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STRUCTURAL PROPERTIES OF PHOSPHOLIPIDS IN THE RAT LIVER INNER MITOCHONDRIAL MEMBRANE

A ^{31}P-NMR STUDY

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Summary

1. The ^{31}P-NMR characteristics of intact rat liver mitochondria, mitoplasts and isolated inner mitochondrial membranes, as well as mitochondrial phosphatidylethanolamine and phosphatidylcholine, have been examined.

2. Rat liver mitochondrial phosphatidylethanolamine hydrated in excess aqueous buffer undergoes a bilayer-to-hexagonal (H_{II}) polymorphic phase transition as the temperature is increased through 10°C, and thus prefers the H_{II} arrangement at 37°C. Rat liver mitochondrial phosphatidylcholine, on the other hand, adopts the bilayer phase at 37°C.

3. Total inner mitochondrial membrane lipids, dispersed in an excess of aqueous buffer, exhibit ^{31}P-NMR spectra consistent with a bilayer arrangement for the majority of the endogeneous phospholipids; the remainder exhibit spectra consistent with structure allowing isotropic motional averaging. Addition of Ca^{2+} results in hexagonal (H_{II}) phase formation for a portion of the phospholipids, as well as formation of 'lipidic particles' as detected by freeze-fracture techniques.

4. Preparations of inner mitochondrial membrane at 4 and 37°C exhibit

^{31}P-NMR spectra consistent with a bilayer arrangement of the large majority of the endogenous phospholipids which are detected. Approx. 10% of the signal intensity has characteristics indicating isotropic motional averaging processes. Addition of Ca^{2+} results in an increase in the size of this component, which can become the dominant spectral feature.

5. Intact mitochondria, at 4°C, exhibit ^{31}P-NMR spectra arising from both phospholipid and small water-soluble molecules (ADP, P_i, etc.). The phospholipid spectrum is characteristic of a bilayer arrangement. At 37°C the phospholipids again give spectra consistent with a bilayer; however, the labile nature of these systems is reflected by increased isotropic motion at longer (at least 30 min) incubation times.

6. It is suggested that the uncoupling action of high Ca^{2+} concentrations on intact mitochondria may be related to a Ca^{2+}-induced disruption of the integrity of the inner mitochondrial phospholipid bilayer. Further, the possibility that non-bilayer lipid structures such as inverted micelles occur in the inner mitochondrial membrane cannot be excluded.

Introduction

The inner mitochondrial membrane is one of the most metabolically active membrane systems observed in nature. This is reflected by the presence of over 70 enzymes associated with the respiratory chain and various transport proprocesses. The fact that these enzymes are membrane associated, and the apparent requirement for an intact membrane in order for substrate oxidation to be coupled to phosphorylation of ADP, has proved a major complication in the quest for a detailed understanding of this system which represents a primary problem in membrane biology.

From the viewpoint of the lipid biochemist, the inner mitochondrial membrane is particularly intriguing. This is not only because of the demonstrated lipid requirement for oxidative phosphorylation to occur [1] and for function of individual components of the respiratory chain [2], but also because of a unique lipid composition. In particular, this organelle membrane is the only mammalian membrane containing (unsaturated) cardiolipin as a major lipid component [3]. This localization, as well as the distinctive ability of cardiolipin to adopt the hexagonal (H_{II}) phase in the presence of divalent cations [5,6], implicity suggests that cardiolipin may satisfy requirements other than maintenance of bilayer structure. Similarly, by analogy with previous results [4], the unsaturated nature of the endogenous phosphatidylethanolamine [3] suggests that this component (40% of the membrane phospholipid) would prefer the hexagonal (H_{II}) arrangement at 37°C. The demonstrated ability of mitochondria to accumulate high divalent cation concentrations [7] leads to the remarkable possibility that over 60% of the endogeneous phospholipids would adopt a non-bilayer arrangement in the absence of other constraints.

