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EFFECTS OF PHOSPHOLIPIDS IN LIVER MITOCHONDRIA OSMOTIC PROPERTIES AND BINDING OF CATIONS

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SUMMARY

1. The function of phospholipid in determining the permeability and the cation-binding properties of the mitochondrial membrane has been investigated.

2. Mitochondria depleted of phospholipid by treatment in acetone, water and NH_3 show a loss of their osmotic characteristics. When the phospholipid content is restored, the osmotic behaviour of the mitochondria reappears both in ionic and in sucrose media. However, the reconstituted mitochondria cannot be rendered ion permeable by treatment with valinomycin (or gramicidin) at alkaline pH.

3. Depletion of phospholipids causes a loss of the capacity of mitochondria to bind cations. The binding of cations is restored together with the restoration of the phospholipid content.

4. Among the phospholipids tested, phosphatidyl ethanolamine is the most effective in restoring the osmotic properties as well as the cation-binding capacity of mitochondria.

INTRODUCTION

Several mitochondrial enzymes and also the electron carriers have been shown to have a phospholipid requirement for maximal activity (for a review, *cf.* ref. 1). However, the role played by the phospholipid in stimulating the enzyme activities is still matter of conjecture.

Following another line of thought, much interest has been devoted to understanding the role of phospholipids in establishing the physical properties of artificial and natural membranes.

The diffusion of ions[2–4] and the binding of cations[5–8] have been widely investigated in artificial membranes.

In the present paper, attention has been focused on two aspects of the role of phospholipids in the mitochondrial membrane: the diffusion of ions and the binding of cations. It will be shown that depletion of the phospholipid causes a loss of the mitochondrial membrane properties of semipermeability and capacity to bind Ca^{2+}. Both properties reappear when the phospholipid content of the mitochondria is restored. Among the phospholipids present in mitochondria, phosphatidyl ethanol-

Abbreviation: EGTA, ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N'*-tetraacetic acid.

amine is more effective than other phospholipid in restoring the osmotic as well as the ion-binding property.

A preliminary report of the present work has already been presented[9].

METHODS

Rat-liver mitochondria prepared in 0.25 M sucrose, 5 mM Tris–HCl (pH 7.5), and 0.5 mM ethyleneglycol-bis-(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) were used throughout. EGTA was omitted in the final suspension of mitochondria.

Phospholipid-depleted mitochondria were prepared according to the method of FLEISCHER, FLEISCHER AND STOECKENIUS[10], using a mixture of water–acetone (90:10, v/v), and NH_3 at 0°.

The phospholipid content of the depleted mitochondria was restored by addition of commercial phospholipid preparations suspended in water by sonication under N_2 and in the presence of 0.5 mM EDTA to minimize lipid peroxidation. After sonication, the phospholipid microdispersion was centrifuged for 30 min at 150000 × *g* in order to remove large phospholipid particles, and only the supernatant was used for the reconstitution. Phospholipid-depleted mitochondria corresponding to 20 mg mitochondrial protein, were incubated at 0° in a medium containing in 2 ml: 0.25 M sucrose, 10 mM Tris–HCl (pH 7.4), and 50 mg phospholipid; after an incubation time of 10 min, the mitochondria were sedimented at 20000 × *g* and washed twice with the sucrose–Tris medium at 0°, in order to remove the unbound phospholipid present in the supernatant.

The total phosporus content of mitochondria was determined, after ashing the samples with concentrated H_2SO_4, by the isobutanol–benzene extraction procedure as proposed by LINDBERG AND ERNSTER[11].

Binding experiments were carried out in 2 ml of medium at 0°, and the added mitochondria (about 1 mg protein/ml) were preincubated for 5 min in the presence of antimycin and rotenone. After an incubation of 5 min in the presence of the radioactive ion (specific activity $^{45}Ca^{2+}$, 45000 counts/min per μmole), the mitochondria were sedimented at 20000 × *g* for 10 min. The pellets were washed twice in cold sucrose, dried, and solubilized in 1 M formic acid; aliquots of the formic acid solution were analysed for radioactivity. The amount of radioactivity found in the pellet was corrected for the radioactivity present in the extramitochondrial space, determined with [^{14}C]dextran-carboxyl.

