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The Relation of Phospholipid and Membrane Structure in Mitochondrial Electron Transport Particles

Ronald Berezney, Y. C. Awasthi, Linda K. Funk, and F. L. Crane

Department of Biological Sciences,
Purdue University, Lafayette, Indiana 47907

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Introduction

In the past, attempts have been made to study the changes in membrane structure and function upon removal of phospholipids by different procedures. Fleischer *et al.*[1] have studied the effect of acetone extractions, involving varying degrees of lipid depletions, on the structure and the electron transport system of intact mitochondria as well as submitochondrial particles. Though the structural changes observed during these treatments can be attributed to the removal of lipids, changes occurring due to the effect of organic solvents cannot be ruled out. Thus a milder approach for removal of lipids has always been called for. Digestion of mitochondrial and other subcellular membranes with phospholipase A has been reported frequently.[2–4] These digestions have been aimed at removal of lipids,[5] separation of outer and inner membrane of mitochondria,[6] and also for the differential extraction of certain membrane bound enzymes.[2–4] Petrushka *et al.* have reported that venom phospholipase A treatment causes drastic structural changes in mitochondria.[7]

In these previous studies, however, no analysis was made of the individual phospholipids. With this in mind, we have begun a systematic analysis of the correlation between sequential breakdown of individual membrane phospholipids and the subsequent removal of the resultant lysocompounds on the structure and function of electron transport particles (ETP).

We have previously reported the action of phospholipase A on ETP membrane structure and the release of NADH dehydrogenase.[8,9] The present study attempts to correlate specific phospholipid hydrolysis and removal of the lysophosphatides with changes in the structure of ETP membrane.

Materials and Methods

Materials

Beef heart mitochondria (BHM) were prepared according to the method of Löw and Vallin.[10] Electron transport particles (ETP) were obtained by sonic disruption of mitochondria suspended in 0·25 M sucrose 0·05 Tris-chloride pH 7·4 at a protein concentration of 30 mg/ml. Sonic disruption was accomplished with a Branson sonifier set at 6–7 A for two 3-min intervals in a vessel submerged in a salt ice bath. The supernatant obtained by centrifugation at 27,000 × *g* for 15 min was centrifuged at 105,000 × *g* for

45 min. The pellet (ETP) was washed twice with 0·25 M sucrose 0·05 M Tris chloride pH 7·4 by homogenization with a glass homogenizer fitted with a teflon pestle and centrifugation at 105,000 × *g* for 45 min.

Phospholipase A was purified from *N. naja* venom according to the method of Cremona and Kearney.[11] *N. naja* venom and BSA (Type F) were purchased from Sigma Chemical Co.

Methods

Phospholipids were extracted by the method of Folch *et al.*[12] as modified by Rouser and Fleischer.[13] The extent of phospholipid hydrolysis was determined by quantitative TLC of the lipid extracts on double thickness (0·5 mm) silica gel-G plates in the solvent systems $CHCl_3$:MeOH:H_2O::100:40:6 and $CHCl_3$:MeOH:7N.NH_4OH::65:30:4. Phosphorus was estimated on well resolved spots by the method of Chen *et al.*[14] The average phosphorus content of phospholipid was taken to be 4%. Protein estimations were done according to the modified Biuret procedure of Yonetani[15] or according to Lowry *et al.*[16] Phospholipase A digestions were carried out in sucrose 0·25 M Tris-HCl 0·01 M buffer pH 7·4 at 30° for 90 min. Washing with BSA was carried out by homogenizing the ETP pellet with 1% BSA in sucrose 0·25 M Tris-HCl 0·01 M pH 7·4 and centrifuging the pellet at 122,000 *g* for 20 min. Phospholipid micelles were prepared according to methods reported earlier.[9]

In preparation for electron microscopy, samples were fixed in the cold with 2·5% formaldehyde, buffered with 0·1 M phosphate pH 7·4 and post-fixed in 1% O_sO_4 in an acetate-veronal buffer. After dehydration in acetone, material was embedded in Epon based on a procedure by Luft.[17] Ultrathin sections were post-stained with 0·25% lead citrate in 0·1 N NaOH for 3 min. Samples were also prepared by negatively staining with 2% phosphotungstate, pH 7·0, according to the method of Cunningham and Crane.[18] Specimens were observed and photographed with a Philips EM 200 or EM 300.