The present work is directed towards obtaining an understanding of the structural properties of phospholipids in the intact mitochondrial membrane and various derivatives employing ^{31}P-NMR [8] techniques. Four systems have been employed, namely hydrated preparations of individual isolated phospho-

lipids and total extracted lipids, the isolated inner mitochondrial membrane and intact mitochondria. Particular attention has been paid to the metabolic stability of the protein-containing system, as well as the influence of Ca^{2+}.

Materials and Methods

Mitochondria were isolated from 1-day-fasted Wistar rats according to established techniques [9]. Up to 0.3 ml of ^2H_2O buffer containing 150 mM NaCl, 10 mM Tris-acetic acid (p^{2}H 7.0) and 0.2 mM EDTA was added to the pelleted mitochondria to obtain a total volume of 1.0 ml. This was transferred to a 10 mm tube for ^{31}P-NMR studies. The protein content was determined by using the method of Lowry et al. [32] and found to be approx. 180 mg/ml, whereas the total phosphorus was approx. 40 μmol/ml as determined by using the method of Fiske and SubbaRow [10]. A lipid extraction procedure as described by Folch et al. [11] established that 63% of the phosphorus was contained in phospholipid.

Mitoplasts (inner membrane plus matrix) were prepared according to the method of Parsons and Williams [9] and the pellet resuspended in 0.3 ml 10 mM NaCl/1 mM Tris-acetic acid (p^{2}H 7.0)/0.2 mM EDTA. The phosphorus content was determined as 28.8 μmol/ml, and the protein as 125 mg/ml.

Inner membrane ghosts were prepared from isolated mitoplasts by a modification of the osmotic lysis procedure for intact mitochondria followed by Caplan and Greenawalt [12]. Briefly, mitoplasts (from approx. 50 g of liver, six rats) were suspended in 50 ml of distilled H_2O at 4°C and subsequently centrifuged (17 500 rev./min; 40 000 × g) for 10 min. This procedure was repeated twice. The pellet was then resuspended in the 10 mM NaCl buffer employed previously for the mitoplasts, and then transferred to a 10 mm tube for ^{31}P-NMR studies. The ratio of non-phospholipid to phospholipid phosphorus in this inner membrane preparation was obtained as less than or equal to 0.1, and the protein-to-phospholipid ratio was approx. 2.7.

Total lipids were extracted from the mitoplasts and the intact mitochondria employing the procedure of Bligh and Dyer [13] with 100 mM NaCl and 50 mM EDTA present in the aqueous phase. Total inner membrane lipids in the chloroform phase were dried under nitrogen gas and then placed under high vacuum for 1 h. Subsequently, the lipids were hydrated for ^{31}P-NMR studies in 0.8 ml of aqueous buffer (10% ^2H_2O) containing 100 mM NaCl, 10 mM Tris-acetic acid (pH 7.2) and 1 mM EDTA.

Phosphatidylcholine and phosphatidylethanolamine were isolated from total mitochondrial lipids employing silicic acid and carboxymethyl cellulose [14] column chromatography. The lipid thus obtained was greater than 99% pure as indicated by thin-layer chromatography. ^{31}P-NMR studies were conducted on approx. 30 μmol phospholipid hydrated in the 100 mM NaCl buffer employed for the total inner membrane lipids.

Respiratory control ratios employing succinate as substrate were obtained using standard oxygraph techniques. In all cases where Ca^{2+} was added, appropriate aliquots from a 1 M or 0.1 M $CaCl_2$ stock solution were added.

^{31}P-NMR studies were performed on a Bruker WP 200 (operating at 81 MHz) or a WH 90 (operating at 36.4 MHz) Fourier transform spectrometer. Both

machines were equipped with temperature control and proton decoupling facilities. High-power proton decoupling was employed in all situations. Except where otherwise indicated, 91.0 MHz spectra were obtained from 1000 transients, employing a 90° radio frequency pulse and 0.8 s interpulse time with a sweep width of 20 kHz. 36.4 MHz spectra were taken employing a 15 kHz sweep width under similar conditions, giving results equivalent to those obtained at 81.0 MHz, but with reduced sensitivity. All free induction decays were exponentially filtered resulting in a 50 Hz line-broadening.

