was kindly supplied by Dr. Hideo Hayashi.

to the function of a complex is the conformational cycle by which substrate molecules enter and leave the central cavity of the complex. This conformational cycle will clearly be influenced by the lipid environment which bathes the complex on both sides. Thermal energies would play an important role in such conformational cycles

and the fluidity of the hydrocarbon chain will perforce affect the thermally-activated conformational transitions required for transport or other functions.

Fusion of membranes. Green *et al.*[103] have shown that two membranous sheets of bilayer phospholipid can fuse to form a characteristic three tiered fusion "membrane". Divalent metals such as Mg^{2+} or basic proteins such as cytochrome *c* and protamine are required to initiate the fusion process. Electron microscopically, each of the two membranous sheets prior to fusion show double tiered structures in osmium-stained thin sections, whereas the fusion "membrane" shows three tiers of staining centers (see Fig. 6) with the middle tier much more electron dense than the two outer tiers. Brucker *et al.*[103a] have interpreted this fusion of phospholipid membranous sheets in terms of a phase transition in the state of the phospholipid on one side of each of the two apposed bilayer sheets (see Fig. 7) with formation of a hybrid "membrane"

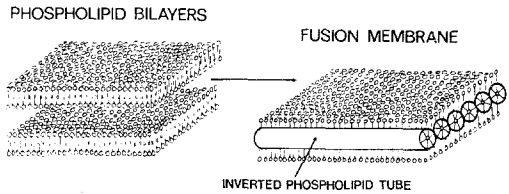

Figure 7. Mechanism of fusion of phospholipid membranous sheets and formulation of the structure of the hybrid structure formed by fusion (see item 2 of addendum).

arising from the two membranous sheets participating in fusion. In the center tier the phospholipid is assumed to be arranged in a concentric and tubular fashion with the polar heads interiorly directed and the aliphatic fatty chains exteriorly directed. The tubular cylinder of phospholipid presents a continuous hydrophobic surface which is apposed to the monolayer of phospholipid molecules on both sides.

Exactly the same kind of fusion may take place between two apposed biological membranes (either identical or nonidentical membranes), since the same reagents are required for fusion of membranes as for fusion of phospholipid sheets, namely Mg^{2+} or basic proteins. Moreover, the electron microscopic appearance of the hybrid membrane formed by fusion of two apposed membranes is indistinguishable from the hybrid structure formed by fusion of two phospholipid sheets. A triple tiered hybrid membrane is formed with an electron dense central tier. According to Brucker *et al.*,[103a] the structure of the fusion membrane can be formulated as shown in Fig. 8. Implicit in this formulation is the postulate of a phase transition in the state of phospholipid triggered by Mg^{2+} or basic proteins (only half the total phospholipid is involved in this transition), and then a rearrangement both of proteins and phospholipids to accommodate to this phase transition.

There are two fundamental processes involved in membrane fusion which merit close attention: (a) the rearrangement of complexes from a double tier to a single tier pattern; and (b) the rearrangement of a membrane from a double tier to a triple tier pattern. We shall consider each of these two processes separately.

If we consider a complex as a set of eight linked bimodal proteins, this set can assume a linear arrangement (in the fusion membrane) or a double tier arrangement (in each of the two membranes prior to fusion). Figure 9 shows diagrammatically this transition of a complex from the linear to the double tier arrangement. To achieve this type of arrangement, the links between the bimodal proteins must be relatively stable. Since membrane fusion is a reversible process[6,103] it would appear that complexes can readily undergo this type of reversible rearrangement.

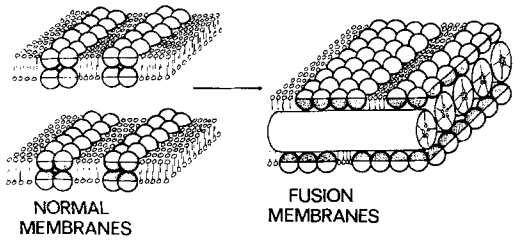

Figure 8. Mechanism of fusion of apposed membranes and formulation of the structure of the hybrid membrane formed by fusion.

