BBA 75425

THE RELATION BETWEEN PHOSPHOLIPASE ACTION AND RELEASE OF NADH DEHYDROGENASE FROM MITOCHONDRIAL MEMBRANE

Y. C. AWASTHI, F. J. RUZICKA AND F. L. CRANE
Department of Biological Sciences, Purdue University, Lafayette, Ind. (U.S.A.)
(Received October 10th, 1969)

SUMMARY

Phospholipase A from *Naja naja* shows different rates of hydrolysis of phospholipids. Both free and membrane-bound phospholipids are digested in the order phosphatidylethanolamine, lecithin and cardiolipin. Release of NADH dehydrogenase is correlated with hydrolysis of cardiolipin. Inability of *Crotalus adamanteus* venom to release NADH dehydrogenase is attributed to its inert nature toward cardiolipin. Selective inhibition of several acceptor sites on NADH dehydrogenase is observed during phospholipase A and phospholipase C treatment. Although products of digestion, fatty acids and lysophosphatides, can account for certain inhibitory effects they do not induce release of the dehydrogenase.

INTRODUCTION

In recent years phospholipase A from *Naja naja* (cobra) venom has become a powerful tool in the hands of enzymologists for the differential extraction of certain membrane-bound enzymes which are otherwise difficult to obtain in purified form by conventional methods of isolation and purification. Extraction of choline dehydrogenase from rat-liver mitochondria[1], α-glycerophosphate dehydrogenase from pig-brain mitochondria[2], NADH dehydrogenase from the electron transfer chain of bovine cardiac mitochondria[3], D-lactate cytochrome reductase from yeast[4], and β-hydroxybutyric dehydrogenase from beef-heart mitochondria[5] are a few of the examples where phospholipase A has been successfully used.

Other phospholipases including phospholipase A from other sources of venom than *N. naja* have been found in some instances to be ineffective in the solubilization of these enzymes or to require different extraction conditions. KING *et al.*[6] have shown that NADH dehydrogenase from cardiac mitochondria could not be solubilized when several purified forms of phospholipase A derived from *Crotalus adamanteus* venom were used. Also phospholipase D from cabbage and phospholipase A from *C. adamanteus* were found ineffective in solubilizing choline dehydrogenase from rat-liver mitochondria[1].

Apparent lack of linear correlation between the activities of different phospholipase A fractions obtained by column chromatography of crude boiled cobra venom

Abbreviation: DCIP, dichlorophenolindophenol.

and their extracting capability for the respiratory chain-linked NADH dehydrogenase from electron transport particles of beef-heart mitochondria observed by KING *et al.*[6] has cast a shadow of a doubt on the role of phospholipase A as the active constituent of venom responsible for the release of this enzyme from the membrane. The inability of *C. adamanteus* phospholipase A to solubilize the respiratory chain-linked NADH dehydrogenase from beef-heart mitochondria has also been pointed out by these authors as "circumstantial evidence" against the role of phospholipase A as the agent for solubilization. Recently cobra venom phospholipase A purified according to the method of CREMONA AND KEARNEY[7] has been shown to consist of six isoenzymes purified by electrofocusing which have different phospholipase A activities and different capacity for extraction of NADH dehydrogenase and other enzymes[8]. However, there is no linear correlation between phospholipase A activity and the solubilizing power of these isoenzymes.

Phospholipase A digestion has also been used to show a role for phospholipid in the NADH dehydrogenase system of beef-heart mitochondria. Acetone extraction which had been used successfully for the removal of phospholipid and restoration of the succinate system[9] could not be applied to the NADH system. LESTER AND FLEISCHER[10] found that NADH-ubiquinone reductase was irreversibly lost by acetone extraction. However, they reported NADH-ferricyanide activity to be equal or better than activity of the unextracted material. Using short-term digestions of electron transport particles with phospholipase A followed by multiple bovine serum albumin washes, FLEISCHER and co-workers[11,28,29] and MACHINIST AND SINGER[12] showed the essentiality of phospholipid for the reduction of ubiquinone and cytochrome *c* by NADH. Long-term digestion with phospholipase A caused irreversible inactivation of ubiquinone and cytochrome *c* reductase activity.

The current investigation is aimed at resolving the nature of the solubilizing agent responsible for the release of the NADH dehydrogenase of beef-heart mitochondria. In order to study the binding of the enzyme to the membrane, the specificity of phospholipase A attack on isolated lipids of beef-heart mitochondria and membrane-bound phospholipids was determined. Also the effect of treatment of submitochondrial particles with other phospholipases and products of phospholipase A digestion were studied in order to understand the changes on the biochemical and structural integrity of the mitochondrial membrane which occur as a result of the breakdown of phospholipid. The effect of phospholipases on the structure of the mitochondrial membrane will be published in a subsequent paper.