Results

^{31}P-NMR techniques are employed in this work to determine the macromolecular organization of phospholipids (for a review, see Ref. 8). Briefly, phospholipids in (large) bilayer structures give rise to asymmetric ^{31}P-NMR spectra with a low-field shoulder and high-field peak separated by approx. 40 ppm. Hexagonal (H_{II}) phase phospholipids, on the other hand, give rise to ^{31}P-NMR spectra with reversed asymmetry which are a factor of the two narrower ones. Finally, phospholipids in small bilayer structures where tumbling or lateral diffusion produces isotropic motional averaging give rise to narrow symmetric spectra. As noted in Discussion, similar symmetric spectra are obtained from phospholipids in non-bilayer arrangements such as inverted micellar [16] and would also be expected from cubic or rhombic [17] structures; which complicates interpretation of such lineshapes.

As suggested in Introduction, it was expected that fully hydrated rat liver mitochondrial phosphatidylethanolamine would prefer the hexagonal (H_{II}) arrangement at 37°C. That this is the case is illustrated in Fig. 1b. A transition from the bilayer to the hexagonal (H_{II}) phase for this phospholipid is observed in the region of 10°C (Fig. 1a). Rat liver mitochondrial phosphatidylcholine, on the other hand, exhibits 'bilayer' ^{31}P-NMR spectra as shown in the spectra of Fig. 1c obtained at 37°C.

Fig. 2a illustrates the spectral behaviour at 4 and 37°C of fully hydrated preparations of the total lipids extracted from the inner mitochondrial membrane. A large 'isotropic' component is observed which is superimposed on a broader 'bilayer' component. It may be noted that the amplitude of the narrow component grows on heating to 37°C, and that the bilayer spectrum has better definition at 4°C than at 37°C. As shown in Fig. 2b, the addition of Ca^{2+} (which results in precipitation of the lipid dispersion) causes marked changes in the spectral behaviour. In particular, a hexagonal (H_{II}) component is apparent, and the isotropic component is reduced in intensity. Higher concentrations of Ca^{2+} (and longer incubation times) result in a re-emergence of the narrow spectral component at the expense of both hexagonal (H_{II}) phase and bilayer phospholipids. The fraction of the ^{31}P-NMR signal with the lineshape typical of extended bilayers was 65, 73, 63 and 54% for Ca^{2+} to phospholipid ratios of 0, 0.2, 0.4 and 0.6, respectively.

As briefly indicated above, the origin of narrow isotropic ^{31}P-NMR spectral components is difficult to ascertain. However, in previous work [16], correlations have been observed between the occurrence of narrow spectral ^{31}P-NMR components and the detection of 'lipidic particles' [18] in freeze-fracture

