review

Role of lipids in the structure and function of biological membranes

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ABSTRACT The concept of biological membranes as vesicular or tubular continua built up of nesting repeating units has been systematically explored and some of the relevant experimental work has been assembled. The bulk of the data have been drawn from studies on the mitochondrion, which is assumed to be a model for membranes generally.

The repeating units of membranes are composite macromolecules containing both protein and lipid. The unit of the mitochondrial inner membrane is tripartite; the basepiece is the membrane-forming element. The four complexes of the electron transfer chain represent the different species of basepieces in the inner membrane. The repeating units of the outer mitochondrial membrane have a different form and size and a completely different set of enzymes (the enzymes of the citric and fatty acid oxidation cycles).

The repeating units of the inner mitochondrial membrane are capable of forming membranes spontaneously. This membrane-forming capability is absolutely dependent on the presence of lipid. Evidence is presented for the view that lipid restricts the number of binding modalities and thus compels a two-dimensional alignment of repeating units. In absence of lipid three-dimensional stacking takes place, and the aggregates thus formed are, in effect, bulk phases. The membrane may be looked upon as a device for molecularizing repeating units, and it is this molecularization which underlies the essentiality of lipid for electron transfer. The theory of lipid requirement for enzymic activity is developed.

The reconstitution of the electron transfer chain is shown to be essentially a membrane phenomenon rather than an expression of direct chemical interaction between the different parts of the electron transfer chain.

KEY WORDS membrane · mitochondria · structure · phospholipid · micelles · enzymes · electron transport · lipid dependence · disruption · bile salts · reconstitution · lipid–protein binding · "structural protein" · cation–phospholipid interaction

INTRODUCTION

PHOSPHOLIPIDS and their amphipathic structural analogues are among the essential molecules of the cell. This essentiality derives from the key role subserved by bimodal molecules in membrane structure and functions; and it is basically at the elucidation of this role that the present review is aimed.

A universal feature of all cell membranes yet examined is the presence in relatively large amount of some amphipathic molecule, be it sulfolipid (1), phospholipid (2, 3), or glycolipid (4). In the membranes of animal cells, phospholipids are the predominant species of amphipathic molecules; in the membranes of plant cells, sulfolipids and glycolipids are on a par with phospholipids as far as relative amounts of each are concerned (4). There is great variability not only in respect to the nature of the polar sector of the amphipathic molecule but also in respect to the hydrocarbon or nonpolar sector. The latter sector may contain a fatty acyl, fatty aldehydic (2), fatty alcoholic (5), or even an isoprenyl residue (5). The residues in the hydrocarbon sector must contain a sufficient number of carbon atoms in a linear chain to reduce the solubility of the bimodal molecule in water to negligible amounts. The critical number of carbon atoms per residue appears to be about 18 (6).

Bimodal molecules of the kind specified above aggregate in water to form micelles and these micelles may be looked upon as a type of macromolecule. Thus, in aqueous media, the reactive unit is the phospholipid micelle and not the individual molecule of phospholipid. To interact with proteins, phospholipids must be presented in micellar state (6). The properties of a given species of phospholipid may be profoundly altered by association with phospholipids of other species in a mixed micelle.

Thus, the composition of a micelle may modulate the properties of the component phospholipids (P. Jurtshuk, unpublished studies). The micellar state of phospholipid and other bimodal molecules will be implicit in the discussions that follow.

Much of our present knowledge about lipids in membrane systems is drawn from studies of the mitochondrion and the chloroplast. It is hoped that the emergent principles found for these two membranes will serve as useful guidelines for the study of membrane systems generally.

THE MEMBRANES OF THE CELL

Although the concept of a membrane is familiar to everyone concerned with the biology and chemistry of living systems, the precise definition of a membrane in physicochemical terms has been elusive until very recently. The classical Danielli-Davson model of the membrane (7) and its latter-day successor, the "unit membrane" hypothesis of Robertson (8, 9), have focused attention primarily on a bimolecular phospholipid leaflet as the backbone of membrane structure (Fig. 1). The designation "backbone" is used advisedly to stress the notion implicit in these models that the essential properties of the membrane are derived primarily from the bimolecular phospholipid micelle sandwiched between two layers of protein. These above-mentioned models of the membrane, which have gained wide acceptance among biologists, involve the following assumptions: (*a*) the membrane is a lamellar structure a few molecules thick; (*b*) the phospholipids which make up the membrane continuum are bound to one another hydrophobically; (*c*) the polar sectors of the phospholipid molecules appose the protein layers, and the paraffinic sectors of the phospholipid molecules nest together; and (*d*) the protein layers have the form of extended polypeptide chains.

The concept of the membrane as a paucimolecular structure has been supported by electron micrographs (10) of sectioned cells after fixation with osmium tetroxide or potassium permanganate. Cellular membranes in preparations which have been examined after fixation by these reagents (11, 12) are found to be composed of three layers with dimensions that have been rationalized in terms of the Danielli-Davson or Robertson "unit membrane." Although these models are still widely accepted, the results of physical and chemical studies of isolated membrane systems, together with evidence from other quarters [see review by Green and Perdue (12)] have led some authors (4, 13) to question the validity of the assumptions on which these models are based. In particular, milder methods of fixation and visualization of biological specimens, such as negative staining and freeze-etching, have revealed, in membranes, repeating structures which were not recognizable with the older methods of fixation.

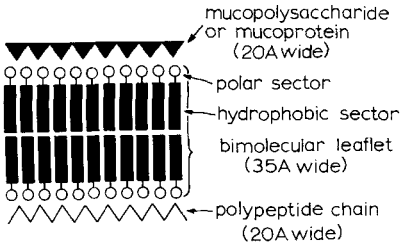


FIG. 1. Diagrammatic representation of the unit membrane model of Robertson (9).

The available evidence points to a model of the membrane which is more in line with biochemical experience. The membrane is visualized as a continuum, one particle thick, made up of fused or nesting repeating particles (14, 15)—in other words, a unimacromolecular film in which the repeating particles are lipoprotein macromolecules, all identical or nearly identical in form and size, though comprised of a number of chemically and functionally different species. The membrane is the expression of the component repeating units and of their pattern of alignment. No structured elements other than the repeating units need be invoked to account for all the macromolecules contained within membrane systems.

Each membrane has, according to this view, its own distinctive repeating unit—distinctive in form, size, and functionalities. But despite the individuality of the repeating units of a given membrane, there appear to be architectural principles which apply to the repeating units of all membranes thus far examined. The repeating units, independent of the origin of the membrane, appear to be lipoprotein macromolecules (for which the range of molecular weights extends from about 100,000 to over a million). In many membranes the repeating unit is a composite of a basepiece linked to a projecting headpiece. The basepieces of repeating units make up the membrane continuum, i.e., the nesting of adjacent repeating units that underlies membrane formation involves only the basepieces. The projecting headpiece of one repeating unit does not make contact with the projecting headpiece of adjacent repeating units. Lipid in the form of phospholipid, or some other bimodal molecule, is an intrinsic part of repeating units and makes a major contribution to the membrane-forming capacity of these units (12).

COMPOSITION OF MEMBRANES

Lipid Composition

The isolated membranes from all types of cells—animal, plant, and microbial—contain about 20–30% by weight

of bimodal molecules (2–4). The total lipid content is highly variable from membrane to membrane but the content of bimodal molecules is not.

The fatty residues of the phospholipids of animal and plant mitochondria are generally polyunsaturated (16) whereas the phospholipids of bacterial membranes are generally monounsaturated (5). The fatty residues in each membrane appear to have a distinctive pattern of unsaturation; this pattern may vary widely from one membrane to another within the same cell.

