PRELIMINARY NOTES

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Action of phospholipase A on mitochondrial cristae

Sequential digestion of membrane phospholipids by phospholipase A results in a differential release of a portion of proteins in the membranes[1-4]. A selective association between lipid and protein is indicated from these studies. Significant breakdown in the membrane structure occurs after almost complete digestion of phospholipids or after subsequent removal of lysophosphatides under milder digestion conditions where no cardiolipin is digested.

When beef-heart mitochondrial cristae (electron transport particles) are exposed to increasing concentrations of purified *Naja naja* venom phospholipase A, the phosphatides are digested in the following order: phosphatidyl ethanolamine, phosphatidyl choline and diphosphatidyl glycerol (cardiolipin). This digestion sequence is not inherent to membrane, since a mixture of same phospholipids isolated from membrane is digested in a similar sequence. Chromatographically pure individual phospholipids of membrane also reveal the same order of specificity towards phospholipase A. Under carefully controlled conditions of digestion, most of the phosphatidyl ethanolamine and phosphatidyl choline of the membrane can be converted into their respective lyso compounds without any significant breakdown of cardiolipin. Co-related to the digestion of phospholipids by phospholipase A is an increasing release of membrane protein in the supernatant obtained after centrifugation of the membrane suspension at 122000 $\times$ *g* for 45 min.

The specific release of respiratory-chain-linked NADH dehydrogenase is correlated with the digestion of membrane cardiolipin. Cytochrome *c* which has been reported to be released under certain conditions of digestion[5] is not released under the conditions employed here even after digestion of the entire phosphatidyl ethanolamine, phosphatidyl choline and more than 70 % of cardiolipin which together constitute about 95 % of the total electron transport particle phospholipid.

The majority of lysophosphatides produced during hydrolysis of phospholipids remain in the membrane and are not released in the supernatant on centrifugation of the membrane suspension at 122000 $\times$ *g* for 45 min. At a stage of membrane digestion when nearly 70 % of total phospholipids (phosphatidyl ethanolamine and phosphatidyl choline) are converted to lysophosphatides, only 3 % of these lyso compounds are dispersed in the suspension. Even after digestion of nearly 95 % of total phospholipids (entire phosphatidyl ethanolamine and phosphatidyl choline and 70 % cardiolipin) of the membrane, only 14 % of total phospholipid phosphorus is found in the supernatant. On the other hand, 83 % of total fatty acids produced during phospholipase A digestion (pH 7.4) are in the supernatant. These fatty acids presumably are not present in free state since they cannot be effectively removed from the supernatant by solvent extractions without significant lowering of its pH (<4) by acidification. When phospholipase A-treated membrane having nearly 85 % of phospholipids in lyso form was treated with lysophosphatidase, 50 % of the total

