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CYTOCHROME *c* SPECIFICALLY INDUCES NON-BILAYER STRUCTURES IN CARDIOLIPIN-CONTAINING MODEL MEMBRANES

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Summary

(1) The effect of cytochrome *c* addition on the phospholipid structure of liposomes composed of cardiolipin, phosphatidylserine, phosphatidylglycerol, phosphatidylcholine or phosphatidylethanolamine in a pure form or in mixtures was investigated by ^{31}P-NMR and freeze-fracture techniques.

(2) Cytochrome *c* specifically induces the hexagonal H_{II} phase and possibly an inverted micellar structure of part of the phospholipids in cardiolipin-containing model membranes.

(3) These results are compared with the effect of Ca^{2+} on cardiolipin and are discussed in relation to the structure and function of the inner mitochondrial membrane.

Introduction

The nature, specificity and functional consequences of lipid-protein interactions remain fundamental problems in membrane biology. To date, most investigators have focussed their attention on the influence of membrane fluidity on protein function, with the precept in mind that the composition and physical state of local 'annular' lipids may play functional regulatory roles. However, as we have indicated elsewhere [1], such an approach has not proved particularly fertile. In spite of intensive effort, there remains no unambiguous evidence that specific local lipids play regulatory roles in vivo.

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In this work, we approach the problem of the functional role of lipids and lipid-protein interactions from a different point of view. It is our contention that the ability of certain membrane lipids to adopt non-bilayer configurations is vital to many functional abilities of biological membranes, including fusion, flip-flop as well as certain transport processes [1,2]. It has been shown that the bilayer/non-bilayer preferences of various pure and mixed lipid systems may be sensitive to such factors as temperature, divalent cation concentration and pH (for review, see Refs. 1 and 2) and that in some biological membranes the presence of protein also appears to influence the polymorphic preferences of the lipid component [3–6]. Clearly, an ability of membrane protein to modulate the local structures of the lipid matrix would provide a new perspective on lipid-protein interactions and their functional consequences.

The model system chosen to explore these possibilities consists of cytochrome *c* and cardiolipin. The major reasons for this choice are straightforward to summarize. First, cytochrome *c* is an easily isolated, well characterized protein which experiences strong interactions with negatively charged membrane phospholipids. For example, in addition to its well defined role in electron transport, this highly basic protein is known to bind electrostatically to negatively charged phospholipids (for review see Ref. 7 and references therein), to penetrate (partially) lipid bilayers [8,9] and monolayers [10,11], to enhance the cation permeability of lipid bilayers [11], to decrease the transition temperatures and enthalpy of phospholipid gel-liquid crystalline transitions [9] and can induce lateral phase separation in mixed lipid systems [12,13].

Given that cytochrome *c* is associated with the inner mitochondrial membrane, and that cardiolipin is the major negatively charged lipid residing in this membrane, the choice of cardiolipin follows naturally. It is also a natural choice from the point of view of our interest in the functional roles of non-bilayer lipids, as cardiolipin adopts the hexagonal (H_{II}) phase in the presence of various divalent cations [14,15].

The particular problems we address are two-fold, concerning whether cytochrome *c* can induce non-bilayer structures in pure and mixed lipid systems containing cardiolipin, and whether such effects are specific. We show that cytochrome *c* can induce hexagonal (H_{II}) and possibly inverted micellar structures in such systems, but not in analogous lipid systems where cardiolipin is replaced by other negatively charged phospholipids. These results are discussed in terms of the functional roles of cardiolipin in the inner mitochondrial membrane.

Materials and Methods

Experimental materials. Cytochrome *c* * (type VI) from horse heart was obtained from Sigma (St. Louis, MO). This material was in the oxidized form as no reduced form could be detected spectrophotometrically. The sodium salt of cardiolipin from bovine heart was obtained from Sigma (St. Louis, MO). Phosphatidylcholine was obtained from egg and soya lipids by standard chromatographic techniques. Phosphatidylethanolamine was obtained employing the

* Unless otherwise stated, cytochrome *c* refers to the oxidized form of cytochrome *c*.

phospholipase D-catalyzed base-exchange procedure [16] from soya phosphatidylcholine. Phosphatidylserine and phosphatidylglycerol were similarly prepared [18] from egg phosphatidylcholine. After isolation, the latter lipids were converted into the sodium salt form [17]. All lipids were chromatographically pure.

Preparation of lipid dispersions and lipid-cytochrome c recombinants. 50 μmol of lipid were dried (from chloroform) under N_2. The resulting lipid film was stored overnight under high vacuum to remove traces of solvent and was subsequently dispersed at 20°C in 1.0 ml of an aqueous buffer (100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0) by vortex mixing. Cytochrome *c* was added to these liposomes either as the dry protein or dissolved in 0.2 ml of the buffer. Identical results were obtained for both protocols. Alternatively, in some cases the lipids were dispersed in 1.0 ml of the above buffer containing an appropriate amount of cytochrome *c*. It was noted that the pH of the buffer dropped to approx. 6.0 after addition of 36 mg cytochrome *c*. Readjusting the pH with NaOH to 7.0 did not affect the ^{31}P-NMR characteristics. Thus, for most of the preparations, the pH was not readjusted after cytochrome *c* addition. In certain experiments, cytochrome *c* was reduced by a 10-fold molar excess of ascorbate. This procedure was performed either prior to addition of the cytochrome *c* to liposomes, or subsequently. As indicated by increased absorbance at 550 nm and the shift in the peak from 410 to 416 nm, it could be concluded that in both cases the reduction was completed within 5 min.

