BBA 4228

THE ROLE OF LIPIDS IN MITOCHONDRIAL ELECTRON TRANSFER AND OXIDATIVE PHOSPHORYLATION*

D. E. GREEN AND S. FLEISCHER

Institute for Enzyme Research, University of Wisconsin, Madison, Wisc. (U.S.A.)

(Received July 2nd, 1963)

SUMMARY

1. The mitochondrion has been fragmented into a fraction containing the elementary particles (the seat of the electron-transfer chain) and a fraction containing the structural protein. On the basis of high-resolution electron microscopy and detailed chemical studies, the mitochondrion is pictured as a structural protein–phospholipid matrix to which are affixed many thousands of elementary particles.

2. The essentiality of lipid for electron transfer can be demonstrated by extracting mitochondria with aqueous acetone to remove lipid. Restoration of activity is achieved by adding back both coenzyme Q (Q) and phospholipid. A phospholipid requirement has been demonstrated in three segments of the electron-transfer chain; succinate $\longrightarrow$ Q; $QH_2 \longrightarrow$ cytochrome *c*; reduced cytochrome *c* $\longrightarrow O_2$.

3. Phospholipids can be oriented in water to form water-clear micelles. The micelle is the key to an understanding of the role of phospholipid and of the interaction of phospholipid with mitochondrial proteins. Two types of interaction have been characterized. The first is ionic, and occurs between acidic phospholipids and basic proteins like cytochrome *c*. The second, mainly hydrophobic in nature, involves the interaction of structural protein with all phospholipids tested, acidic as well as basic.

4. The role of phospholipid in electron transport, oxidative phosphorylation and the energy-linked transport of ions across membranes is discussed and a generalized formulation of the molecular structure and properties of membranes is given.

INTRODUCTION

The mitochondrion is a structured, subcellular unit that fulfills two transducing functions: (1) the conversion of energy released by oxidation to the bond energy of ATP, and (2) the conversion of energy released by oxidation to the translocation of solutes from outside to inside the mitochondrion (osmotic work). Lipid is an essential ingredient of this transducing machine and it is the purpose of the present communication to point up the how and where of lipid participation in mitochondrial structure and function.

Abbreviation: Q, coenzyme Q or ubiquinone.

* This is the revised and extended version of a paper read at the 7th International Conference on Biochemical Problems of Lipids, Birmingham 1962, published in A. C. FRAZER, *Biochemical Problems of Lipids*, Elsevier, Amsterdam, 1963, p. 325.

The mitochondrion, whatever the source, has the gross structure shown schematically in Fig. 1 (refs. 1, 2). It consists of structured elements (external envelope and internal cristae) and of spaces between these structured elements. We shall be concerned only with the properties of the structured elements; the spaces, presumably, are filled with a fluid containing salts, other solutes and possibly some soluble enzymes.

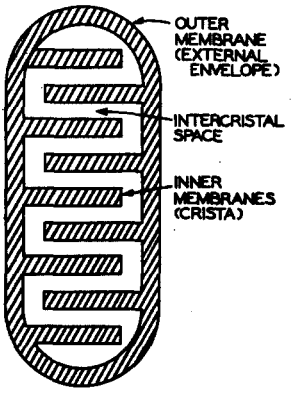


Fig. 1. Diagrammatic representation of a mitochondrion. The cross-hatched areas represent the structured portions of the mitochondrion while the clear areas represent the spaces filled with aqueous fluid in which soluble components are dissolved.

High-resolution electron microscopy and the development of ingenious techniques for the staining, mounting and examination of specimens in the electron beam have augmented enormously our knowledge of the molecular details of the structured elements of the mitochondrion[3]. Fig. 2 contains an electron micrograph of a section of the mitochondria of retinal rods prepared by FERNANDEZ-MORAN[4]. From an extensive combined study based on electron-microscopic and biochemical techniques carried out jointly by FERNANDEZ-MORAN and our group[5,6] the following interpretation can be made of the available information and evidence. The structured elements of the mitochondrion (both external envelope and internal cristae) are made up of three parts: (1) the elementary particles[5,6], (2) the primary dehydrogenase complexes[7], and (3) the structural protein–lipid network[8]. The relation among these parts is represented diagrammatically in Fig. 3. The primary dehydrogenase complexes are probably associated with the outer membrane (not shown); the elementary particles with the inner membrane and cristae. Each elementary particle is made up of a head piece, a stalk, and a base piece. The mesozone consists of the structural protein–lipid network covered by a thin layer of the fused base pieces of the elementary particles. The material of the structural protein–lipid network is not readily visualized on the electron micrographs shown. It is best seen in negatively stained whole mounts of mitochondria.

In specimens of mitochondria negatively stained with phosphotungstate, the elementary particle can be seen to consist of two parts[5,81]: a spherical head piece (80–100 Å in diameter) and a cylindrical stalk (30 × 50 Å) which is attached at one end to the head piece and at the other to the mesolayer (*cf.* Fig. 3). The isolated elementary particle is spherical in shape but shows no evidence of a stalk. The molecular weight of the isolated and purified elementary particle is about $2 \cdot 10^6$ (ref. 6). Nonethe-

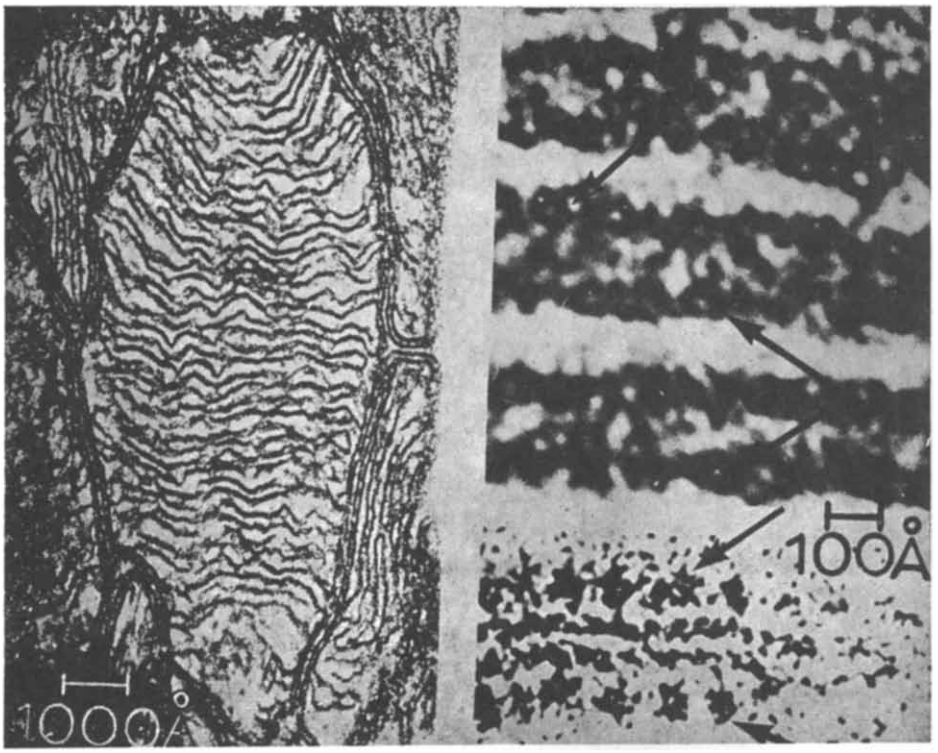


Fig. 2. Electron micrograph of sections of retinal rod. The photograph on the left-hand side shows a complete section of a single mitochondrion surrounded by segments of other mitochondria. The photographs on the right-hand side (upper right) show cristae at high magnification. Subunits of the elementary particles are clearly visible in these photographs. The arrows point to individual particles that are particularly distinct. The sections shown were fixed with osmium tetroxide. For purposes of comparison the photograph on the right-hand side, bottom portion, is an electron micrograph of a segment of a crista of beef-heart mitochondria negatively stained with phosphotungstate. This photograph clearly demonstrates paired arrays of elementary particles (pointed to by the arrows). Which comprise a head piece, a base piece and a stalk. The photograph was prepared and kindly provided by H. FERNANDEZ-MORAN.

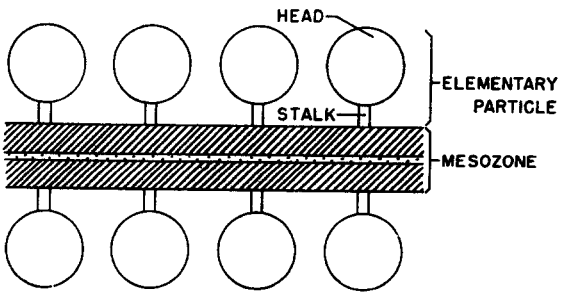


Fig. 3. Diagrammatic representation of the arrangement of parts in the mitochondrial cristae. The elementary particles are shown lined up on both sides of the mesolayer. The elementary particle is connected to the mesolayer by means of a stalk which is clearly visible in negatively stained mitochondria. The approximate diameter of the elementary particle is 80–100 Å. The distance between stalks is 112 Å. The base piece of the elementary particle is buried in the outer layers of the mesozone.

less, the short diameter of the isolated particle in negatively stained phosphotungstate preparations is 120 Å (ref. 5). The long diameter of the isolated particle is 160 Å.

Each elementary particle contains the complete electron-transfer chain for the oxidation of succinate or DPNH by molecular oxygen[6]. In the intact mitochondrion, electrons enter the elementary particle not by way of DPNH but rather by way of the intermediates of the citric acid cycle (pyruvate, α-ketoglutarate, malate and isocitrate) as schematized in Fig. 4. The primary dehydrogenase complexes are in fact the instruments for drawing paired electrons from the various citric-acid-cycle substrates (succinate excepted) or from β-hydroxybutyrate, fatty acids, etc. and introducing them into the electron-transfer chain of the elementary particle through bound DPNH as an intermediate. When the elementary particles are separated from these complexes, electrons can then enter the chain only by way of succinate or external DPNH.

The relative proportions of protein and lipid in each of the three parts of the mitochondrial membrane are given in Table I (ref. 9). The network of the structural protein accounts for some 60 % of the total protein; the elementary particle for about 20 % and the primary dehydrogenase complexes for the balance. Lipid, however, is associated largely if not exclusively with the elementary particles and with the structural protein sandwich layer.