The ratio of neutral lipid to bimodal lipid is another variable feature of membranes. The value of this ratio is very low in mitochondria (<0.05) and can be as high as 1–2 in other membranes such as the microsomal and erythrocytic membranes (2, 17, 18). Also, the composition of the neutral lipid is highly variable from membrane to membrane. In some membranes, cholesterol is a major component (e.g., the erythrocytic and microsomal membranes); in others, cholesterol is present only in traces (18, 19).

We have enumerated above the four alternative chemical classes of nonpolar residues found in bimodal molecules. There are wide deviations in the distribution of these different residues in the phospholipids of different membranes. For example, the phospholipid of heart muscle mitochondria is rich in fatty aldehyde residues (2) whereas the phospholipid of liver mitochondria is devoid of such residues (20). These structural idiosyncracies may be expressions more of the cell type than of the organelle.

The polar nature of the bimodal molecules is yet another variable feature of membranes. Bovine heart mitochondria contain predominantly three phospholipids—phosphatidyl ethanolamine, phosphatidyl choline, and cardiolipin (2) (Table 1). No other membrane of animal cells is known to contain significant amounts of cardiolipin. Phosphatidyl serine, which is absent from mitochondria, is found in high concentration in the membranes of the outer segments of retinal rods (21).

TABLE 1 PHOSPHOLIPID COMPOSITION OF BOVINE HEART MITOCHONDRIA*

	% total phosphorus
Phosphatidyl choline	37
Phosphatidyl ethanolamine	31
Cardiolipin	16
Phosphatidyl inositol	10
	μmoles/μmole P
Plasmalogen	0.4
Double bonds	3.2
Fatty esters	1.8

* Data of Fleischer, Klouwen, and Brierley (2).

Out of the welter of variations in detail among lipids of membranes, three facts emerge which probably have universal application. The first is the invariable presence of bimodal molecules in all membrane systems. The second is the relatively high concentration of bimodal molecules in membranes (approaching ten bimodal molecules per molecule of protein). The third is the constancy and uniqueness of the lipid of each membrane, suggestive of genetic determination.

Protein Composition

The technology for fractionating membrane systems has been slow in development and highly empirical. With the discovery of new reagents (bile salts and other detergents) and physical methods (sonication, gradient centrifugation) it has become possible to extract and separate from one another the different enzymic systems of mitochondria and other membranes (22). Most of these reagents and some of these conditions are directed toward the weakening of hydrophobic interactions, followed by the fractionation of the proteins by classical methods. The rationale of these empirical fractionating procedures has been obscure, but now, with a better understanding of the ultrastructural parameters, it is possible to achieve a better insight into the nature of these techniques. Let us consider one membrane system, the mitochondrion, from this standpoint.

The outer and inner membranes of the mitochondrion can be effectively separated from one another by controlled exposure of mitochondria to the action of phospholipase (23). The outer membrane is selectively weakened by the digestive process or by the products of digestion, such as lysolecithin, until it gradually falls apart. In this way the still intact inner membrane can be separated from the shredded and fragmented outer membrane by differential centrifugation (23).

The inner mitochondrial membrane contains the entire electron transfer chain as well as the enzymes of oxidative phosphorylation (24). The various complexes comprising the electron transfer chain can be separated from one another by fractionation with bile salts and ammonium sulfate or acetate. Thus, complex I can be isolated free from II, III, and IV; complex II, free from I, III, and IV, etc. (25–28). In addition, conditions have been found for isolating sets of complexes, e.g. I–III, II–III, or III–IV (29–31). The bonds that hold I–III together are apparently stronger than the bonds that hold I and II together. Thus, there may be a measure of selectivity in the association of repeating units. In principle any complex can combine with any other complex or with another unit of the same species. But it may be that given a choice, preference will be shown in the selection of the associating partner complex.

Each complex is a composite of 8–10 subunit proteins (32). Of these only 4–5 are known to function as oxida-

tion–reduction components (in the form of cytochromes, flavoproteins, or metalloproteins) (33). The rest are colorless proteins (core proteins) without any known prosthetic groups other than sulfhydryl groups. The core proteins are intimately linked to the catalytic proteins of each complex; their separation can be achieved under conditions which lead to depolymerization of the complex (32) or, in some cases, to the breaking of disulfide bonds (I. Silman, unpublished studies). The core proteins appear to be a sort of template on which the various catalytic proteins are arranged.

A large body of circumstantial evidence suggests that each sector of a membrane repeating unit is a composite of a set of catalytic proteins and of a noncatalytic organizing protein. The two moieties are usually present in 1:1 proportions by weight. The core protein(s) appears to be the organizing protein(s) of the basepieces of the inner membrane. The classical structural protein[1] isolated by Criddle and coworkers (34, 35) appears to be the organizing protein of the headpieces of the inner membrane. How many such organizing proteins are present in the mitochondrial membranes is still unknown. Our working assumption is that each sector of a repeating unit is built up by the attachment of catalytic proteins to a nucleus of the organizing protein. This nucleus could consist of one or more molecules of the organizing protein.

When the term noncatalytic is applied to the organizing proteins such as core protein and structural protein, the implication is merely that these proteins are not concerned in metabolic reactions such as electron transfer, citric cycle oxidations, etc. The organizing proteins are undoubtedly concerned in catalytic events not encompassed by the category of metabolic processes.

The notion of multiple organizing proteins, of which the classical structural protein is only one species, came too late to head off the ambiguity of nomenclature implicit in the identification of structural protein with all noncatalytic proteins of membranes. Thus when we speak of the separation of the electron transfer chain from structural protein (36), we are talking operationally. The structural protein in this instance refers to a set of noncatalytic proteins that are impurities in the preparations of the electron transfer complexes.

Organizing proteins, such as the structural protein of Criddle and coworkers (34, 35), have been shown to be present both in the inner and outer mitochondrial membranes (A. Lauwers, unpublished studies) and in each of several other isolated membranes (plasma membrane of the erythrocyte, chloroplast membrane, and microsomal membrane) (44). The similarity in gross physical and chemical properties found among organizing proteins from different membrane systems suggests that proteins of this class, regardless of source and cell, are very similar, in the same sense that cytochrome *c* from whatever source has a characteristic molecular pattern. Woodward and Munkres (36*a*, 36*b*) have carried out a fascinating study of the structural protein in the mitochondrion of a strain of *Neurospora*. The amino acid composition of this protein is virtually identical with that of the structural protein isolated from bovine heart mitochondria by the method of Criddle, Bock, Green and Tisdale (35). Mutants (cytoplasmic or nuclear) of the yeast can be obtained in which the amino acid composition of the structural protein is altered by the substitution of a single amino acid by another. Such mutants exhibit profound differences in the properties of in situ mitochondrial enzymes such as malic dehydrogenase.

The catalytic membrane proteins are clearly "designed" for interaction with one another and with the organizing protein. In other words we must visualize membrane proteins in terms of a large set of properties which are required for participation in membrane interactions. Each protein has its place in a repeating unit. It must be capable of binding specifically to invariant neighbor proteins and possibly to lipid. All these features must be built into the molecule. There are in fact several markers that characterize some, if not all, of the membrane proteins: the tendency to form polymers, the capacity to combine with lipid, and the tendency to combine with other proteins of the membrane.