Results

The Selective Digestion of ETP Phospholipids by Phospholipase A

Previously, this laboratory has reported that purified *Naja naja* venom phospholipase A digests ETP membrane phospholipids in the order: phosphatidyl ethanolamine (PE), phosphatidyl choline (PC) and phosphatidyl diglycerol (PDG) (cardiolipin).[8,9] By digesting with low levels of phospholipase A (0·05 μg/mg ETP protein), most of the phosphatidyl ethanolamine and phosphatidyl choline are hydrolyzed to their respective lysophosphatides without any effect on cardiolipin (Table I). Digestion with higher levels of phospholipase A (0·1–12 μg/mg ETP protein) causes increasing hydrolysis of cardiolipin to a maximum of approximately 90% (Table I). The state of the membrane phospholipid can therefore be differentially modified according to the amount of phospholipase A used in digestion.

Table I defines four compositional states of the phospholipid in the membrane according to the specific hydrolysis of the phospholipids, and the subsequent removal of the lysophosphatides by BSA washings.

In the partial lyso state, both phosphatidyl ethanolamine and phosphatidyl choline are essentially converted to lysocompounds, whereas cardiolipin is unaffected. The

TABLE I. Phospholipid compositional states in ETP membranes

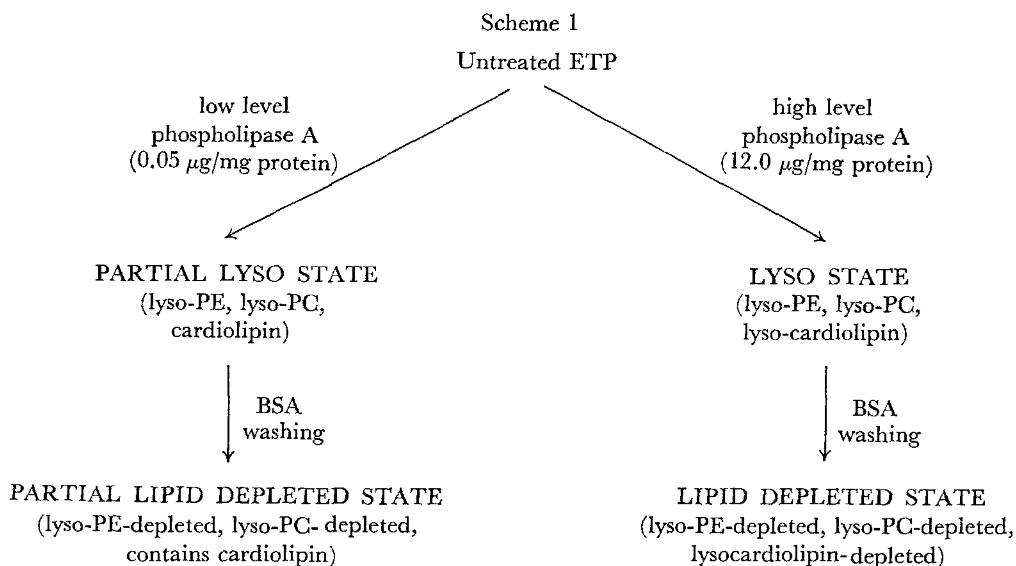
Phospholipid compositional state	Treatment of the membrane	% Individual phospholipid converted to lysophosphatides			% Lysophosphatides* removed from membrane
		PC	PE	DPG	
Partial lyso state	Digestions† with low levels (0·05/μg/mg protein) of phospholipase A	68	98	0	2·7
Lyso state	Digestions with higher levels of phospholipase A (0·10–12 μg/mg ETP protein)	100	100	25–90	5–14
Partial lipid depleted state	Partial lyso state followed by BSA washing‡ to remove lysophosphatides	—	—	—	48
Lipid depleted state	Lyso state followed by BSA washing to remove lysophosphatides	—	—	—	60–70

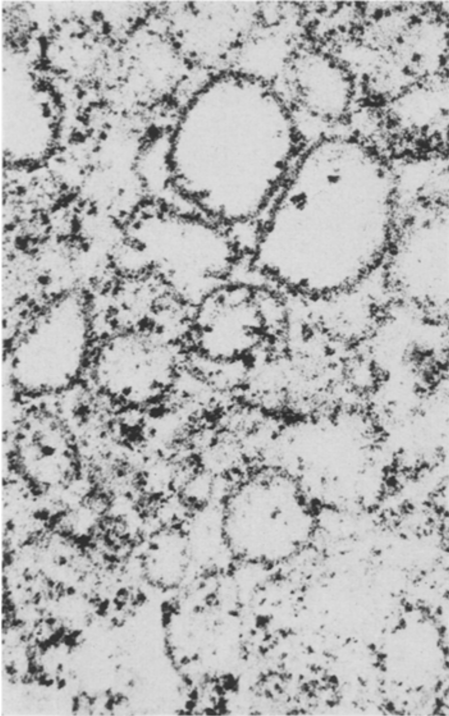
* Removed in soluble form in the supernatant obtained by centrifuging the digest at 122,000 × g for 30 min.
† Digested at 30°, in sucrose (0·25 M) Tris-HCl (0·01 M) pH 7·4 for 90 min.
‡ One percent BSA in sucrose (0·25 M) Tris-HCl (0·01) M pH 7·4.