The maneuver which a complex must undergo during membrane fusion is highly informative about the possible mechanism of the biogenesis of complexes. Given a linear set of eight linked bimodal proteins, this set will automatically assume a double tier pattern when exposed to bilayer phospholipid and will revert to a linear pattern when exposed to inverted, concentric phospholipid. The precise order of bimodal molecules in an octet set may be crucial for assembly of a membrane-forming complex. Unless this precise order is achievable, the resulting set may not have the properties of a membrane-forming complex.

The inner mitochondrial membrane has 90 Å headpieces projecting periodically from its matrix side.[71] In fact, the headpieces are so closely clustered that we may consider the membrane covered with a mosaic wall of projecting 90 Å headpieces. Yet two inner membranes in presence of Mg^{2+}, protamine, or cytochrome *c* can approach one another on the headpiece side and achieve fusion. During fusion the 90 Å headpieces disappear and the proteins of the headpiece appear to be incorporated into the fusion membrane. At first glance this may seem an improbable maneuver. But the rationale of the maneuver

becomes more obvious when the headpiece is considered to be a complex (probably an octet). During fusion this complex appears to undergo a transition from a double tier to a single tier arrangement in precisely the same fashion as the intrinsic complex. There is one basic difference between the extrinsic and intrinsic complexes. The plane dividing each bimodal protein into polar and nonpolar halves is rotated 90° in the intrinsic as compared to the extrinsic complex as shown in Fig. 10.

The second of the fundamental features of fusion membranes is that membranes can exist in two states—the classical, bilayer state and the

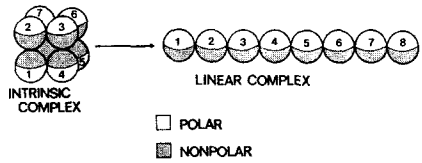

Figure 9. Transition of intrinsic membrane complexes from the double-tiered to the single tier pattern.

fusion triple tier state. In the classical state paired bimodal proteins pack together in the same continuum with paired phospholipid molecules. The orientation of the paired molecules, whether protein or phospholipid, is at right angles to the plane of the membrane. In the fusion membrane the pairing is eliminated because half the phospholipid is in the concentric inverted state and half in the orientation of the bilayer. The bimodal proteins originally paired are now lined up in linear array on one or the other side of the central hydrophobic tier of inverted phospholipid.

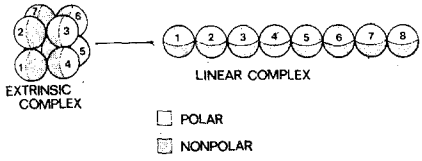

Figure 10. Transition of the ATPase complex of the projecting headpiece of the mitochondrial inner membrane from the double-tiered to the single-tier pattern.

The plasma lipoproteins appear to be constructed in the same fashion as fusion membranes.[6] These lipoprotein particles have a central core of inverted phospholipid covered by a thin shell of bimodal protein.[106] The proteins appear to be flattened discs (10 Å thick) with one side polar and the other side nonpolar. The polar face is directed exteriorly and the nonpolar face is directed interiorly to the inverted

phospholipid core. The bimodality of the apolipoproteins appears to be a consequence of a helical arrangement in which the side chains on one side of the helix are polar and the side chains on the other side are non-polar.[105]

The structure of myelin. Myelin is a membrane which is generally used as a yardstick in assessing any membrane model. This has its amusing side in that myelin is about as "far out" a membrane as can be found and for myelin of all membranes to be used as a yardstick without appreciation of its idiosyncracies appears to be unwarranted to say the least. Nonetheless, in view of the widely claimed importance which attaches to myelin, it is all the more necessary to show how our unitization model which allegedly has universal applicability can rationalize the properties of this membrane.