The electron-transfer chain is made up of two flavoproteins (succinic dehydrogenase (F_S) and DPNH dehydrogenase (F_D), four cytochromes (cytochromes *a*, *b*, c_1

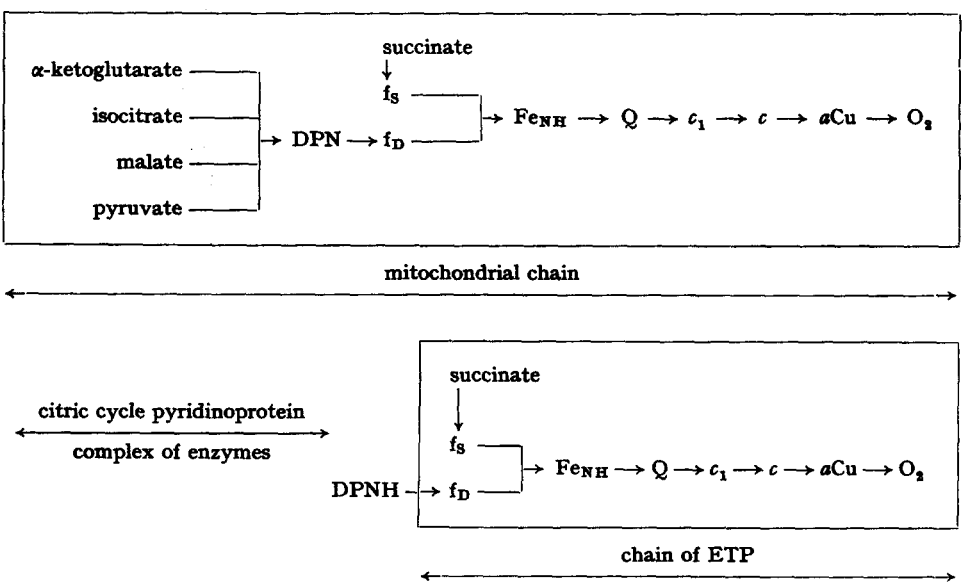


Fig. 4. The relation between the electron-transfer sequence of the elementary particle and of the sequence of the elementary particle in combination with the primary dehydrogenase complex. The citric-acid-cycle pyridinoprotein–enzyme complexes constitute in large measure the primary dehydrogenase complexes. These complexes generate DPNH from various substrates (α-ketoglutarate, isocitrate, etc.) which then serves as the source of electrons for the electron-transfer chain of the elementary particle. ETP is a particle containing arrays of elementary particles still attached to the structural protein sandwich layer. The electron-transfer chain shown in ETP is abbreviated for reasons of simplicity. Cytochrome *b* and some of the non-heme iron proteins have been deliberately omitted from the scheme.

TABLE I

THE RELATIVE PROPORTIONS OF PROTEIN AND LIPID IN THE MITOCHONDRIAL MEMBRANE*

	% of total mitochondrial protein	% of total mitochondrial lipid
Elementary particle	20	25
Structural protein in mesolayer	60	75
Primary dehydrogenase complexes	20	0

* Inferred from unpublished experiments of P. BLAIR, D. E. GREEN AND S. FLEISCHER.

and *c*), three clusters of iron atoms (not associated with heme and designated as non-heme iron), one cluster of copper atoms, and finally coenzyme Q (ubiquinone[7]). These 11 different oxidation–reduction species[10] are arranged in the electron-transfer chain in the sequence and in the proportions shown in Fig. 5 (ref. 82).

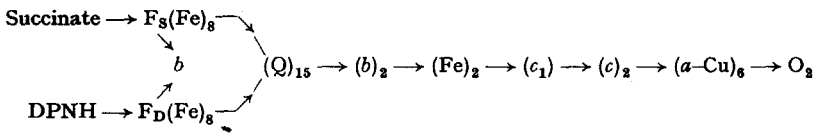


Fig. 5. The formulation of the electron-transfer chain from succinate and DPNH to molecular oxygen. The symbol Fe refers to non-heme iron which is localized at three different sites in the chain. The letters *a*, *b*, c_1 and *c* refer to the cytochromes, Q to coenzyme Q and Cu to copper.

The elementary particle is a unit made up of four primary enzymic complexes (*cf.* Table II for the designation and composition of the complexes). These four complexes appear to be the ultimate units of electron transfer and oxidative phosphorylation[11,12]. Each of the complexes accounts for a specific segment of the electron-transfer chain that is self-contained. The elementary particle can be resolved into these four primary complexes and in turn the isolated complexes under suitable conditions, can recombine in the correct molecular proportions (1:1:1:1) to reconstitute the functionally intact particle with its complete electron-transfer chain[11,13].

TABLE II

THE FOUR COMPLEXES OF THE ELECTRON-TRANSFER CHAIN*

Complex	*Designation*	*Reaction catalyzed*	*Composition*
I	DPNH–coenzyme Q reductase	DPNH → Q	$F_D(Fe)_8$
II	Succinate–coenzyme Q reductase	Succinate → Q	$F_S(Fe)_8$ *b*
III	QH_2–cytochrome *c* reductase	QH_2 → cytochrome *c*	$(b)_2(Fe)_2(c_1)$
IV	Cytochrome oxidase	Reduced cytochrome *c* → O_2	$(a\text{-}Cu)_6$

* *cf.* HATEFI *et al.*[11].

LIPID COMPOSITION OF MITOCHONDRIA AND DERIVATIVE PARTICLES

Our studies on the lipids of mitochondria[14] have been confined to an examination of beef-heart mitochondria which we are able to prepare in homogeneous suspensions

on a large scale[15]. These mitochondria contain about 26% by weight of lipid while the four primary complexes derived from the elementary particle, the elementary particle itself, and the structural protein mesolayer contain about 30% by weight of lipid (*cf.* Table III; refs. 14). Thus, lipid is uniformly distributed in all but the primary dehydrogenase complexes[14].

TABLE III

LIPID CONTENT OF MITOCHONDRIAL PARTICLES*

	$\frac{lipid\ (g)}{lipid\ (g) + protein\ (g)} \times 100$
Mitochondria	26
Succinate–coenzyme Q reductase	26
DPNH–coenzyme Q reductase	22
QH_2–cytochrome *c* reductase	29
Cytochrome oxidase	27
Succinate–cytochrome *c* reductase	29
DPNH–cytochrome *c* reductase	27

* Data of FLEISCHER *et al.*[14].

TABLE IV

COMPOSITION OF MITOCHONDRIAL LIPID*

	% *total P*	
Phosphatidylcholine	37	
Phosphatidylethanolamine	31	
Cardiolipin	16	Phospholipid accounts for > 90% of total lipid
Phosphatidylinositol	10**	
	μmoles/μmole P	
Plasmalogen	0.4	
Double bonds	3.2	
Fatty acid esters	1.8	

* Data of FLEISCHER *et al.*[14].

** More recent data (S. FLEISCHER, B. FLEISCHER AND G. ROUSER, unpublished results) suggest that the phosphatidylinositol content is substantially lower than 10%.

The composition of whole mitochondrial lipid is summarized in Table IV (ref. 14). The lipid derived from submitochondrial particles has essentially the same composition. In excess of 90% of the total lipid is phospholipid. The balance is the neutral lipid fraction, the principal constituents of which are Q, cholesterol, carotenoid and glyceride esters[17]. Four phospholipids, in the proportions shown in Table IV make up the phospholipid fraction, and the proportions of these phospholipids appear to be the same or similar in the complexes, in the structural protein network, and in the whole mitochondrion[14]. The net charge of total mitochondrial phospholipid will be negative since each phospholipid contributes at least one negative charge (cardiolipin contributes two). Lecithin and phosphatidylethanolamine in addition

contribute a positive charge but nonetheless there is an excess of negative charges in whole mitochondrial phospholipid of which lecithin and phosphatidylethanolamine account for some 70 %.

The hallmark of mitochondrial phospholipid is a high degree of unsaturation—about 3.2 double bonds per atom of phospholipid P (refs. 14, 18). TAPPEL *et al.*[19] have shown that the lipids of mitochondria generally are highly unsaturated and, as we shall see later, this unsaturation has considerable importance.

The phospholipids of beef-heart mitochondria contain a high proportion of plasmalogen[14,17] but this appears to be an idiosyncrasy of heart tissue since mitochondria from other sources have been reported not to contain major amounts of plasmalogen[20]. The plasmalogen content of beef-heart mitochondria has no apparent functional significance since plasmalogen-free phospholipid preparations can substitute for beef-heart mitochondrial preparations of phospholipid in restoring the activity of mitochondria from which phospholipid has been extracted[21,22].

LIPID AND ELECTRON-TRANSFER ACTIVITY

A mixture of 90 % acetone and 10 % water (v/v) will extract the bulk of the phospholipid from mitochondria and submitochondrial particles (some 85 %)[23]. Such extracted particles lose the capacity to carry out electron transfer and this capacity can in large measure be restored by adding back mitochondrial phospholipid and Q to the particles (*cf.* Fig. 6; refs. 21–25). It can be demonstrated that extracted particles exposed to phospholipid dispersions rebind the phospholipid, and once recombination has been accomplished, the reconstituted particles no longer show a requirement for

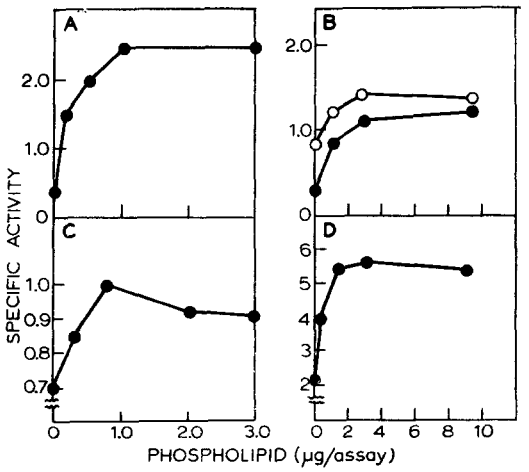


Fig. 6. The activity of various extracted particles as a function of the concentration of added phospholipid. Data of BRIERLEY *et al.*[25]. A, cytochrome oxidase; B, succinate cytochrome *c* reductase (O—O, Q_2; ●—●, Q_{10}); C, succinate–coenzyme Q reductase; D, reduced coenzyme Q–cytochrome *c* reductase.

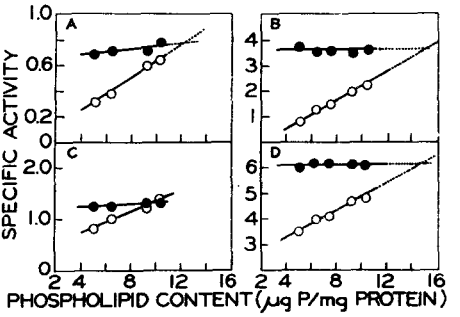


Fig. 7. Activity of various extracted particles (A, succinoxidase; B, cytochrome oxidase; C, succinate–coenzyme Q reductase; D, reduced coenzyme Q–cytochrome *c* reductase) as a function of the concentration of reincorporated phospholipid after interaction of the extracted particles with added phospholipid. The closed circles refer to unextracted mitochondria and the open circles refer to extracted mitochondria. Both unextracted and extracted mitochondria were exposed to the same amount of phospholipid. Data of BRIERLEY *et al.*[25].

phospholipid (*cf.* Figs. 7 and 8). An unambiguous requirement for lipid has been demonstrated in the following reactions: succinate $\longrightarrow O_2$; succinate $\longrightarrow$ Q; reduced Q $\longrightarrow$ cytochrome *c*; reduced cytochrome *c* $\longrightarrow O_2$ (refs. 22–25). Such requirement is demonstrable both in the intact mitochondria and in the isolated enzymic complexes that make up the respiratory chain. The DPNH–coenzyme Q reductase is the only complex for which a lipid requirement has yet to be demonstrated. The extreme sensitivity of the activity of this complex to solvents such as acetone has made it impossible to demonstrate a lipid requirement by conventional methods.