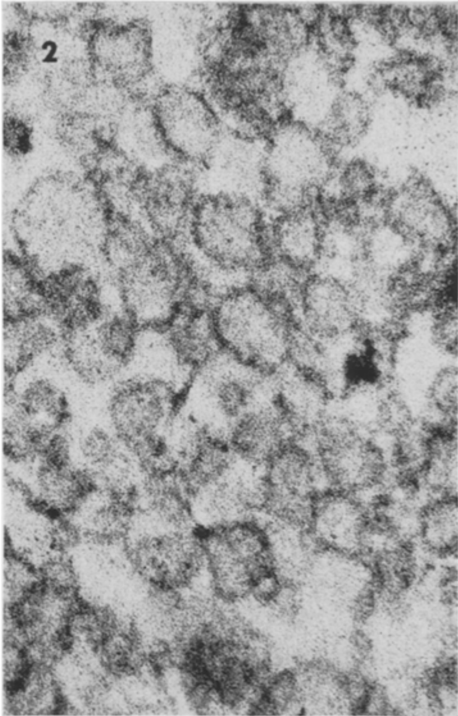
partial lyso state is therefore defined as a lyso-PE, lyso-PC, DPG (cardiolipin) state. A lyso state is created by use of higher levels of phospholipase A, which for these studies represents a level of 12 μg/mg ETP protein. The lyso state may be described as the nearly total hydrolyzed state in which all three components are largely converted to their lysophosphatides (lyso-PE, lyso-PC, lyso-DPG).

The lysocompounds formed in the partial lyso and lyso states can be effectively removed by washing with a 1% solution of bovine serum albumin (BSA) (see Methods).

Scheme 1



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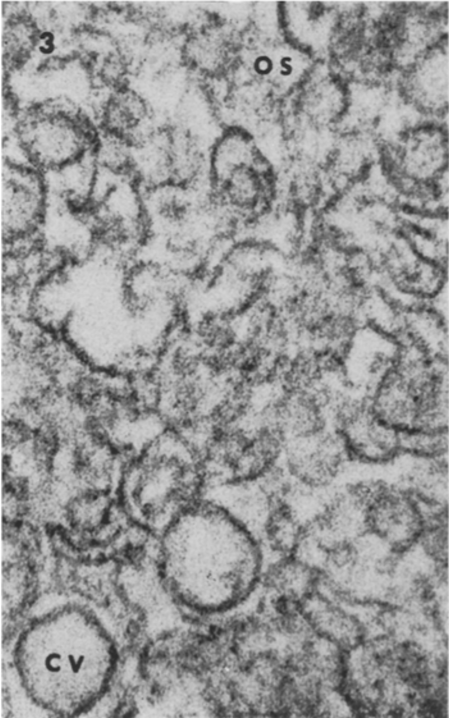
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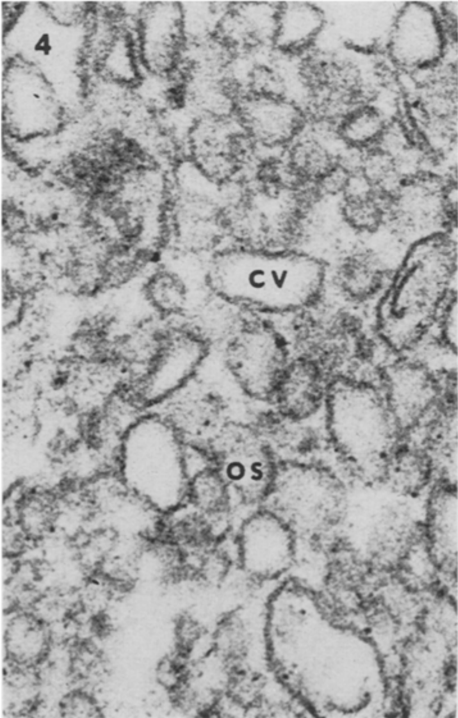
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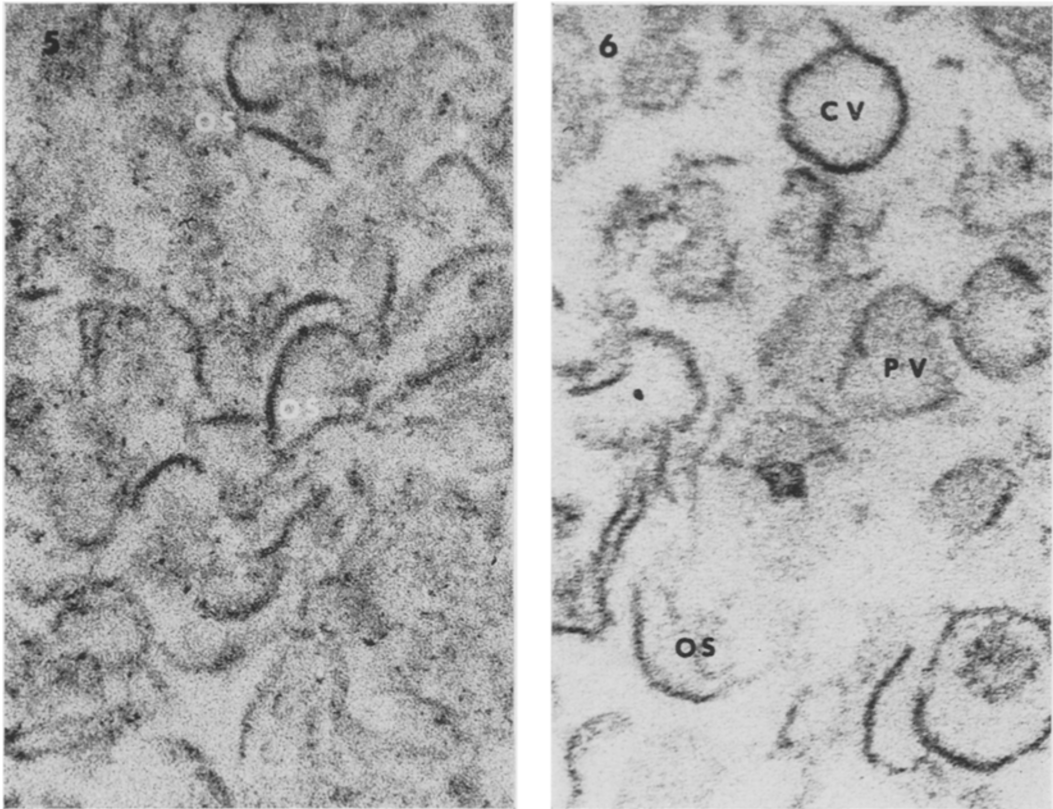


