Non-bilayer Lipids and the Inner Mitochondrial Membrane

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Introduction

In the last decade the fluid mosaic model (Singer and Nicholson 1972) of biological membranes has become generally accepted as it provided a rationale for many structural and functional features of membranes. More recently, however it has become increasingly clear that this model is incomplete for reasons relating to lipid composition as well as functional abilities of biological membranes. First, although the chemical variation in membrane lipids is enormous, it is surprising that most of them can be divided into only two groups on structural grounds: The lipids of the first group, including PC* and sphingomyelin, will organize themselves in bilayers when they are in the fully hydrated state (bilayer lipids). It is obvious that this property has greatly contributed to the bilayer concept of biological membranes. In contrast, the lipids in the second group do not form bilayers when they are dispersed in excess buffer (non-bilayer lipids). This group includes major lipids such as PE*, monoglucosyl and monogalactosyl diglyceride and CL[4] (in the presence of Ca^{2+}) (for review and references see Cullis and de Kruijff 1979). These lipids prefer the hexagonal H_{II} phase (Fig. 1). This phase consists of cylinders of lipids surrounding long aqueous channels. The unique feature of the H_{II} phase both from a structural and functional point of view is that it allows polar lipids to be organized in a low energy configuration inside a hydrophobic environment. The reason for the abundant presence of these non-bilayer lipids in membranes is difficult to understand in terms of membrane models in which the bilayer is suggested to be the only organization available to the lipids.

The second property of biological membranes not explained by present membrane models is of a functional nature. In a large number of membrane mediated processes, part of the lipids would appear to temporarily have to leave the bilayer configuration. Clear examples are all forms of membrane fusion and transbilayer movements of lipids (flip-flop). These processes have as a common characteristic that in some intermediate state part of the lipids must reside within the hydrophobic environment of the membrane. These two considerations have led us to suggest an alternative model of biological membranes (Cullis and de Kruijff 1979) in which non-bilayer lipids and the non-bilayer structures they allow play active functional roles.

In this article we illustrate the various aspects of non-bilayer structures employing the lipids of the inner membrane of the mammalian mitochondrion. It will be shown that this membrane contains a large fraction

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[4] *Abbreviations:* PC, phosphatidylcholine; PE, phosphatidyl ethanolamine; CL, cardiolipin; NMR, nuclear magnetic resonance

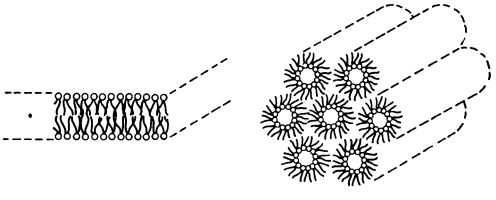

Fig. 1. Schematic representation of the bilayer and the hexagonal H_{II} phase

of lipids which when hydrated (in isolated form) will adopt various non-bilayer phases under a variety of different physiological conditions. The implications of these findings for the structure of the inner mitochondrial membrane will be discussed especially in relation to Ca^{2+} transport, protein insertion and cytochrome oxidase activity.

Structural Properties of Inner Mitochondrial Lipids

One Lipid Systems

PC, PE and CL are the main lipids found in the inner membrane of the mammalian mitochondrion in beef heart mitochondria, for example, amounting to 39, 33 and 25 mol% respectively of the total lipids (Krebs et al. 1979). When these lipids in isolated form are dispersed in excess buffer they will undergo a process of self-association, thereby forming large aggregates, the structure of which will depend on the type of lipid and the conditions. This polymorphic phase behaviour of phospholipids can be conveniently monitored by ^{31}P NMR (Cullis and de Kruijff 1979). In the case of lipids organized in extended bilayers, the proton decoupled ^{31}P NMR spectrum has a characteristic asymmetrical lineshape with a low field shoulder and a high field peak. Such a spectrum is observed for an aqueous dispersion of inner mitochondrial PC at 37 °C (Fig. 2) demonstrating that this lipid is organized in extended bilayers as has been found for all other long chain PC's tested so far. Phospholipids organized in the hexagonal H_{II} phase have a ^{31}P NMR spectrum of a reduced width and a reversed asymmetry. Furthermore, the dominant spectral feature has a characteristic chemical shift. Inner mitochondrial PE in excess buffer at 37 °C shows such a spectrum (Fig. 2) demonstrating that this lipid is organized in the hexagonal H_{II} phase at 37 °C. In agreement with data on other natural PE's (Cullis and de Kruijff 1978) at lower, non-physiological, temperatures the bilayer phase is preferred (Fig. 2).

Cardiolipin, the third main inner mitochondrial lipid when dispersed in a salt solution at neutral pH forms extended bilayers (Rand and Sengupta 1972; Cullis et al. 1978) as is illustrated in Fig. 3. In addition to a preferential localization in the inner mitochondrial membrane this lipid has another very distinctive property in that it is the only major negatively charged membrane phospholipid which will adopt the hexagonal H_{II} phase in the presence of various divalent