The movement of water was followed either with photometric or gravimetric measurements. In the former case, the change in absorbance was followed with an Eppendorf photometer at 546 nm. In the latter case, the water content of mitochondria was calculated by subtracting from the centrifuged pellet weight, the dry weight (obtained in vacuum over P_2O_5) and the [^{14}C]dextran space.

Spectrophotometric measurements were carried out at room temperature with a split-beam spectrophotometer built in the workshop of the Johnson Foundation, University of Pennsylvania (Philadelphia, Pa., U.S.A.). The difference spectrum, reduced *minus* oxidized, was recorded in the region between 400 and 650 nm. The reduced state of the respiratory carriers was obtained by the addition of sodium dithionite.

All chemicals and solvents used were of reagent grade. DL-α-Lecithin (β,γ-

dipalmitoyl-DL-α-lecithin) and L-α-lecithin (β,γ-dipalmitoyl-L-α-lecithin) were purchased from Sigma Chemical (St. Louis, Mo., U.S.A.); high-purity cholesterol S.C.W., lecithins (egg, bovine and soy), phosphatidyl ethanolamine (α-amino N: <0.02; iodine N: 78), phosphatidyl inositol (α-amino N: 0.1; inorganic P: <0.05), cardiolipin, and cholic acid were from the Nutritional Biochemical (Cleveland, Ohio, U.S.A.). Synthetic phosphatidyl ethanolamines (β,γ-dipalmitoyl-L-α-cephalin and β,γ-dipalmitoyl-DL-α-cephalin) were from Mann Res. Lab. (New York, N.Y., U.S.A.). $^{45}Ca^{2+}$ was obtained from The Radiochemical Centre (Amersham, England), and [^{14}C]dextran-carboxyl (mol. wt. 15000–17000) from New England Nuclear (Boston, Mass., U.S.A.).

Protein was measured by the biuret method.

RESULTS AND DISCUSSION

Difference spectra of phospholipid-depleted mitochondria

Recently FLEISCHER, FLEISCHER AND STOECKENIUS[10] reported that treatment of mitochondria with acetone resulted in an almost complete depletion of their phospholipid, while the electron micrographs revealed no changes in the mitochondrial structure. Since this technique can be a useful means of analysing the effect of phospholipids on the mitochondrial properties, a further characterization of the phospholipid-depleted mitochondria may be necessary. In Fig. 1 are reported the difference spectra (reduced *minus* oxidized) of untreated and phospholipid-depleted mitochondria. The major differences reside in the absorption bands of cytochromes *a* and a_3. The γ-band of cytochrome a_3 in the Soret region was absent in the depleted mitochondria. The α-band of cytochrome *a* at 605 nm was also absent and was replaced by another band absorbing at 584 nm, which is presumably an altered form of cytochrome *a*. On the other hand the extinction of the α- and γ-bands of the cytochromes *b*, *c*, and c_1 were higher, as compared per mg mitochondrial protein, in the depleted than in the normal mitochondria. This is presumably due to loss of matrix proteins during depletion.

From the experiment of Fig. 1, we conclude that the phospholipid-depleted mitochondria retain not only their unit membrane structure but also their content of electron carriers except for cytochromes *a* and a_3, which may be present in a modified form. This is in agreement with the observations of FLEISCHER, FLEISCHER AND STOECKENIUS[10] on the restoration of electron transport by rebinding of phospholipids.