LIPID–PROTEIN INTERACTIONS IN MEMBRANES

Electrostatic Bonding

Phospholipid micelles can interact electrostatically with basic proteins such as cytochrome *c* to form stable complexes (6, 37–39). This can be visualized as an interaction between an acidic polyelectrolyte[2] (the phospholipid micelle) and a basic polyelectrolyte (the basic protein). Each molecule of cytochrome *c* carries 8 net plus charges, all of which are engaged in the interaction with acidic groups of the phospholipid (6). The micelle–protein complex is "soluble" in hydrocarbon solvents, such as heptane, as well as in water and will partition between the two solvents. Thus, the complex can exist with the hydrocarbon residues of the phospholipid molecules facing the exterior (in heptane) or with the polar sectors of the phospholipid molecules facing the exterior (in water). In heptane, cytochrome *c* would be buried in the interior

[1] The possibility that structural protein is a denatured form of a previously catalytic protein is one that has yet to be conclusively excluded.

[2] The predominant acidic phospholipid in mitochondrial lipid is cardiolipin. Phosphatidyl ethanolamine has a partially acidic character whereas lecithin has none.

of the micelle; in water it would be on the exterior surface of the micelle (6). The electrostatic interaction of phospholipid micelles with cytochrome *c* has physiological significance since there is evidence that the active form of cytochrome *c* within the mitochondrial electron transfer chain is a complex with phospholipid (40, 41).

Divalent metal ions can also interact with phospholipid micelles to form stable micellar salts (42). The phosphoric ester residues of acidic phospholipids (cardiolipin, phosphatidyl inositol, and to some extent phosphatidyl ethanolamine) participate in such electrostatic interactions. As we shall see later, this capacity of phospholipid micelles to interact with cations may be highly relevant to the problem of membrane swelling and ion translocation.

Electrostatic interaction in membranes between phospholipids and basic proteins has been demonstrated in a few instances, but it appears not to be a major determinant of the bonds which hold the membrane continuum together.

Hydrophobic Bonding

Lipid is bonded to protein in membranes predominantly through hydrophobic interactions (6, 37, 43). Such interactions are relatively unaffected by the ionic strength of the medium, show a large temperature coefficient, and are greatly influenced by the number of carbon atoms in the paraffinic chain (the longer the chain—up to about C_{18}—the more effective the interaction). Any phospholipid, acidic or zwitterionic, can bind to membrane proteins. The reactive partners in hydrophobic interactions are the phospholipid molecules in micellar form, and protein in the form of a macromolecular complex. Membrane proteins can bind only a limited and defined amount of phospholipid.[3] The number of sites or areas in the protein where lipid can be bound is apparently a constant; and thus, the binding has a precise stoichiometric character (6).

Polymeric structural protein binds mitochondrial phospholipids to form a complex containing 20–25% of phospholipid by weight (44). Unlike its complex with cytochrome *c*, the structural protein–phospholipid complex is not dissociated by high concentrations of salt. When dialkyl phosphates having different side chains were tested for their ability to combine with structural protein, the extent of binding was found to depend solely on the length of the hydrocarbon residues. The best binding was obtained with alkyl phosphates that had 16–18 carbons in the hydrocarbon chains (6).

Since only the paraffinic sector of the phospholipid participates in hydrophobic interactions, it should be possible to demonstrate that the polar (or charged) sectors of the bound phospholipid molecules are available for another type of interaction. This is found indeed to be the case. The acidic groups of mitochondrial phospholipids bound to structural protein are available for interaction with cytochrome *c*. Up to 90% of the theoretical number of acidic groups in the bound phospholipid are titratable with externally added cytochrome *c*. The missing 10% may be inaccessible to cytochrome *c* for steric reasons or may represent the small percentage of phospholipid molecules that are bound electrostatically to the membrane protein (6).

The nature of the binding of lipid and protein has been studied by Brown (45) in the cell membrane of the halophilic bacterium, *H. halobium*. This membrane is stable in solutions of high ionic strength (3–5 M NaCl). When the ionic strength is decreased (less than 1 M NaCl), the membrane dissociates into its subunit lipoproteins (43). The interaction of lipid and protein in the whole membrane and in its disaggregated form was found to be stable under a wide range of salt concentration (0–5 M NaCl) and pH (1.4–11.7). Furthermore, succinylation of the α- and ϵ-NH_3 groups of the proteins with succinic anhydride did not dissociate the lipoprotein complex. These studies excluded any significant contribution of electrostatic bonding between lipid and protein in this membrane (43).

The shape of phospholipid molecules will be greatly influenced by the degree of unsaturation of the fatty chains (each double bond of the *cis* configuration leads to a kink in the hydrocarbon chain). Such irregularities in shape should influence both the extent and speed of binding. It might be expected that highly kinked phospholipid molecules would not be able to fit into certain sites in the protein that are accessible to less highly kinked molecules. Collins has found, however, (personal communication) that only the kinetics of binding is influenced by the fatty acid composition (certain alterations in fatty acid composition lead to a faster rate of binding) but the total amount of phospholipid bound was unaffected. This would suggest some degree of plasticity of the binding sites as well as of the phospholipid molecules to account for the fact that binding sites can accommodate either monounsaturated or polyunsaturated phospholipid molecules.

MICELLAR STATES OF PHOSPHOLIPIDS

Phospholipid molecules in water readily align with one another to form paracrystalline aggregates called micelles (49). Some means of dispersion is required to facilitate this micellization process; suitable procedures are dialysis of the phospholipid in a butanol–cholate mixture against water (47), or sonication (48). This is particularly

[3] The binding of cardiolipin is an exception to this rule. Apparently there are reasons other than stoichiometry that account for the open-ended character of the binding of cardiolipin to membrane protein (see reference 6).

true for phospholipid molecules of biological origin which have essentially no solubility in water. Micellar solutions of phospholipids of natural origin can be dialyzed for weeks without any loss of bound phosphorus (47). Once formed, the micelle is a highly stable system.

The size of the micelle differs for different phospholipid preparations (6), but, in general, phospholipid molecules spontaneously form micelles of rather large size (molecular weights of 140,000 or more) when prepared by the butanol–cholate procedure. These micelles usually take the form of a coiled three-dimensional spiral—the spiral representing a continuously coiled bimolecular leaflet. The width of a leaflet depends on the phospholipids present in the micellar mixture, the degree of unsaturation of the phospholipid molecules, and the nature of the cationic counterions (E. Munn, personal communication).

Divalent ions can profoundly affect the size and three-dimensional pattern of the micellar domains as well as the spacing of the phospholipid molecules within a bimolecular leaflet. For example, calcium ions induce a remarkable change in the micellar state of mitochondrial phospholipid. The coiled spiral form of the micelle is converted to a concave disc and the spacings of the leaflet are visibly altered (E. Munn, personal communication). The effect of divalent ions is representative of that of a group of molecules that can modulate the state and size of phospholipid micellar domains. Molecules such as valinomycin and gramicidin, which have profound effects on oxidative phosphorylation, have been found to modify the capacity of phospholipid micelles to bind monovalent ions (50).

In addition to the lamellar arrangement, phospholipids have also been shown by Luzzati and Husson (51) and by Stoeckenius (52) to form hexagonal arrays in water systems. In this arrangement, the polar sectors form the core of a cylinder in which the nonpolar groupings are free to interact with neighboring cylinders.

Lucy and Glauert (53) have discovered that lecithin, as well as mixtures of lecithin and steroid molecules such as cholesterol, forms globular micelles which can assume tubular and helical structures. The globular elements (40 A in diameter) were interpreted in terms of micromicelles which are the repeating units of the macromicelle. The concept of a repeating unit offers a new constructional possibility that is precluded in the bimolecular leaflet configuration. Three-dimensional arrays of a variety of shapes can be built with these repeating units (sheets, tubes, helices, and vesicles). Lucy and Glauert further postulated that biological membranes have a globular structure in which phospholipid micromicelles form the backbone of the membrane (53, 54). In their model, protein macromolecules are inserted into spaces within the phospholipid micelle. The notion of the micromicelle as an intrinsic part of the repeating units of membranes has many merits and deserves close study.