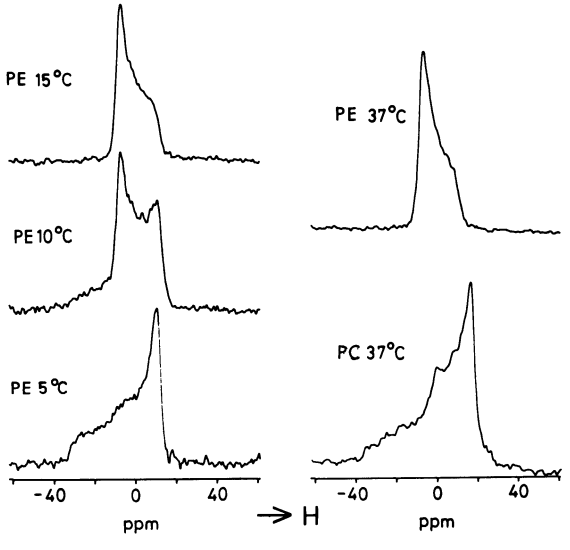

Fig. 2. 81 MHz ^{31}P NMR spectra of aqueous dispersions of rat liver inner mitochondrial PE and PC. 30 μmoles of phospholipid was hydrated with 1.0 ml 100 mM NaCl, 10 mM Tris-acetic acid (pH 7.2) and 1 mM Edta. Reproduced with permission from Cullis et al. 1980

cations (Rand and Sengupta 1972; Cullis et al. 1978). The preference of CL for the H_{II} phase in the presence of equimolar Ca^{2+} is illustrated in Fig. 3. Since the mitochondrion can actively accumulate large amounts of Ca^{2+} into the matrix space (Nicolls and Crompton 1980; Carafoli 1979) and CL is preferentially located on the inner monolayer of the inner mitochondrial membrane (Krebs et al. 1979) (thus facing the matrix space) we have the remarkable possibility that for this biomembrane the majority (60%) of its lipids will *not* prefer the bilayer but rather the H_{II} phase configuration under physiological conditions. Furthermore, this phase behaviour can be expected to be strongly Ca^{2+} dependent.

At this point of our structural analysis two important and related questions should be considered. In the first place, does the H_{II} phase occur in mixtures of the inner mitochondrial lipids. Secondly, is the presence of H_{II} phase in a membrane compatible with the functioning of that membrane. Intuitively, it seems difficult for instance to reconcile extended areas of H_{II} phase with the barrier function of the membrane. Therefore, we have to consider the possibility that the H_{II} forming tendency of PE and CL can be expressed in alternative, possibly functionally, more relevant, non-bilayer structures.

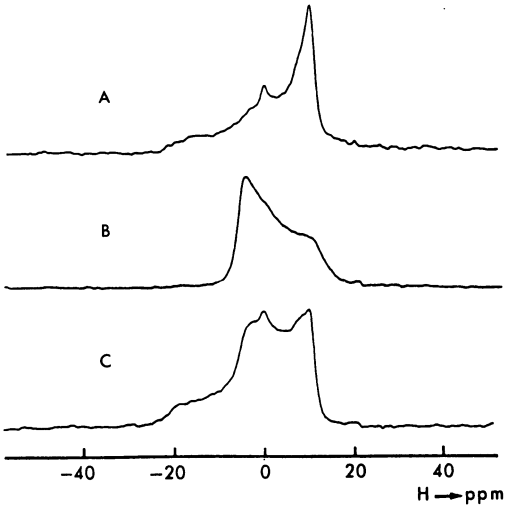

Fig. 3. 81 MHz ^{31}P NMR spectra at 30 °C of an aqueous dispersion of cardiolipin (*A*) in the presence of Ca^{2+} (*B*) and cytochrome c (*C*). 50 μmoles beef heart cardiolipin was dispersed in 1.0 ml 100 mM NaCl, 10 mM Tris/HCl, 0.2 mM Edta pH 7.0. In *B* 0.1 ml 1 M $CaCl_2$ and in *C* 0.2 ml buffer containing 36 mg oxidized cytochrome c was added

Mixed Lipid Systems

The major binairy mixtures of the inner mitochondrial membrane lipids are PC-PE, PC-CL and PE-CL mixtures. The results obtained with the one lipid systems indicate that these lipid mixtures will have different polymorphic phase properties, therefore, we will discuss them in turn. It should be noted that as the ^{31}P NMR measurements require substantial amounts of lipids (the isolation of which from the inner mitochondrial membrane is rather tedious), these experiments employ more readily available natural PE's and PC's which have a very similar phase behaviour to inner mitochondrial lipids.

In mixtures of unsaturated PC and PE the PC component will stabilize a bilayer structure for the PE component (Cullis and de Kruijff 1979; Cullis and de Kruijff 1978). When this bilayer stabilization by PC is monitored by ^{31}P NMR an unexpected intermediate situation is encountered. Instead of gradually going from a "hexagonal H_{II}" to a bilayer spectrum with increasing PC concentration, a narrow symmetrical resonance at the chemical shift position of phospholipids undergoing rapid isotropic motion is observed as an intermediate (Cullis and de Kruijff 1979; Cullis and de Kruijff 1978). This "isotropic" signal can become the dominant spectral feature in the ^{31}P NMR spectrum (de Kruijff et al. 1979). In mixtures of PC and mitochondrial CL in the absence of divalent cations, as expected, only the lamellar phase is observed (de Kruijff et al. 1979). Addition of Ca^{2+} to these mixtures results again in an unexpected intermediate situation. Instead of a mixture of bilayer and H_{II} phase an isotropic ^{31}P NMR signal is observed for a fraction of the phospholipids (de Kruijff et al. 1979).