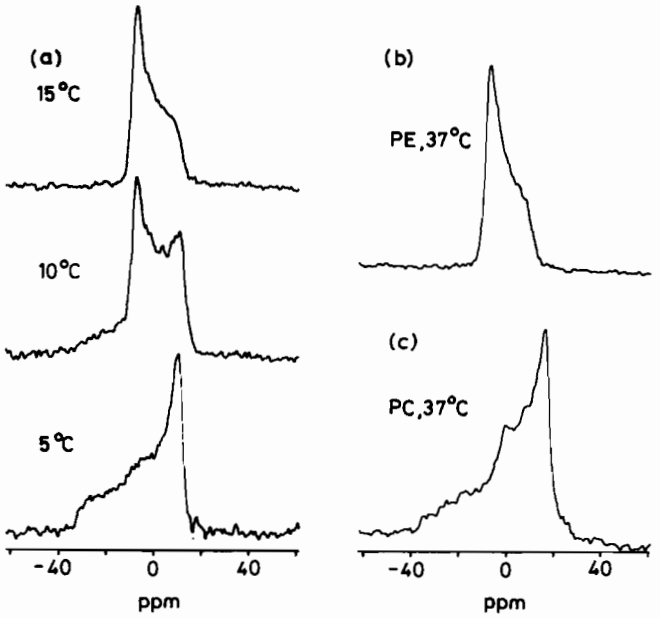

Fig. 1. 81.0 MHz proton decoupled ^{31}P-NMR spectra of: (a) hydrated rat liver mitochondrial phosphatidylethanolamine in the temperature interval 5—15°C; (b) rat liver mitochondrial phosphatidylethanolamine (PE) at 37°C; (c) hydrated rat liver mitochondrial phosphatidylcholine (PC) at 37°C. The buffer employed contained 100 mM NaCl, 10 mM Tris-acetic acid (pH 7.2) and 1 mM EDTA.

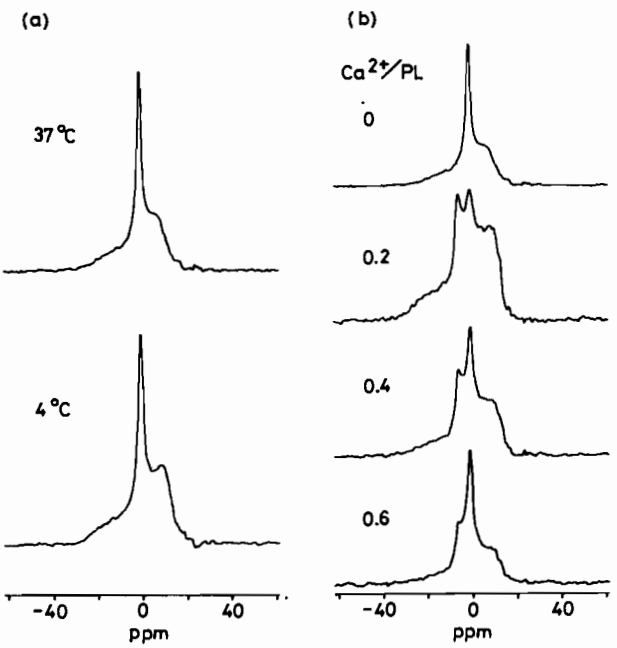

Fig. 2. 81.0 MHz ^{31}P-NMR proton decoupled spectra of the total extracted lipids obtained from rat liver inner mitochondrial membrane dispersed in excess aqueous buffer: (a) at 4 and 37°C; (b) in the presence of increasing amounts of Ca^{2+} at 37°C. The ratio, R = Ca^{2+}/PL, refers to the molar ratio of Ca^{2+} added to phospholipid (PL) present. Other conditions as for Fig. 1.