Mitochondria extracted under standard conditions contain 15–18% residual phospholipid, predominantly the acidic phospholipid cardiolipin; such extracted mitochondria respond equally to all four mitochondrial phospholipids (*cf.* Fig. 8, ref. 22) When extraction is more complete (< 8 % residual phospholipid), then a pronounced requirement for an acidic phospholipid is demonstrable (unpublished studies, S. FLEISCHER). Phospholipid alone among natural lipids (not replaceable by detergents) could fully satisfy the requirement for activity[22,23].

One of the primary dehydrogenase complexes—the β-hydroxybutyric dehydrogenase—can be isolated in a form the activity of which shows an absolute requirement for lecithin (*cf.* Fig. 9; ref. 26). The higher the degree of unsaturation in the fatty acid residues the more effective is the lecithin in restoring activity[26]. Thus, a synthetic lecithin with two saturated fatty acid residues is barely active whereas a synthetic lecithin with one oleyl residue can induce full activity. However, the concentrations of the monooleyl lecithin required are higher than those of a lecithin of natural source

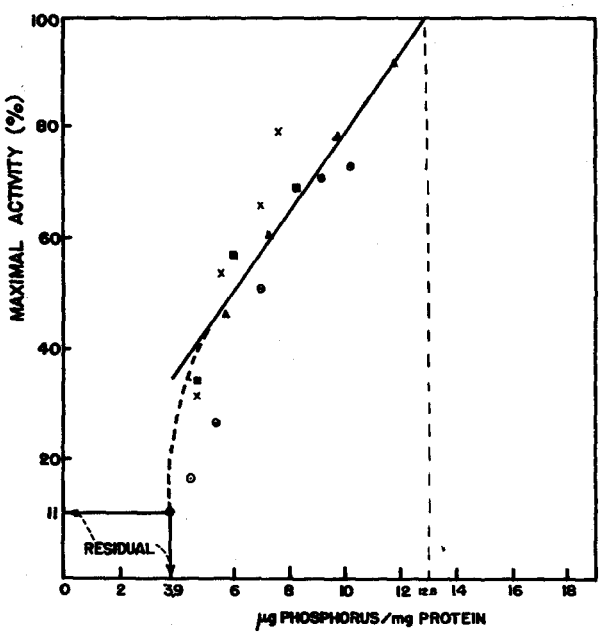


Fig. 8. The reactivation of phospholipid-deficient mitochondria (mitochondria extracted with aq. acetone) by four different phospholipid preparations. The enzymic activity measured was succinate–cytochrome *c* reductase activity. Percentage of maximal activity is plotted as a function of the phospholipid content after the rebinding of varying amounts of phospholipid. By this criterion, the four phospholipid preparations are not significantly different. Data of FLEISCHER *et al.*[22].

(*e.g.*, lecithin prepared from mitochondria or from Asolectin, a commercial product derived from soybean) which has some three times as many double bonds as does monooleyl lecithin. This apodehydrogenase can form a complex with lecithin that can be obtained in highly purified form; the purified complex no longer shows a requirement for added lecithin[48].

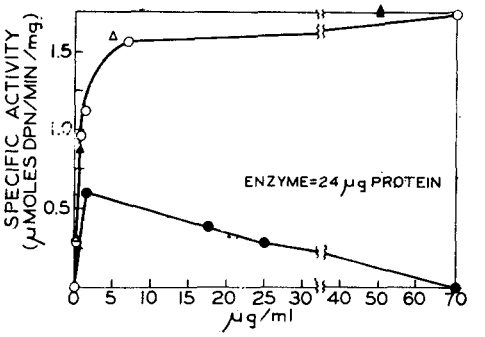


Fig. 9. The activity of the β-hydroxybutyric apodehydrogenase of beef-heart mitochondria as a function of the concentration of various phospholipid preparations. The mitochondrial cofactor is essentially a preparation of lecithin isolated from submitochondrial particle fragments. Data of JURTSHUK *et al.*[26]. ○, mitochondrial cofactor; ▲, egg lecithin; △, beef lecithin (commercial); ●, saturated lecithin (synthetic L-(α)-dimyristol).

MICELLAR STATE OF PHOSPHOLIPID

The phospholipid has to be presented to the acetone-extracted mitochondria or to the β-hydroxybutyric apodehydrogenase in a special state which may be described as a molecularly dispersed micellar or myelinic state[21]. Individual phospholipids or mixtures thereof (providing the fatty acid residues are unsaturated) can be molecularly dispersed in water by two techniques: (1) sonication[28,29], and (2) slow-rocking dialysis of a butanol–cholate mixture of the phospholipid against water[21]. Such dispersions have the appearance in electron micrographs shown in Fig. 10 (ref. 30). An interpretation of the electron micrograph is given in Fig. 11. In essence the molecular dispersion of phospholipids takes the form of bimolecular arrays tightly coiled in three dimensions. The interior of each bimolecular array is hydrophobic—this being the locale of the paraffinic fatty acid residues, whereas the outside layers of such arrays are hydrophylic—these being the locale of the charged phosphate ester and, where applicable, of the charged nitrogenous base, as well as the locale of the polar groups of the glycerol or polyalcohol moiety of the phospholipid molecule. The phospholipid micelle is highly stable over a wide range of conditions. These micellar solutions are essentially optically clear[21]. Sedimentation studies show a wide distribution of molecular size[31] but even in the smallest micellar array several hundreds of phospholipid molecules are involved. The molecular weight of the phospholipid micelle can reach $5 \cdot 10^5$ to $1 \cdot 10^6$ (ref. 31).

Micelles containing several different kinds of phospholipids are not merely mixtures of the micelles of one particular phospholipid. This is readily demonstrable by the use of DEAE-cellulose columns (N^+). Such columns readily bind micelles of acidic phospholipids but not micelles of lecithin[32]. When lecithin is a component of a micelle containing all the mitochondrial phospholipids the column holds up not

Fig. 10. Electron micrograph of lecithin micelles prepared according to FLEISCHER AND KLOUWEN[21], negatively stained with phosphotungstate. The technique for preparing the mounts examined in the electron beam was devised by Dr. H. FERNANDEZ-MORAN, who kindly provided the photograph. The arrows point to the areas in which phosphotungstate accumulates.

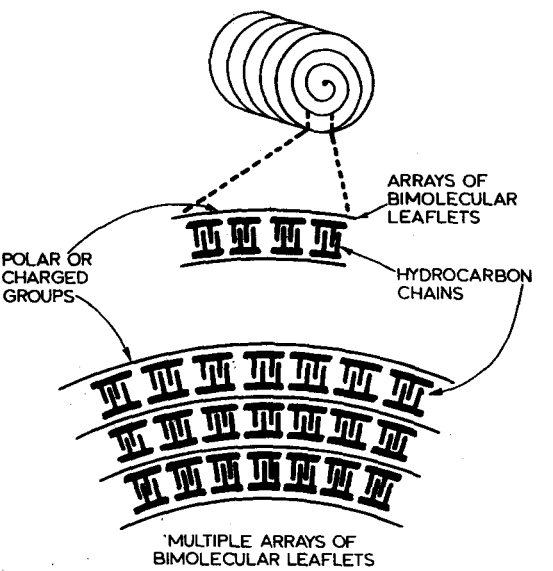


Fig. 11. Interpretation of the electron micrographs of lecithin micelles.

only the acidic phospholipids but lecithin as well. This shows clearly that all the phospholipids are part of the same micellar system since lecithin is bound only by virtue of its association with acidic phospholipids[32].

The available evidence suggests that in the interaction of phospholipid micelles with acetone-extracted mitochondria or with the β-hydroxybutyric apodehydrogenase, individual molecules are not involved. The micelle without redistribution of the component phospholipid molecules interacts with the protein; the lipid composition of the protein–lipid complex thus formed is identical with that of the original micelle before interaction[32]. Light-scattering studies and dialysis studies have established that even at high dilution, phospholipid micelles do not dissociate significantly to free molecules of phospholipid (S. FLEISCHER AND V. A. K. SARMA, unpublished observations).

EQUILIBRATION OF PROTEIN-BOUND PHOSPHOLIPID WITH EXTERNAL PHOSPHOLIPID

The mitochondrion or the elementary particle can be fragmented into the various complexes of the electron-transfer chain by bile acids in the presence of salts or by *tert.* amyl alcohol[33]. FLEISCHER AND BRIERLEY[34] have demonstrated that under the conditions which lead to the separation of the four enzymic complexes, one from the other, there is complete and rapid exchange of the bound phospholipids with externally added phospholipid. This exchange demonstrates that, under conditions that lead to fragmentation, the bonds between protein and phospholipid are so weakened that bound phospholipid can be displaced by externally added phospholipid, and conversely that bound phospholipid can be displaced to merge with the pool of phospholipid external to the particle. When the concentration of bile acid or inorganic salts or both is reduced below the critical point at which fragmentation takes place, no exchange of phospholipid can be observed.

The available evidence suggests that lipid–protein bonds are the predominant links that hold together the various complexes of the elementary particle and that these bonds are also partially responsible for the attachment of the elementary particle to the structural protein–lipid mesolayer.

THE NATURE OF THE BONDING OF PROTEINS TO PHOSPHOLIPIDS

The key to an understanding of the role and state of lipid in mitochondria is the nature of the bonds between lipid and protein. It was toward this end that we embarked on a systematic study of protein–phospholipid interactions in model systems[35]. Two main types of interaction were observed. The first type—ionic in character—is exemplified by the interaction of cytochrome *c* and other basic proteins with acidic phospholipids or with a mixture of phospholipids containing acidic phospholipids[32]. The second type—non-ionic and largely hydrophobic—is exemplified by the interaction between the structural protein of mitochondria (and of other membrane systems) with phospholipids whether acidic or zwitterionic[36].

1. Ionic interaction

Basic proteins (lysozyme, polylysine, cytochrome *c*, etc.) form complexes with acidic phospholipids or with a phospholipid mixture containing acidic phospholipids, but

not with lecithin (a zwitterion). We have studied the interaction of cytochrome *c* with phospholipid micelles in considerable detail. The broad outlines of these studies will be sketched to illustrate the guiding principles that underlie electrostatic interaction.

Interaction in aqueous solution

That the interaction between phospholipid micelles and cytochrome *c* in water leads to a complex is demonstrable by passage of the reaction mixture through Sephadex G-75 to remove uncombined cytochrome *c* (the complex comes through the column) or by high-speed centrifugation (the complex sediments out as a red button). The molecular proportions of cytochrome *c* and phospholipid in the complex are determined exclusively by considerations of charge neutralization[32].

Cytochrome *c* is a soluble, basic protein (isoelectric point pH 10.7) of molecular weight 12 000. According to the amino acid composition as determined by MARGOLIASH, SMITH AND TUPPY[37,38], the protein contains 34 charged groups at physiological pH (7.4). 21 are positive charged (19 lysine and 2 arginine) whereas 13 are negative charged (3 aspartic acids, 9 glutamic acids and one additional terminal carboxyl group). Glycine, the terminal amino group is acetylated. There is, therefore, a net positive charge of 8 per molecule of cytochrome *c*.