Agreement is general that C.N.S. myelin arises by the fusion of two loops of the plasma membrane of the Schwann cell.[107] The fusion membrane then is wrapped concentrically around the axon with the ribbons of fused membranes being tightly apposed one to another in laminar concentric rings. How unique is this capability of the myelin membrane for undergoing fusion, and how unique is the capability of fused membranes to form laminar concentric rings? The capacity for undergoing fusion appears to be a general property of membranes and the capacity of fused membranes to form laminar sets depends entirely on providing the necessary ingredients in the medium—either divalent metals such as Mg^{2+} or basic proteins such as protamine.[103] Myelin contains such a basic protein which accounts for about 25% of the total protein content.[108] It is of interest to note that in demyelinating diseases where the ribbons of fused membranes tend to separate, the fundamental lesion in many cases is a deficiency of the basic protein.[109] The cristae of the inner mitochondrial membrane can be induced to form laminar sets of fused membranes (by addition of Mg^{2+} or protamine) which are indistinguishable from the laminar sets of myelin.[6, 103] At least with regard to the lamination of myelin, we are dealing with a property which is inducible under appropriate conditions in other membranes.

We have mentioned in a previous section that fusion is a reversible process. Indeed sonication of myelin apparently leads to the defusion of the concentric ribbons of fused membranes to the two partner membranes.[6, 103] This defusion process is recognizable by the transition from a triple-tiered to a pair of double-tiered membranes.

Myelin, like all other biological membranes, can be depolymerized to lipoprotein repeating units given the proper detergent, and these units will generate vesicular membranes *de novo* when the detergent is removed by appropriate means.[110] As we have emphasized many times in this article, the capability for *de novo* membrane formation is the essence of biological membranes because it implicates complexes as

the units of membrane structure, and the complex is the heart of the structure-function unitization model.

The myelin membrane has one feature which to many investigators is so overriding that all evidence bearing on the normalcy of myelin is swept aside. This feature concerns the ratio of phospholipid to intrinsic protein. In most membranes this ratio is about 1:1;[86] in myelin the ratio is about 2:1 when the value for intrinsic protein is corrected for the content of basic protein.[112] It is this apparent surplus of phospholipid which is taken to be a token of a new constructional principle for myelin. There are, however, other compositional features of myelin which have to be taken into account in evaluating the phospholipid–protein ratio. Firstly, myelin has almost as much cholesterol as phospholipid.[113] Secondly, myelin has an unusually high level of galactolipids equivalent to about half the level of phospholipid.[114] The effect of both the cholesterol and the galactolipid with its complement of C_{24} fatty acid residues is to decrease fluidity and increase rigidity of the membrane.[115] Myelin is less fluid a membrane than other membranes despite the high phospholipid to protein ratio and this appears to be due to multiple causes—laminar fusion and a high content of cholesterol and galactolipid.

The only atypical feature that needs rationalization is the high phospholipid to protein ratio in myelin membranes. How can such a low protein content be reconciled with the lipoprotein repeating unit postulate? Experimentally the lipoprotein repeating units are demonstrable in myelin[110] and this must mean that the protein domains in myelin have to be stabilized by considerably larger lipid domains (domains of phospholipid and galactolipids) than are necessary for other membranes. But this apparent discrepancy may have to be viewed in somewhat different light. If as would be predictable, the galactolipids are not randomly dispersed in the phospholipid domain but rather are clustered and intimately associated with complexes[115a] then the orderly association of protein and lipid domains could be maintained. The protein domains in effect become expanded by this postulated tight association with galactolipids. Such an association would be reasonable since the galactolipids by virtue of the C_{24} chain length of the hydrocarbon tails would be expected to pack more readily with proteins than with phospholipids.

The role of internal cavities and channels in intrinsic membrane complexes. The cavity-channel postulate invoked for the construction of intrinsic complexes is consistent with the experimental observations suggesting that membranes contain selective macromolecular channels for the facilitated or energy-linked transmembrane transport of polar molecules,[117] and also provides a rationale for the selective entry of substrate molecules into the interior of complexes where controlled chemical interactions between substrate and enzymic active sites can

take place. Phenomena are known in mitochondria which hitherto have been without explanation and these phenomena can be readily rationalized in terms of the cavity-channel concept.