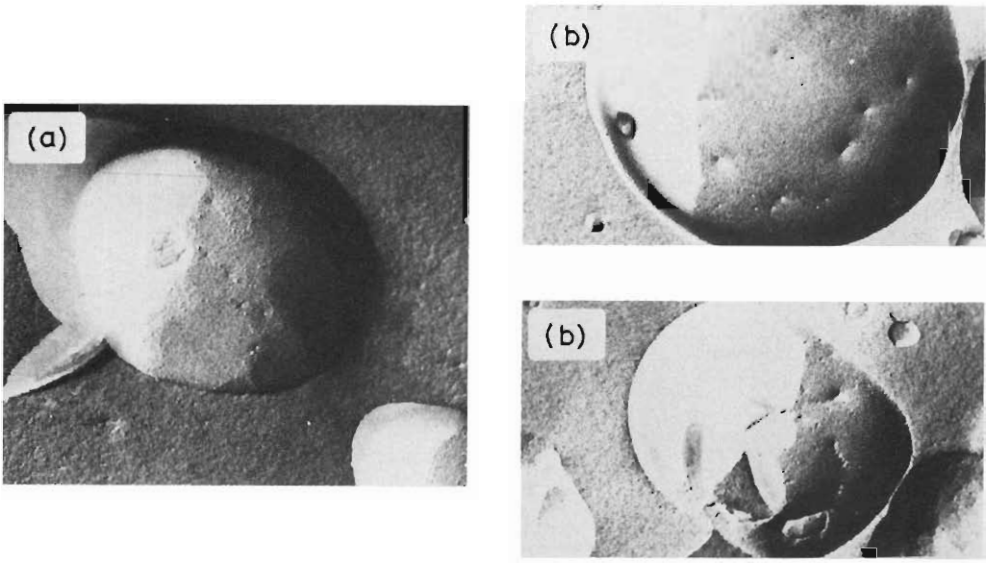

Fig. 3. Freese-fracture micrographs of: (a) hydrated total inner mitochondrial membrane lipids and (b) total inner mitochondrial lipids in the presence of Ca^{2+} (Ca^{2+}/phospholipid ratio 0.5). Quench temperature was 20°C. Magnification, X 53 720.

micrographs. As shown in Fig. 3, the freeze-fracture faces of total inner mitochondrial membrane lipids can exhibit similar features in the presence of Ca^{2+} (Fig. 3b). It should be noted that the absence of such particles when Ca^{2+} is not present (Fig. 3a) does not preclude the possibility that similar structures contribute to the narrow components of the spectra of Fig. 2a. For example, the freezing process may result in their removal.

The ^{31}P-NMR behaviour of inner mitochondrial membrane ghosts is illustrated in Fig. 4. Clearly, at both 4 and 37°C (Fig. 4a) the bulk (greater than 90%) of the phospholipid observed gives rise to the familiar bilayer spectrum. A small component at a position corresponding to isotropic motional averaging is also apparent. This membrane system is fairly stable, as incubation at 37°C for up to 2 h resulted in only a slight increase in the component experiencing more isotropic motion (results not shown). The progressive addition of Ca^{2+} (Fig. 4b) is observed to result in corresponding increase in isotropic motional averaging for the inner mitochondrial membrane phospholipids, as indicated by the increasing predominance of a broad symmetric peak at higher Ca^{2+} contents.

^{31}P-NMR spectra obtained from mitoplasts at 4°C were qualitatively similar to those obtained for intact rat liver mitochondria (see below). In particular, small metabolite (ADP, P_i etc.) ^{31}P-NMR resonances are superimposed on the broad asymmetric phospholipid spectrum. At 37°C, the behaviour is again similar to that of intact mitochondria, with strong increases in the amplitude of the resonance corresponding to P_i at longer (greater than 30 min) incubation times.

It has been previously noted [20] that endoplasmic reticulum microsomal membrane preparations are freely permeable to Mn^{2+} at 37°C. A similar situation is obtained with the mitoplasts, as addition of Mn^{2+} resulted in total

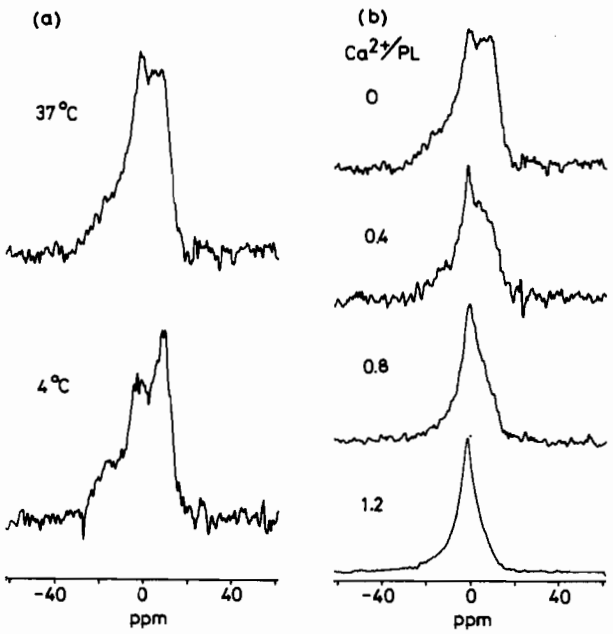

Fig. 4. 81.0 MHz proton decoupled ^{31}P-NMR spectra of isolated rat liver inner mitochondrial membrane: (a) at 4 and 37°C; (b) in the presence of various amounts of Ca^{2+} at 37°C, where the ratio R = Ca^{2+}/PL refers to the molar ratio of Ca^{2+} to endogeneous phospholipid (PL). For details of sample preparation see Materials and Methods.

elimination of the phospholipid and small molecule resonances (results not shown).