Some of the key outstanding problems are: how are phospholipids packed into membrane proteins hydrophobically? What kind of micellar arrangement applies to membrane lipoprotein systems? Where is the lipid localized in the repeating units? To what extent is lipid involved in the binding of one repeating unit to its neighbors?

EVIDENCE FOR MACROMOLECULAR REPEATING UNITS IN MEMBRANES

The functional units of the mitochondrion have long been known to be macromolecular complexes (22). The very fact that such complexes could be isolated from the mitochondrial membrane made it almost certain that counterpart structures to that of the isolated complexes would be found in the membrane in question.

Negative staining (55), with its attendant advantage in resolution, has given us new insights into the ultrastructure of membranes. Fernandez-Moran was the first to demonstrate the presence of repeating structures in the inner membrane of mitochondria (56). The repeating unit of the inner membrane (elementary particle) was shown by Fernandez-Moran, Oda, Blair, and Green (57) to consist of a headpiece (80–100 A in diameter) attached by means of a stalk to a basepiece in the membrane itself. The basepiece can exist as a rectangular unit (115 A × 45 A) or as a spherical unit, 90–100 A in diameter (58). This pioneer observation has since been confirmed in many laboratories (59–62) and extended to other membranes (63–66). There can be little doubt at the present time that all membranes are built up of repeating units, the size and form of these units being characteristic of a given membrane. The negative staining technique Fernandez-Moran used to render the elementary particle visible is not the only technique that can be used for this purpose. The freeze-etching technique of Moor and Muhlethaler (67), which is by far the mildest procedure yet devised for electron microscopic examination of biological specimens, fully confirms the existence of globular repeating units in the membranes to which this technique has been applied. X-ray studies of the membrane of the outer segments of the retinal rods (68), and of the chloroplast membranes (69) have established the presence, in the membrane continuum, of repeating units which roughly correspond to the repeating units observed under electron microscopy. While there are still uncertainties about the characteristics of dimension and shape of the repeating units of membranes, the existence of such units is no longer seriously challenged by electron microscopists.

IDENTIFICATION OF MEMBRANE REPEATING UNITS WITH ENZYME COMPLEXES

If, as now appears highly probable, the units of ultrastructure in membranes can be identified with the functional units of integrated enzymic processes, then we have reached the stage of correlating ultrastructure and enzymology—a correlation that has enormous potential. This identification has been made thus far in only one membrane (the inner mitochondrial membrane). The experimental difficulties that had to be overcome before this first identification could be established are likely to crop up in all other efforts aimed at identification.

The specific question that we shall be considering in the present section is the relation of the basepiece of the repeating units of the inner mitochondrial membrane to the electron transfer chain. Several important advances had to be made before the problem of identification could be narrowed down to one clearly defined possibility. First, it had to be established that there were two mitochondrial membranes (23), and that the electron transfer chain was localized exclusively in the inner mitochondrial membrane (23), and exclusively in the basepieces of the repeating units of this membrane (36). Second, the minimal mass of the electron transfer chain had to be known for a decision to be reached whether a single basepiece could accommodate one complete chain (70). The probable molecular size of a complete chain (stripped of all components that do not contribute to the electron transfer process) is now known to exceed the probable molecular size of a basepiece by such a large factor that no risk is involved in assuming that the identification problem reduces to the equation of the molecular size of a basepiece with that of a complex of the electron transfer chain. The third development was the demonstration that the individual complexes as well as the complete chain could be stripped of structural protein without loss in enzymic function (36). Studies on identification could then be limited to particles that were free from structural protein. Finally, the examination of the electron transfer complexes under electron microscopy has made it possible to correlate the ultrastructure of the complexes with their molecular weights (D. G. McConnell and J. Perdue, unpublished observations; see also reference 74).

Structure of the Electron Transfer Chain

The transfer of electrons from the ultimate donors (NADH and succinate) to molecular oxygen is mediated in mitochondria by an electron transfer chain made up of four component and separable complexes (I, II, III, and IV). The complex may be defined as the unit of integrated electron transfer. Each complex has a distinctive set of catalytic proteins (Fig. 2) in constant molecular proportions. The enzymic unit can exist at two levels of complexity:

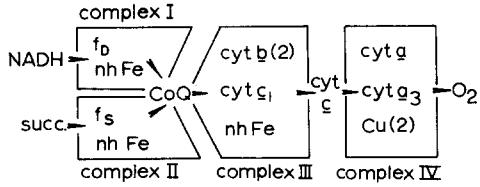


FIG. 2. The sequence, composition, and stoichiometry of the four complexes of the mitochondrial electron transfer chain: *nhFe*, nonheme iron; f_D, acid-extractable flavin; f_s, acid-nonextractable flavin.

There is one molecule of nonheme iron protein in complexes I, II, and III with an electron spin resonance signal (probably 2 atoms of iron per molecule of protein). In addition there are some six atoms of iron in complexes I and II which do not show an ESR signal.

(*a*) catalytic protein + core protein + lipid + "structural protein"

(*b*) catalytic protein + core protein + lipid

At each of the two levels, the integrated electron transfer reaction characteristic for a given electron transfer complex can proceed. The morphological unit, however, will change during this stepwise peeling process—if not in shape, at least in size and density. The important point is that the comparison of the morphological unit and the functional unit must be made at the same level of complexity since there are two different levels. Only three of the four complexes have been brought to an acceptable stage of purification. Complex I has yet to be isolated free from "structural protein."

A particle (EP_2) has been isolated from the inner membrane of bovine heart mitochondria which contains the fixed components of the complete electron transfer chain in the same molecular proportions as in the intact mitochondrial chain (36). In this particle, which contains exclusively basepieces of the inner membrane, the four complexes are stripped of "structural protein."

For some time it was thought that the intact chain contains one molecule of each of the four complexes. But new evidence points to a stoichiometry of 1:1:1:3 for the actual molecular proportions of I, II, III, and IV. Thus one complete electron transfer chain of bovine heart mitochondria is made up of a set of six molecules in all four component complexes (71).

The Mass of a Complex

The minimal molecular weights of complexes II, III, and IV can be computed on the basis of the content of some functional group in the complex, the assumption being made that there is one molecule of each such group per molecule of complex. Acid-nonextractable flavin is the group in question for complex II, cytochrome c_1 for

complex III, and cytochrome *a* or a_3 for complex IV (since cytochrome *a* and a_3 are both estimated as heme *a*, the minimal molecular weight of complex IV is defined as the unit containing two molecules of heme *a*). The estimate of the minimal molecular weight thus arrived at takes into account only the protein moiety of the complex. Lipid accounts for about 36% of the total mass of a complex (36). Thus the estimate of minimal molecular weight based on the content of the appropriate functional group has to be normalized for 36% lipid. This normalized estimate is 340,000 for complex II, 418,000 for complex III, and 260,000 for complex IV (Table 2).

TABLE 2 MOLECULAR WEIGHTS OF ELECTRON TRANSFER COMPLEXES

Complex	Presence of Structural Protein	Method of Determination: Light Scattering* −Lipid	Light Scattering* +36% Lipid	Minimum Mol. Wt. −Lipid	Minimum Mol. Wt. +36% Lipid
I	+	—	—	750,000	1,170,000†
II	0	—	—	220,000	340,000‡
III	0	270,000	420,000	268,000	418,000
IV	0	230,000	360,000	166,000	260,000§

* Data of Tzagoloff, Yang, Wharton, and Rieske (72).