CL-PE mixtures are of particular interest for two reasons. In the first place both lipids are preferentially localized in the inner monolayer of the inner mitochondrial membrane (Krebs et al. 1979). Secondly it can be expected that CL will stabilize the bilayer configuration of PE but that this stabilization will be extremely Ca^{2+} sensitive. This is illustrated in Fig. 4 for a 2:1 mixture of soya PE [which adopts the H_{II} phase above 10 °C (de Kruijff and Cullis 1980)] and CL where, in the absence of Ca^{2+} predominantly a bilayer structure is observed at 30 °C. Addition of a small amount of Ca^{2+} ($Ca^{2+}/CL = 0.05$) has a dramatic effect on the spectrum in that a large isotropic peak is formed at the expense of the bilayer signal (Fig. 4). With higher amounts of Ca^{2+} as expected only the H_{II} phase is formed. The picture which emerges from these experiments is that in a mixed system of a bilayer and a non-bilayer lipid, the non-bilayer preference of the latter lipid is not expressed in formation of the H_{II} phase but in an intermediate structure in which the phospholipids can undergo rapid (on the NMR time scale) isotropic motion. This situation is also observed for the total inner mitochondrial lipids. A large isotropic ^{31}P NMR signal is observed superimposed on a bilayer lineshape (Cullis et al. 1980). Addition of Ca^{2+} increases the isotropic component and also induces the formation of some H_{II} phase (Cullis et al. 1980).

^{31}P NMR cannot give precise information on the structure of this "isotropic" intermediate structure. Since these systems are macroscopically large the isotropic signal cannot originate from small vesicles in which vesicle tumbling or lateral diffusion of the lipids would provide an isotropic averaging mechanism. Non-bilayer structures such as inverted micelles or lipids, undergoing rapid lateral diffusion around curved bilayer surfaces in large structure are the most likely candidates for this "isotropic" signal.

Freeze-fracturing provides a less ambiguous interpretation of the structure of the "isotropic" phase. In these systems large numbers of small (60–120 Å, depending on the system) uniformly sized particles and pits

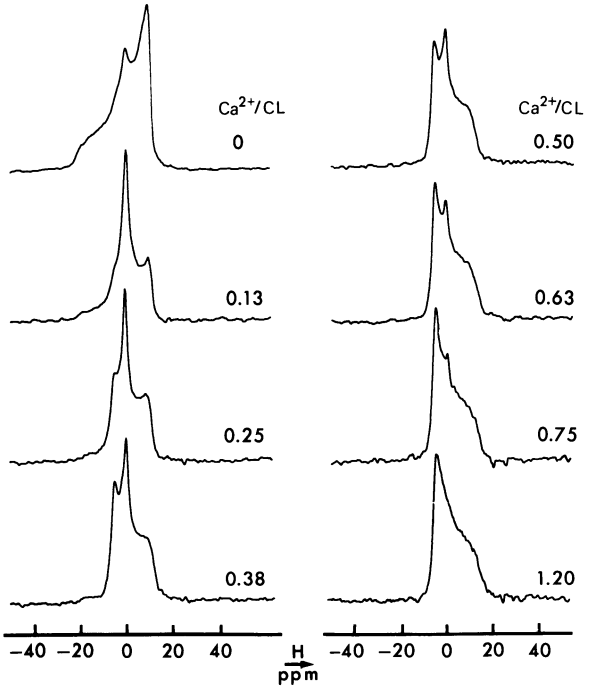

Fig. 4. Effect of Ca^{2+} addition on the 81 MHz ^{31}P NMR spectra of soya PE-CL (2:1) mixed liposomes. To 50 μmoles phospholipid dispersed in 1.0 ml of 100 mM NaCl, 10 mM Tris/HCl, 0.2 mM Edta pH 7.0 aliquots of a 100 mM $CaCl_2$ solution were added to give the Ca^{2+}/cardiolipin ratios indicated in the figure