Analytical methods. All ^{31}P-NMR experiments were performed at 30°C at 81 MHz, under conditions of proton decoupling as described before [17]. Before the ^{31}P-NMR measurement, 10% of the ^2H_2O analogue of the buffer was added to the sample. Typically, 1000 transients were recorded with a 1 s interpulse time employing 10-μs 90° pulses. An exponential filtering, resulting in a 50 Hz linebroadening, was applied to all free induction decays. Freeze-fracture studies were carried out as described elsewhere [19] on samples to which 25% (by volume) glycerol was added to prevent freezing damage. The samples were quenched from 20°C. Lipid phosphate was determined according to the method of Fiske and Subbarow [20] after perchloric acid destruction of the lipids. Oxidized or reduced cytochrome *c* was quantitated by measuring the absorbance at 410 and 416 nm, respectively [7].

Results

Effect of cytochrome c on cardiolipin liposomes

In order to develop a suitable system for the NMR and freeze-fracture studies, we first investigated the kinetics (Fig. 1) and concentration dependence (Fig. 2) of cytochrome *c* binding to cardiolipin liposomes. Fig. 1 shows that the binding is fast and nearly quantitative. Furthermore the resulting recombinant must be a large structure as the phospholipid could be quantitatively recovered by low-speed centrifugation. As shown in Fig. 2, cytochrome *c* binds stoichiometrically to cardiolipin until a plateau at approx. 1.4 mg cytochrome *c* bound/5 μmol cardiolipin is reached. This corresponds to approx. 43 mol cardiolipin/mol cytochrome *c*. In order to ascertain whether the bound cytochrome *c* is localized only on the outher layer of the liposomes, experiments

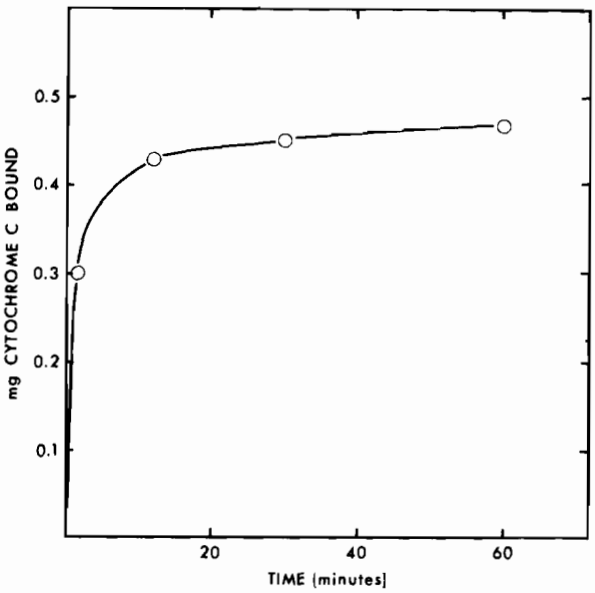

Fig. 1. Time course of the cytochrome *c* binding to cardiolipin liposomes. To 2.7 ml 100 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA, pH 7.0, buffer containing 2.5 μmol cardiolipin, 0.6 mg cytochrome *c* dissolved in 0.3 ml of buffer was added. The mixture was incubated for various times whereafter the samples were spun for 20 min at 27 000 × *g* at 4°C and the amount of cytochrome *c* and phospholipid remaining in the supernatant were determined. No detectable amounts (less than 2%) of phospholipid were present in the supernatant in any of the samples.

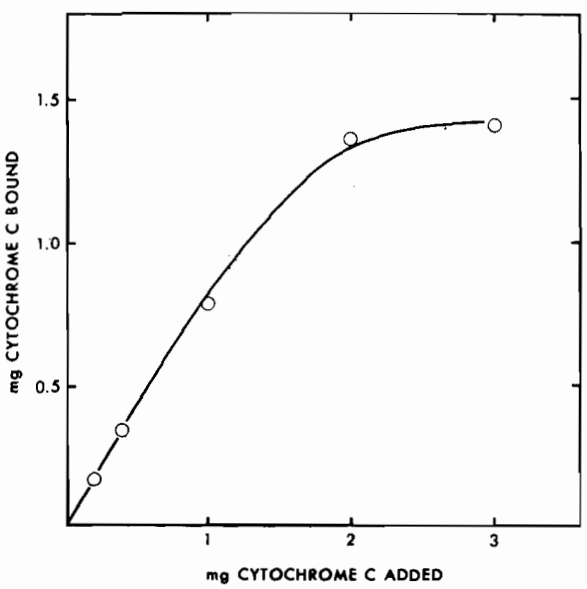

Fig. 2. Stoichiometry of the cytochrome *c* binding to cardiolipin liposomes. Experimental conditions as described in the legend of Fig. 1 except that 5 μmol cardiolipin and varying amounts of cytochrome *c* were used. The incubation time was 30 min. No detectable amounts of phospholipid were present in the supernatant in any of the samples.