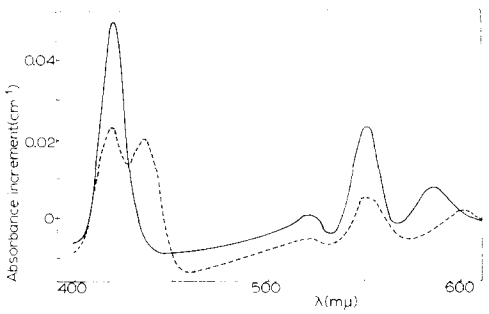

Fig. 1. Difference spectra of untreated and lipid-depleted liver mitochondria. 7 mg of mitochondrial protein, either untreated (·····) or phospholipid depleted (——), were added to a medium containing 0.25 M sucrose and 5 mM Tris–HCl (pH 7.5). The difference spectra (reduced *minus* oxidized) were obtained with the addition of $Na_2S_2O_4$ in one sample. Final volume 3 ml. Temp., 24°.

In view of the minor alterations of the mitochondrial structure, it would seem that this treatment lends itself to the investigation of the specific effects related to the presence of phospholipids.

The osmotic properties of mitochondria

Isolated mitochondria behave as perfect osmometers, when suspended in sucrose or in KCl (refs. 12–14). Therefore when the weights of the mitochondrial pellets after centrifugation are plotted against the reciprocal of the osmolarity, a straight line is obtained with the intercept giving the nonosmotic volume. Since the absorbance of a mitochondrial suspension is proportional to the water content of the mitochondria, the weight of the pellets can be replaced by the reciprocal of the absorbance. The values reported in the abscissae of Figs. 2 and 3 are the reciprocals of the differences in absorbance induced by the addition of increasing amounts of KCl. The Δabsorbance was calculated in respect to the value at the lowest KCl concentration.

In separate experiments, however, it was established that the correspondence between absorbance and gravimetric measurements did occur under our experimental conditions.

When the permeability of isolated mitochondria was increased to both K^+ (by the addition of valinomycin) and Cl^- (by the alkalinization of the medium), the osmotic behaviour was lost (Fig. 2A). Fig. 2B shows that phospholipid-depleted mitochondria did lose their osmotic properties in a way similar to that of mitochondria treated with valinomycin at pH 8.8. However, when the phospholipid content of the mitochondria was restored, by readding the phospholipids, as described in METHODS, the osmotic behaviour of the mitochondria also reappeared, Fig. 2C. Thus KCl was again an impermeant solute, and the osmotic volume of the mitochondria decreased

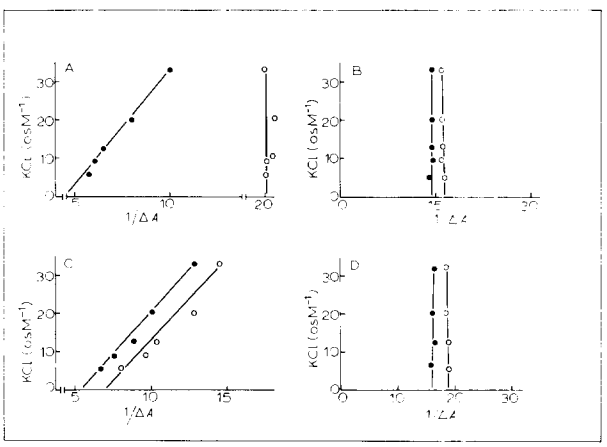

Fig. 2. The effect of varying KCl concentrations on the absorbance of untreated mitochondria (A), lipid-depleted mitochondria (B), phospholipid-restored mitochondria (C), and microdispersion of phospholipid (D). Experimental conditions were as follows: mitochondria (1.5 mg protein), lipid-depleted mitochondria (1.7 mg protein), phospholipid-restored mitochondria (1.3 mg protein) and 8 mg of phospholipids microdispersed in water were added to a medium containing 5 mM Tris–HCl (pH 7.4) (●—●) or 5 mM Tris–HCl (pH 9), 1 μg valinomycin (○—○), 3 μM rotenone, and the KCl concentrations indicated in the ordinates. The mixture of phospholipid used in C and D had the following composition (w/w): 30% phosphatidyl ethanolamine, 30% cardiolipin, 35% lecithin, and 5% phosphatidyl inositol. Final volume, 2 ml. Temp., 24°.

in proportion to the increase of the KCl concentration in the medium. However, in contrast to the intact mitochondria, it was not possible to abolish the osmotic behaviour of the reconstituted mitochondria by the addition of valinomycin or increase of pH. Similar results were obtained in the presence of gramicidin and NaCl; osmotic behaviour was also observed in sucrose media.