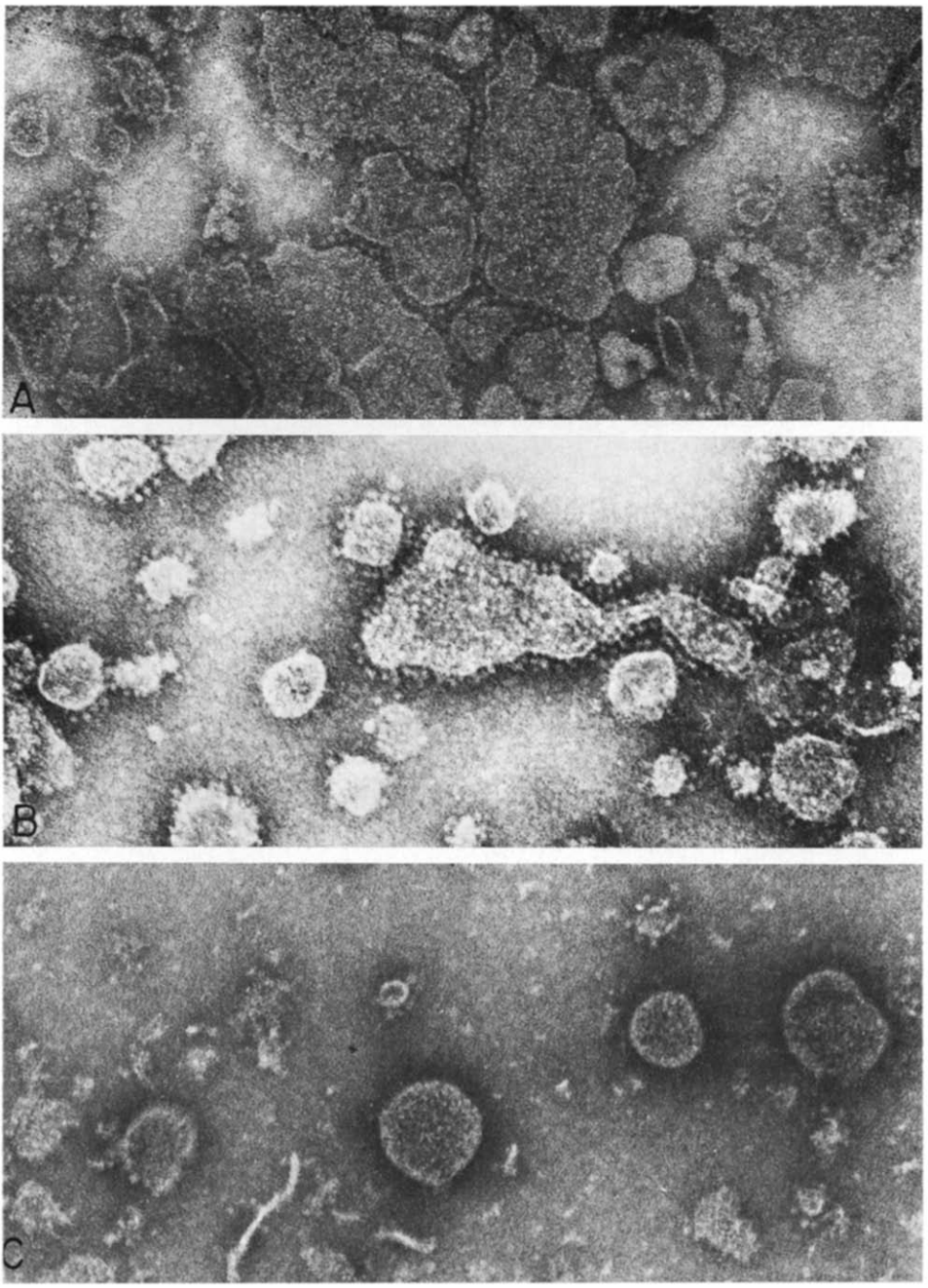

Fig. 1. Samples negatively stained with phosphotungstate[20]. A. Electron transport particle vesicles. B. Electron transport particle vesicles digested with low levels of phospholipase A. C. Electron transport particle vesicles digested with high levels of phospholipase A. Final magnifications × 123000.

cytochrome *c* present in the membrane was released in the supernatant. By lysophosphatidase treatment, 48% of total phospholipid phosphorus could be removed from the membrane and some damage was observed to the membrane structure.

As evident from electron microscopic studies there seems to be no noticeable change in membrane structure on low level digestions with phospholipase A sufficient to cause almost complete breakdown of phosphatidyl ethanolamine and phosphatidyl choline (Figs. 1A and 1B). Apparently lysocompounds which are still present in the membrane maintain membrane structure. Removal of lysocompounds from this partially digested membrane causes noticable damage to the membrane structure, including the loss of the 90 Å F_1 head pieces[6]. Similar membrane damage and loss of 90 Å F_1 head pieces is also observed when the membrane is digested with higher levels of phospholipases A where cardiolipin digestion occurs (Fig. 1C). Removal of lysophosphatides by bovine serum albumin wash[7] from the cardiolipin digested membranes results in no further damage to membrane structure.