TABLE I

CYTOCHROME *c* BINDING TO CARDIOLIPIN LIPOSOMES

A dry film of 4.5 μmol of cardiolipin was either hydrated with (sample 1) 1.0 ml cytochrome *c* solution (2 mg cytochrome *c*/ml buffer) or with (sample 2) 1.0 ml buffer. After vortex mixing, the samples were incubated at 25°C for 10 min, whereafter 1.0 ml buffer and 1.0 ml cytochrome *c* solution (2 mg cytochrome *c*/ml buffer) were added to samples 1 and 2, respectively. After 10 min incubation at 25°C, the samples were centrifuged at 4°C for 20 min at 27 000 × *g*, whereafter the amount of cytochrome *c* remaining in the supernatant was determined.

	mg
Cytochrome *c* added	2.00
(1) Cytochrome *c* bound when present during liposome formation	1.69
(2) Cytochrome *c* bound when added after liposome formation	1.66

were performed where cytochrome *c* was present in the buffer during preparation of the cardiolipin liposomes. Table I shows the comparable binding is observed which suggests that cytochrome *c* added to preformed cardiolipin liposomes is able to penetrate to the inner bilayers of the liposome. Further experiments, suggested by the observation that cytochrome *c* binding can be enhanced by decreasing the ionic strength [21], were conducted employing a 10 mM NaCl analogue of the buffer. In general, it was then observed that substantial amounts of cardiolipin remained in the supernatant after centrifugation. Freeze-fracture results indicate that this lipid is most likely in the form of small vesicles. As the presence of small structures greatly complicates the inter-

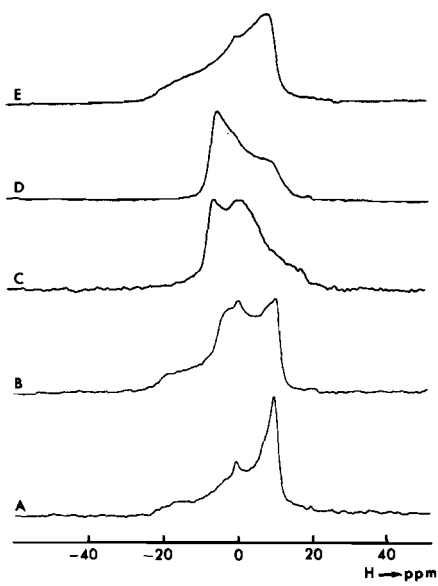

Fig. 3. ^{31}P-NMR spectra of cardiolipin in the presence of cytochrome c and Ca^{2+}. (a) 50 μmol of cardiolipin in 1.0 ml buffer; (b) 10 min after addition of 0.2 ml buffer containing 36 mg cytochrome *c*; (c) as b 10 min after the addition of 100 μl of 1 M $CaCl_2$; (d) 50 μmol of cardiolipin in 1.0 ml buffer 10 min after the addition of 100 μl 1 M $CaCl_2$; (e) 50 μmol of cardiolipin hydrated with 1.0 ml of buffer containing 36 mg cytochrome *c*. The 0 ppm position in this and subsequent figures represents the chemical shift position of the signal form sonicated egg phosphatidylcholine vesicles.