^{31}P-NMR spectra obtained at 4 and 37°C of intact rat liver mitochondria are illustrated in Fig. 5a. The 4°C spectrum consists of a strong phospholipid component with a well-defined low-field shoulder in the region of −20 ppm, characteristic of phospholipids in a liquid crystalline bilayer configuration. Superimposed on this broad bilayer spectrum are ^{31}P-NMR signals arising from small phosphorus-containing molecules, which can be assigned to sugar phosphates, P_i and ADP [19]. This system was stable for times of the order of hours at 4°C. At 37°C the major spectral components arise from sugar phosphate and P_i, with a broad underlying phospholipid component. Two features are apparent. The ADP resonances are depleted, and the inorganic phosphate peak is increased in amplitude relative to the situation at 4°C. This presumably arises due to ADP ⇌ ATP + AMP phosphate-exchange processes, with subsequent hydrolysis of the ATP by the endogenous inner mitochondrial ATPase. This ATPase is expected to be active, due to the uncoupled nature of the mitochondria at 37°C. This was reflected by the fact that incubation of these mitochondria at 37°C for 5 min or longer resulted in loss of respiratory control, reducing respiratory control ratios from more than 6 to less than 2. This may be compared to the minimum time to obtain a ^{31}P-NMR spectrum with an adequate signal-to-noise ratio, which was of the order of 10 min at 81.0 MHz.

In order to characterize the possible effects of uncoupling and biodegradation on the ^{31}P-NMR spectra obtained from intact anaerobic mitochondria, the

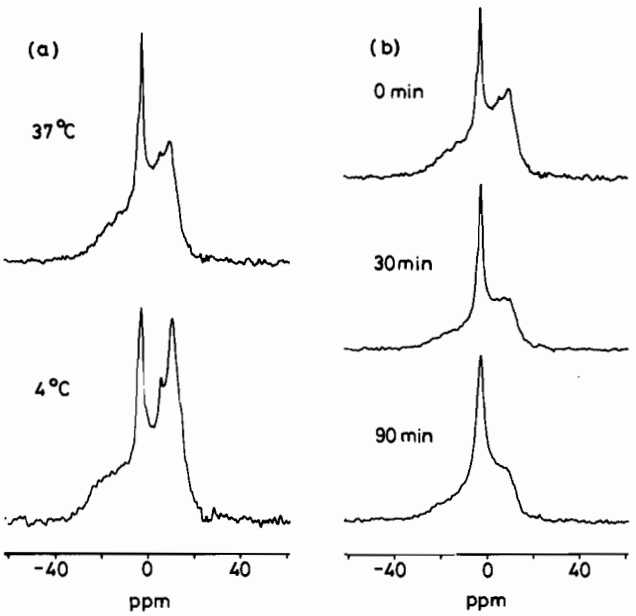

Fig. 5. 81.0 MHz ^{31}P-NMR proton decoupled spectra of intact rat liver mitochondria: (a) at 4 and 37°C and (b) as a function of incubation time at 37°C. For other details see Materials and Methods.

time course of the spectra at 37°C was investigated, as illustrated in Fig. 5b. Two effects are seen. First, the amplitude of the composite peak in the region of 0 ppm increases with time, continuing the trend noted in Fig. 5a. Second, more importantly, the underlying phospholipid signal is observed to undergo a significant change in lineshape, moving away from a shape consistent with bilayer structure towards a lineshape indicating strong isotropic motional averaging effects. This is reflected by the high-field phospholipid peak in the region of 10 ppm which becomes less distinct and is shifted to lower field as time goes on. Similar effects have been noted elsewhere for rat liver slices [20]. These results, when correlated with other biochemical data such as loss of respiratory control, emphasize the labile nature of such systems, and indicate that extreme caution must be taken before results obtained for long (greater than 5 min) incubation times at 37°C can be reliably related to the in vivo lipid organization.