In view of the results obtained with phospholipid micelles[15], the question may by raised as to whether the phospholipids need to be bound to mitochondrial ghosts in order to give rise to vesicles capable of osmotic behaviour. This suggestion, however is not supported by the experiment reported in Fig. 2D; in the absence of mitochondrial ghosts, the increase of the KCl concentration in the medium was unable to cause an increase in absorbance of the phospholipid microdispersed in water. Also, osmotic vesicles were not obtained when the phospholipids were bound to serum albumin.

According to FLEISCHER *et al.*[16] the main phospholipid constituents of the mitochondrial membrane are lecithin, 43.4 %, phosphatidyl ethanolamine, 34.5 %, and cardiolipin, 17.2 %. Minor components, including phosphatidyl inositol, make up 4.9 %. In the experiment reported in Fig. 3, the above-mentioned phospholipids were tested for their individual ability to restore the osmotic properties of the phospholipid-depleted mitochondria. The amount of phospholipid rebound to the mitochondria was

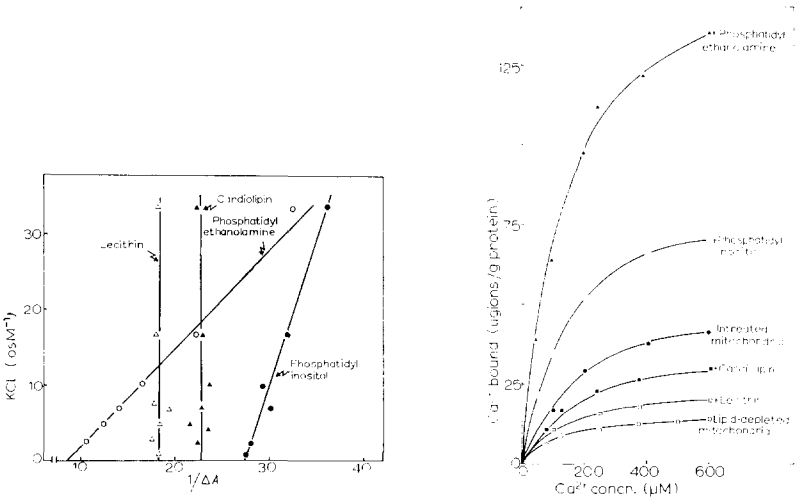

Fig. 3. Dependence of the osmotic behaviour of the reconstituted mitochondria on the phospholipid composition. Experimental conditions were as in Fig. 2C except that instead of a mixture, the individual phospholipids indicated in the figure were rebound to the mitochondrial membrane. The P bound with the various phospholipids was about the same (± 20 %). The amount of mitochondrial protein was: 1.7, 1.8, 2 and 2.1 mg with phosphatidyl ethanolamine, cardiolipin, lecithins, and phosphatidyl inositol, respectively. Final volume, 2 ml. Temp., 20°.

Fig. 4. Relationship between Ca^{2+} binding and phospholipid composition of mitochondria. Experimental conditions: extraction and rebinding of phospholipid was carried out as described in METHODS. The restoration of the phospholipid content was obtained using the individual phospholipids indicated in the figure. The amount of phospholipid in each preparation was similar to that described in Table I. Ca^{2+} binding was measured in a 0.25 M sucrose medium containing 5 mM Tris–HCl (pH 7.4), 5 μM rotenone, 1 μg antimycin A, and the Ca^{2+} concentrations as reported in the abscissa. Protein concentrations were: untreated mitochondria, 3.1 mg; lipid-depleted mitochondria, 2.8 mg; phospholipid restored with phosphatidyl ethanolamine, 3 mg; restored with lecithin, 3.4 mg; restored with cardiolipin, 2.8 mg; and restored with phosphatidyl inositol, 2.4 mg. Final volume, 2 ml. Temp., 4°.