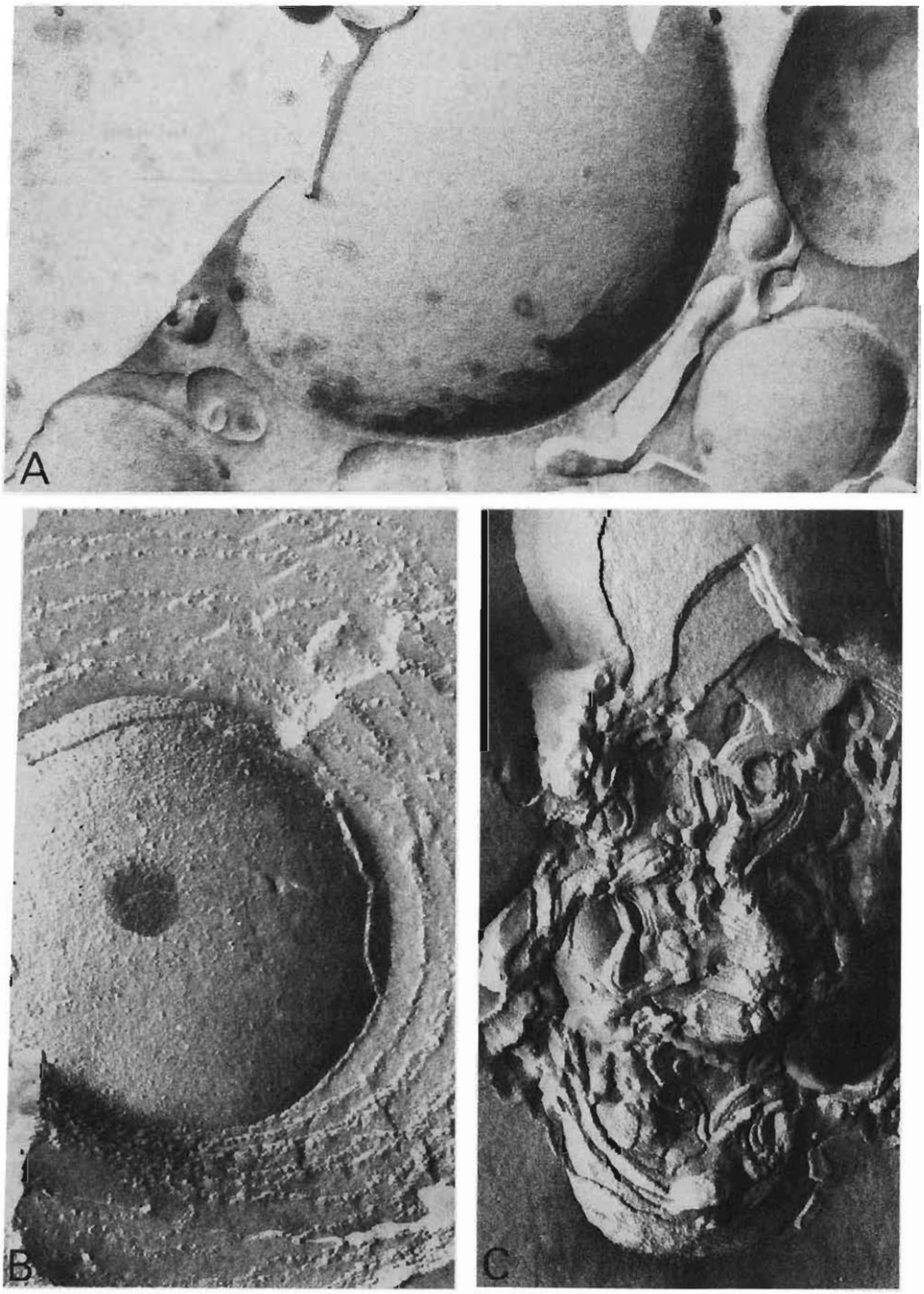

Fig. 4. Freeze-fracturing of cardiolipin liposomes in the absence (A) and presence (B, C) of cytochrome *c*. Samples were prepared as described for Fig. 3a and b. Magnification, ×100 000.

pretation of the ^{31}P-NMR data, we employed the 100 mM NaCl buffer in all subsequent experiments.

The ^{31}P-NMR spectrum of cardiolipin liposomes in the 100 mM NaCl buffer

(Fig. 3A) has the characteristic asymmetrical lineshape indicative of phospholipids in extended bilayers [1]. This is also evidenced by the large smooth fracture faces as observed by freeze-fracturing (Fig. 4A). The origin of the small narrow resonance at the 0 ppm position of phospholipids undergoing isotropic motion is unclear. Small vesicles free in solution are unlikely to be involved as all the phospholipid is quantitatively pelleted by low-speed centrifugation. Alternative sources include entrapped small vesicles or possibly a small amount of non-bilayer phase. The latter possibility would be consistent with the occasional observation of lipidic particles in freeze-fracture pictures of similar preparations (Leunissen-Bijvelt, J. and Verkleij, A.J., unpublished observations). The addition of excess Ca^{2+} to these liposomes induces the hexagonal H_{II} phase as evidenced by the characteristic spectrum of a reversed asymmetry and reduced width (Fig. 3D) in agreement with previous results [14,15]. It should be noted that the dominant spectral features of lipids in the bilayer phase, the hexagonal H_{II} phase and in phases allowing isotropic motion occur at characteristic chemical shift positions. Addition of excess cytochrome *c* to the cardiolipin liposomes reproducibly (six different experiments) induces an increase in the isotropic component and the appearance of an H_{II} component most clearly seen as a shoulder on the low-field side of the isotropic peak (Fig. 3B). The sum of the isotropic and H_{II} signals is approx. 25% of the total signal intensity. Doubling the cytochrome *c* concentration or adding reduced cytochrome *c* gave very similar results. Furthermore, incubation of the cytochrome *c*-cardiolipin dispersions for up to 3 h did not result in any spectral changes.

The slight differences in chemical shift position of the H_{II} phase component in the spectra in Fig. 3B and D might originate from differences in length or diameter of the H_{II} cylinders or alternatively might be caused by changes in the rate of lateral diffusion of the lipids.

It is of interest to compare these results with results obtained employing freeze-fracture techniques, which show three important features (Fig. 4b, c). Firstly, in the presence of cytochrome *c*, the lipids are still mainly organized in extended bilayers, but, surprisingly, as cytochrome *c* is thought to be an extrinsic membrane protein, on most of the fracture faces very small (approx. 50—70 Å diameter) particles are present (Fig. 4B). Secondly, in cross-features of multilayered liposomes, the bilayers often exhibit a particulate appearance. The size of the particles is similar to those found on the fracture face (Fig. 4B). Furthermore, the presence of these particles in the inner shells supports the conclusion reached from the binding experiments that cytochrome *c* can penetrate to the inner bilayers of preformed cardiolipin liposomes. Finally, in some regions of the fracture faces (Fig. 4C), long parallel lines characteristic of the hexagonal H_{II} phase [15,22,24] are observed. The diameter of the cylinders is approx. 60 Å.

When cytochrome *c* was present during formation of the cardiolipin liposomes, freeze-fracturing showed very similar features except that no region of hexagonal H_{II} phase was detected. ^{31}P-NMR spectra of these preparations gave variable results. In some cases, spectra similar to those shown in Fig. 3B were observed except that no H_{II} component was present. In other cases, no sharp isotropic signal was observed, but the linewidth of the resonance was much increased. An example of such a spectrum is shown in Fig. 3E. The differences

in linewidth are not understood. They could possibly arise from structures of different size. In all cases, however, the ^{31}P-NMR spectra are consistent with increased motion of part of the phospholipids.