Antimycin inhibits electron transfer of Complex III at exceedingly low concentrations—equivalent to one or two molecules of antimycin per complex.[118] The various effects of antimycin A and the characteristics of this inhibition are summarized in Table I. The simple notion that antimycin can selectively enter the hypothetical cavity of Complex III provides a sufficient basis for rationalizing all the effects of antimycin summarized in the table. The rationalizations are also listed in the same table. Here is a case in which one of the most carefully studied phenomena of mitochondriology defied explanation until rationalized in terms of the cavity-channel concept.

TABLE I. The interaction of antimycin A with Complex III and rationalization of the inhibition phenomena in terms of the cavity–channel principle

Phenomenon	Rationalization
1. Cleavage of Complex III into particulate cytochrome *b* and soluble cytochrome c_1 by exposure to taurodeoxycholate in a temperature-dependent reaction.[129]	1. Taurodeoxycholate must enter the internal cavity of Complex III in order to achieve cleavage. At low temperatures entry is interdicted.
2. Antimycin prevents cleavage of Complex III by taurodeoxycholate.[129]	2. Antimycin once in the cavity prevents entry of taurodeoxycholate into the cavity.
3. Antimycin prevents electron transfer from cytochrome *b* to cytochrome c_1 but not the reduction of cytochrome *b*.[118]	3. Antimycin prevents the conformational change by which cytochrome *b* is brought close enough to cytochrome c_1 for electron transfer to take place.
4. Antimycin can completely inhibit electron transfer in Complex III when the molar ratio of antimycin: Complex III is 1. No covalent link is formed between antimycin and Complex III.[129]	4. Antimycin is not removable from the cavity once it has entered, except by disruption of the complex.
5. Reduction of the complex prevents inhibition by antimycin.[129]	5. Reduction of the complex leads to a closure of the cavity and the cavity becomes inaccessible to antimycin.
6. Reduction of the complex prevents cleavage by taurodeoxycholate.[129]	6. The cavity in the reduced complex becomes inaccessible to taurodeoxycholate.
7. The potential of cytochrome *b* in Complex III is altered when antimycin is present in the complex.[130]	7. Antimycin changes the environment of the cavity and thereby modifies the oxidation-reduction potential of cytochrome *b*.

The 90 Å headpieces of the inner membrane are linked to the membrane via a cylindrical stalk (30 Å diameter and 50 Å long).[71] The stalk is capable of extension from the membrane and collapse into the membrane.[119] This must mean that the stalk can move in and out of a sleeve in the membrane. The headpiece-stalk projections are known to be linked to a membrane-forming complex.[22,120] Thus the three parts, the intrinsic complex in the membrane, the stalk, and the extrinsic complex of the headpiece, form one integrated unit, called the oligomycin sensitive ATPase.[120] It would appear, therefore, that the stalk fits into a cavity in the interior of the intrinsic complex in the membrane and that there is some control device which regulates the extent to which the stalk penetrates into the cavity. When the stalk is pushed deep into the cavity, the headpiece collapses on the membrane; when the stalk is extruded from the cavity, the headpiece projects away from the membrane.

Oligomycin inhibits ATPase activity only when the headpiece is attached to the membrane via the stalk.[121,120a] It has no effect on the ATPase activity of the headpiece itself (F_1). It has also been known that the presence of the stalk is essential for oligomycin-sensitivity and that oligomycin sensitivity is controlled by some protein which is neither in the headpiece nor in the stalk.[122] This protein, according to Beechey,[122] appears to be in the membrane. A viable explanation for this phenomenon is that oligomycin can selectively enter the channel of the complex which serves as the sleeve for the stalk. When oligomycin is in the cavity, the stalk is perforce extruded and the headpiece assumes a conformation incompatible with ATPase activity. When the headpiece is detached from the membrane and the stalk, then oligomycin can exert no effect on ATPase activity. The Beechey protein which appears to control oligomycin sensitivity may be one of the proteins in the intrinsic complex to which the stalk is anchored.