Figures 1–6. Electron micrographs of sectioned ETP membranes in the various phospholipid compositional states. Final magnification of all figures 108,000×. CV, closed vesicle, OS, open sheet, PV, partially closed vesicle.

Figure 1. Control ETP.

Figure 2. Partial lyso state ETP. Vesicles are predominantly closed as in the control.

Figure 3. Lyso state ETP. Although some vesicles are closed (CV), many appear as open sheets (OS) of various sizes.

Figure 4. Partial lipid depleted state ETP. In this state most of the membranes appear as closed vesicles (CV) but some are open sheets (OS).

Figure 5. Lipid depleted state ETP. Here the membranes are almost totally in the open sheet form (OS).

Figure 6. Lipid depleted state ETP plus added phospholipid. Addition of phospholipid causes a significant amount of vesicle formation from the open sheet form in Fig. 5. Closed vesicles (CV), partially closed vesicles (PV) and open sheets (OS) can be seen.

Washing with BSA converts the partial lyso state to a partial lipid depleted state. This compositional state is depleted of lysophosphatidyl ethanolamine and lysophosphatidyl choline but retains cardiolipin. In a similar fashion BSA washing converts the lyso state to a lipid-depleted state in which more than 70% of the lysocompounds of all three major phospholipids are removed from the membrane. These four compositional states of phospholipid in ETP membranes were routinely prepared together for analysis. Scheme 1 illustrates their preparation.

ETP Membrane Structure in the Partial Lyso State

ETP membranes in the partial lyso state and in an untreated state are shown in section in Figs. 1 and 2. Both fractions appear similar in general membrane morphology.

The vesicles are predominantly closed and there are no noticeable changes in vesicle shapes or sizes. The membranes in the partial lyso state, however, appear to stain slightly darker than the control ETP membranes. There is also no evident change in the surface structure as seen by negative staining (Figs. 7 and 8). Both the control and partial lyso state ETP had the characteristic ETP surface substructure consisting of projecting 90 Å particles (headpieces) attached by stalks to the membrane surface. There were no observable distortions in the headpieces or their attachments to the membrane surface. It therefore appears that conversions of PE and PC to their respective lyso phosphatides has no obvious effect on membrane structure.