MATERIALS AND METHODS

Materials

Beef-heart mitochondria were prepared according to the method of LÖW AND VALLIN[13]. Electron transport particles were obtained by sonic disruption of mitochondria suspended in 0.25 M sucrose–0.05 M Tris·HCl buffer (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 2–3-min intervals in a vessel submerged in a salt–ice bath. The supernatant obtained by centrifugation at 27000 × *g* for 15 min was recentrifuged at 105000 × *g* for 45 min. The pellet (electron transport particles) was washed twice with 0.25 M sucrose–0.05 M Tris·HCl buffer (pH 7.4) by homogenization with a glass

homogenizer fitted with a Teflon pestle and centrifugation at 105000 × *g* for 45 min. The electron transport particles were resuspended in the same buffer and stored at −20°.

Phospholipids used in this study were isolated from beef-heart mitochondria. Extraction of lipids was carried out by the procedure of FOLCH *et al.*[14], modified by ROUSER AND FLEISCHER[15] and neutral lipids were removed by precipitating phospholipids in cold acetone at −20° (ref. 16). O_2-free N_2 was bubbled through all stages and evaporation of solvents was carried out at temperatures not above 20°. Lecithin, phosphatidylethanolamine, and cardiolipin were obtained from beef-heart mitochondria in chromatographically pure form by preparative thin-layer chromatography with double thickness (0.5 mm) Silica gel G plates. Lipid samples were streaked on plates under a current of O_2-free N_2, run in solvent system chloroform–methanol–water (10:40:6, by vol.) and dried under a current of N_2. Good separations of lecithin, phosphatidylethanolamine, and cardiolipin were observed and usually one separation gave pure samples of lipids.

Lipids were dispersed in micellar form by sonicating suspensions of lipids in 0.01 M Tris·HCl buffer (pH 7.4) for 4–5-min intervals in an atmosphere of pure N_2. The dispersed samples were centrifuged at 105000 × *g* for 60 min and subsequently used as substrate for the phospholipases. The homogenous dispersions consisted of chromatographically (thin-layer chromatography) pure samples in the case of the individual lipids. No observable deterioration was observed with mixed phospholipids during micellar formation.

Phospholipase A was purified from *N. naja* venom according to the method of CREMONA AND KEARNEY[7]. After acid precipitation, the solution was applied to a Sephadex G-75 column and 5-ml fractions were collected. The highest activity fractions (manometric assay) were combined, lyophilized, and reapplied to another Sephadex G-75 column. 5-ml fractions were collected and the highest activity fractions were used in the phospholipase A treatment.

Methods

Phosphorus was estimated by the method of CHEN *et al.*[18] and protein was assayed by the modified biuret procedure of YONETANI[19]. Unless specifically noted, all digestions with phospholipases were carried out at 30° in 0.06 M Tris·HCl buffer (pH 7.4). The rate of hydrolysis of lipids by phospholipase A was measured in two ways: (a) spectrophotometric determination of ester groups[17] in case of purified lipid micelles and (b) quantitative thin-layer chromatography on Silica gel G in case of electron transport particles using solvent systems chloroform–methanol–water (100:40:6, by vol.) and chloroform–methanol–7 M NH_4OH (65:30:4, by vol.). Aliquots of electron transport particle digests containing at least 20 mg lipids were extracted (Folch procedure) under a pure N_2 atmosphere and the solvent removed below 20°. The dried samples were redissolved in a known volume of solvent and 1–1.5 mg of lipid (based on phosphorus determination) were spotted on plates and phosphorus analyses were carried out on scrapings of well resolved spots developed by iodine vapour. Purified lipids were taken to have a 4% phosphorus content[20]. The rate of hydrolysis of lipids by phospholipase C was measured by estimating phosphorus release in aqueous phase and also by thin-layer chromatography following decrease

of parent phospholipids. Fatty acids were estimated by titrating the residues of diethyl ether-soluble fractions in neutral ethanol with 0.01 M KOH.