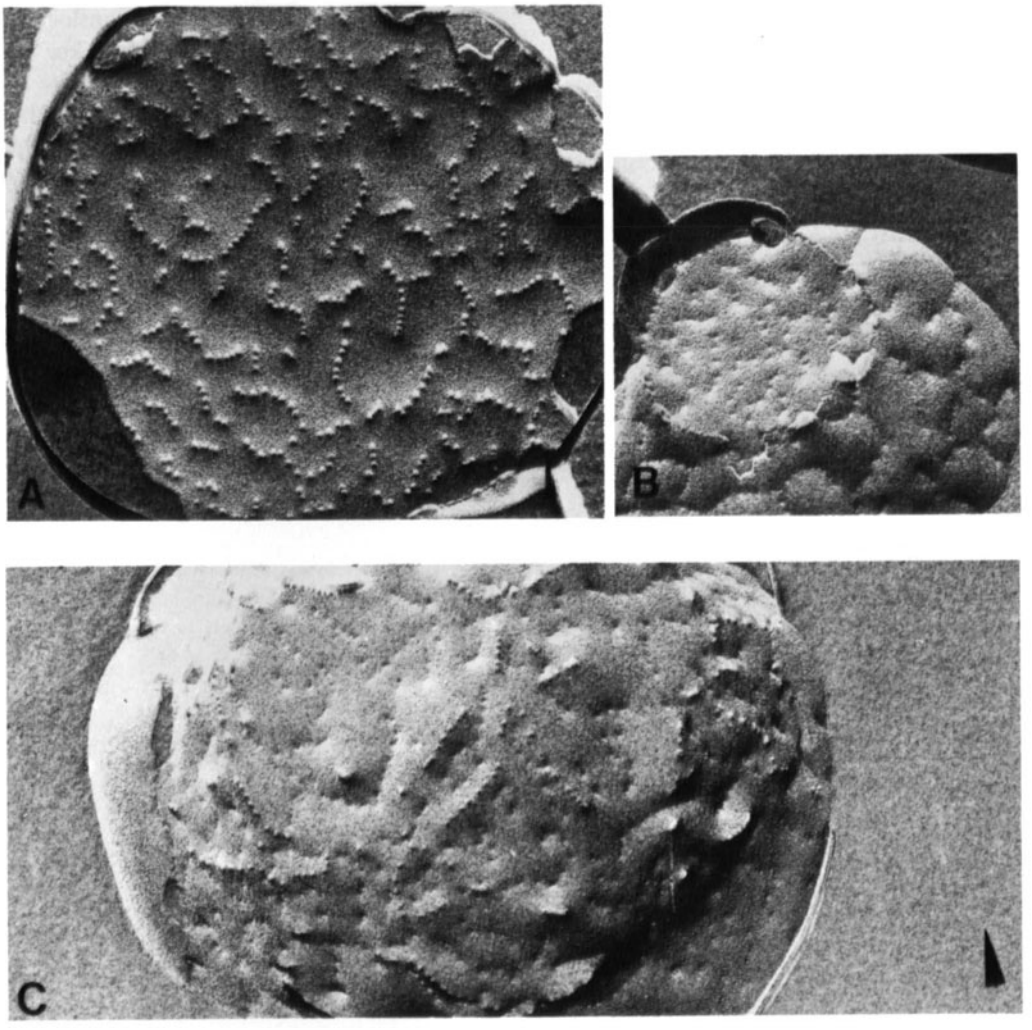

Fig. 5 A – C. Freeze-fracture pictures of Ca^{2+} containing egg PC/CL (1:1) large unilamellar vesicles. Magnification about 100 000×. For details see Verkleij et al. 1979

are often present (de Kruijff et al. 1979; Verkleij et al. 1979; de Kruijff et al. 1980) on the fracture faces. The observation of these intramembranous "lipidic particles" (these systems contain no protein), an example of which is shown in Fig. 5 for PC-CL (1:1) bilayers in the presence of Ca^{2+}, together with the ^{31}P NMR data suggest that the "isotropic" intermediate structures are intra bilayer inverted micelles (Fig. 6 A). Freeze-fracturing indicates that the inverted micelles can be both randomly dispersed or linearly arranged in the lipid bilayer. The linearly arranged particles appear to be localized on the nexus of intersecting bilayers. This situation might be encountered in multilayered liposomes in which upon fusion of bilayers inside the liposomes a honey-comb network of bilayers can be formed (see Fig. 6 B). This latter structure, in which two bilayers are joined via inverted micelles might be relevant for the possible sites of contact between outer and inner mitochondrial membrane (Blok et al. 1971).

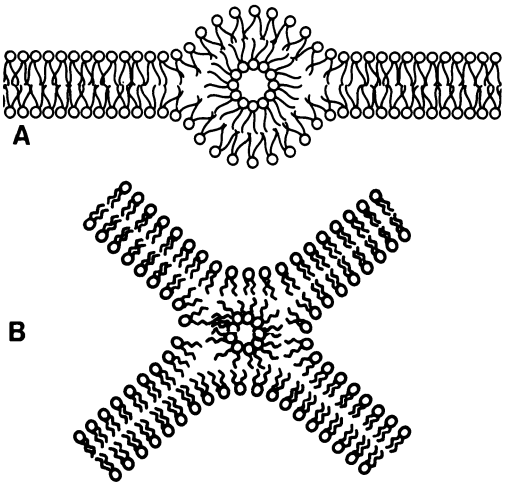

Fig. 6 A, B. Intrabilayer inverted micelles as models for the lipidic particle. In **A** the inverted micelle is located inside a single bilayer in Fig. **B** it is shown at the nexus of two intersecting bilayers

Inner Mitochondrial Lipids and Transbilayer Transport

Since the lipidic particle appears to be the favoured non-bilayer structure in mixtures of inner mitochondrial lipids, it is useful to consider the way this structure can be formed in a bilayer. In addition to its occurrence as an intermediate in bilayer fusion (Verkleij et al. 1979; Verkleij et al. 1980) it can be envisaged that within a bilayer inverted micelles can be formed by local high concentrations of the H_{II} lipid (Cullis and de Kruijff 1979). This could result in an invagination of the bilayer resulting in the formation of the inverted micelle which can be dissociated in the opposite or the original monolayer. This model predicts two properties of the inverted micelle containing bilayer. In the first place phospholipid flip-flop which in pure lipid bilayers under equilibrium conditions is extremely slow (Rothman and Lenard 1977) should be increased (Cullis and de Kruijff 1977). Secondly, when the inverted micelle is formed as a result of divalent cation-CL interactions (Fig. 7) the divalent cation is translocated across the membrane. In this case the inverted micelle would act as an ionophore for the divalent cation. In Fig. 8 it is shown that for mixed PC-CL bilayers both the PC flip-flop and the Mn^{2+} permeability are greatly increased when lipidic particles are present in the bilayer, supporting the above model (Gerritsen et al. 1980).

The increased divalent cation permeability in the CL containing bilayer is particularly interesting as the mitochondrion is capable of a very rapid Ca^{2+} uptake (Nicholls and Crompton 1980; Carafoli 1979). Our data suggest that non-bilayer structures may be involved in this Ca^{2+} uptake. To elaborate further on this suggestion we studied the ef-