ETP Membrane Structure in the Lyso State

In contrast to the partial lyso state, ETP membranes in the lyso state show considerable modification in both general membrane morphology and substructure. The most obvious change in section is the apparent breakage of a considerable portion of the closed membrane vesicles (Fig. 3). Although many of the vesicles still appear intact, some are broken into pieces of various sizes. These appear as open sheets which are either curved in a comma-like fashion, wavy, or are fairly straight thin rods. The darker staining of the membranes is even more pronounced, giving the membranes a thicker appearance. Membrane substructure is also altered in the lyso state. In negative staining (Fig. 9), the membrane vesicles and pieces are almost totally stripped of the projecting 90 Å headpiece-stalk complexes. The membrane edges are either jagged, indicating the breakage of the vesicles, or have a smooth PTA repellent surface.

ETP Membrane Structure in the Partial Lipid Depleted State

The partial lipid depleted membranes are altered in structure in a manner similar to the changes observed in the lyso state, but not as pronounced. In section (Fig. 4), most of the membranes appear to be intact closed vesicles, however a small proportion of the vesicles are not closed but are opened into sheets. The appearance of these opened vesicles are similar to those in the lyso state being membrane pieces curved like commas, wavy or straight. Darker staining of the membranes is also evident. The negatively-stained appearance (Fig. 10) shows a portion of the vesicles damaged to various degrees ranging from small pieces missing to the breakup into large chunks. The characteristic 90 Å headpiece-stalk complexes are also largely gone. It therefore appears that the partial lyso state to partial lipid depleted state transition results in considerable membrane alteration.

ETP Membrane Structure in the Lipid Depleted State

The structural modifications observed in the lipid depleted state are similar to those in the lyso state but even more pronounced. A sectioned view (Fig. 5) indicates the total absence of closed vesicles. All the membrane vesicles appear to have broken into pieces of membranes. These broken pieces have the same characteristic shapes and sizes as in the lyso and partial lipid depleted states. The membranes also stain much darker than the control ETP, as noticed for the other states, resulting in an apparent thickening of the membranes. It is important to emphasize, however, that this highly lipid-depleted state still has a membrane structure (Fig. 5). Figure 11 confirms the broken nature of the vesicles in surface view. Broken vesicles are seen with sizes ranging from large membrane

fragments to small pieces. The 90 Å headpiece-stalk complexes are also conspicuously absent.

ETP Membrane Structure in the Lipid Depleted State to which Phospholipid was Added

Addition of total mitochondrial phospholipid to the lipid depleted ETP membranes (20 μg P/mg ETP protein) results in a partial transformation from open sheet, non-vesicular membranes to closed vesicles (Fig. 6). Many of the membranes still in the open sheet form appear in the process of reforming closed vesicles. The membranes, however, retain the property of staining much darker than control ETP.

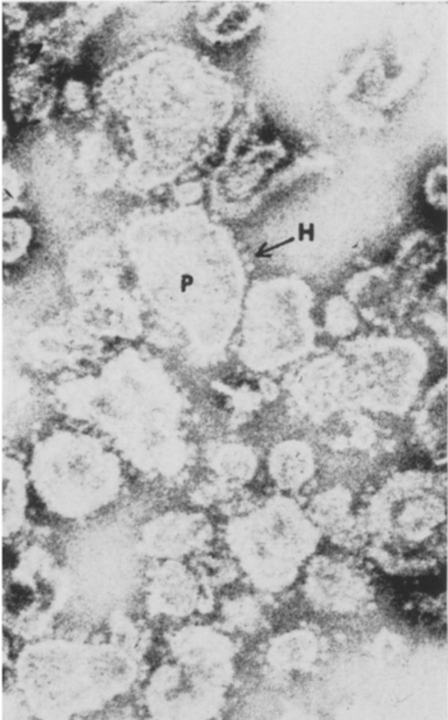
Surface view of the fraction by negative staining (Fig. 12) appears similar to that before phospholipid addition (Fig. 11). Some of the vesicles appear more intact which is consistent with the sectioned view (Fig. 6) showing intact closed and nearly closed vesicles. Very significantly the 90 Å headpiece-stalk complexes were still not visible.