The acidic phospholipids (cardiolipin, phosphatidylinositol) bear one negative charge per atom of phosphorus. Experimentally, we observe a value of 8–9 for the ratio moles of phospholipid P per mole of cytochrome *c*, when complexes of cytochrome *c* with acidic phospholipid are formed[32]. Lecithin—a zwitterion that bears a net charge of zero—does not react at all with cytochrome *c*. On the other hand, phosphatidylethanolamine—the salt of a strong acid and a weaker base—interacts to a very limited extent[39]. The value of the P:cytochrome *c* molar ratio for the complex of phosphatidylethanolamine and cytochrome *c* is about 100. When micelles of mixed phospholipids interact with cytochrome *c*, the values of the P:cytochrome *c* molar ratios for the complexes formed reflect the proportion of acidic phospholipids in the micelle. When the proportion of acidic phospholipids is relatively low, the P:cytochrome *c* molar ratio is correspondingly high.

As might have been expected for an electrostatic interaction, the complex is dissociated by salt and its formation is prevented by salt. For example, 0.4 M KCl decreases complex formation to more than 95 % (ref. 32).

Extraction of a cytochrome c complex into heptane

A highly charged protein such as cytochrome *c* is insoluble in non-polar solvents. Yet WIDMER AND CRANE[40] and DAS *et al.*[39] have reported a form of cytochrome *c* which is "soluble" in isooctane or heptane. We have investigated this phenomenon in some detail[32].

The complex of cytochrome *c* and phospholipid formed in water can be extracted into heptane or isooctane by addition of ethanol (30 %, v/v) to the aqueous solution[32,33,40]. When the organic phase in which the complex has been extracted is removed by evaporation, the solvent-free complex is found to be insoluble in water. There are two stages in the formation of the heptane-soluble complex[32]. The first is the formation of a cytochrome *c*–phospholipid complex in the aqueous phase by

ionic interaction (Eqn. 1). The second is the change in orientation from that of the complex in aqueous solution to that of the complex in heptane solution (Eqn. 2).

$$\text{Cytochrome } c + \text{phospholipid} \longrightarrow \text{cytochrome } c\text{–phospholipid (polar orientation)} \quad (1)$$

$$\text{Cytochrome } c - \text{phospholipid} \longrightarrow \text{cytochrome } c\text{–phospholipid (non-polar orientation)} \quad (2)$$

It is the cytochrome *c*–phospholipid complex in the non-polar orientation that is insoluble in water.

The additional step of extracting the phospholipid–cytochrome *c* complex into the heptane from water introduces several complications into the study of the interactions between phospholipids and cytochrome *c*. Since these complications involve facets which can easily obscure the ionic parameters of the heptane-soluble complex, a few comments are in order.

The polar orientation of phospholipid micelles in water (a hydrophobic interior and a polar exterior) must be changed by inversion to a non-polar orientation of the complex (polar interior and a hydrophobic exterior). In heptane the complex must contain cytochrome *c* and the ionic groups of the phospholipid in the interior screened from the solvent by the hydrocarbon portion of the phospholipid. The inversion of the aqueous complex to the heptane complex is enhanced by the presence of ethanol, acetone, or isobutyl alcohol[32].

The complex of cardiolipin and cytochrome *c* is not readily extracted into heptane; instead the complex concentrates largely at the interface between the aqueous and organic phases. When cardiolipin is mixed with an equal amount of lecithin or phosphatidylethanolamine (equal in terms of P content), the extraction of the resulting complex with cytochrome *c* becomes practically quantitative[32]. Apparently the number of cardiolipin molecules required for the charge neutralization of cytochrome *c* is too small to provide a complete screen for the polar interior of the complex in heptane. Additional phospholipid is required for the stabilization of the heptane complex. Supplementation of cardiolipin with lecithin which is completely inert ionically for the purposes of complex formation or with phosphatidylethanolamine which is largely inert in that regard, solves the problem of adequate shielding; the complexes between cytochrome *c* and these mixtures of phospholipids are readily extracted into and are extremely stable in heptane[32].

Apart from the complications of the shielding problem and the problem of inversion of the complex it is clear that the same basic principles of ionic interactions (Eqn. 1) are also operative in the heptane system. The stoichiometry of phospholipid and cytochrome *c* in the complex is likewise based upon charge neutralization. Lecithin alone is ineffective for the conversion of cytochrome *c* to a heptane-soluble complex since there is no ionic interaction between the two. Salt prevents the extraction of the cytochrome *c* into heptane because the complex of cytochrome *c* and phospholipid (Eqn. 1) cannot be formed in presence of salt.

Interaction of phospholipid micelles with chemically modified cytochrome c and cytochrome c from different sources

Auxiliary evidence for ionic interaction comes from studies with chemically modified cytochrome *c* and with cytochrome *c* from different sources. When cytochrome *c* is succinylated or acetylated so as to tie up the basic groups (*cf.* TAKEMORI *et*

al[41].) then the capacity for forming a phospholipid–cytochrome *c* complex is completely lost. However, when the amino groups of the lysine residues are replaced by guanido groups, the derivative cytochrome retains its capacity to interact with phospholipids[32].

Bacterial and plant cytochrome *c*'s unlike yeast cytochrome *c*, are neutral or acidic proteins. None of these are capable of forming phospholipid–cytochrome *c* complexes[32].

A variety of basic proteins (histone, protamine, polylysine, RNAase and lysozyme) have the same capacity to interact in aqueous solution with acidic phospholipid micelles as mammalian cytochrome *c*[32]. The phospholipid–protein complex can then be extracted into non-polar solvents by reorientation of the aqueous complex. The fundamental interaction between these basic proteins and acidic phospholipids is electrostatic in nature.

Definition of charge neutralization

In the above discussion we have been considering exclusively ionic interactions between two polyelectrolytes—the protein and the phospholipid micelle. All of the charges in both of these polyelectrolytes are, of course, neutralized at the pH at which the reactions are carried out. Neutralization by the small counterions is is ignored in the context of the interaction between the protein and the micelle. Charge interaction between the two polyelectrolytes would take precedence over charge interaction with the small counterions. Thus the term "charge neutralization" as used above is intended to imply exclusively the neutralization of the charge of one polyelectrolyte by the opposite charges of the paired polyelectrolyte.

Net positive charge of cytochrome c

In most of the ionic interactions between cytochrome *c* and acidic phospholipids that we have studied, the number of acidic phospholipid molecules ionically bound to cytochrome *c* corresponds to the net positive charge of the protein at the pH of the reaction. Thus at pH 7.4 only 8 of the 21 positive charges in the protein participate in the ionic processes leading to complex formation with the phospholipid. Precisely why in most interactions the net positive charge of the protein is the determinant of stoichiometry and not the total positive charge is not clearly understood. We have found conditions under which the molar ratio of cardiolipin to cytochrome *c* corresponds to the interaction of all the positive groups in the protein with the negative groups of the phospholipid. Thus, at least under special circumstances complete charge neutralization of all the positive groups in cytochrome *c* can be achieved[32].

Inversion of phospholipid micelles

Although it is not strictly germane to the problem of ionic interaction of basic proteins with phospholipid micelles, the parallelism between the behavior of phospholipid micelles and that of the phospholipid–protein complex should be noted. Phospholipid micelles can also be extracted from water into organic solvents by addition of alcohol and other reagents, and the same kind of charge in orientation discussed above for the extraction of the phospholipid–cytochrome *c* complex into heptane appears to apply with equal force to the extraction of the phospholipid micelle into heptane[32].

2. Non-ionic interaction of mitochondrial structural protein with phospholipid micelles

Mitochondrial structural protein combines spontaneously with phospholipid micelles to form complexes[35,43]. We have studied the nature of these interactions in some detail[36]. The indications are that the forces involved in complex formation are largely if not exclusively hydrophobic in nature.

Structural protein can form a complex with all phospholipids tested—acidic as well as zwitterionic. Thus both the lecithin and mitochondrial phospholipid (in micellar state) bind to the protein to an equal extent (about 10 μg of phospholipid P per mg protein). The binding between lecithin and these two proteins was our first indication that the interaction of phospholipids with the structural protein was very different in character from the electrostatic type of interaction. The impression was further borne out by the failure of salt to dissociate or to prevent the formation of the structural protein–phospholipid complex[36].

Structural protein alone does not combine with cytochrome *c*. However, when structural protein has combined with acidic phospholipid, the resultant binary complex can combine with cytochrome *c* to form a ternary complex. The molar ratio of phospholipid to cytochrome *c* in the ternary complex is not very different from that for the complex of cytochrome *c* and the same acidic phospholipid. This finding establishes that in the binding of the phospholipids to structural protein the ionic groups of the phospholipid are not involved in the interaction; these groups are thus available for conferring hydrophilic properties on the complex[36].

Salt dissociates cytochrome *c* from the ternary complex containing structural protein, phospholipid and cytochrome *c*. The released cytochrome *c* is water-soluble while the residual binary complex (of structural protein and phospholipid) remains particulate and is undistinguishable from the starting binary complex[36].

To evaluate the importance of the hydrophobic fatty acid residues of the phospholipid for the bonding to structural protein, we studied the bonding of synthetic alkyl phosphates to structural protein. The chain length of the alkyl phosphates determines the extent of bonding of the molecule to structural protein. The longer the side chain, the better is the bonding. Dialkyl phosphates bond to a much greater extent than monoalkyl phosphates. Dioctyl phosphate bonds to about half the extent of lecithin (which contains two fatty residues C_{16} and C_{20}). Ditridecyl phosphate bonds almost as well as lecithin. Complexes of structural protein and alkyl phosphates can react with cytochrome *c* to form a ternary complex in much the same fashion as the complex of structural protein and phospholipid. From the stoichiometry of bonding it can be deduced that the phosphate groups in the structural protein–alkyl phosphate complex are completely titrable by cytochrome *c*. Salt dissociates cytochrome *c* from this ternary complex whereas salt has no effect on the dissociation of the binary complex containing structural protein and the alkyl phosphate[36].

The model interaction of alkyl phosphates with structural protein provides the most decisive lines of evidence for the importance of hydrophobic bonding in the analogous interaction of structural protein and phospholipid. These may be summarized as follows. (1) The bonding with structural protein is dependent on the chain length of the alkyl phosphate. (2) The phosphate group of the structural protein–alkyl phosphate complex is available for ionic interaction with cytochrome *c*. (3) The link of cytochrome *c* to alkyl phosphate in the ternary complex is severed by salt whereas

under exactly the same conditions salt has no effect on the link between structural protein and the alkyl phosphate.

The bonding of phospholipid to structural protein is analogous to that of the alkyl phosphates in that (1) it is not dissociated by salt, and (2) the phosphate groups in the structural protein–phospholipid complex are also capable of bonding cytochrome *c*. From such considerations the basically hydrophobic character of the bonding of phospholipid to structural protein can be deduced[36].