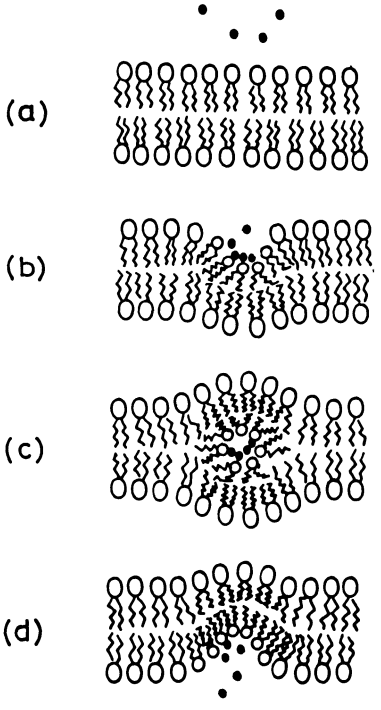

Fig. 7 a – d. A model of facilitated transport of Ca^{2+} (or other divalent cations) via formation of an intermediate intrabilayer inverted micellar cation-CL complex (Fig. 2 c). The headgroups of CL interacting with the cation are depicted as being smaller in order to indicate a reduction in the area per phospholipid molecule in the headgroup region arising from the Ca^{2+}-CL interaction (see Cullis et al. 1980)

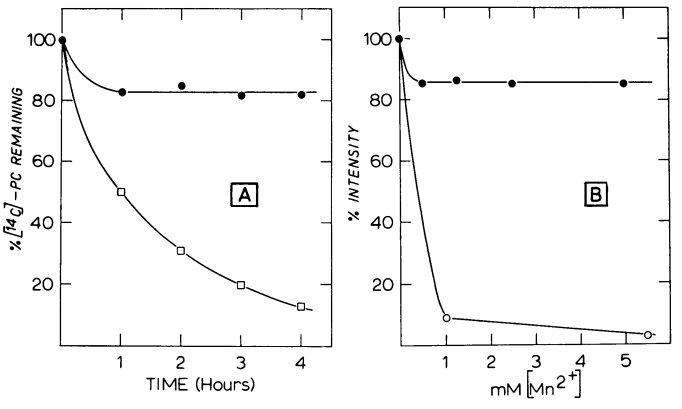

Fig. 8. PC flip-flop (**A**) and Mn^{2+} permeation (**B**) through PC/CL (1:1) liposomes (●—●) containing no lipidic particles and PC/CL (1:1)-Ca^{2+} vesicles prepared by an ether injection method which have bilayers containing large numbers of lipidic particles (○—□). The PC flip-flop is measured by determining the fraction of ^{14}C-PC exchangeable with PC-exchange protein. The Mn^{2+} permeability is measured as a reduction in the ^{31}P NMR signal intensity of the phospholipids after the addition of aliquots of the paramagnetic cation. In the multilayered liposomes approximately 15% of the molecules are located in the outer monolayer. For details see Gerritsen et al. 1980. Reproduced with permission from Gerritsen et al. 1980

fect of ruthenium red, a potent inhibitor of the Ca^{2+} transport in mitochondria, on the polymorphic phase behaviour of cardiolipin (Cullis et al. 1980). Addition of equimolar Ruthenium red to cardiolipin bilayers had no effect on the ^{31}P NMR lineshape. However, its presence completely blocked the Ca^{2+}-induced bilayer to H_{II} transition of CL (Cullis et al. 1980).

Table 1. Phospholipid mediated Ca^{2+} uptake into chloroform

	Amount of Ca^{2+} taken up (μmoles)[a]
Cardiolipin	4.7
Cardiolipin + Ruthenium red (1:1)	0.5
Phosphatidic acid	10.7
Phosphatidylserine	1.1
Phosphatidylcholine	0.4
No Lipid	0.0

[a] Phospholipid corresponding to 6 μmoles of phosphorus was dissolved in chloroform. Subsequently 4 ml 100 mM NaCl, 10 mM $CaCl_2$ (containing 1 μCi $^{45}Ca^{2+}$), 10 mM Tris/HCl pH 7.4 was added. In some cases 6 μmoles of Ruthenium red was added to the aqueous buffer. The resulting two phase system was shaken for 3 h at 20 °C when after the amount of $^{45}Ca^{2+}$ in the chloroform phase was determined. For more details see Cullis et al. 1980

If divalent cation transport across membranes could proceed via inverted micelles as ionophores it can be expected that lipids forming these structures also would be able to translocate the divalent cation into a bulk phase of low dielectric contrast such as chloroform (Cullis et al. 1980). Using the techniques developed by Green and coworkers (Tyson et al. 1976) we studied the uptake of $^{45}Ca^{2+}$ from an aqueous solution into chloroform for various membrane lipids. Table 1 shows both cardiolipin and phosphatidic acid [which can adopt under some cases the H_{II} phase in the presence of divalent cations (Papahadjopoulos et al. 1976)] can partition $^{45}Ca^{2+}$ into the organic phase (Cullis et al. 1980). Also in full agreement with a proposed role of CL in Ca^{2+} transport in mitochondria ruthenium red blocks the Ca^{2+} uptake into chloroform by cardiolipin as was reported before (Tyson et al. 1976).

Cardiolipin Specific Formation of Non-bilayer Structures by Cytochrome c

Cytochrome c is a small (MW 12,000), nearly spherical (radius of gyration 12–14 Å) highly basic (8 net positive charges at neu-

tral pH) inner mitochondrial protein which is involved in electron transport between cytochrome b, c_1 and cytochrome oxidase. Since it experiences strong electrostatic interactions with negatively charged phospholipids (for ref. see de Kruijff and Cullis 1980) and cardiolipin is the only main negatively charged phospholipid in the membrane we became interested in the question as to whether cytochrome c would show a specific interaction with cardiolipin and in particular whether this interaction would result in the formation of non-bilayer structures.