The addition of excess Ca^{2+} to the cardiolipin-cytochrome *c* reconstitutes causes marked increases in the hexagonal H_{II} component of the ^{31}P-NMR spectra (Fig. 3C). This demonstrates that even in the presence of excess cytochrome *c*, Ca^{2+} is still able to induce the Ca^{2+}-cardiolipin hexagonal H_{II} phase complex. We return to this apparent ability of Ca^{2+} to compete with cytochrome *c* for cardiolipin in subsequent sections.

Effect of cytochrome c on phosphatidylserine, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine dispersions

The addition of cytochrome *c* to liposomes prepared of phosphatidylserine and phosphatidylglycerol, under conditions similar to those used for the cytochrome *c*-cardiolipin systems, caused immediate precipitation of all the lipids and virtually all the cytochrome *c* as was observed for cardiolipin. However, in marked contrast to the situation observed for cardiolipin, only very minor changes, e.g., a slight increase in the width at half-height in the ^{31}P-NMR spectrum of the cytochrome *c* complex formed with these lipids, were observed (Fig. 5). The ^{31}P-NMR lineshape of the phospholipids shows that in all cases the lamellar phase is maintained. Furthermore, the spectra demonstrate that the local order of the phosphate region of the phospholipids is not appreciably affected by the cytochrome *c* binding, since no significant changes in the distance between the high-field peak and the low-field shoulder, which is a measure of this order [25], are observed. Finally, it was found that cytochrome *c* did not bind to neutral zwitterionic lipids and had no effect on the ^{31}P-NMR spectra arising from aqueous dispersions of egg phosphatidylcholine or soya phosphatidylethanolamine.

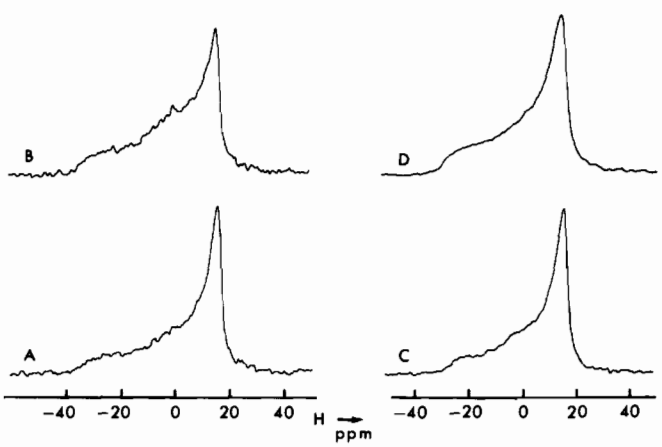

Fig. 5. ^{31}P-NMR spectra of 50 μmol phosphatidylserine (A, B) or phosphatidylglycerol (C, D) dispersed in 1.0 ml buffer before (A, C) and after (B, D) the addition of 36 mg cytochrome *c* in 0.2 ml buffer to the liposomes.

Effect of cytochrome c on mixed lipid systems

Phosphatidylcholine/cardiolipin mixtures. Cytochrome *c* addition to egg phosphatidylcholine/cardiolipin (2 : 1) mixed liposomes under conditions as described in the legend of Fig. 3 gave very similar results to those represented in Fig. 3a and b for pure cardiolipin, in that an isotropic and H_{II} phase component in the ^{31}P-NMR spectrum were induced. The sum of both signals was approx. 20% of the total signal intensity.

Phosphatidylethanolamine/cardiolipin mixtures. Prior to examining the effects of cytochrome *c* on the structural preferences of mixed phosphatidylethanolamine/cardiolipin systems, a characterization of the behaviour of the lipid system alone was required. This is an interesting lipid system for two reasons. Firstly, a recent investigation [26] suggests that these compounds may be the major lipid components of the inner monolayer of the inner mitochondrial membrane. Secondly, (rat liver) mitochondrial phosphatidylethanolamine prefers the hexagonal (H_{II}) phase above 10°C [27] which implies that if cardiolipin stabilizes a bilayer arrangement in the mixed system, this structure should be very sensitive to the presence of Ca^{2+}.

The latter suspicions were confirmed on investigation of soya phosphatidylethanolamine/cardiolipin (2 : 1) dispersions. In these systems, it is observed (Fig. 6, $Ca^{2+}/CL = 0$) that the cardiolipin stabilizes a bilayer configuration for the bulk of the phosphatidylethanolamine (which prefers the H_{II} configuration at 30°C [28]). The small isotropic component could arise from small lamellar structures or non-bilayer lipid structures such as inverted micelles. The addition