Assays of quinone reductases were carried out at 30° by following the oxidation of NADH at 340 mμ after the addition of quinone in the presence of antimycin A and cyanide. The assay system (3 ml) contained 30 μmoles Tris·HCl buffer (pH 7.4), 0.5 μmole NADH, 0.5 μg antimycin A, and 0.3 μmole KCN. 0.3 μmole of quinone (in 10 μl absolute ethanol) was added to start the reaction. 0.12 mμmole piericidin A was added when applicable. 1.0 mg cytochrome *c* was substituted for the quinone and antimycin A was omitted when NADH–cytochrome *c* reductase was assayed. NADH–ferricyanide reductase assays were carried out at fixed ferricyanide concentration (1.7 mM) under assay conditions described by MINAKAMI *et al.*[21]. Succinate–cytochrome *c* reductase was assayed according to the method of TISDALE *et al.*[22] and succinate–dichlorophenolindophenol (DCIP) reductase by the method of TAKEMORI AND KING[23].

Chemicals

Piericidin A was a gift of Dr. K. Folkers. Ubiquinone-2 was kindly provided by Dr. A. F. Wagner of Merck, Sharp, and Dohme Research Laboratories. *N. naja* venom, *C. adamanteus* venom, phospholipase C Type I (*Clostridium welchii*), phospholipase D (cabbage), oleic acid, lysophosphatidylethanolamine (enzymatically prepared; one spot on thin-layer chromatography), and cytochrome *c* (Type III) were purchased from Sigma, duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) was obtained from Aldrich and juglone (5-hydroxy-1,4-naphthoquinone) was purchased from K and K Laboratories.

RESULTS

In order to study the specificity of phospholipase A from *N. naja* venom, phospholipid micelles from beef-heart mitochondria were incubated with different levels of phospholipase A. Both mixed phospholipids as well as chromatographically-pure samples of lecithin, phosphatidylethanolamine, and cardiolipin (diphosphatidylglycerol) isolated from beef-heart mitochondria were treated with phospholipase A at 30°. It was observed that when phospholipids were digested with a low level of enzyme (0.3 μg enzyme protein per mg lipid), the rate of breakdown of phosphatidylethanolamine was higher than that of lecithin while cardiolipin was completely inert to the enzyme under these conditions. However, when the enzyme concentration was raised to 3.0 μg enzyme protein per mg lipid, some hydrolysis of cardiolipin was also observed. When mixed mitochondrial phospholipids were incubated with phospholipase A at 0.3 μg enzyme protein per mg lipid, it was observed that only phosphatidylethanolamine and lecithin were hydrolyzed. The rate of breakdown of lipids followed the same order as in the case of the isolated lipids. With increasing enzyme levels, hydrolysis of cardiolipin was also observed but in all cases cardiolipin hydrolysis was observed only after all of the phosphatidylethanolamine and most of the lecithin were converted into lyso compounds. Table I presents the amount of hydrolyzed lipids of mixed phospholipids at different enzyme concentrations.

Thus having established that phospholipase A from *N. naja* venom had the specificity of attack on beef-heart mitochondria phospholipids in the order phos-

TABLE I

HYDROLYSIS OF MIXED MITOCHONDRIAL PHOSPHOLIPIDS BY *N. naja* VENOM PHOSPHOLIPASE A

Lipid micellae were incubated with desired amount of phospholipase A at 30° for 60 min in 0.06 M Tris·HCl buffer (pH 7.4) and amount of lipids cleaved was tracked through quantitative thin-layer chromatography of the lipids in digestion mixture at the end of reaction.

Enzyme concentration (μg enzyme protein per mg lipid)	*Percentage of total individual phospholipid cleaved*		
	Phosphatidyl-ethanolamine	*Phosphatidyl-choline*	*Diphosphatidyl-glycerol*
0.3	95	68	0
2.0	100	100	25
9.0	100	100	68
12.0	100	100	90

phatidylethanolamine>>lecithin>>>cardiolipin, the effect of this enzyme on the membranous lipids of electron transport particles of beef-heart mitochondria was studied. One might envisage that the same order of preference would not be followed necessarily by phospholipase A in the case of membrane-bound lipids since steric factors dependent on the relative positions of these lipids in membranes could cause variations of susceptibility of these lipids to phospholipase A. When electron transport particles were digested with low levels of phospholipase A (0.05 μg phospholipase A per mg electron transport particle protein) for 60 min, 98% of the phosphatidyl-ethanolamine and 70% of the lecithin were broken down (Table II). There was no observable hydrolysis of cardiolipin at this level of enzyme at the end of the digestion (60 min). When the digest was cooled and centrifuged (30 min, 122000 × *g*) and the pellet and supernatant were assayed for lipid and protein content, it was found that only 2.6% of the phospholipid and 5.7% of the original protein remained in the supernatant. Although more than 70% of the total lipids of mitochondrial electron transport particles were converted to lysophosphatides, these lyso compounds tend to stick to the membrane rather than be dispersed in the supernatant as one would assume due to their detergent nature. However, most of the fatty acids released (Tables III and IV) during enzymatic hydrolysis remained in the supernatant. Furthermore, the supernatant obtained following the low level treatment of electron