Interacting cytochrome c (oxidized or reduced) with a variety of different negatively charged membrane phospholipids revealed a specific change in the ^{31}P NMR spectrum indicative of the formation of the H_{II} phase and an isotropic phase (de Kruijff and Cullis 1980; see Fig. 3 C) only when CL was present in the bilayer. This result was confirmed by freeze fracture electron microscopy where the H_{II} phase structure and numerous small (< 60 Å diameter) particles are observed on the fracture face of the bilayer (Fig. 9). These latter structures are highly unusal for a protein which is believed to be extrinsic. These data, together with the notion that cytochrome c upon interaction with negatively charged phospholipids can form isooctane soluble complexes (Das and Crane 1964) which upon rehydration give rise to H_{II} phase (Borovjagin and Moshkov 1974) can be rationalized as is shown in Fig. 10. Upon electrostatic interaction with cardiolipin the lipid molecule will adopt a shape permitting the bilayer to invaginate thereby allowing cytochrome c partly to "penetrate" the bilayer (without interacting to a great extent with the acyl chains of the lipids!). This process can finally result in an intra bilayer inverted micellar or short H_{II} configuration in which the cytochrome c is present. This complex can dissociate in a way analogous to that described in Fig. 7 on either side of the bilayer permitting the cytochrome c to move across the bilayer. This latter process has been observed in both model membranes (de Kruijff and Cullis 1980) and in the membrane of the submito-

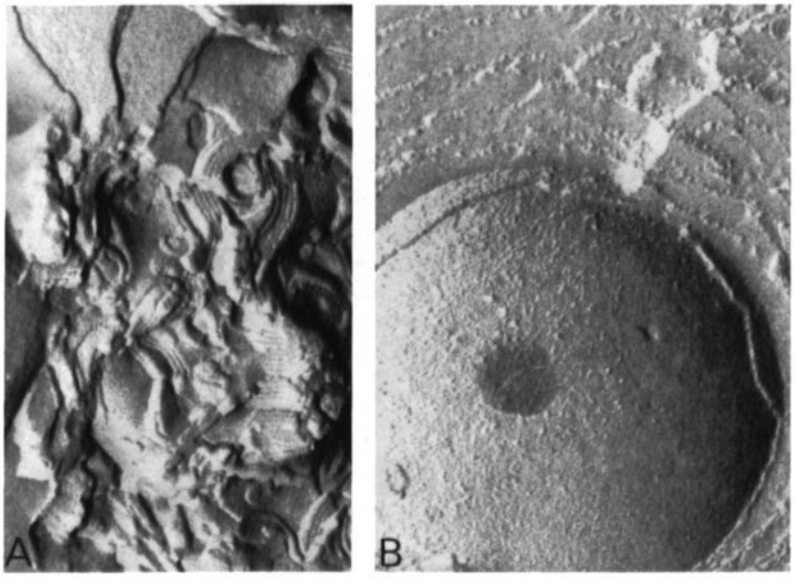

Fig. 9 A, B. Freeze fracturing of CL liposomes after the addition of cytochrome c. Details as in legend of Fig. 3 C. Next to the areas of H_{II} phase (**A**) multilayered bilayer vesicles are observed on the bilayers of which (including the innermost) numerous small particles are visible (**B**). This is also evident in cross-fractures of the bilayes. Magnification about 100,000×

chondrial particle (Nicholls 1974). The possibility of the cardiolipin specific formation of a low energy intra bilayer configuration of cytochrome c offers new possibilities for the involvement of lipids in cytochrome oxidase activity which will be discussed in more detail on p. 570.

sical structure probing techniques such as x-ray have, to our knowledge, not been successfully applied to the mitochondrion. This most likely is related to the extreme biochemical instability of this metabolically very active membrane. This will become clear in the following sections in which we

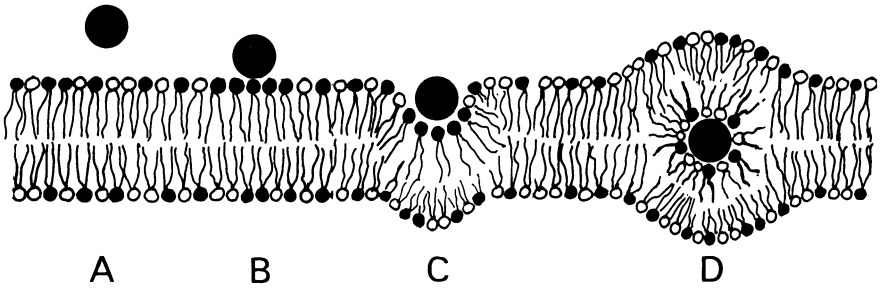

Fig. 10 A–D. Model for the formation of intrabilayer non-bilayer structures by cytochrome c in CL (dark lipids) containing bilayers. Soluble cytochrome c (**A**) binds to the bilayer surface, thereby clustering the CL (**B**) which results in bilayer invagination (**C**) and subsequent formation of cytochrome c containing inverted micelles or H_{II} phases (**D**)

Phospholipid Structure in the Inner Mitochondrial Membrane

Despite the fact that the mitochondrion has been an extremely popular system for many membrane scientists it is surprising to discover how little is actually known about the precise structure of the inner mitochondrial membrane, in particular with respect to its membrane lipids. The question of the structure of the lipid component of this membrane becomes even more pertinent in view of our findings that the lipids can adopt non-bilayer structures and that both Ca^{2+} and cytochrome c will promote the formation of these structures.

Although thin sectioning electron microscopy indicates a trilamellar structure typical of a bilayer structured membrane, considerable controversy exists about the interpretation of these pictures (Sjostrand 1978). Freeze-fracturing studies have given firm evidence that extended areas of bilayer exist in this membrane (Hackenbrock 1977). However, quantitative information on the extent of this structure is not available. Clas-

will summarize our own structural ^{31}P (Cullis et al. 1980) NMR studies on the isolated inner membrane ghost (p. 567) and the intact mitochondrion (p. 568).