about the same for the various phospholipids. When cardiolipin was used for the reconstitution of the membrane, the permeability to ions was not restored. Similar results were also obtained with lecithins from various sources. On the other hand, addition of phosphatidyl inositol, and to an even greater extent of phosphatidyl ethanolamine was largely able to restore the impermeability of the membrane and thus the osmotic properties of the mitochondria. The experiments reported in Figs. 2 and 3 have been carried out with phospholipids prepared from various sources (*cf.* the list of phospholipids reported in METHODS). However, their capacity to restore the osmotic properties of the mitochondria when tested either as a mixture of various phospholipids or individually was not dependent on the source of the material.

The binding of cations

Another essential feature of artificial and natural membranes is the capacity to bind cations. According to recent reports, this property appears to be mainly dependent on the presence of phospholipids[5–8]. In regard to mitochondrial membranes, data have been reported concerning the binding of Ca^{2+} in various mitochondrial spaces[17–18], the interaction among various cations in the binding process[19], the role of the surface binding on the process of aerobic Ca^{2+} translocation[20]. Furthermore the anionic sites to which Ca^{2+} becomes bound under anaerobic conditions have been shown to be phospholipid[19].

Further characterization of the role of phospholipid in cation binding is reported below. Fig. 4 shows that depletion of phospholipid resulted in a decrease of about 80 % in the mitochondrial capacity to bind Ca^{2+}. When cardiolipin or lecithin was rebound to the mitochondria, there was a slight increase in the amount of Ca^{2+} bound. Mitochondria reconstituted with phosphatidyl inositol, on the other hand, were able to bind an amount of Ca^{2+} 50 % higher than normal mitochondria, whereas the amount of Ca^{2+} bound was 3 times higher in the case of phosphatidyl ethanolamine.

In Table I the amount of Ca^{2+} bound per g protein is related to the amount of phospholipids present in the membrane. It is seen that treatment with acetone decreases the P content of mitochondria from 524 to 61 μgatoms/g protein, whereas the addition of individual phospholipids brought the P content to about 60 % of the original values. This was the maximum value of phospholipid P which could be bound

TABLE I

RELATIONSHIP BETWEEN PHOSPHOLIPID CONTENT AND Ca^{2+} BINDING

Experimental conditions as in Fig. 4 except for the Ca^{2+} concentration which was 200 μM. The P values indicate the total phosphorus *minus* a certain amount of phosphorus (68 μgatoms/g protein) which is not extractable with chloroform–methanol (2:1, v/v) and is probably not lipid phosporus.

Sample	*Ca^{2+} bound (μgions/g protein)*	*Phosphorus bound (μgatoms/g protein)*	*Ca^{2+}/P ratio*
Untreated mitochondria	24	513	0.047
Lipid-depleted mitochondria	4	74	0.054
Mitochondria reconstituted			
with phosphatidyl ethanolamine	81	352	0.230
with lecithin	15.6	271	0.058
with cardiolipin	19.2	229	0.083
with phosphatidyl inositol	34	300	0.110

to lipid-depleted mitochondria under these conditions. In fact reconstitution experiments in the presence of increasing amounts of phospholipid indicated that this value was an expression of saturation of mitochondrial binding sites by phospholipid.

The Ca^{2+} bound was only 4 μgions/g protein in the depleted mitochondria, but increased to 81 μgions/g protein in the mitochondria restored with phosphatidyl ethanolamine. The Ca^{2+}/P ratio was about 0.05 in the untreated and depleted mitochondria; it remained between 0.058 and 0.083 in the mitochondria restored with lecithin and cardiolipin; it rose to 0.11 and 0.23 in the mitochondria restored with phosphatidyl inositol and phosphatidyl ethanolamine, respectively.

The Ca^{2+} binding analyzed above was independent of metabolism and occurred in the presence of rotenone and antimycin A; it therefore differs from the energy-dependent accumulation of Ca^{2+}. However, a close relationship between nonenergy-dependent binding of Ca^{2+} and energy-dependent translocation of Ca^{2+} into mitochondria has been shown elsewhere[20].