† The minimum molecular weight of complex I is calculated from the flavin content reported by Hatefi, Haavik, and Griffiths (25).

‡ The minimum molecular weight of complex II is calculated from the flavin content reported by Ziegler and Doeg (26).

§ The minimum molecular weight of complex IV is based on preparations containing 12 mμmoles of heme *a* per mg of protein [see Kopaczyk and Green (36)] with the assumption of two hemes per molecule of the complex.

The estimate of the average minimal molecular weight of a complex can also be made from the data for the composition of EP_2. If we assume one molecule of cytochrome *c* (or of acid-nonextractable flavin) per chain, the minimal molecular weight of the chain of EP_2 is 1.72×10^6 (normalized for 36% lipid) and the minimal molecular weight per complex is $1.72 \times 10^6 \div 6 = 2.87 \times 10^5$ (36).

Whether the minimal molecular weights are the same as the actual molecular weights can be determined by physical measurements. From light-scattering measurements of complexes III and IV, solubilized with bile salts, the estimated molecular weights of these two complexes are about 360,000 and 420,000 respectively (72) (See Table 2). Several uncertainties in the measurements limit the precision of the estimates of molecular weight. With due allowance for these uncertainties, the estimates of molecular weight from light-scattering data make it possible to equate the minimal molecular weights of the complexes based on compositional data with the actual molecular weights.

Electron Microscopy of the Complexes and the Chain

Examination in the electron microscope of negatively stained specimens of the complexes referred to above reveals the existence of spherical particles with diameters of 80–100 A (70). Since the repeating units are polyhedral rather than spherical, the volume of a sphere 100 A in diameter would be a close approximation to the volume of the repeating unit. If the particle contains 36% lipid, its density would be 1.24 (phospholipid has a density of 1.09 and protein a density of 1.33). A sphere 100 A in diameter with a density of 1.24 would have a mass of 390,000 (57)—a value in good agreement with the estimate of the mass of a complex arrived at from compositional data. On the basis of such agreement the identification of a basepiece with a complex becomes highly probable. The data are not precise enough to specify the exact mass of a basepiece but they are sufficiently reliable to exclude more than one molecule of complex per basepiece or more than one basepiece per complex.

Racker and coworkers (46) have presented convincing evidence for the identification of a complex with ATPase activity with the projecting headpieces of the mitochondrial inner membrane repeating units. The isolated complex (73) is spherical (80 A in diameter), contains no lipid, and has a molecular weight of 280,000 (46). The ATPase can recombine with a preparation of the inner membrane stripped of headpieces. Such reconstituted preparations now show evidence of projecting headpieces.

MEMBRANE FORMATION BY REPEATING UNITS

The repeating units of all membranes tested (inner and outer mitochondrial membranes, microsomal membrane, erythrocyte membrane, bacterial membrane) show the capacity to realign spontaneously and form vesicular membranes (15, 74, 75) (Fig. 3). In the case of the mitochondrial inner membrane, structure is destroyed by addition of bile salts. This is a form of displacement reaction:

$$(\text{–repeating unit–repeating unit–})_n \xrightarrow{\text{bile salt micelle}} n(\text{repeating unit–bile salt micelle})$$

In the presence of bile salts, the repeating units are no longer capable of nesting with one another. The membrane continuum is broken up into individual repeating units which are complexed with the bile salt micelle. This phenomenon is reversible since removal of bile salt from the solubilized membrane allows the repeating units to realign spontaneously and re-form hollow vesicular membranes. Thus the repeating units have the

capacity to form a membrane continuum in the absence of any external directing influence. Electron micrographic examination of such reconstituted structures, after fixation with osmium tetroxide and sectioning of Epon-impregnated specimens, shows clearly that these are hollow vesicles whose structured periphery is made up of a layer of repeating units, one particle thick (74). By all criteria these are reconstituted membranes indistinguishable from the membrane structures obtained by isolation of membrane fragments. This phenomenon of vesicularization by disaggregated repeating units provides evidence that the membrane is a special polymeric state of the component repeating units.

An unexpected feature of this phenomenon was the finding that the capacity for membrane formation is retained by the purified enzyme complexes (15, 74). The isolated electron transfer complexes can be maintained in a dispersed state in the presence of bile salts. When the concentration of bile salts in the suspending medium is lowered, the enzyme complexes become less soluble and aggregate. We have found that the removal of the bile salts from the complexes results in the formation of membranes. Thus, in this case, aggregation and membrane formation are synonymous. By shadow-casting and thin sectioning it has been demonstrated that the membranes are vesicular (Fig. 4); that is, they are enclosed systems similar to the membranes found in preparations of the electron transfer particles (74). The subunits in the structure of the membranes are particles with the same dimensions as those seen in the original dispersed preparations. These experiments demonstrate that each electron transfer complex has a property separate from its enzymic function, namely the capacity to organize into membranes, the subunits of which are the enzyme molecules themselves.

Fleischer, Brierley, Klouwen, and Slautterback (76) have reported that the integrity of the mitochondrial membrane was preserved even after extraction of over 85% of the phospholipids. Cunningham, Hall, Crane, and Das (58), however, have found that extraction of mitochondria with polar solvents that remove phospholipids results in a complete disarray of the membrane. There is really no contradiction between these observations from two different laboratories. When lipid is removed without mechanical agitation of the extracted mitochondria the original arrangement of repeating units can persist. Once this arrangement is disturbed, the disarrayed repeating units are no longer capable of reforming membranes. Instead, huge random conglomerates of repeating units form when extracted particles are subjected to sonic irradiation.