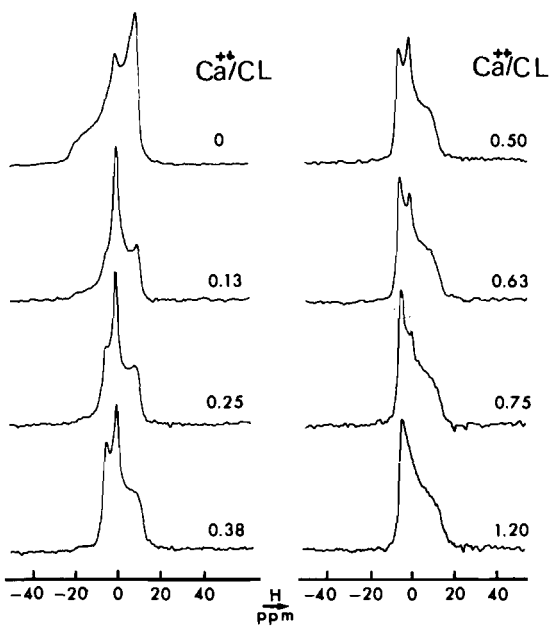

Fig. 6. Effect of Ca^{2+} addition on the ^{31}P-NMR spectra of soya phosphatidylethanolamine/cardiolipin (2 : 1) mixed liposomes. To 50 μmol phospholipid dispersed in 1.0 ml of the 100 mM NaCl buffer, aliquots of 100 mM $CaCl_2$ were added to give the Ca^{2+}:cardiolipin ratios indicated in the figure. After vortex mixing, the ^{31}P-NMR spectrum was recorded. For each sample the spectrum was recorded again 2 h after incubation at 30°C with the same results.

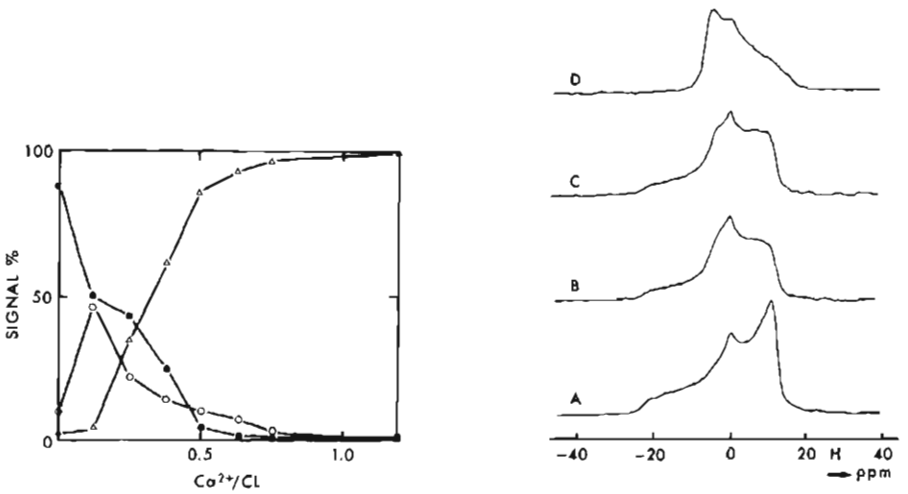

Fig. 7. Effect of Ca^{2+} on the amount of bilayer (●———●), isotropic (○———○) and hexagonal H_{II} (△———△) signal in the ^{31}P-NMR spectra of soya phosphatidylethanolamine/cardiolipin (2 : 1) liposomes. Details as in the legend of Fig. 6.

Fig. 8. Effect of cytochrome *c* addition on the ^{31}P-NMR spectrum of soya phosphatidylethanolamine/cardiolipin (2 : 1) liposomes. 50 μmol phospholipid were dispersed in 1.0 ml of 100 mM NaCl buffer, (a) without cytochrome *c*; (b) 10 min after the addition of 0.2 ml buffer containing 36 mg of oxidized cytochrome *c*; (c) 10 min after reducing cytochrome *c* by adding 300 μl of 100 mM ascorbate solution; (d) as b except that 20 μl of 1 M $CaCl_2$ were added prior to recording the NMR spectrum. When cytochrome *c* was reduced prior to addition to the liposome spectra very similar to that shown in c were obtained.

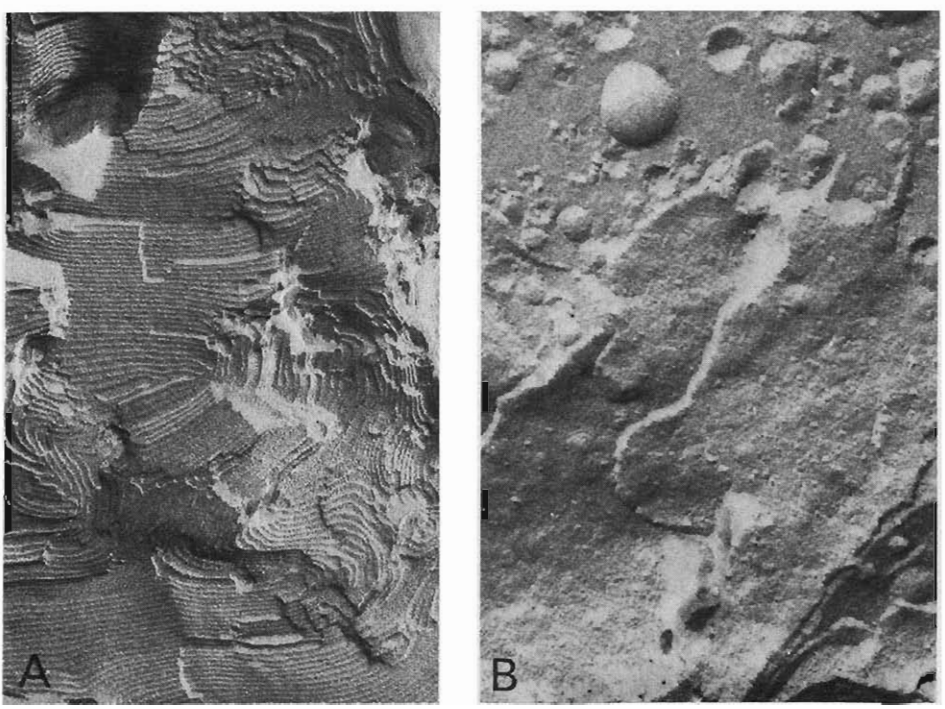

Fig. 9. Freeze-fracturing of soya phosphatidylethanolamine/cardiolipin (CL) (2 : 1) liposomes in the presence of (A) Ca^{2+} and (B) cytochrome *c*. Samples were prepared as described for Figs. 6 (Ca^{2+}/CL = 1.20) and 8b. Magnification, ×100 000.