PROPERTIES OF STRUCTURAL PROTEIN

Structural protein in its monomeric form has a molecular weight of about 23000 (ref. 42). At neutral pH it polymerizes to form a water-insoluble aggregate. The reagents or conditions which solubilize the structural protein are in fact the reagents or conditions that monomerize the polymeric aggregate. These are dilute alkali (pH 11–12), 66% acetic acid and 1% dodecyl sulfate. The structural protein of mitochondria appears to be a single component which shows a high degree of homogeneity by all physical criteria[42].

TABLE V

AMINO ACID COMPOSITION OF STRUCTURAL PROTEIN*

Amino acid	*μmoles amino acid per 100 mg of protein*	*Moles of amino acid per mole of structural protein*
Asp	58	12.8
Thr	37	8.1
Ser	37	8.1
Pro	20	4.4
Glu	55	12.1
Gly	69	15.2
Ala	74	16.3
Val	48	10.5
Met	18	4.0
Ileu	44	9.7
Leu	68	15.0
Tyr	28	6.2
Phe	39	8.6
Lys	56	12.3
His	11	2.4
Arg	36	7.9
Amide-NH_2	90	9.7
Try	29	6.5
Cys (as cysteic acid)	68	10.3

* Data of T. GERRITSEN reported in the paper of CRIDDLE *et al.*[42].

The amino acid analysis (*cf.* Table V) points up several features: (1) the preponderance of positive charges (lysine, arginine and histidine) over negative charges (glutamic and aspartic acids) after allowing for the carboxyl groups tied up in amide linkage; (2) the relatively high proportions of amino acids with hydrophobic side chains (isoleucine, leucine, methionine, valine, phenylalanine, tyrosine); (3) the relatively high proportion of amino acids with charged groups. The calculated isoelectric point lies between pH 10 and 11.5 (ref. 44). How many of the sulfhydryl groups in

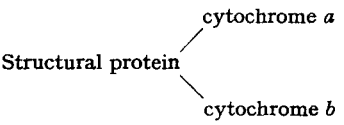
the structural protein are free is still undetermined and this uncertainty limits better precision in the calculation of the isolelectric point.

Cytochromes *a* and *b* and an iron protein isolated from Complex III of the electron-transfer chain have several properties in common with those of structural protein. In particular the monomers of these cytochromes and of the non-heme iron protein tend to form insoluble polymeric aggregates at neutral pH. These aggregates can be depolymerized by the same reagents as were effective in monomerizing structural protein[42,45].

When the monomer of the structural protein is allowed to react with the monomer of the two cytochromes listed above, simple molecular compounds are formed with the following compositions:

Structural protein – cytochrome *b*

Structural protein – cytochrome *a*



These compounds are water-soluble; their molecular weights correspond to the molecular weight of the monomeric form of structural protein plus that of the cytochrome or cytochromes. The fact of water solubility suggests that the respective hydrophobic regions of the cytochrome protein and of the structural protein coalesce, and that the charge density in the resulting compound is sufficiently high to ensure water solubility[42].

Lipid-free structural protein is best prepared by the improved procedure of RICHARDSON *et al.*[43] which minimizes damage to the protein. This type of preparation has been used for most of the studies on lipid–protein interactions reported in this review[35,36].

ROLE OF LIPID IN MITOCHONDRIAL ELECTRON TRANSFER

At one time it was possible to think in terms of a specific role for lipid in the electron-transfer process. But as more evidence accumulated, it became clear that lipid is not like a coenzyme in the sense of fulfilling one function but rather like water in the sense of providing a medium which has multiple functions. Lipid is an integral part of the mitochondrial structure, and there is hardly a mitochondrial function that does not reflect this participation.

Lipid as a bridge

Most of the proteins of the mitochondria (except for those of the primary dehydrogenase complexes) have a pronounced hydrophobic character, and when free of lipid, form water-insoluble polymeric arrays (*e.g.* cytochrome *a* (ref. 46), cytochrome *b* (ref. 47) and iron protein[45]). The hydrophobic proteins of the electron-transfer chain require lipid as a bridge to the water phase. The link of phospholipid with these proteins is hydrophobic[36]. Thus, the charged or polar end of the phospholipid is free to effect solubilization of the lipid-protein array. The amphipathic character of phospholipids makes possible this function of bridging the protein (via hydrophobic groups) to the water (via the free polar and ionic groups).

Catalytic role of lipid

The absolute essentiality of lipid for electron transfer in the mitochondrial chain[22] and also for the activity of the β-hydroxybutyric dehydrogenase[26] could be explained in several ways. First, lipid might serve as a form of coenzyme. But this is ruled out by the relatively high concentrations of lipid required. Second, lipid might influence the conformation of the enzymic protein with which it is associated. This possibility cannot at present be excluded but it is pertinent to point out that the lipid requirement for the electron-transfer chain can be satisfied by any one of four different phospholipids with vastly different chemical structures[22]. A role in determination of exact protein conformation would be expected to impose a high degree of specificity on the structure of the phospholipid. All the available evidence now points to the conclusion that among other functions phospholipid fulfills the function of providing a non-aqueous medium or, more precisely, a medium of low dielectric constant in which reactions abetted by such a medium can proceed. Presumably, the oxidation–reduction groups of the electron-transfer chain are so oriented as to abut into or fit within the phospholipid layer that envelopes each of the complexes.

Study of the β-hydroxybutyric dehydrogenase has given valuable insight into the rationale of lipid requirement[26,27]. This dehydrogenase is undistinguishable from any of a large number of pyridinoprotein enzymes in all but one respect, via its requirement for lipid. In other known respects there is nothing to choose between the β-hydroxybutyric dehydrogenase and any other soluble pyridinoprotein—none of which requires added lipid. The net result of this comparative survey has been the conclusion that all functionally active pyridinoprotein enzymes contain a hydrophobic region which is requisite for the interaction of substrate with DPN^+. The β-hydroxybutyric dehydrogenase is unique in that the essential hydrophobic region (*i.e.*, lecithin in this case) is detachable from the protein whereas the hydrophobic regions of other pyridinoprotein enzymes consist of cluster of non-polar amino acid residues which form an integral part of the structure of the protein.

$$\text{Substrate (reduced)} + E\begin{matrix}S\\|\\S\end{matrix} \longrightarrow \text{substrate (oxidized)} + E\begin{matrix}SH\\\\SH\end{matrix} \qquad (1)$$

$$E\begin{matrix}SH\\\\SH\end{matrix} + DPN^+ \longrightarrow E\begin{matrix}S\\|\\S\end{matrix} + DPNH \qquad (2)$$

Fig. 12. Two stages in the interaction of DPN^+ with pyridinoproteins. Unpublished studies of P. JURTSHUK, I. SEKUZU AND D. E. GREEN. ES_2, vicinal dithiols bound to the apoprotein. The interaction of the dithiol enzyme with DPN^+ undoubtedly involves prior binding of DPN^+ to some site in the enzyme protein adjacent to the dithiols.

There are two chemical reasons why the interaction of substrate with DPN^+ by pyridinoprotein enzymes should require a hydrophobic milieu. First, the interaction proceeds in two steps (*cf.* the scheme shown in Fig. 12) both of which would be facilitated by a medium of low dielectric constant. In such a medium the directing influence of the charged nitrogen atom in the pyridine ring would be enormously augmented. Second, the binding of the amide group by a metal is greatly facilitated in a medium of low dielectric constant (Zn is an essential component of the enzyme)[48].

Micellar lipid as a solvent

Water-insoluble molecules such as cholesterol, Q and neutral lipids may be presumed to exist in the form of solute within the micellar array[49]. The available evidence suggests that these molecules are localized and oriented within the hydrophobic sector of the phospholipids. Within this sector there is the possibility of extensive molecular movement. For example Q associated with the DPNH–Q reductase becomes available as an electron acceptor for succinate–Q reductase when the particles associated with these two activities react to form a single unit[11].

It bears repeating that the lipid of the mitochondrion is a molecularly dispersed phase[49], not a bulk phase. This molecular dispersion facilitates the rapid equilibration of solutes between hydrophobic and aqueous phases of the mitochondrion.

ROLE OF LIPID IN OXIDATIVE PHOSPHORYLATION

The oxidation–reduction reactions of electron transfer are the instruments for the coupling of oxidation to synthesis of ATP. A proposed generalized mechanism of oxidative phosphorylation is summarized in Fig. 13 (ref. 50). The essential steps are: (1) the formation of a high-energy intermediate during the oxidation of DPNH, reduced Q and reduced cytochrome *c*, each reaction being catalyzed by the appropriate primary enzymic complex (I, III and IV)[51–53]; (2) the transfer of the coenzyme from the acceptor group of Complex I, Complex III and Complex IV, respectively, to the respective acceptor groups of the factors (F_1, F_2 and F_3)[51–53]; (3) the displacement of the coenzyme group by inorganic phosphate and then the displacement of the factor by ADP leading to the synthesis of ATP[54]. All the primary complexes concerned with the formation of the high-energy bond and also the transfer reactions leading up to the synthesis of ATP appear to require protection from the hydrolytic effect of water. Conservation of the high-energy bond may well be incompatible with the presence of water. Presumably it is this vital function that the phospholipid wrapping of each complex fulfills in the mitochondrion. The elaborate weaving of lipid into the fabric of the mitochondrion facilitates the objective of forming high-energy compounds in a non-aqueous medium.

GRIFFITHS AND CHAPLAIN[55,84] have isolated a phosphorylated derivative of DPNH which is not an intermediate of oxidative phosphorylation, but is probably a

Substrate (reduced)* + complex (oxidized)** ⟶ substrate (oxidized) ~ complex (reduced) (1)

Substrate (oxidized) ~ complex (reduced) + factor*** ⟶ substrate (oxidized) ~ factor + complex (reduced) (2)

Substrate (oxidized) ~ factor + P_i + ADP ⟶ ATP + substrate (oxidized) + factor (3)

Fig. 13. Generalized mechanism of oxidative phosphorylation. Unpublished studies of A. SMITH, G. WEBSTER AND M. HANSEN[50].

* DPNH or reduced Q or reduced cytochrome *c*.

** Complex I with DPNH; III with reduced Q; and IV with reduced cytochrome *c*. I, III and IV are, respectively, DPNH–Q reductase, reduced Q–cytochrome *c* reductase and cytochrome oxidase.

*** There are three factors involved in the coupled phorphorylations catalyzed by I, III and IV respectively. These are the DPN^+, Q and cytochrome *c* factors.

derivative of the intermediate. This phosphorylated intermediate is highly unstable in water at neutral pH[54]. Special conditions have had to be imposed in the isolation to overcome the stability problem. This is the first direct proof of the thesis that high-energy intermediates have to be formed in a non-aqueous medium or at least in a medium of low dielectric constant.