Inner Membrane Ghost

The isolated inner mitochondrial membrane was first studied in order to avoid complications caused by the presence of small phosphorous containing molecules present in the matrix.

Inner mitochondrial membrane ghosts, prepared by osmotic lysis of mitoplasts show ^{31}P NMR spectra which do not change when the membranes are incubated for up to 30 minutes at temperatures ranging from 4–37 °C (Cullis et al. 1980). Since the data accumulations take on the order of 10 min we feel confident that we obtain information on the "native" membrane. The ^{31}P NMR spectrum of these membranes at 4 °C has the characteristic line shape of phospholipids organized in extended bilayers (Fig. 11). At the 0 ppm position a small ($\pm$ 10% of the total intensity) signal characteristic of phos-

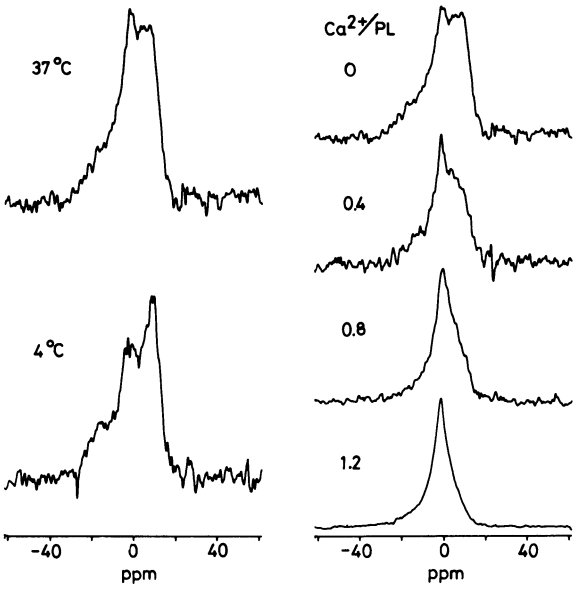

Fig. 11. 81.0 MHz ^{31}P NMR spectra of isolated rat liver inner mitochondrial membranes. The Ca^{2+} titration was done at 37 °C. The amount of Ca^{2+} is expressed as a molar ratio to total inner mitochondrial phospholipid (*PL*). Reproduced with permission from Cullis et al. 1980

pholipids undergoing isotropic motion is observable. Increasing the temperature to 37 °C results in a spectral change where the isotropic component has grown at the expense of the bilayer component. Due to the fact that both resonances overlap, no exact quantification on the amount of isotropic signal can be given. Incubating the membranes at 37 °C with increasing amounts of Ca^{2+} results in a marked increase in the amount of isotropic signal (Fig. 11). The experiments demonstrate that the majority of the phospholipids in the isolated inner mitochondrial membrane are localized in extended bilayers but that a small fraction of the phospholipids undergoes rapid isotropic motion at 37 °C. This fraction is increased in the presence of Ca^{2+}. The exact nature of the structures giving rise to this motion is unknown.

Intact Mitochondrion

Since the inner mitochondrial membrane is quantitatively by far the most abundant membrane in the mitochondrion we attempted to study its phospholipid organization in the intact mitochondrion by ^{31}P NMR. The ^{31}P NMR spectrum of rat liver mitochondria is composed of a bilayer component on top of which narrow resonances of various phosphorous containing molecules such as Pi, ATP and ADP are present (Cullis et al. 1980). Incubating these mitochondria for times as short as 5 min at 37 °C results in large spectral changes, mainly because of ATP hydrolysis, resulting in a growth of the phosphate peak which makes it virtually impossible to say anything of the structure of the membrane phospholipids. These problems can be partially circumvented by using more stable beef-heart mitochondria, 20 mm sample tubes containing 8 ml of mitochondrial suspension (50 mg protein/ml) thereby cutting down the accumulation time to approximately 1 min, and by keeping the mitochondria (in the presence of succinate) well oxygenated by continuously injecting H_2O_2 to the catalase containing stirred sample. Under these conditions we were able to obtain ^{31}P NMR spectra at 37 °C and 0 °C which remained stable for up until 10 min (Fig. 12). At 0 °C, except for two small resonances originating most likely from inorganic phosphate and a sugar phosphate, the spectrum consists entirely of the typical bilayer spectrum and demonstrates that at this

temperature all phospholipids are organized in extended bilayers. At 37 °C a similar spectrum is observed with the additional feature of a small but significant broad isotropic peak centered at 0 ppm. At the same time the definition of the low field shoulder became less clear. Both of these observations demonstrate increased isotropic motion of a small part of the membrane phospholipids. Finally, incubating the intact beef-heart mitochondria with 100 mM Ca^{2+} results in a large increase in the isotropic component.

Functional Implications

The occurrence of non-bilayer structures in the hydrated preparation of inner mitochondrial lipids and the observation of isotropic motion for part of the inner mitochondrial phospholipids at 37 °C in the intact system suggests that non-bilayer structures might occur in this membrane in vivo. It is therefore useful to consider how the mitochondrion functionally may benefit from these structures. In this discussion we will speculate on the involvement of non-bilayer lipids in three areas of mitochondrial functioning, e.g. (1) Ca^{2+} uptake, (2) protein insertion and (3) cytochrome oxidase activity.