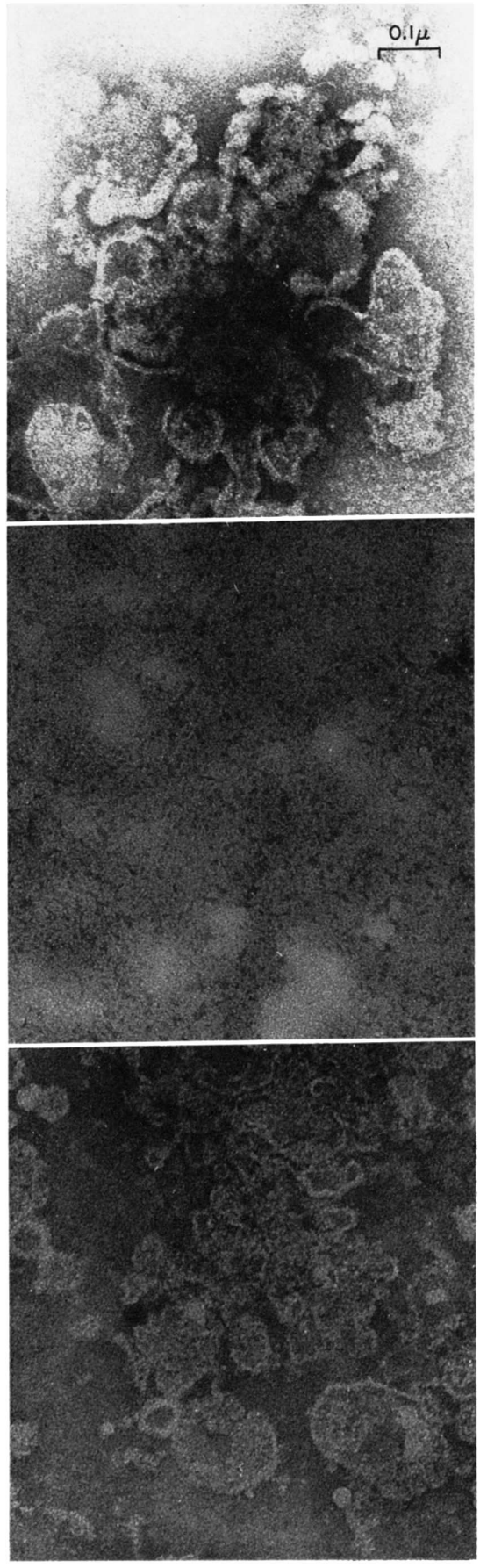
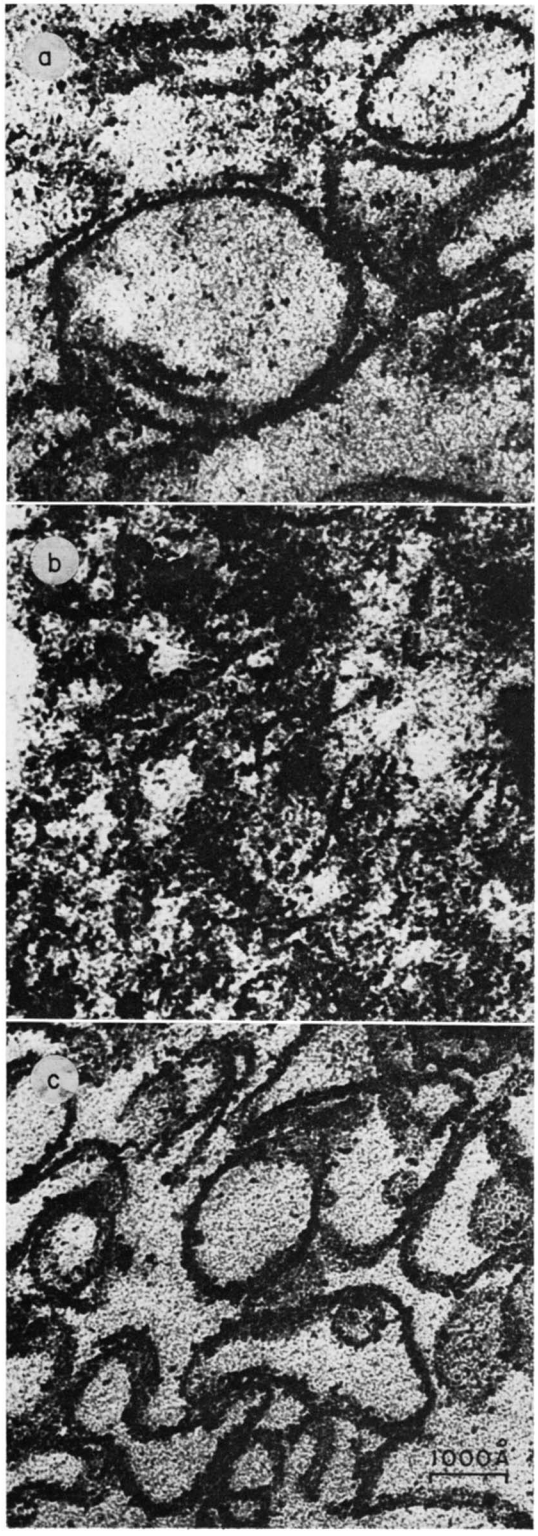


FIG. 3 (right). Membrane formation by the repeating units of the microsomal membranes of bovine liver: top, original membrane; middle, solubilized with cholate; bottom, cholate removed. Electron micrographs of negatively-stained specimens (phosphotungstate). Unpublished studies of D. G. McConnell and A. Tzagoloff.

FIG. 4 (below). Membrane formation by the repeating units of cytochrome oxidase: *a*, original cytochrome oxidase; *b*, cytochrome oxidase treated with cholate and ammonium sulfate to remove lipid; *c*, lipid-depleted cytochrome oxidase reconstituted with whole mitochondrial phospholipids. The three preparations were dialyzed and washed to remove residual bile salts. Specimens were fixed in osmium tetroxide, dehydrated, impregnated with Epon, and sectioned (74).



The role of phospholipid in vesicle formation has been studied with purified preparations of cytochrome oxidase. The cytochrome oxidase used in these studies contained about 30% of phospholipid. The enzyme complex can be depleted of its phospholipid complement by refractionation with ammonium sulfate in the presence of high concentrations of cholate (41). Such preparations aggregated upon removal of the residual bile salts but did not form membranes. Membrane formation in the phospholipid-depleted enzyme could be induced by adding back whole mitochondrial phospholipids (Fig. 4) (74). It should be stressed that lipid alone does not form vesicular membrane but rather forms tightly coiled laminar structures that are readily distinguishable from the true membranes.

When a vesicular membrane in a salt-free medium is subjected to prolonged sonic irradiation, the membrane structure is torn apart and a fraction consisting of the dispersed repeating units of the membrane can be isolated by differential centrifugation (Fig. 5). Addition of salt to this fraction induces the repeating units to re-form membranes. The ability of repeating units to remain in a dissociated state appears to be a function of the ionic strength of the medium. This phenomenon may be related to the observation of Brown that the membrane of the halophilic organism, *H. halobium*, readily dissociates into its repeating units at low ionic strength (45).

The basepieces of repeating units are the actual membrane-forming elements, since basepieces from which projecting knobs have been detached retain completely their capacity for vesicularization (36). Moreover,

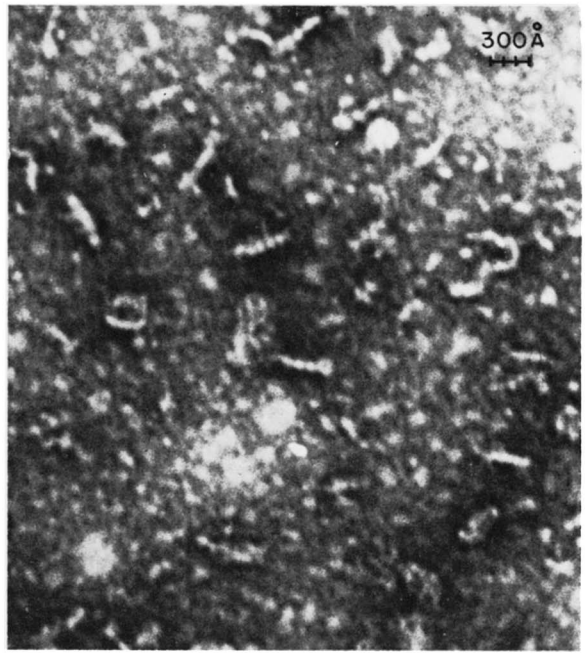


FIG. 5. Electron micrograph of electron transfer particles after extended exposure to sonic irradiation and removal of residual membranes. Note ribbons of particles each about 100 A in diameter. Specimen was negatively stained with phosphotungstate (unpublished data of D. G. McConnell and A. Tzagoloff).

"structural protein" can be completely removed from the complex that contains catalytic protein and core protein, and the residue, free from "structural protein," still shows an unimpaired capacity for membrane formation (36). Apparently the capacity for membrane formation is a property of catalytic and core protein in combination.

When tubular membranes such as the cristae of the mitochondria are disaggregated into the component repeating units, the membranes that re-form are always vesicular. Apparently, then, special conditions have to be imposed to achieve tubularization of the repeating units rather than vesicularization. The fact that nucleic acid serves as a directive influence for tubularization of the virus coat proteins (77) suggests that some kinds of template might be needed to induce repeating units to form tubular membranes.