TABLE II

HYDROLYSIS OF ELECTRON TRANSPORT PARTICLE PHOSPHOLIPIDS BY LOW LEVELS OF PHOSPHOLIPASE A

0.05 μg phospholipase A per mg electron transport particle protein at 30°, in 0.06 M Tris·HCl buffer (pH 7.4).

Phospholipids	*Percent hydrolyzed at different intervals*				
	0 min	*15 min*	*30 min*	*45 min*	*60 min*
Phosphatidylethanolamine	0	76.2	88.8	89.6	98.7
Lecithin	0	24.8	67.5	68.6	68.6
Cardiolipin	0	0	0	0	0

TABLE III

DISTRIBUTION OF PROTEINS AND LIPIDS IN SUPERNATANT AND PELLET AFTER LOW-LEVEL PHOSPHOLIPASE A TREATMENT

Pellet obtained by centrifuging digest at 122000 × *g* for 30 min. 0.05 μg phospholipase A per mg electron transport particle protein at 30° for 60 min in 0.06 M Tris·HCl buffer (pH 7.4).

Fractions	*Total protein (mg)*	*Protein (%)*	*Total phospholipid (mg)*	*Phospholipid (%)*	*Free fatty acids (%)*
Electron transport particles	400	100	187	100	—
Pellet	366	91.5	176.5	94.4	20
Supernatant	23	5.7	4.8	2.6	80

TABLE IV

DISTRIBUTION OF PROTEIN AND LIPIDS IN PELLET AND SUPERNATANT AFTER HIGHER-LEVEL PHOSPHOLIPASE A TREATMENT

Pellet obtained by centrifugation at 122000 × *g* for 30 min, 3 μg phospholipase A per mg electron transport particle protein at 30° for 60 min in 0.06 M Tris·HCl buffer (pH 7.4).

Fractions	*Total protein (mg)*	*Protein (%)*	*Total phospholipid (mg)*	*Phospholipid (%)*	*Free fatty acids (%)*
Electron transport particles	400	100	187	100	—
Pellet	340	85	156	83.9	17
Supernatant	48	12	26.5	14.1	83

transport particles by phospholipase A was found to have no NADH–ferricyanide reductase activity as would be expected if NADH dehydrogenase were solubilized. The NADH–ferricyanide activity was almost completely recoverable in the pellet and was not lost during the digestion.

With increasing levels of phospholipase A, more effective hydrolysis of lipids was observed. Lecithin and phosphatidylethanolamine were almost completely broken down and degradation of cardiolipin was evident. At a level of 0.1 μg phospholipase A per mg electron transport particle protein, 22.5 % of the cardiolipin was also broken down along with the entire phosphatidylethanolamine and lecithin. Cardiolipin breakdown in all experiments of electron transport particle digestion with phospholipase A was accompanied by release into the supernatant of the NADH dehydrogenase as determined by NADH–ferricyanide reductase activity. Table V shows the cardiolipin breakdown during these digestions and also the release of NADH–ferricyanide reductase activity into the supernatant. Distribution of protein and lipids in the pellet and supernatant after high level phospholipase A treatment of electron transport particles is shown in Table IV. It is remarkable that even after more than 95 % of the phospholipid is broken down, loss of lipid from the pellet in terms of phosphorus does not exceed 14 % (Table IV). About 12 % of the protein is in the solubilized form when 57 % of the total NADH–ferricyanide reductase activity is in the supernatant.

TABLE V

BREAKDOWN OF CARDIOLIPIN *versus* RELEASE OF NADH–FERRICYANIDE REDUCTASE IN THE SUPERNATANT BY PHOSPHOLIPASE A

Electron transport particles were digested by phospholipase A at 30° for 60 min in 0.06 M Tris·HCl buffer (pH 7.4). Ferricyanide activity was assayed in supernatant obtained by centrifuging the digest at 122000 × *g* for 30 min.