Discussion

We arbitrarily have defined four states related to the degree of hydrolysis or removal of the phospholipid in the membrane. One of the states, the partial lyso state, results in no structural change, whereas all three other states have similar structural changes to various degrees. The structural modifications observed in the lyso state are correlated with the conversion of cardiolipin to its lysophosphatides. Similar structure change is found in the partial lipid depleted state, where the phosphatidyl choline and phosphatidyl ethanolamine lysophosphatides are removed from the membrane, but the cardiolipin remains intact. Since hydrolysis of cardiolipin on the one hand, and removal of the lysophosphatides of phosphatidyl choline and phosphatidyl ethanolamine on the other, result in similar structural changes, the specific structure of intact ETP vesicles can be modified by either the cleavage of cardiolipin, or the removal of the cleaved molecules of phosphatidyl choline and phosphatidyl ethanolamine. Just the cleavage of phosphatidyl choline and phosphatidyl ethanolamine, even though they represent about 80% of the total membrane phospholipid, does not result in overt changes in membrane structure.

The specific correlations made between the nature of the phospholipids in phospholipase A treated ETP and the resultant structural changes, point to the role of cardiolipin in maintenance of both general morphology and the specific substructure of the membrane vesicles. The question arises, however, as to why structural changes similar to those caused by cardiolipin cleavage or its removal (lyso and lipid depleted states respectively) are found in the partial lipid depleted state where phosphatidyl choline and phosphatidyl ethanolamine are removed but cardiolipin is not affected. It is suggested that removal of the two predominating phospholipids cause steric shifts in cardiolipin which has effects on ETP membrane structure similar to that caused by the cleavage of fatty acids from cardiolipin. The lack of structural changes in the partial lyso state points to the ability of the phosphatidyl choline and phosphatidyl ethanolamine lysophosphatides to stabilize cardiolipin so that the characteristic ETP structure is maintained.

These studies also indicate a key role for protein in maintaining membrane structure. This view is contrary to the unit membrane model of membrane organization. If the ETP membrane consists of a bilayer backbone of phospholipid covered by protein, then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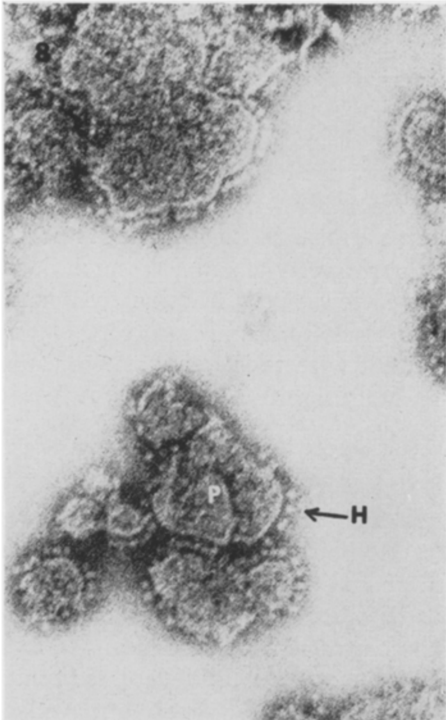
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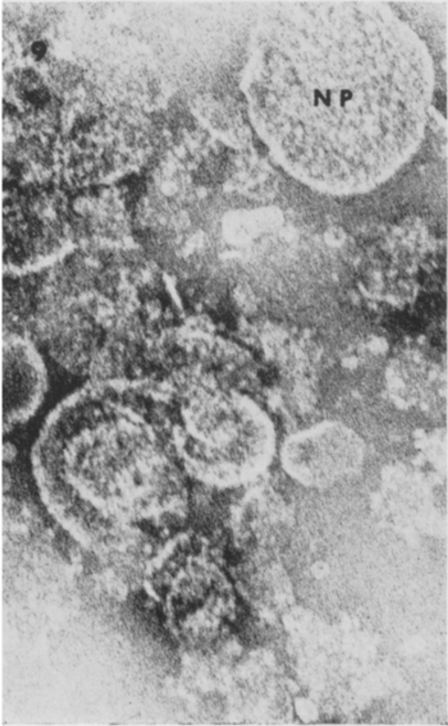
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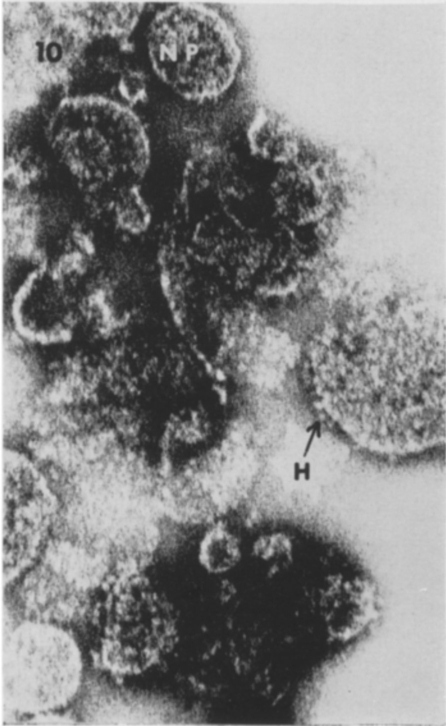
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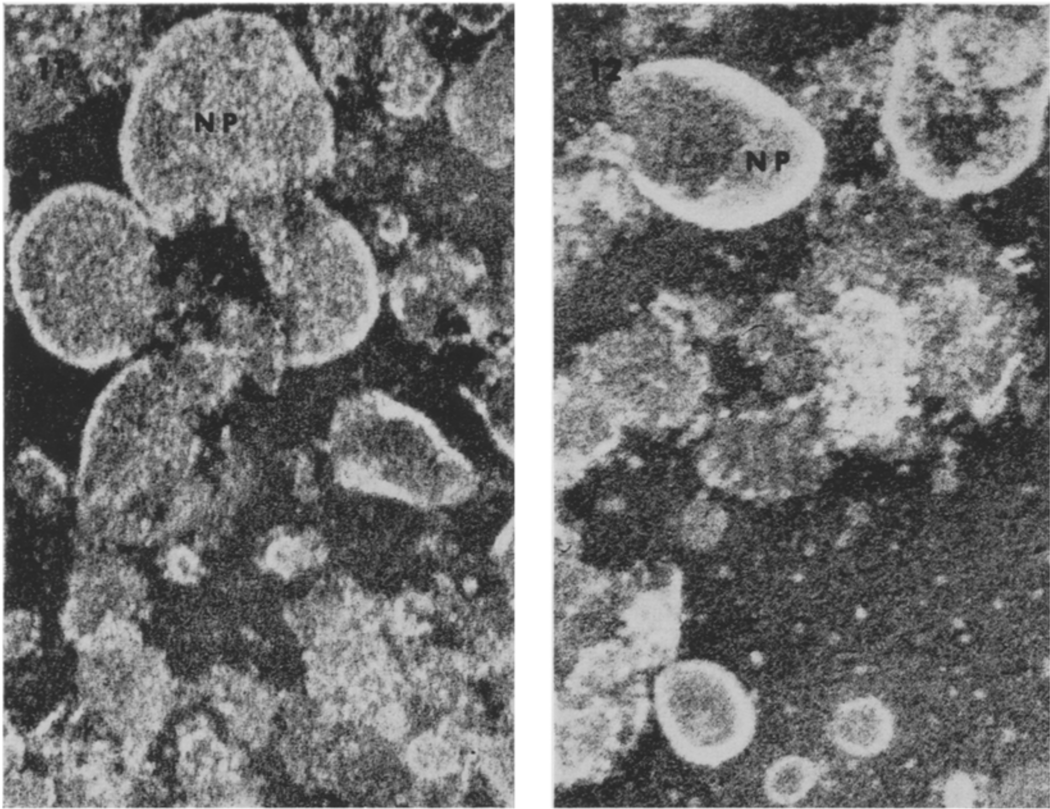