Why phospholipid is essential for membrane formation is an intriguing question. In a membrane each repeating particle is probably attached to four or six other particles, and this attachment is only in one plane. No stacking is permitted in this kind of arrangement. Apparently lipid has the effect of excluding three-dimensional stacking or association, and restricting the association of nesting particles to one plane (Fig. 6). In addition, lipid may determine the curvature of the plane of association, which could account for the formation of spherical structures.

If the effect of lipid is to prevent three-dimensional stacking of the repeating units this may occur via a mechanism such that, wherever lipid binds to the repeating units, interaction at that site with other repeating units is excluded. If lipid were localized on the surfaces of the base particles that are parallel to the plane of membrane formation, then association by hydrophobic interaction with other base particles could take place in one plane only. Lipid would thus compel association of repeating particles in one plane only. The presence of negatively charged lipid would prevent both hydrophobic and electrostatic interactions with other repeating units at right angles to the plane.

Lipid systems tend to form spherical structures in aqueous media by virtue of the principle that maximal stability is achieved when the surface area is minimal. Whether this principle accounts for the tendency of membrane repeating units to form vesicular membranes is still an open question.

REQUIREMENT OF LIPID FOR ENZYMATIC ACTIVITY OF MEMBRANE PROTEINS

A large body of evidence bearing on the essentiality of phospholipid for some of the key mitochondrial functions has been accumulated during the past decade. It is now possible not only to recognize the basis for this essentiality but also to make predictions about processes in other membrane systems that should be lipid dependent. We shall first survey the literature relating to mitochondria and then consider the principles which should make it possible to extrapolate from a particular membrane to membranes generally.

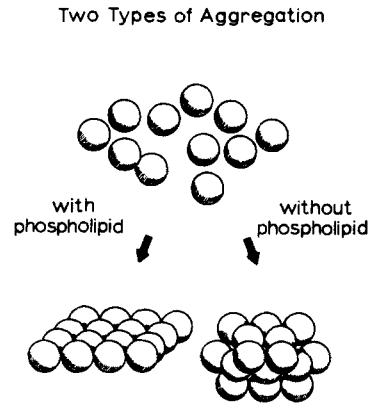


FIG. 6. The possible role of phospholipid in determining the mode of aggregation that leads to membrane formation.

The early studies of Edwards and Ball on phospholipase-treated mitochondria (78) strongly suggested that lipid was essential for the electron transfer process. The demonstration was more difficult than was originally appreciated. It was Fleischer and his colleagues (76) who first established the rigorous criteria that had to be satisfied for an unambiguous demonstration of essentiality. It was necessary to show not only a loss of activity with removal of lipid but also a restoration of activity when lipid was reintroduced into the extracted particles. In many cases loss of activity could be a consequence not of the removal of lipid but of the accumulation of split products of phospholipid hydrolysis. Lester and Fleischer (79) introduced the technique of extracting lipid with acetone–water 9:1 and of using micellized phospholipids in reconstitution studies.

As far as the electron transfer process is concerned, the essentiality of lipid has been established in studies both of whole mitochondria (76) and of the isolated complexes of the chain (41, 80–82). Lipid has been extracted by exposure of particles to phospholipase digestion (83, 84), to acetone, or to bile salts in presence of ammonium sulfate (41). These three alternative methods for extracting lipid have all been used successfully to demonstrate essentiality of lipid for the electron transfer process. It can now be stated that lipid is required for electron transfer in each of the four complexes of the electron transfer chain.

Restoration of electron transfer in lipid-extracted particles can be achieved with micellized mixtures of

phospholipids (e.g., Asolectin,[4] mitochondrial phospholipids) or with individual phospholipids in micellized form (76). Thus it is not necessary to replace the native set of phospholipids with the identical mixture. There are some indications in the literature that surfactants such as Tween-80 can substitute in part for phospholipids (81).

The electron transfer process is not the only mitochondrial function that is lipid dependent. E. Bachmann has found (unpublished studies) that ion translocation in phospholipase-digested inner membrane particles from bovine heart mitochondria can be restored specifically by addition of micellized phospholipids. It is becoming increasingly clear that all integrated functions of the mitochondrial inner membrane are lipid dependent. The problem is now the technical one of removing lipid without irreversibly inactivating the enzymic function under examination. Controlled phospholipase digestion may be the most promising tactic for the study of labile functions such as oxidative phosphorylation.

It is not only the electron transfer process in the inner mitochondrial membrane or processes dependent on electron transfer such as ion translocation that are lipid dependent but also other enzymic processes. The particulate enzyme that catalyzes the transfer of phosphoryl choline from cytidine diphosphocholine to α,β-diglyceride (cytidine diphosphocholine transferase) is localized both in the inner and outer mitochondrial membranes (R. E. McCaman and D. W. Allmann, manuscript in preparation). This enzyme shows a complete requirement for micellized phospholipid (85). Apparently, then, the essentiality of lipid may be generally independent of the nature of the enzyme catalysis.

The outer mitochondrial membrane catalyzes several integrated sequences (23, 86, 87) (citric cycle oxidations, fatty acid oxidation, fatty acid elongation, etc.) but thus far only two activities localized in the outer membrane have been shown to require lipid. One is the transferase activity referred to above and the other is the β-hydroxybutyric dehydrogenase activity (88, 89). Why lipid essentiality is limited in scope for enzyme complexes localized in the outer mitochondrial membrane and general for enzyme complexes localized in the inner mitochondrial membrane raises some fundamental issues which we shall consider later on.

Studies on the reconstitution of lipid-depleted cytochrome oxidase (lipid removed by fractionation with cholate and ammonium sulfate) have shown that the spectral characteristics of the phospholipid-depleted preparation are indistinguishable from those of the native preparation (41). Clearly, the quaternary structure of cytochrome oxidase can not be modified by lipid extraction since such modifications have been reported to cause shifts in the absorption bands (90). It is unlikely that the phospholipid contributes significantly to the over-all structure of the enzyme complex. The amount of phospholipid required for reconstitution is so large that any catalytic role becomes highly improbable, all the more so in view of the lack of specificity for the chemical nature of the phospholipid that is used for reconstitution.

There are two aspects of lipid essentiality—a general aspect that applies to all reconstitution studies and a specific aspect that applies exclusively to a particular system or complex. For example, the β-hydroxybutyric dehydrogenase is specific for lecithin (89); the cytidine diphosphocholine transferase is specific for (or at least prefers) lysophosphatides (85); cytochrome oxidase shows a requirement for some acidic phospholipid (41, 80, 82). These are the specific aspects of lipid essentiality and mirror the enzymic idiosyncracies of the complex under study. The requirement of cytochrome oxidase for acidic phospholipids has been rationalized by Tzagoloff and MacLennan (41) and by Brierley and Merola (80) in terms of the necessity to convert the electron donor (reduced cytochrome *c*) to a phospholipid complex thereof. The true substrate of the enzyme complex is the phospholipid complex of cytochrome *c* and not the free protein. Figs. 7 and 8 show the pertinent data on this point. Thus in this instance phospholipid serves not only in the general role but also in the specific role of activating the substrate by forming a phospholipid complex.

The general role of phospholipid appears to be to control membrane formation. In the form of membranes, the enzymic complexes of the inner membrane are active. In absence of lipid, membranes cannot be formed and the lipid-depleted enzymic complexes can exist only as a bulk phase (in which state their activity is no longer measura-

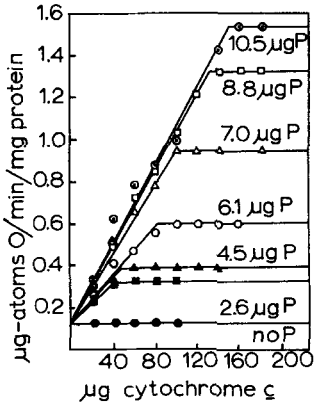


FIG. 7. The amount of cytochrome *c* that is saturating in a given enzymic assay of cytochrome oxidase as a function of the phospholipid concentration [Tzagoloff and MacLennan (41)].