ROLE OF LIPID IN TRANSPORT ACROSS THE MITOCHONDRIAL MEMBRANE

The mitochondrion has the capacity to move ions across the membrane against a concentration gradient. The following ion pairs of physiological interest have been shown to be concentrated in the mitochondrial interior $Mg^{2+} + P_i$, $Ca^{2+} + P_i$, and $Mn^{2+} + P_i$ (refs. 56–59). These paired ion movements are energized by a high-energy intermediate generated either by the electron-transfer process (oxidation of substrate) or from ATP by a substitution reaction[56]. For each pair of electrons moving through the chain 3 molecules of P_i are concentrated together with 6 molecules each of Mg^{2+}, Ca^{2+}, or Mn^{2+} (ref. 60). In the mitochondrial interior there is deposition of $Mg_3(PO_4)_2$, $Ca_3(PO_4)_2$ or $Mn_3(PO_4)_2$ (refs. 56, 60). For each molecule of P_i deposited 1 H^+ is released as a consequence of the deposition reaction[56,57]. These ion translocations are assumed to be catalyzed by a translocase located in the mesolayer of the external mitochondrial membrane[57]. The translocase is presumed to exist in two states: (a) in combination with a molecule of P_i and 2 atoms of the divalent ion (Mg^{2+}, Ca^{2+}, or Mn^{2+}); (b) not combined. Only in the state where it is combined with the appropriate ion pairs is the translocase capable of undergoing a conformational change induced by the high-energy intermediate (actually there are three high-energy intermediates). This conformational change leads to the movement of ions across the membrane. Precisely how this conformational change takes place and what is the nature of the conformational change is still unknown.

Lipid probably participates in at least two ways in the phenomenon of energized ion movements. First the structural protein–lipid network of the mesolayer in the external envelope of the mitochondrion serves as a permeability barrier to the unrestricted flow of solutes. Some ions and neutral solute molecules are completely barred from entrance or exit whereas others may penetrate this barrier but the rate is severely circumscribed. In addition solubility in the mesolayer would be an important determinant of the rate with which organic molecules could penetrate the membrane barrier. Large polar molecules would in general penetrate slowly if at all whereas small non-polar molecules would penetrate more freely.

The barrier imposed by the mesolayer to the movement of ions has the consequence that selective movements of ions that are energy-driven cannot be described in terms of passive diffusion through the membrane. The precise stoichiometry of the process of ion transport in the mitochondrion and the exact relation between the amount of high-energy intermediate generated and the amount of ions accumulated argues for an enzymic mechanism. The concept of a translocase was developed from these considerations.

The translocase has to be visualized as a macromolecule comparable to actomysin[61] or rhodopsin[62]. These macromolecules undergo a profound conformational change when impinged upon, respectively, by ATP (ref. 61) and light[62]. It is of interest that the ATP-induced contraction of actomyosin requires the presence of Mg^{2+}

(ref. 63). The translocase would have to undergo a conformational change of a vectorial character such that before the change the attached ions would be on the outside of the mitochondrion while after the conformational change the attached ions would be on the inside of the mitochondrion.

The mitochondrion is known to be capable of cyclical contraction and swelling. OHNISHI AND OHNISHI[64-66] brought this phenomenon to a head by isolating from mitochondria a protein fraction capable of an ATP-induced conformational change (measured by light-scattering changes) and of ATPase activity. BLAIR[67] has established that a component associated with the structural protein–lipid network has contractile properties (as measured by viscosity changes induced by ATP) as well as ATPase activity. These properties disappear when lipid is extracted from the complex[83]. Our working hypothesis is that the structural protein–lipid network has both contractile potentialities which may be expressed as contractions or swelling, and translocase properties which are expressed in terms of ion transport. In these two potentialities lipid undoubtedly plays an essential role.

THE MESOLAYER OF STRUCTURAL PROTEIN AND LIPID IN THE MITOCHONDRIAL MEMBRANE

The mesolayer of the mitochondrial membrane has been identified as the locale of the structural protein–lipid network on the basis of the following evidence. The trilaminar structure of the membrane is unaltered by sonication of mitochondria—a treatment which leads to the release of the primary dehydrogenase complexes[68]. The primary dehydrogenase complexes are thus eliminated from consideration as fixed components of the trilaminar membrane structure. The electron-transfer chain can be cleanly separated from structural protein[6]. The purified chain in the form of the elementary particles corresponds closely in dimensions and shape to the corresponding particles attached to the mesolayer (plus base piece)[5]. By exclusion, only the mesolayer can be the locus of the structural protein. The amount of structural protein that can be isolated from mitochondria corresponds to some 50–60 % of the total protein of the mitochondrion (the percentage is 65–75 % for sonicated mitochondrial particles that have been separated from the soluble protein released by sonication)[6]. It can be estimated from electron micrographs that the elementary particles could not possibly account for this percentage of the total protein. Again by exclusion, the mesolayer has to be identified as the locale of the structural protein–lipid network.

When modified structural protein is lipid-free, it exists as an insoluble polymer in water at neutral pH[42]. After interaction of the protein with lipid the physical properties of the resulting complex are altered in several respects. Electron micrograph studies of H. FERNANDEZ-MORAN and also of D. SLAUTTERBACK (unpublished observations privately communicated) show that the characteristic periodicities and structural pattern both of the structural protein and of the micellar lipid disappear when reaction has taken place. The complex which is formed has structural characteristics found neither in the starting protein nor the starting micellar lipid. Such extensive alteration of the structural pattern undoubtedly reflects the fact that the complex contains the two reacting partners in an altered chemical state. Lipid in interacting with the protein appears to modify the polymeric form of the protein;

conversely protein appears to modify the micellar form of the lipid. Under favorable conditions the emergence of new physical properties can be seen visually. For example when cardiolipin is allowed to react with the structural protein (to the extent of 50 μg P per mg protein) the resultant complex when sedimented is gelatinous in appearance in contrast to the structural protein which is somewhat granular in appearance[36]. The ready sedimentability of the complex (some minutes in a clinical centrifuge) has to be compared with the difficult sedimentability of the cardiolipin micelle (greater than 1 h at 50000 rev./min)[21]. These physical changes apply only to structural protein which has been modified by depolymerization with alkali. Undenatured structural protein[43], by contrast, does not change appreciably in physical characteristics as a result of combination with phospholipid.

In the previous section the evidence that the interaction of structural protein with phospholipid is hydrophobic in character has been fully discussed. Leaving aside cardiolipin, all the other phospholipids tested (lecithin, soy bean phosphatides, mitochondrial phospholipid) combine with structural protein in about the same molar proportions[36]. This comes to 7–8 molecules of phospholipid (expressed as P equivalents) per molecule of structural protein. Each molecule of structural protein thus contains approx. 7–8 surface locales for hydrophobic interaction with phospholipid molecules can be consummated. Cardiolipin reacts in any molar ratio with structural protein. This particular reaction involves parameters such as the polyvalent character of cardiolipin that are irrelevant in the present context. Any satisfactory interpretation of the arrangement of parts in the structural protein–lipid network must account for both the stoichiometric and hydrophobic character of the interaction of structural protein with phospholipid.

The electron microscope has revealed another feature of the mesolayer. Under appropriate conditions of preparation, mounting and fixation of the specimen to be examined, the mesolayer appears to be bifurcated into parallel rows separated by a space[5]. When mitochondria are allowed to swell the width of the space between the parallel rows in the mesolayer increases. This evidence suggests that the mesolayer is built up of two parallel layers that under some conditions appear as one continuous layer and under other conditions as two separate layers. Whatever the nature of the mesolayer its structure must allow for this splitting down the middle and separation of the two-component layers.

The relation of the walls of a crista (visualized as a tube) to the material in the interior (mesozone) has still to be defined. Our present interpretation is that the walls (40-Å thick) are made up of the fused base pieces of the elementary particles while the mesozone proper is the locale of the structural protein network. The internal contents of the cristae appear to slide relative to the wall. Some sections of crista have nothing but wall; others are enormously distended. This variable width of the crista (seen clearly in negatively stained preparations) is not fully understood; it has important implications for an understanding of the role of structural protein in mitochondrial structure[5].

When mitochondria are extracted with acetone to remove lipid (some 85 % of the phospholipid is thus removed), there is no apparent change in the basic trilaminar structure of the mitochondrion[22]. Clearly the structural pattern of the membrane is not dependent on the presence of lipid. Lipid apparently can be removed and reinserted without affecting the structural integrity of the mitochondrial membranes.

One further piece of evidence has to be adduced before we can consider a possible model for the structure of the mesolayer. The attachment of the elementary particle to the mesolayer can be severed by exposure of the mitochondrion to high salt concentration[67]. This would argue for an electrostatic link between one of the components of the mesolayer and the elementary particle.

The diagrammatic representation of the gross structure of the mesolayer shown in Fig. 3 is based on the various properties of the structural protein–lipid complex summarized above[69]. The stalk by which the head piece of the elementary particle is attached to the mesolayer is probably one of the complexes. The stalk is visible only under special circumstances in the mounting and fixation of the specimen. It may not extrude from the mesolayer in the natural structural pattern of the membrane. The link of the stalk with the head piece of the elementary particle is assumed to be hydrophobic just as is the link of structural protein with phospholipid.

The key to any molecular model of the structural protein–lipid network is the central fact that extraction of lipid (> 90%) does not alter the trilaminar character of the mitochondrial membranes. It would be predicted from the model shown in Fig. 14 that the removal of lipid would lead to a shrinkage of the mitochondrion. No such shrinkage is observed. A model in which no shrinkage would be predicted is shown in Fig. 15. The structural protein network is built up of nesting discs—each disc made up of two flattened monomers linked end to end. The hollow core of the disc would be filled with lipid. Extraction of lipid from this polymeric array would not alter the gross structural pattern.

The molecular dimensions of structural protein and phospholipid are not incompatible with these models. Two molecules each of structural protein and phospholipid could be lined up in linear array underneath the base piece of each elementary particle. Assuming structural protein to be a spherical particle with a density of 1.37 the estimated diameter for a molecular weight of 22000 would be 37 Å. The diameter of a phospholipid molecule (short dimension) would be about 20 Å (ref. 71). The distance between the stalks of adjacent elementary particles would thus come to 114 Å— a value in good agreement with the values reported by various investigators (refs. 72, 73).

MEMBRANE SYSTEMS

It is now possible to recognize some universal features of membrane systems that can be stated as follows. All membrane systems yet examined appear to subserve the same functions: provision of a permeability barrier, active transport of ions, contractility and ATPase activity. All membranes yet examined show the same or very similar structural pattern: a trilaminar structure, a mesolayer of structural protein and lipid (predominately phospholipid), micellar character of the lipid, polyunsaturated fatty acids in the phospholipids and paired arrays of particles equivalent to the elementary particles of the mitochondrion.

The electron micrographs of membrane systems examined at high resolution and with suitable staining techniques such as the negative phosphotungstate method invariably show a trilaminar character and unmistakable evidence of paired arrays of particles (we shall refer to these as elementary particles) attached to a central layer (we shall refer to this as the mesolayer)[69]. The total width of the three layers

may vary from membrane to membrane, but the extent of variation is not too great (±30%).

RICHARDSON *et al.*[74] have isolated in high yield from three membrane systems (red blood corpuscle, microsomes and chloroplasts) proteins that closely resemble the structural protein of the mitochondrion. These proteins combine hydrophobically with phospholipid micelles to form a protein–lipid complex[85,86]. The characteristics of these interactions are in the main respects identical with those for the interaction of mitochondrial structural protein with phospholipid though there are some differences of a minor nature. All these "structural proteins" are insoluble in water at neutral pH and can be solubilized by the same reagents that depolymerize the structural protein of the mitochondrion[42,74].