The rod outer segment membrane and the unit of photoreception. The rod outer segment membrane would appear to be one of the most highly organized membranes as judged by several criteria—electron microscopy, cooperativity, and crystallinity. It is completely classical in respect to some fundamental properties—depolymerizability to lipoprotein structures which can form vesicular membranes *de novo*,[123] the capacity for assuming a crystalline pattern,[124] and a normal ratio of protein to lipid (about 1:1).[125] These properties would suggest that the rod outer segment membrane conforms in basic respects to the structure-function unitization model, namely that the membrane is built up of complexes and associated bilayer phospholipid, and that there are alternating domains of protein and phospholipid.

This simple picture is in conflict with a body of evidence which suggests that the rod outer segment membrane is highly fluid,[126] that rhodopsin molecules are randomly arranged in the membrane,[127] that

there is an asymmetric distribution of rhodopsin molecules on the two sides of the membrane (hence like pairing would be excluded),[128] that rhodopsin molecules not only have freedom of translation but they can spin at high speed during photoexcitation.[128a,128b] It is our present view that these observations will eventually be rationalized without compromise of the structural principles we consider to be universal for biomembranes. Some fundamental studies on the structure of the rod outer segment membrane have yet to be carried out. Until then a final decision will have to be deferred.

IV. A Critique of the Singer–Nicolson Fluid Mosaic Model of Biological Membranes

Singer and Nicolson[8] have recently proposed a model of membrane structure which in one important respect is opposed to the structure-function unitization model which we have presented in the present communication. In respect to certain molecular features such as hydrophobic bonding of protein and phospholipid, the bimodal character of intrinsic membrane proteins, the bilayer character of the phospholipid, and the distinction between intrinsic and extrinsic membrane proteins, the two models are in agreement. But the models diverge sharply in respect to the organization of the intrinsic membrane proteins. According to the fluid mosaic model, there is only one continuum, namely bilayer phospholipid. The intrinsic proteins are randomly interspersed in the bilayer lipid. The proteins, like the lipids, are capable of moving freely through the membrane so that there is no fixed position in the membrane for any molecule. The only restriction imposed by the fluid mosaic model on the movement of proteins is the flipping of a protein molecule from one side of the membrane to the other. It is to be noted that the notions of complexes and protein domains are essentially rejected for membranes generally although Singer and Nicolson admit the possibility that certain specialized membranes such as the inner membrane of the mitochondrion may have sets of intrinsic proteins rather than individual intrinsic proteins.

The phenomena on which we have based the unitization model provide the most powerful refutation of the basic thesis of the fluid mosaic model—phenomena such as lipoprotein repeating units, *de novo* membrane formation, crystalline membranes, the ultrastructural evidence of repeat structure in membranes, viral membrane formation and induction of intrinsic membrane enzymes. As we see it, an acceptable model of the membrane must deal with and provide a satisfactory rationale for all membrane phenomena. This neglect of critical membrane phenomena appears to be a major deficiency of the fluid mosaic model.

What were the compelling lines of evidence which led Singer and

Nicolson to the formulation of the fluid mosaic model? Basically there were three lines of evidence: first, the x-ray data of Blasie *et al.*[127] on the circular symmetry of the rod outer segment membrane and the inference of randomness in the arrangement of rhodopsin in the membrane; second, the immunochemical evidence that the proteins in the red blood corpuscle membrane are randomly distributed[130]; and third, a miscellaneous collection of observations which point to the conclusions that proteins in membranes have high mobility and can move around freely.[8]

With respect to the assumption of a high degree of mobility for intrinsic proteins in the membrane, we have pointed out that only a special group of intrinsic proteins, namely the glycoproteins, may show this mobility. The fluid mosaic model may be restricted in application to the intrinsic proteins concerned in immunochemical reactions.

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Addendum

1. Enzymes which are intrinsic to the membrane continuum may be either polymeric complexes or mobile, through-membrane monomeric proteins like the glycoproteins which are localized in the lipid phase.

2. Space limitation has made it necessary to compress our discussion of the fusion phenomenon to the point that a balanced treatment of the uncertainties still remaining in the model of the fusion membrane could not be provided. The fusion phenomenon has opened a new door to membranology and the tentative character of the molecular interpretations given in the text should be borne in mind.