[4] The Asolectin used in these studies was a commercial preparation from soybeans and was obtained from Associated Concentrates, Woodside, L. I. This preparation was analyzed by Dr. Sidney Fleischer who found it to be composed primarily of lecithin, cephalin, and liposterol in about equal proportions.

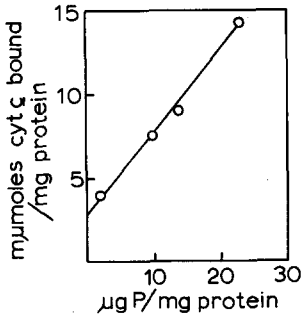


FIG. 8. Binding of cytochrome *c* as a function of the phospholipid content of a cytochrome oxidase preparation (Tzagoloff and MacLennan, 41). *P*, represents phospholipid.

ble). It is to be noted that the conditions for assay of activity (high dilution) automatically lead to the formation of membranes when the enzymic complex has its normal quota of lipid.

The lipid-free complexes of the chain exist as highly polymerized, water-insoluble particles—in fact, these extracted particles form a bulk phase in water. The exercise of enzymic activity requires molecularization of the complexes, and membrane formation achieves just that. When lipid is introduced into such particles, the capacity for membrane formation is restored. Within a membrane, the complexes are aligned in a molecular film, and are readily accessible to the solute molecules of the suspending aqueous medium.

The enzymes in the outer membrane, with one exception, show no requirement whatsoever for lipid (23, 96). This lack of requirement for lipid is readily explicable in terms of the properties of most of the enzymic complexes of the outer membrane. With but two known exceptions, these complexes have measurable water solubility; thus, the advantage of molecularization by membrane formation does not apply. The enzymic complexes of the outer membrane are already dispersed; no improvement in this dispersion can be achieved by inducing membrane formation by addition of lipid. The β-hydroxybutyric dehydrogenase is known to form water-insoluble polymers (88); for this enzyme, lipid is an absolute requirement because membrane formation provides the only device for molecularization of the enzyme. The variability in respect to lipid requirement for the activity of membrane-localized complexes is thus related to their solubility in water.

From these considerations applicable to the enzyme complexes of the mitochondrion it is now possible to predict that lipid will be essential for the complexes of any membrane that polymerize to water-insoluble aggregates when lipid-free. Whenever the molecularization of a complex is lipid dependent, lipid will be essential for activity. For the most part this molecularization will take the form of membrane formation (the possibility of other molecularizing modalities cannot be excluded in all known cases).

RECONSTITUTION AND MEMBRANE FORMATION

Hatefi and his colleagues (91–93) and King independently (94, 95) discovered the reconstitution of the electron transfer chain from the component complexes. This phenomenon can readily be explained as follows. Complex I catalyzes the oxidation of NADH by coenzyme Q; complex III, the oxidation of reduced coenzyme Q by cytochrome *c*. If these two complexes are merely mixed together and tested immediately, the combination of the two will not catalyze the oxidation of NADH by cytochrome *c*. However, if the two complexes are first solubilized with bile salts before they are mixed together, and then the bile salts are in effect removed from the mixture by dilution with 50 volumes or more of water, the resultant emergent particle now has the capacity to carry out a reaction which neither of the original particles possessed. This emergence of a new activity achieved by the interaction of two particles is known as reconstitution. Close inspection of the phenomenon discloses that the conditions for achieving reconstitution are identical with the conditions for inducing membrane formation. The two particles are mixed in concentrated solution, and by virtue of residual bile salts in each of the particles, the membrane repeating units are disaggregated and scrambled. Dilution of this concentrated mixture with water reduces the concentration of bile salts to the point where vesicularization can take place. Membranes are formed in which the two complexes can now be present in the same membrane as next door neighbors, and thus the appropriate conditions for the interaction of I with III are established. Membrane formation brings both complexes into the same "coordinate system" where they can interact readily with one another. In the example just cited we have considered only the reconstitution of an activity that requires two complexes. The reconstitution of the complete electron transfer chain would involve the participation of the four complexes of the chain.

DYNAMIC PROPERTIES OF MEMBRANES

Physiological studies provide impressive evidence of the capacity of membrane systems to undergo phase transitions. Amoeboid movement, pinocytosis, rhythmic contraction, membrane depolarization in nerve excitation—all these and other phenomena testify to the dynamic properties of biological membranes. To what extent can the repeating unit concept of membranes account for and explain these dynamic properties? In essence this reduces to the question whether or not repeating units are cap-

able of phase transitions while they are still part of the membrane continuum—transitions of the kind postulated by Kavanau (96) in his monograph on membrane systems. A few experimental observations suggest that repeating units indeed are capable of a variety of phase transitions.

The inner mitochondrial membrane is capable of translocating ions into the interior space by an energized process. Divalent ions, such as Ca^{++}, Mg^{++}, and Mn^{++}, are moved with their counterions (particularly phosphate) across the membrane into the interior space (97–100). This translocation appears to be an expression of the operation of a repeating unit which, when energized by a high energy intermediate, can turn inside out as it were; in the process, ions taken up from the exterior space are translocated into the interior space and deposited there. Such a translocation would necessitate a major phase transition in the repeating particle. Ion translocation may be another variant of the same kind of conformational change which underlies high energy bond formation coupled to electron transfer. The transfer of electrons through a complex triggers a conformational wave that leads to the formation of a high energy bond; ion translocation would be essentially the reverse of this process except that the conformational change would lead to the movement of ions and not to the transfer of electrons.

Mitochondria can undergo reversible swelling and shrinking (101, 102) and this phenomenon is in large measure a reflection of phase transitions in the inner mitochondrial membranes. Metal ions, such as Ca^{++}, play a key role in the swelling and shrinking of mitochondria. The interaction of these metal ions with the phosphate groups in phospholipid, bound to the repeating units of membranes, may lead to phase transitions in the lipid–protein domain; these transitions profoundly influence the amount of water contained within the volume enclosed by the membrane. Electron micrography has shed light on the nature of these phase transitions in the repeating units of the inner mitochondrial membranes. As soon as metallic ions have interacted with the phospholipid of the repeating units, the tubules of the inner membrane disappear, and one expanded vesicular membrane is formed (E. G. Munn and G. Blondin, unpublished studies). The interaction of metallic ions with the phospholipid apparently changes the characteristic shape of the repeating units which no longer can nest within the restriction of tubular membranes. Rearrangement of the membrane leads to the disappearance of the individual tubules, which expand into large vesicular membranes by the paying out of the dislocated repeating units. The volume of the mitochondrion is increased 3- to 5-fold by this arrangement and the amount of water contained within the membranes is correspondingly increased.

In addition to the change in shape of repeating units (the major factor contributing to mitochondrial swelling), metallic ions may also affect the size and volume of repeating units—yet another set of factors that can induce mitochondrial swelling. Any increase in the size and volume of repeating units would automatically increase the radius of a vesicular membrane and, correspondingly, the amount of entrained water. The contribution of this type of change to mitochondrial swelling is considerably smaller than that of change of shape.

Tubular membranes, according to Caspar and Klug (77), inevitably require a helical disposition of the repeating units. The possibility of helix–nonhelix transitions within tubular membranes is one which is worthy of careful exploration.

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