The presence of lipid in the membrane systems examined so far has been established whenever tested. The amount varies from about 25 to 50 % by weight[75,85-87]. The lipid in these membranes contains a high proportion of phospholipid, the fatty acids of which are undoubtedly polyunsaturated.

HULTIN *et al.*[76] have isolated from red blood corpuscles a particle originally associated with the stroma protein (structural protein of the red blood corpuscle) which contains the two oxidizing enzymes of the glycolytic cycle (lactic and triose-phosphoric dehydrogenases), ATPase and triose phosphate isomerase. This elementary particle contains about 30% by weight of lipid. The isolation of a second elementary particle makes it possible to recognize a principle of broad generality. The key physiological function of all membranes is that of ion regulation, a process that requires energizing by ATP or some high-energy intermediate. The elementary particles are the instruments for generating the high-energy intermediates. The mitochondrial elementary particle contains the electron-transfer chain for generation of high-energy intermediates. The corresponding particle of the red blood corpuscle contains the oxidizing enzymes of the glycolytic system which generate ATP by electron transfer between triose phosphate and pyruvate mediated by DPN^+ (ref. 76). The microsomal membrane (probably the sarcotubular membrane in the case of muscle) contains yet another type of elementary particle the essential components of which are DPNH dehydrogenase (a flavoprotein) and cytochrome *b* (refs. 77, 78). How ATP or a high-energy intermediate is generated in the microsomal particle remains to be determined.

The sarcotubular membrane of skeletal muscle has been shown to bind Ca^{2+} in a fashion similar to the mitochondrion[79]. This accumulation requires the presence of ATP (ref. 79). The red blood corpuscle membrane accumulates K^+ in an ATP-supported reaction. By inference from physiological experiments, active transport of ions can be invoked for the cell membrane and for the membranes of the kidney tubules, the nerve cell and the intestinal mucosa. Thus, enough evidence is on hand to consider active transport of ions as a universal feature of membranes.

The structural protein–lipid network and contractility appear to be two sides of the same coin. The OHNISHI's have isolated a contractile protein from the membranes of red blood corpuscles as well as from mitochondria[64-66]. These proteins show ATPase activity. PACKER *et al.*[80] have isolated a contractile protein from chloroplasts. It will be of interest to determine how generally the relationship between contractile proteins and membranes will apply.

FORMULATION OF THE MOLECULAR STRUCTURE OF THE MITOCHONDRIAL MESOLAYER

Enough information of a physical and chemical nature is now to hand to permit the first tentative effort to formulate the structure of the mitochondrial mesolayer in chemical terms. The formulations shown in Figs. 14 and 15 are based on evidence which has been considered at one point or another in this review. We shall summarize here all the lines of evidence pertinent to this formulation.

Evidence from electron microscopy

(1) The micellar character of phospholipid in the sense of continous arrays of phospholipid molecules is not recognizable in the mesolayer. These arrays either are seen in cross section or are buried within protein.

(2) The random character of polymeric structural protein is not recognizable in the mesolayer of osmium stained mitochondrial cristae[5].

(3) Electron micrograph studies of H. FERNANDEZ-MORAN and independently of D. SLAUTTERBACK (unpublished studies) have shown that when structural protein interacts with micellar phospholipid, the characteristic lamellar pattern of the micelles (*cf.* Fig. 10) is no longer in evidence. The state of the lipid is thus clearly altered during the interaction, but at present information is lacking as to the corresponding changes, if any, sustained by the structural protein.

(4) The cylindrical stalks which attach the mesolayer to the elementary particle are 30 × 50 Å (refs. 5, 72). The third dimension of the cylinder has yet to be evaluated.

(5) The mesolayer is variable in width and the distance between stalks is about 112 Å (refs. 5, 72, 73).

(6) The diameter of the elementary particle is about 80–100 Å (refs. 5, 72).

Evidence from chemical studies

(1) Under suitable experimental conditions 1 molecule of structural protein can interact hydrophobically with 8 molecules of phospholipid[36]. This may be the limit of combination under the particular experimental conditions employed. It can be inferred from some of our analytical data that the lipid content of the mesolayer is about 30% by weight[14] and on this basis it would follow that the upper limit for the molar ratio may be > 8 (perhaps as high as 12).

(2) The ionic groups of the phospholipid attached to the structural protein are free to interact with cytochrome *c* (ref. 36).

(3) The link of the elementary particle with the mesolayer is partly electrostatic in nature (ruptured by strong salt) and partly hydrophobic (ruptured by detergents plus salt)[67].

Dimensions of the structural protein and phospholipid

If we assume that the structural protein is spherical in shape, the diameter for a spherical molecule of 22000 molecular weight[42] would be about 38 Å assuming a density of 1.37. This value is calculated from the equation

$$\text{molecular weight} = 0.315\, d^3 \cdot \text{density}$$

where d = diameter (Å) and density is expressed as g/cm^3. The dimensions of the lecithin molecule according to VANDENHEUVEL[71] are 4 × 10 × 30 Å. We shall assume

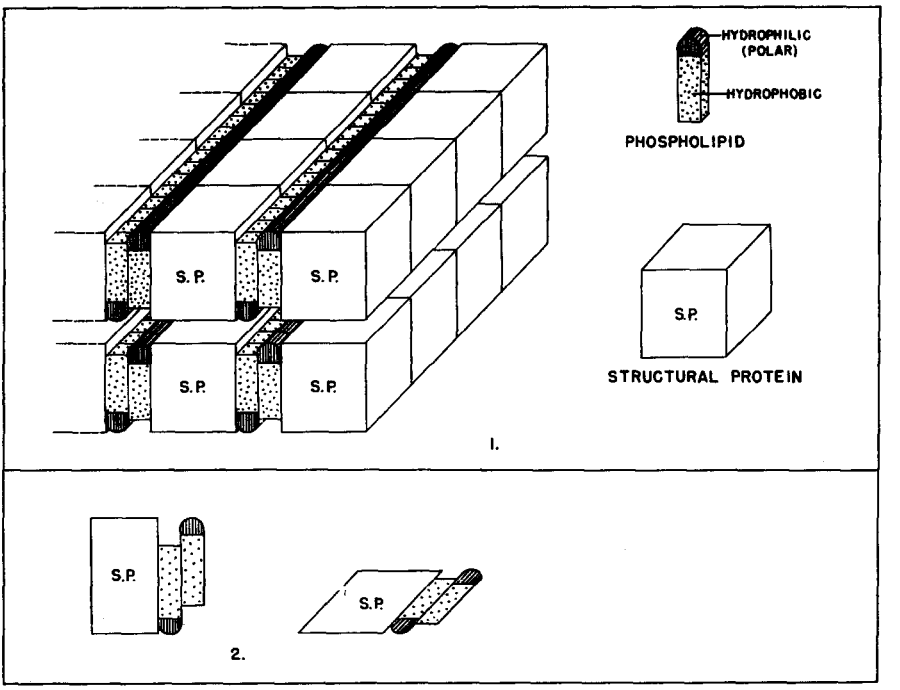


Fig. 14. Diagrammatic representation of the structural protein–lipid network of the mesolayer; 1, three-dimensional representation of the network; 2, alternative modes of nesting of the structural protein (S.P.) with phospholipid.

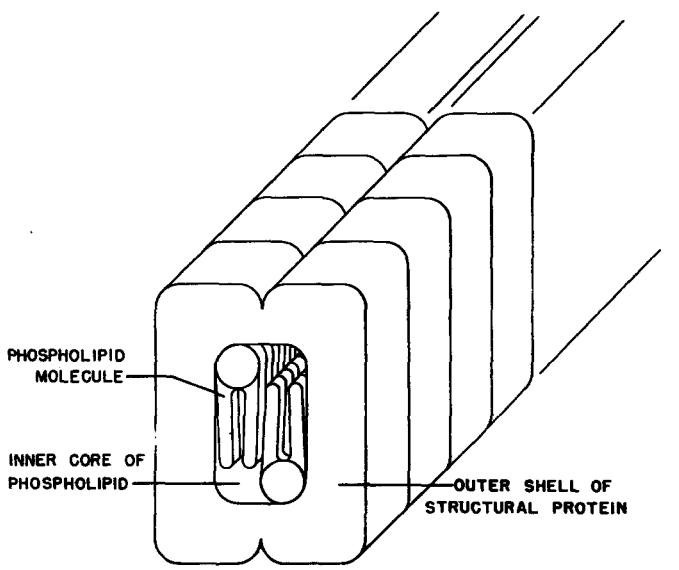


Fig. 15. Diagrammatic representation of the disc hypothesis of the structural protein–lipid network.

that all the other phospholipids approximate these dimensions. As a first approximation, cardiolipin may be assigned the dimensions of $4 \times 18 \times 30$ Å *i.e.* about twice the length of lecithin.

Review of lipid function in the mitochondrion

The essentiality of lipid for the exercise of organized electron transfer activity must be taken to mean that the integrated reactions of the chain depend upon the presence of lipid. The interaction of any individual protein in a complex with external electron acceptors or donors can go on in absence of lipid, but electron flow between the protein components of the four complexes of the chain requires lipid. Our present picture of the structure of the complexes of the chain is similar to that shown in Fig. 15 for the arrangement of the structural protein–lipid network. The oxidation–reduction proteins of each complex (4–6 molecules) are arranged on the periphery while lipid is present in the core of the disc. The constant ratio of protein to lipid in the electron transfer chain and in the structural protein network appears to point to a universal mode of protein–lipid interaction in the mitochondrion perhaps of the type shown in the formulation in Fig. 15. The essentiality of lipid for electron flow between the protein components of each complex may be interpreted in terms of lipid as a medium in which the functional groups are immersed and properly oriented for interaction. The hydrophobic sector of the lipid core would serve the additional function of providing a medium of low dielectric.

The bulk of the proteins of the electron transfer chain form water-insoluble polymers by hydrophobic interaction. Paradoxically, it is lipid in the form of phospholipid that confers the property of water solubility on the mitochondrial proteins. The hydrophobic faces of the protein are shielded from water by phospholipid while the polar faces of the phospholipid provide the link with the aqueous medium. Lipid-free mitochondria are utterly water insoluble and difficult to disperse in water. By contrast mitochondria with a normal complement of lipid are readily dispersable in water to fine suspensions.

The available evidence suggests that mitochondrial phospholipid is micellar in structure and that the micelle is the unit in the interaction of phospholipid with protein.

The hydrophobic interaction of structural protein and of the complexes of the electron transfer chain with phospholipid micelles has opened the door to new and interesting possibilities of molecular arrangements. The formulation shown in Figs. 14 and 15 are harbingers of the more precise models that can now be constructed. More electron microscope data are badly needed to fill in many of the details, but at least the black box of membranes has been finally opened for biochemical inspection.

The unique physical properties characteristic of phospholipid of the micellar organization which allows a hydrophobic medium to exist side by side with an aqueous environment.