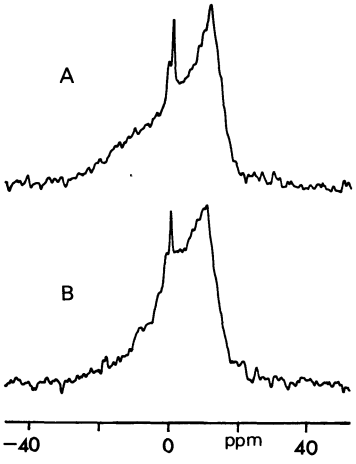

Fig. 12. 81.0 MHz ^{31}P NMR spectra of intact beef heart mitochondria at *A* 0 °C and *B* 37 °C. The sample consisted of 8 ml mitochondria (50 mg protein/ml) in 0.25 M sucrose pH 7.4 suspension containing 50 mM Na-succinate and 1 mg catalase which was placed in a 20 mm NMR tube and was kept continuously oxygenated by injecting at a rate of 100 µl/min a 3% H_2O_2 solution into the stirred suspension

Ca^{2+} Transport

Many cellular processes and enzymes are sensitive to and often regulated by Ca^{2+} present in the cytosol. It has been postulated that the mitochondrion can act as a regulator of this Ca^{2+} pool (Nicholls and Crompton 1980) since it is capable of a very fast energy-dependent Ca^{2+} uptake and release. The molecular details and biochemical nature of this process are not very well understood. However our results show that Ca^{2+} induces the formation of lipidic particles in CL containing bilayers, that these particles facilitate flip-flop and divalent cation transport, that ruthenium red blocks the formation of non-bilayer phases by Ca^{2+} in CL containing bilayers and that this inhibitor of Ca^{2+} transport in mitochondrion also blocks the uptake of Ca^{2+} in an organic phase by CL. We therefore propose that non-bilayer structures (most likely inverted micelles) formed as a result of the Ca^{2+}-CL interaction are involved during some stage of the Ca^{2+} transport process across the inner mitochondrial membrane.

Protein Insertion

Many different proteins of the inner mitochondrial membrane such as cytochrome c and several subunits of the cytochrome oxidase are synthesized on free ribosomes and are subsequently transported to and inserted in the inner mitochondrial membrane. Furthermore it is known that cytochrome c can move across the inner mitochondrial membrane (Nicholls 1974) which process might be related to the turnover of this protein. Although it is clear that protein factors will be involved in these processes, we suggest that inverted non-bilayer structures could provide a low energy pathway for the insertion

and translocation of these proteins. Our own data on the CL specific cytochrome c induced non-bilayer phases support this hypothesis. Furthermore, it is a remarkable observation that many if not all of the "integral" inner mitochondrial membrane proteins which are synthesized on endoplasmic reticulum bound ribosomes are like cytochrome c, basic proteins with a relativey low hydrophobicity (Iyengar M. R. and Iyengar C. L. 1980). Examples of such proteins include: creatine kinase, the ADP-ATP porter, subunit V of the ATPase and subunits VI and VII of cytochrome oxidase (Iyengar M. R. and Iyengar C. L. 1980). Furthermore in model experiments it has been demonstrated that for incorporation of isolated cytochrome c oxidase in a bilayer cardiolipin (in the presence of phosphatidylcholine) or a mixture of another (bilayer type) negatively charged lipid with unsaturated phosphatidylethanolamine is required (Eyton et al. 1977). Saturated PE's (which cannot adopt the H_{II} phase (Cullis and de Kruijff 1978) inhibit the proper incorporation of cytochrome oxidase (Eyton et al. 1977). In addition, it has been shown that the mitochondrial ^{31}Pi-ATP exchange reaction only can be properly reconstituted in mixed PC/PE systems (Racker et al. 1975) in which as we have demonstrated bilayer structures can occur.

Cytochrome Oxidase Activity

Cytochrome oxidase has been recently demonstrated to have an absolute requirement for cardiolipin for activity (Fry and Green 1980; Vik and Capaldi 1980). We demonstrated that cytochrome c, the substrate for cytochrome oxidase, shows a very specific and unique interaction will cardiolipin resulting in the formation of an intra membrane cytochrome c-cardiolipin complex. We, therefore, propose that the formation of this complex is important for the electron transport between cytochrome c and cytochrome oxidase. Possible modes of interaction between cytochrome c in this complex and cytochrome oxidase are shown in

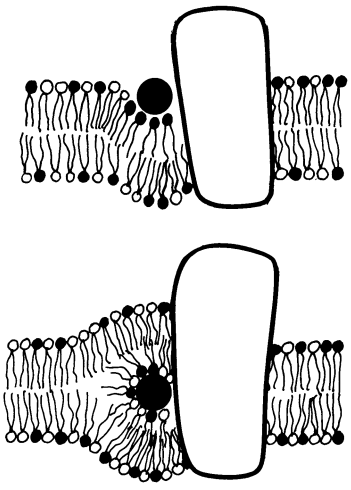

Fig. 13. Two possible ways in which cytochrome c-CL non-bilayer complexes could interact with cytochrome oxidase

Fig. 13. By extrapolation to the intact membrane we suggest that cytochrome c could be involved in lateral intramembrane electron transport between cytochrome bc_1 and cytochrome oxidase. Although recent data (Schneider et al. 1980) indicate that the cytochromes bc_1 and c and cytochrome oxidase from one free diffusable complex, others (Roberts and Hess 1977) have reported that cytochrome c is a highly diffusible membrane component in the process of electron transfer.

Concluding Remarks

The abundant occurrence of non-bilayer lipids in the inner mitochondrial membrane offers exiciting new dimensions to our understanding of the functioning of the mitochondrion. Future research will be directed towards answering two fundamental questions; (1) what is the role of non-bilayer lipids in the coupling between electron transport and ATP synthesis and (2) what will be the structural organization of the non-bilayer lipids under conditions of active oxidative phosphorylation.

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