Figures 7–12. Electron micrographs of negatively stained (phosphotungstic acid) samples of the various ETP membrane fractions. Final magnifications 140,000×. H, 90 Å projecting headpiece; P, emembran in which 90 Å headpieces are seen projecting from the membrane edges; NP, membrane in which projecting headpieces are not seen.

Figure 7. Control ETP. The membranes have many projecting headpieces (H) as seen along the edges of the vesicles (P).

Figure 8. Partial lyso state ETP. Membranes are virtually identical in appearance to the control.

Figure 9. Lyso state ETP. Projecting headpieces are nearly totally absent (NP).

Figure 10. Partial lipid depleted state ETP. Projecting headpieces are seen (H), but much less frequently than in control ETP. Many membranes have no projecting headpieces (NP).

Figure 11. Lipid depleted state ETP. As in Fig. 9, projecting headpieces are seen only rarely (NP.)

Figure 12. Lipid depleted state ETP plus added phospholipid. Addition of phospholipid does not cause the reappearance of projecting headpieces (NP).

the membrane should collapse upon removal of the lipid. Fleischer *et al.*[1] found similar lack of collapse after lipid removal by extracting ETP vesicles with acetone and ammonium hydroxide. They found that "unit membrane" structure is maintained even after removal of 95% of the phospholipid. It has been argued, however, that treatment of membranes with organic solvents may fix the protein layers on the surface of the membranes so that they do not collapse. The membrane can thus retain its structure even though the phospholipid is extracted. Stoeckenius and Engelman[19] have discounted this fixation hypothesis.

By selectively hydrolyzing phospholipids to their lysophosphatides and removing the lysophosphatides with BSA washing, we have an alternative approach to lipid extraction.