of small amounts of Ca^{2+} has dramatic effects (Fig. 6). Initially, Ca^{2+} induces formation of a structure in which isotropic motional averaging is allowed, whereas higher Ca^{2+} concentrations result in formation of the H_{II} phase. These results are presented in a more quantitative fashion in Fig. 7. Full conversion to the H_{II} phase is observed at a Ca^{2+}:cardiolipin stoichiometry of approx. 1, in agreement with results obtained for pure cardiolipin systems [15]. The freeze-fracture morphology of the H_{II} phase is shown in Fig. 9a.

The addition of reduced or oxidized cytochrome *c* to the phosphatidylethanolamine/cardiolipin (2 : 1) liposomes produced effects similar to those for the pure cardiolipin systems. In particular, an 'isotropic' and a hexagonal (H_{II}) phase lipid component are clearly visible (Fig. 8). Freeze-fracturing (Fig. 9b) shows the presence of extended lipid bilayers but also the presence of some relatively small (500—2000 Å diameter) vesicles. In agreement with the data on pure cardiolipin, numerous very small particles are present on the fracture faces of the bilayers. No H_{II} phases were observed which may arise because, although the quenching was performed at 20°C, the actual fixation could occur at a lower temperature, where phosphatidylethanolamine prefers the bilayer phase. The presence of cytochrome *c* does not prevent the Ca^{2+}-cardiolipin interaction in the phosphatidylethanolamine/cardiolipin liposomes, since Ca^{2+} addition leads to a total transition to the H_{II} phase (Fig. 8d). This is accompanied by a dissociation of the cytochrome *c*-cardiolipin complex and the release of cytochrome *c* from the liposomes as indicated in Table II.

Phosphatidylethanolamine/phosphatidylserine mixtures. In order to ascertain whether the ability of cytochrome *c* to induce non-bilayer lipid structures is specific for cardiolipin, the effects of cytochrome *c* on equimolar mixtures of soya phosphatidylethanolamine with phosphatidylserine were investigated. In the absence of cytochrome *c*, the phosphatidylserine/soya phosphatidylethanolamine system exhibited behaviour consistent with previous work [29]. Hydration of this mixture resulted in bilayer ^{31}P-NMR spectra, and the addition of equimolar Ca^{2+} (with respect to phospholipid) triggered formation of hexagonal (H_{II}) phase.

The addition of 36 mg of cytochrome *c* (in 0.2 ml) to the soya phosphatidylethanolamine/phosphatidylserine dispersion (50 μmol phospholipid) resulted

TABLE II

EFFECT OF Ca^{2+} ON THE CYTOCHROME *c* BINDING TO PHOSPHATIDYLETHANOLAMINE/CARDIOLIPIN (2 : 1) LIPOSOMES

The cytochrome *c* binding to the liposomes was determined 10 min after the addition of 0.2 ml buffer containing 0.4 mg cytochrome *c* to 3.4 ml buffer containing 3.5 μmol phospholipid. In sample 2, 10 μl of buffer containing 10 μmol of $CaCl_2$ were added followed by a 10 min incubation, whereafter the binding was determined. In sample 3, first the Ca^{2+} was added after 10 min followed by the cytochrome *c* addition. After the incubations, the samples were centrifuged at 4° C for 20 min at 27 000 × *g* whereafter the amount of cytochrome *c* in the supernatant was determined.

Sample	nmol cytochrome *c* bound per μmol phospholipid
(1) $-Ca^{2+}$	2.33
(2) $+Ca^{2+}$ (added after cytochome *c* addition)	0.70
(3) $+Ca^{2+}$ (added before cytochrome *c* addition)	0.01

in total precipitation of the phospholipids, as is observed on Ca^{2+} addition, but no change in the characteristic 'bilayer' ^{31}P-NMR spectrum. The subsequent addition of 50 μmol Ca^{2+} (in 50 μl) induced a hexagonal (H_{II}) ^{31}P-NMR component corresponding to at least 50% of the phospholipids.

Discussion

In this work, we have demonstrated that cytochrome *c* experiences strong and specific interactions with cardiolipin in model membrane systems, and that this interaction can result in an H_{II} configuration of the cardiolipin. These results have certain interesting consequences with regard to the structure of cytochrome *c*-cardiolipin complexes and the functional roles of cardiolipin in the inner mitochondrial membrane. We discuss these two areas in turn.