Discussion

Before discussing the data presented in detail, it is worthwhile to point out some of the advantages and limitations of the ^{31}P-NMR technique as applied to studies of phospholipid organization. As we have pointed out elsewhere [8], a major advantage of the ^{31}P-NMR technique is that such studies may be applied, in a straightforward manner, to intact biological membranes and even membranes in intact tissue [21]. This is not true of other presently available techniques. Low-angle X-ray techniques, for example, require that membranes be organized in regular stacked arrays, which often necessitates partial dehydra-

tion of biological membranes with an associated risk of introducing artifacts. In addition, the long exposure times (commonly of the order of hours) required for structural determinations employing X-ray procedures clearly limit the applicability of this technique to labile systems such as mitochondria.

The major disadvantage of ^{31}P-NMR determinations of lipid organization is inherent in the indirect nature of the technique, as motional behaviour which is sensitive to the macroscopic lipid structures adopted is monitored, as opposed to determining the lipid structure directly. Unfortunately, in some cases a variety of potential lipid structures can give rise to similar NMR behaviour. This applies particularly to situations where isotropic motional averaging occurs which gives rise to symmetric spectral components. In such cases NMR techniques alone cannot discriminate between highly curved lamellar structures (i.e., small vesicular systems, or larger systems with an irregular topography containing regions with a small radius of curvature) where lateral diffusion can lead to isotropic averaging, and non-bilayer phases, as the source of this additional motion. This is a regrettable situation, as non-bilayer phospholipid phases occurring as intermediaries between lamellar and hexagonal (H_{II}) phases commonly exhibit such isotropic ^{31}P-NMR spectra [6,8,16] as do certain biological membranes [22,23]. On the basis of such correlations it is tempting to suggest that non-bilayer structures occur in these biological membranes, a conclusion which is not presently justifiable.

The most unambiguous results presented here apply to the behaviour of isolated mitochondrial phosphatidylethanolamine and phosphatidylcholine. The observation that mitochondrial phosphatidylethanolamine prefers the hexagonal (H_{II}) phase at physiological temperatures is in agreement with X-ray [23] and ^{31}P-NMR [8] studies on phosphatidylethanolamines from other sources. Similarly, the observation that endogenous phosphatidylcholine prefers the bilayer configuration is consistent with an affinity of this phospholipid for the bilayer phase, regardless of fatty acid composition [8].

The hydrated total lipids of mitochondria exhibit a large symmetric spectral component superimposed on a broader asymmetric component consistent with bilayer structure. The source of the isotropic motion giving rise to the narrow feature is not known. However, it has been noted that in other model systems consisting of mixtures of bilayer and hexagonal (H_{II}) lipids, an isotropic ^{31}P-NMR component is often accompanied by the appearance of lipidic particles in the freeze-fracture faces [16]. This suggests that phospholpid in these small intrabilayer structures (possibly inverted micelles) contributes to the narrow spectral component. This does not appear to be the case for the total inner mitochondrial membrane lipids, as smooth fracture faces are observed (Fig. 3a). However, it is possible that the freezing process may result in the removal of such structures, which would be consistent with the observation that a freeze-thaw cycle (at -20°C) resulted in the disappearance of the narrow ^{31}P-NMR component at 4°C.