The phospholipid-depleted ETP are clearly membranous. In fact, rather than collapsing the membranes stain darker and appear thicker than untreated ETP membranes. By direct measurement the untreated ETP membranes vary from 70–100 Å. Associated with the apparent increased thickness is the absence of projecting 90 Å headpiece-stalk complexes (Fig. 11). Similarly the lyso state lacks headpiece-stalk complexes (Fig. 10) and also has thicker membranes of 100–120 Å (Fig. 4). Moreover addition of phospholipid to the lipid depleted state does not result in reappearance of projecting headpiece-stalk complexes (Fig. 12) and the membranes remain thick (100–120 Å). Study of ATPase activity in the various phospholipid states as well as protein recovery studies[20] indicate that the headpiece-stalk complexes are still associated with the membrane but in a non-projecting manner.

Consistent with these findings is the possibility that the thicker membranes are due to the collapse of headpiece-stalk complexes which then coat the membrane. Since osmium tetroxide fixation is reported to destroy the integrity of headpiece-stalk complexes, collapse of the headpiece-stalk complexes may induce resistance to degradation during osmium fixation and result in thicker membranes. This might especially be true if the complexes collapsed and became buried in the membrane rather than coating the outside surfaces.

If the headpiece-stalk complexes are buried in the membrane, however, there is no basis for assuming an increase in membane thickness. The proteins of the complexes might easily accommodate space inside the membrane left vacant by the removal of phospholipid as well as by general conformational changes in the membrane caused by the loss of phospholipid.

An alternative explanation for the apparent increase in membrane thickness is based on the observed staining affinities of the membrane fractions. In the lyso state, lipid depleted state and depleted state plus phospholipid, the stain penetrates darkly throughout the membranes and ends sharply at the membrane surfaces. In contrast, control ETP stain lighter and much more diffusely along the membrane surface (Fig. 1). Thus the control membranes may be as thick as the treated membranes but appear thinner because of very low staining affinity along either one or both sides of the membrane. For example poor staining on one side of a binary membrane[21] would give the appearance of a thin membrane in the untreated condition, whereas greater penetration of stain after lipid removal could show both layers of the membrane. These observed differences in staining affinity may be due to changes in membrane conformation caused by changes in phospholipid composition and/or the collapse of the headpiece-stalk complexes.

Fleischer *et al.*[1] have also reported thicker membranes in lipid depleted mitochondria. They similarly point out that the increase in width may not represent an actual increase in membrane thickness but be a result of the removal of the stainable groups of the lipid molecules as well as possible changes in the stainability of the membrane proteins. Since the acetone extractions used by Fleischer *et al.*[1] appear to actually remove the headpiece-stalk complexes from the membrane, the differences in staining and apparent membrane thicknesses which we also observe, may be caused by changes in phospholipid composition without the necessary participation of the collapsed ATPase headpiece-stalk complexes.

A comparison of Figs. 1 and 5 indicates that the most obvious effect of phospholipid removal is vesicle breakage. Addition of phospholipid to the lipid depleted state results

in a partial return to the vesicularized state. Many of the membranes appear as closed vesicles or in the process of closing. It is therefore concluded that phospholipid is required for vesicularization but is not essential for maintaining a membrane structure.

Summary

1. Digestion of ETP membranes with low levels of phospholipase A (0·05 μg/mg ETP protein) converts phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) to their lyso derivatives with no effect on cardiolipin (partial lyso state). There is no loss of projecting headpieces. The membrane vesicles remain closed but the membranes appear to have greater affinity for the stain as judged by their darker appearance.

2. High levels of phospholipase A (12 μg/mg ETP protein) result in hydrolysis of cardiolipin as well as PE and PC (lyso state). The membranes no longer have projecting headpieces, many of the vesicles are opened into sheets, stain darker than control ETP and appear thicker.

3. Removal of lysophosphatides from the partial lyso state results in a partial lipid depleted state. There is a partial loss of projecting headpieces and a small proportion of vesicles are broken into open sheets. The membranes also appear to stain darker.

4. The lipid depleted state is created by removing the lysophosphatides from the lyso state. No projecting headpieces are visible and the membranes are almost totally in the open sheet form. The membranes also show greater affinity for the stain and appear thicker.

5. Addition of phospholipid to the lipid depleted membranes results in a transformation from the open sheet to closed vesicle form. The membranes remain thicker, darkly stained and no projecting headpieces are seen.

6. It is concluded that intact, unhydrolyzed phospholipid is essential for membrane vesicularization but not for maintaining a membrane structure.

7. The apparent increases in membrane thickness may be a result of the collapse of the projecting headpieces, the change in phospholipid composition or the differences in staining affinities of the membranes.

Acknowledgements

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