The ability of cytochrome *c* to induce the hexagonal (H_{II}) phase for cardiolipin suggests that this positively charged protein can actually reside within the aqueous channel of the H_{II} phase. Steric considerations are also consistent with such a possibility as the radius of gyration of cytochrome *c* (12—14 Å [30]) is similar to the radius of the H_{II} channel which can be inferred to be 10—15 Å. It may be noted that cytochrome *c* could fit equally well into inverted micelles, which appear to occur as intermediaries in bilayer-to-hexagonal (H_{II}) transitions (for review see Refs. 1 and 2). Indeed, such structures could be responsible for the 'isotropic' ^{31}P-NMR components observed in the cytochrome *c*-cardiolipin dispersions (see Fig. 3), although other interpretations are possible.

We therefore suggest that in cardiolipin-containing membranes, a certain fraction of cytochrome *c* may be present in an intra-bilayer structure which may be an inverted micelle or an elongated version thereof (corresponding to an H_{II} phase channel). A schematic representation of the formation and dissociation of such a complex is given in Fig. 10. Briefly, we propose that the cytochrome *c* induces a lateral segregation of the endogeneous cardiolipin (Fig. 10a, b), resulting in (local) destabilization of bilayer structure (Fig. 10c) and subsequent adoption of the lowest energy (intra-bilayer) 'inverted' lipid configuration (Fig. 10d). The cytochrome *c* could possibly be released to the opposite site of the bilayer (Fig. 10e—g).

Although this model may appear overly speculative at first sight, there is much evidence which is consistent with the notion of a lipid-soluble cytochrome *c*-cardiolipin complex. In particular, such structures could account for the (small) particles observed in the freeze-fracture faces of cytochrome *c*-cardiolipin bilayers (Fig. 4). Furthermore, the ability of cytochrome *c* to permeate through cardiolipin-containing bilayers would be consistent with an intermediate formation of such complexes (cf. Fig. 9). Other suggestive evidence includes the ability of cytochrome *c* to form complexes with negatively charged lipids which are soluble in non-polar environments such as isooctane [31] and which upon rehydration can give rise to the hexagonal H_{II} phase [32] as well as X-ray studies [8], which suggest an ability of cytochrome *c* to penetrate partially into cardiolipin bilayers.

The localization of both cardiolipin and cytochrome *c* in the inner mitochondrial membrane, and the fact that these components experience strong and specific interactions with one another leads to the suspicion that these interac-

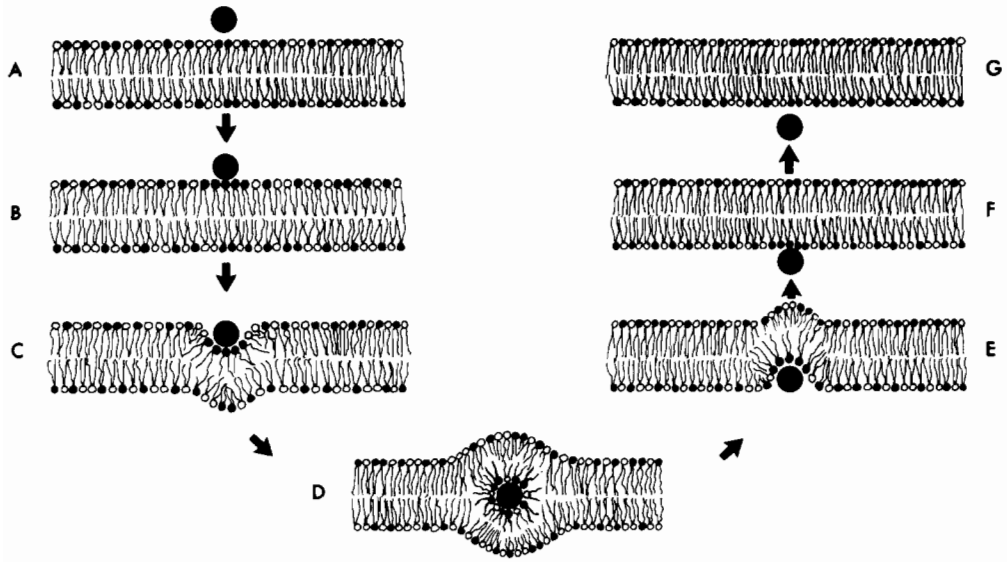

Fig. 10. Schematic representation of the formation of (intra-bilayer) 'inverted' lipid structures by cytochrome c in cardiolipin-containing bilayers. The lipids with the dark head-groups represent cardiolipin, the others might represent other lipids such as phosphatidylcholine or phosphatidylethanolamine. Cytochrome c is represented by the black sphere.

tions may have functional consequences. We discuss three possibilities here. Firstly, the observation that cytochrome *c* trapped inside submitochondrial particles slowly leaks out [7] would be consistent with a transport mechanism facilitated by cardiolipin as indicated in Fig. 9. Secondly, it is possible that cytochrome *c* which is partially or completely buried in the membrane could experience different, possibly enhanced, rates of electron transfer from cytochrome *b*, c_1 to cytochrome oxidase. It may be noted that the cytochrome oxidase binding site for cytochrome *c* is commonly postulated to reside on the outer surface of the inner mitochondrial membrane [7]. The ability of cytochrome *c* to penetrate the membrane suggests that this does not necessarily have to be the case. Finally, the ability of Ca^{2+} to displace cytochrome *c* from membrane systems containing cardiolipin suggests that the membrane concentrations of cytochrome *c* may be modulated by the local Ca^{2+} concentration, which could also be related to the uncoupling action of Ca^{2+} on mitochondria [33].

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