The observation of H_{II} phase formation on addition of Ca^{2+} is consistent with the ability of Ca^{2+} to trigger hexagonal (H_{II}) phase formation in (bilayer) beef heart cardiolipin dispersions [5]. In the presence of Ca^{2+}, the cardiolipin component of the total inner mitochondrial lipids will therefore become an H_{II}-preferring species, in addition to the phosphatidylethanolamine. The iso-

tropic component can be attributed, as least in part, to the occurrence of lipidic particles in the freeze-fracture faces. It may be noted that such particles and corresponding narrow ^{31}P-NMR spectral features are observed in cardiolipin-phosphatidylcholine systems in the presence of Ca^{2+} [6,16]. The gradual increase in the narrow component at higher Ca^{2+} concentrations is not presently understood. It could arise from increased formation of lipidic particles, or the formation of smaller lamellar structures. One point which must be emphasized is that the amount of lipid contributing to the isotropic NMR component is larger than the amount of lipid in lipidic particle structure detected by freeze-fracture. Again, this could arise from a reduction in the number of particles during freezing as well as alternative sources of motional averaging.

The results obtained on the intact mitochondria and the inner mitochondrial membrane illustrate both the power of the NMR technique as well as the limitations. First, at 4°C, the results obtained are consistent with the vast majority of the phospholipid detected being in a liquid crystalline lamellar phase. Second, at higher temperatures (37°C) it appears that the labile nature of these systems, which is reflected by the very rapid loss of respiratory control, is also manifested by a change in the ^{31}P-NMR spectrum of the phospholipids which exhibit time-dependent changes consistent with increased isotropic motion. Whether these changes could be related to an increased membrane permeability to protons (uncoupling) is an intriguing, albeit speculative, possibility that is currently under investigation.

With regard to results obtained for the isolated inner mitochondrial membranes, there are three points of interest. Firstly, the ^{31}P-NMR technique demonstrates that the vast majority (greater than 90%) of the phospholipids detected exhibit motional characteristics consistent with a liquid crystalline bilayer structure. The importance of this observation is implicit in the fact that, to our knowledge, there is only one other study in the literature demonstrating the presence of extended bilayers in the inner mitochondrial membrane. This is the freeze-fracture work of Hackenbrock [25] on inner mitochondrial membranes incubated at −10°C.

Secondly, the observation of a largely bilayer structure for the inner mitochondrial membrane is potentially inconsistent with the observed preference for a large proportion of the total isolated lipids for structures exhibiting isotropic motional averaging, and the preference of the endogenous phosphatidylethanolamine for the H_{II} phase. It is therefore certainly possible that some proteins play structural bilayer-stabilizing roles, as has been suggested for rod outer segment membranes [26]. This possibility is reinforced by recent studies [27] indicating an asymmetric distribution of phospholipids across the inner mitochondrial membrane, with an enrichment of phosphatidylethanolamine and cardiolipin in the inner monolayer. It is interesting to note that the enhanced instability of the inner monolayer this suggests, particularly when Ca^{2+} is present, is similar to behaviour noted for the erythrocyte membrane [28].

Thirdly, the influence of Ca^{2+} on the ^{31}P-NMR spectra obtained from the inner membrane is of interest with regard to the ability of mitochondria to actively accumulate Ca^{2+} [7] and the uncoupling action of high concentrations of Ca^{2+} [29] (in this regard, the permeability of the mitoplasts to Mn^{2+} could arise due to active or facilitated transport, as Mn^{2+} is also accumulated by

mitochondria [29]). Whether the growth of an isotropic NMR component in the inner membrane plus Ca^{2+} ^{31}P-NMR spectra can be attributed to non-bilayer structures such as lipidic particles is clearly debatable. However, the occurrence of such structures in the corresponding isolated lipid preparations is not inconsistent with the proposal. It is of interest, in this regard, that lipidic particles interpreted as inverted intra-bilayer micelles can be logically proposed to facilitate trans-membrane transport processes [31]. Finally, the uncoupling action of high concentrations of Ca^{2+} on mitochondria, in association with the loss of a permeability barrier [29], is also consistent with a Ca^{2+}-induced instability in the bilayer arrangement of endogenous lipids which this study suggests.

Acknowledgements

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