REFERENCES

[1] G. E. PALADE, in O. H. GAEBLER, *Enzymes: Units of Biological Structure and Function*, Academic Press, New York, 1956, p. 185.
[2] F. S. SJOSTRAND, *Rev. Mod. Phys.*, 31 (1959) 301.
[3] H. FERNANDEZ-MORAN, *The Structure of the Eye*, Academic Press, New York, 1961, p. 521.
[4] H. FERNANDEZ-MORAN, *A. Res. Nerv. and Mental Dis. Proc.*, 40 (1962) 235.

[5] H. FERNANDEZ-MORAN, T. ODA, P. BLAIR AND D. E. GREEN, *J. Cell. Biol.*, in the press.
[6] P. BLAIR, T. ODA, D. E. GREEN AND H. FERNANDEZ-MORAN, *Biochemistry*, 2 (1963) 756.
[7] D. E. GREEN, *Comp. Biochem. Physiol.*, 4 (1962) 81.
[8] D. E. GREEN, S. FLEISCHER AND H. FERNANDEZ-MORAN, *Science*, in the press.
[9] P. BLAIR, D. E. GREEN AND S. FLEISCHER, unpublished results.
[10] D. E. GREEN AND S. FLEISCHER, in M. KASHA AND B. PULLMAN, *Horizons in Biochemistry*, Academic Press, New York, 1962, p. 381.
[11] Y. HATEFI, A. G. HAAVIK, L. R. FOWLER AND D. E. GRIFFITHS, *J. Biol. Chem.*, 237 (1962) 2661.
[12] D. M. ZIEGLER, *Biochim. Biophys. Acta*, 41 (1960) 491.
[13] L. R. FOWLER AND S. H. RICHARDSON, *J. Biol. Chem.*, 278 (1963) 456.
[14] S. FLEISCHER, H. KLOUWEN AND G. P. BRIERLEY, *J. Biol. Chem.*, 236 (1961) 2936.
[15] F. L. CRANE, J. L. GLENN AND D. E. GREEN, *Biochim. Biophys. Acta*, 22 (1956) 475.
[16] P. BLAIR, unpublished results.
[17] R. E. BASFORD, *Biochim. Biophys. Acta*, 33 (1959) 195.
[18] R. T. HOLMAN AND C. WIDMER, *J. Biol. Chem.*, 234 (1959) 9.
[19] T. RICHARDSON, A. L. TAPPEL, L. M. SMITH AND C. R. HOULE, *J. Lipid Res.*, 3 (1962) 344.
[20] J. B. WITTENBERG, S. R. KOREY AND F. H. SWENSON, *J. Biol. Chem.*, 219 (1956) 39.
[21] S. FLEISCHER AND H. KLOUWEN, *Biochem. Biophys. Res. Commun.*, 5 (1961) 378.
[22] S. FLEISCHER, G. P. BRIERLEY, H. KLOUWEN AND D. B. SLAUTTERBACK, *J. Biol. Chem.*, 237 (1962) 3264.
[23] R. L. LESTER AND S. FLEISCHER, *Biochim. Biophys. Acta*, 47 (1961) 358.
[24] G. P. BRIERLEY AND A. J. MEROLA, *Biochim. Biophys. Acta*, 64 (1962) 205.
[25] G. P. BRIERLEY, A. J. MEROLA AND S. FLEISCHER, *Biochim. Biophys. Acta*, 64 (1962) 218.
[26] P. JURTSHUK, Jr., I. SEKUZU AND D. E. GREEN, *Biochem. Biophys. Res. Commun.*, 6 (1961) 76.
[27] I. SEKUZU, P. JURTSHUK, Jr. AND D. E. GREEN, *J. Biol. Chem.*, 238 (1963) 975.
[28] R. L. LESTER AND A. L. SMITH, *Biochim. Biophys. Acta*, 47 (1961) 475.
[29] G. ROUSER, *Am. J. Clin. Nutr.*, 6 (1958) 681.
[30] H. FERNANDEZ-MORAN AND S. FLEISCHER, unpublished results.
[31] P. YANG, R. M. BOCK AND S. FLEISCHER, unpublished results.
[32] S. FLEISCHER, S. RICHARDSON, E. MURER, H. TISDALE AND D. GREEN, in preparation.
[33] D. E. GREEN AND R. L. LESTER, *Federation Proc.*, 18 (1959) 987.
[34] S. FLEISCHER AND G. P. BRIERLEY, *Biochim. Biophys. Acta*, 53 (1961) 609.
[35] S. FLEISCHER, S. H. RICHARDSON, A. CHAPMAN, B. FLEISCHER AND H. O. HULTIN, *Federation Proc.*, 22 (1963) 2186.
[36] S. FLEISCHER, S. RICHARDSON, H. O. HULTIN, A. CHAPMAN AND B. FLEISCHER, in preparation.
[37] E. MARGOLIASH AND E. L. SMITH; G. KREIL AND H. TUPPY; E. MARGOLIASH, E. L. SMITH, G. KREIL AND H. TUPPY, *Nature*, 192 (1961) 1121–1127.
[38] E. MARGOLIASH, J. R. KIMMEL, R. L. HILL AND W. R. SCHMIDT, *J. Biol. Chem.*, 237 (1962) 2148.
[39] H. L. DAS, H. HIRATSUKA, J. M. MACHINIST AND F. L. CRANE, *Biochim. Biophys. Acta*, 60 (1962) 633.
[40] C. WIDMER AND F. L. CRANE, *Biochim. Biophys. Acta*, 27 (1958) 1804.
[41] S. TAKEMORI, K. WADA, I. SEKUZU AND K. OKUNUKI, *Nature*, 195 (1962) 456.
[42] R. S. CRIDDLE, R. M. BOCK, D. E. GREEN AND H. D. TISDALE, *Biochem.*, 1 (1962) 827.
[43] S. RICHARDSON, H. O. HULTIN AND S. FLEISCHER, in preparation.
[44] R. M. BOCK, unpublished calculations.
[45] J. RIESKE AND D. MCLENNAN, unpublished results.
[46] K. S. AMBE AND A. VENKATARAMAN, *Biochem. Biophys. Res. Commun.*, 1 (1959) 3.
[47] R. GOLDBERGER, A. L. SMITH, H. TISDALE AND R. BOMSTEIN, *J. Biol. Chem.*, 236 (1961) 2788.
[48] P. JURTSHUK, I. SEKUZU AND D. E. GREEN, *J. Biol. Chem.*, in the press.
[49] S. FLEISCHER AND G. P. BRIERLEY, *Biochem. Biophys. Res. Commun.*, 5 (1961) 367.
[50] A. SMITH, G. WEBSTER AND M. HANSEN, unpublished results.
[51] G. WEBSTER, A. SMITH AND M. HANSEN, *Proc. Natl. Acad. Sci. U.S.*, 49 (1963) 259.
[52] G. B. PINCHOT, *Proc. Natl. Acad. Sci. U.S.*, 46 (1960) 929.
[53] A. L. SMITH AND M. HANSEN, *Biochem. Biophys. Res. Commun.*, 8 (1962) 2.
[54] G. WEBSTER, unpublished results.
[55] D. E. GRIFFITHS AND R. A. CHAPLAIN, *Biochem. Biophys. Res. Commun.*, 8 (1963) 495.
[56] G. BRIERLEY, D. E. GREEN AND E. MURER, *Science*, 140 (1963) 60.
[57] G. P. BRIERLEY, E. BACHMANN AND D. E. GREEN, *Proc. Natl. Acad. Sci. U.S.*, 48 (1962) 1928.
[58] A. L. LEHNINGER, C. S. ROSSI AND J. GREENAWALT, *Biochem. Biophys. Res. Commun.*, 10 (1963) 444.
[59] J. B. CHAPPELL, M. COHN AND G. D. GREVILLE, in B. CHANCE, *Proc. Symp. Energy-Linked Functions of Mitochondria*, Academic Press, New York, 1963, p. 219.
[60] C. R. ROSSI AND A. L. LEHNINGER, *Biochem. Biophys. Res. Commun.*, 11 (1963) 441.
[61] J. GERGELY AND A. KOHLER, *Proc. Conf. Muscle Contraction, Tokyo*, Igaku Shoin, 1957, p. 14.

[62] G. WALD, in W. D. MCELROY AND B. GLASS, *Light and Life*, Johns Hopkins Press, Baltimore, 1961, p. 724.
[63] E. H. BARANYI, K. A. P. EDMAN AND A. PALIS, *Acta Physiol. Scand.*, 24 (1951) 361.
[64] T. OHNISHI AND T. OHNISHI, *J. Biochem. (Japan)*, 52 (1962a) 230.
[65] T. OHNISHI AND T. OHNISHI, *J. Biochem. (Japan)*, 52 (1962b) 207.
[66] T. OHNISHI AND T. OHNISHI, *J. Biochem. (Japan)*, 51 (1962c) 380.
[67] P. BLAIR, unpublished results.
[68] D. M. ZIEGLER, A. W. LINNANE, D. E. GREEN, C. M. S. DAS AND H. RIS, *Biochim. Biophys. Acta*, 28 (1958) 524.
[69] D. E. GREEN AND P. V. BLAIR, *J. Theoret. Biol.*, in the press.
[70] S. FLEISCHER AND D. SLAUTTERBACK, unpublished results.
[71] F. A. VANDENHEUVEL, *Can. J. Biochem. Physiol.*, 40 (1962) 1299.
[72] D. F. PARSONS, *Science*, 140 (1963) 985.
[73] W. STOECKENIUS, *J. Cell Biol.*, 17 (1963) 443.
[74] S. RICHARDSON, H. HULTIN AND D. E. GREEN, *Proc. Natl. Acad. Sci. U.S.*, in the press.
[75] J. J. WOLKEN, *J. Theoret. Biol.*, 3 (1962) 192.
[76] H. HULTIN, S. RICHARDSON AND D. E. GREEN, *Proc. Natl. Acad. Sci. U.S.*, in the press.
[77] D. GARFINKEL, *Comp. Biochem. Physiol.*, 8 (1963) 367.
[78] N. PENN AND B. MACKLER, *Biochim. Biophys. Acta*, 27 (1958) 539.
[79] W. HASSELBACH AND M. MAKINESE, *Biochem. Z.*, 333 (1961) 518.
[80] L. PACKER, R. H. MARCHANT AND Y. MUKOHATA, *Biochem. Biophys. Res. Commun.*, 11 (1963) 429.
[81] H. FERNANDEZ-MORAN, *Science*, 140 (1963) 381.
[82] D. E. GREEN AND D. WHARTON, *Biochem. Z.*, in the press.
[83] P. M. VIGNAIS, P. V. VIGNAIS AND A. L. LEHNINGER, *Biochem. Biophys. Res. Communs.*, 11 (1963) 313.
[84] D. E. GRIFFITHS AND R. A. CHAPLAIN, *Biochem. Biophys. Res. Commun.*, 8 (1963) 501.
[85] F. LEUTHARDT AND B. EXER, *Helv. Chim. Acta*, 36 (1953) 500.
[86] F. KOGL, J. DE GIER, I. MULDER AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 43 (1960) 95.
[87] T. KONO AND S. P. COLOWICK, *Arch. Biochem. Biophys.*, 93 (1961) 520.
[88] K. S. AMBE AND A. VENKATARAMAN, *Biochem. Biophys. Res. Commun.*, 1 (1959) 133.