The solubilization of NADH dehydrogenase[3] appears to be related to the breakdown of cardiolipin and not to the amount of phosphatidyl ethanolamine or phosphatidyl choline converted to their lysophosphatides (Table I). This also indicates that the formation of lysophosphatides and fatty acids by phospholipase A digestion *per se* is not the basis for release of this protein from the membrane. Phospholipase A as purified from *N. naja* venom shows specificity of attack which favors phosphatidyl ethanolamine over phosphatidyl choline in both purified lipids and membranes. This specificity has also been observed by GALLAI-HATCHARD AND GARY[8] in rat-liver plasma membranes. Cardiolipin breakdown occurs slowly and only at comparatively higher levels of *N. naja* venom phospholipase A. This is in agreement with observations

TABLE I

CORRELATION OF PHOSPHOLIPID DIGESTION AND PROTEIN RELEASE FROM CRISTAE MEMBRANES

All digestions with phospholipase A were carried out at 30°, for 60 min in 0.05 M Tris–HCl (pH 7.4) and in absence of exogenous Ca^{2+}. Lipid analysis were carried out by extracting lipids from aliquots of digest by the procedure of FOLCH *et al.*[13] in N_2 atmosphere followed by quantitative thin-layer chromatography on double-thickness silica gel g plates in chloroform–methanol–water (100:40:6, by vol.). Protein was estimated by method of YONETANI[14] and phosphorus by method of CHEN *et al.*[15]. NADH–ferricyanide reductase assays were carried out at fixed ferricyanide concentration (1.7 mM) in 0.05 M Tris–HCl (pH 7.4) under assay conditions of MINAKAMI *et al.*[16]. Cytochrome *c* was determined by reduced-*minus*-oxidized different spectra (ε_{mM} (550–535 nm) = 25.1)[17]. Lysophospholipase was prepared from pancreas by a method of SHAPIRO[18]. Phospholipase A was prepared and purified by chromatography on Sephadex G-75 according to the method of CREMONA AND KEARNEY[19].

Phospholipase A concentration (*μg/mg electron transport particle protein*)	% *Digestion of phospholipids*			% *of membrane protein released*	% *of NADH dehydrogenase released*	% *Cytochrome c released*
	Phosphatidyl ethanolamine	*Phosphatidyl choline*	*Cardiolipin*			
0	0	0	0	2.1	0	0
0.05	98	70	0	5.7	0	0
0.1	100	100	23	—	31	0
0.1	100	100	27	—	38	0
0.8	100	100	36	—	54	0
3	100	100	50	8.2	57	0
9	100	100	71	12.5	63	0
+ lysophospholipase	—	—	—	17.3	—	50

made by DE HAAS *et al.*[9] in the case of snake-venom phospholipase A. However, phospholipase A isolated recently from porcine pancreas shows the reverse order of specificity towards neutral and anionic phospholipids[10]. We find that phospholipase A from *Crotalus adamanteus* venom is still less active towards cardiolipin either in membrane or in isolated form. Inability of *C. adamanteus* venom phospholipase A to extract respiratory-chain-linked NADH dehydrogenase[11] from electron transport particles reported earlier and also observed by us during this study might be correlated to its relatively inactive nature towards cardiolipin. SALACH *et al.*[12] have recently further purified *N. naja* venom phospholipase A by electrofocusing. They find several isoenzymes which differ in their effect on solubilization of NADH dehydrogenase and inhibition of electron transport chain in NADH–Q region. These separate isoenzymes may show differing selectivity for individual phospholipids.

These studies not only show correlation between specific enzyme release and breakdown of particular phospholipid but also indicate that membrane structure is retained even after complete digestion of phosphatidyl ethanolamine and phosphatidyl choline and breakdown of membrane structure occurs only after removal of lysophosphatides and also on digestion of cardiolipin.

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Department of Biological Sciences,
Purdue University,
Lafayette, Ind. 47907 (U.S.A.)

Y. C. AWASTHI
RONALD BEREZNEY
F. J. RUZICKA
F. L. CRANE

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