Several arguments can be raised in support of the suggestion that the effect of phospholipids on the binding of cations reported in the present study is related to the same mechanism of cation binding by untreated, fresh liver mitochondria. First, other anions which could also be bound to phospholipid-depleted mitochondria, such as cholate, did not cause an increase of Ca^{2+} binding. Second, it has been shown that the binding of Ca^{2+} to untreated liver mitochondria and submitochondrial particles can be inhibited by local anaesthetics and univalent cations[19]. Similar results were obtained with the phospholipid-reconstituted mitochondria. Third, the K_m for Ca^{2+} binding was about 100 μM for untreated and phospholipid-reconstituted mitochondria.

From the data presented, it appears that the phospholipids play an important role in determining some physical properties of the mitochondria, *e.g.*, the rates of ion diffusion and the binding of cations. Among the effects of the various phospholipids, those of phosphatidyl inositol, are perhaps of minor relevance due to its low concentration in mitochondrial membranes.

On the other hand, phosphatidyl ethanolamine, which is a major component of mitochondrial phospholipids, was also the most efficient in causing the restoration of osmotic and ion-binding properties of the mitochondria.

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REFERENCES

1 L. M. VAN DEENEN AND G. H. DE HAAS, *Ann. Rev. Biochem.*, 35 (1966) 157.
2 P. MULLER, D. O. RUDIN, H. TI TIEN AND W. C. WESCOTT, *Nature*, 194 (1962) 979.
3 T. E. THOMPSON, in E. LOCKE, *Cellular Membranes in Development*, Academic Press, New York, 1964, p. 83.
4 A. D. BANGHAM, M. N. STANDISH AND J. C. WATKINS, *J. Mol. Biol.*, 13 (1965) 238.
5 M. B. ABRAMSON, R. KATZMAN AND H. P. GREGOR, *J. Biol. Chem.*, 239 (1964) 70.
6 H. S. HENDRICKSON AND J. G. FULLINGTON, *Biochemistry*, 4 (1965) 1599.
7 D. O. SHAH AND J. H. SCHULMAN, *J. Lipid Res.*, 6 (1965) 341.
8 E. ROJAS AND J. M. TOBIAS, *Biochim. Biophys. Acta*, 94 (1965) 394.

9 A. SCARPA AND G. F. AZZONE, *Abstr. Meeting Federation European Biochem. Soc., Prague, 1968*, p. 85.
10 S. FLEISCHER, B. FLEISCHER AND W. STOECKENIUS, *J. Cell Biol.*, 32 (1967) 193.
11 O. LINDBERG AND L. ERNSTER, *Methods Biochem. Anal.*, 3 (1957) 1.
12 K. W. CLELAND, *Nature*, 170 (1952) 497.
13 J. RAAFLAUD, *Helv. Physiol. Pharmacol. Acta*, 11 (1953) 142.
14 H. TEDESCHI AND D. L. HARRIS, *Arch. Biochem. Biophys.*, 58 (1955) 52.
15 A. D. BANGHAM, J. DE GIER AND G. D. GREVILLE, *Chem. Phys. Lipids*, 1 (1967) 225.
16 S. FLEISCHER, G. ROUSER, B. FLEISCHER, A. CASU AND G. KRITCHEVSKY, *J. Lipid Res.*, 8 (1967) 170.
17 J. B. CHAPPELL, M. COHN AND G. D. GREVILLE, in B. CHANCE, *Energy-linked Function of Mitochondria*, Academic Press, New York, 1963, p. 219.
18 C. ROSSI, A. AZZI AND G. F. AZZONE, *J. Biol. Chem.*, 242 (1967) 951.
19 A. SCARPA AND A. AZZI, *Biochim. Biophys. Acta*, 150 (1968) 473.
20 A. SCARPA AND G. F. AZZONE, *J. Biol. Chem.*, 243 (1968) 5132.