Phospholipase A concn. (μg/mg electron transport particle protein)	*Cardiolipin cleaved* (%)	*NADH–ferricyanide reductase solubilized* (%)
0.05	0	0
0.1 (1st treatment)	22.5	30.6
0.1 (2nd treatment)	27.4	38.3
0.8	36.4	54.1
3.0	50.0	57.2
9.0	70.9	62.5

Cardiolipin breakdown at this level of phospholipase A is about 50 %. At higher concentrations of phospholipase A, more solubilization of the dehydrogenase is observed along with more cardiolipin breakdown. Even at the highest levels of phospholipase A used in this study (12 μg phospholipase A per mg electron transport particle protein) release of cytochrome *c* from the pellet was not observed. However, when digestions were carried out in the presence of exogenous Ca^{2+} (0.001 M $CaCl_2$) release of cytochrome *c* was observed.

In order to account for the ineffectiveness of *C. adamanteus* venom in the extraction of NADH dehydrogenase from mitochondrial cristae, the effect of this venom on electron transport particle lipids was also studied. Digestion of electron transport particles was carried out simultaneously by heat-treated crude venoms of *N. naja* and *C. adamanteus*. At 50 μg venom protein per mg electron transport particle protein, *N. naja* venom hydrolyzed all of the phosphatidylethanolamine and lecithin and about 70 % of the cardiolipin whereas *C. adamanteus* venom caused breakdown of only 40 % of the phosphatidylethanolamine and almost an equal amount of lecithin. Neither breakdown of cardiolipin nor release of soluble NADH dehydrogenase was observed with *C. adamanteus*. At higher concentrations of venoms (100 μg venom protein per mg electron transport particle protein), *N. naja* venom caused almost complete breakdown of lipids while *C. adamanteus* venom could cleave approx. 47 % of each of phosphatidylethanolamine and lecithin. Addition of Ca^{2+} (0.001 M $CaCl_2$) which activates phospholipase A from *C. adamanteus* did not allow for the hydrolysis of more than 56 % of the lecithin and 56 % of the phosphatidylethanolamine. *C. adamanteus* was almost completely ineffective in the hydrolysis of cardiolipin and in the release of NADH dehydrogenase (Table VI).

Treatment of electron transport particles with potassium oleate and lysophosphatidylethanolamine at concentrations approximately equivalent to fatty acid and lysophospholipid produced during the phospholipase A digestion, did not cause the solubilization of NADH dehydrogenase. Therefore the formation of lysophosphatides and fatty acids by phospholipase A digestion *per se* is not the basis for the release of this protein from the membrane. Also phospholipase C digestion (30°, 60 min) which caused the breakdown of almost all the lecithin exclusively did not solubilize the NADH dehydrogenase.

TABLE VI

COMPARATIVE EFFECT OF CRUDE HEAT-TREATED VENOMS OF *N. naja* AND *C. adamanteus* VENOMS ON ELECTRON TRANSPORT PARTICLE LIPIDS

Digestions of electron transport particles were carried out at 30° for 60 min in 0.06 M Tris·HCl buffer (pH 7.4).

Venom (*μg*/*mg electron transport particle protein*)	*Lipids hydrolyzed* (%)		
	Phosphatidyl-ethanolamine	*Lecithin*	*Cardiolipin*
C. adamanteus			
50	40.1	39.3	0
100	47.8	46.4	0
100 + 0.001 M Ca^{2+}	55.9	56.0	0
N. naja			
50	100	100	68.5
100	100	100	88
100 + 0.001 M Ca^{2+}	100	100	>90

In addition to determining the extent of phospholipid breakdown and release of NADH dehydrogenase from the mitochondrial cristael membrane, the effect of phospholipase A treatment on several mitochondrial electron transport activities was investigated. By the use of quinones and ferricyanide, a method has been introduced for the resolution of the NADH dehydrogenase as a multi-enzyme complex[24]. Juglone (5-hydroxy-1,4-naphthoquinone), ferricyanide, ubiquinone, and duroquinone (2,3,5,6-tetramethyl-1,4-benzoquinone) are found to react at four sites in the NADH dehydrogenase segment of the electron transport system. Juglone (Site 1) and ferricyanide (Site 2) react before the piericidin A block whereas ubiquinone (Site 3) and duroquinone (Site 4) react after the piericidin A block and before the antimycin A block. Piericidin A has been previously shown to be an effective inhibitor of electron transport in the NADH dehydrogenase segment between flavoprotein and cytochrome.

When electron transport particles were incubated with phospholipase A from *N. naja* (0.05 *μg* phospholipase A per mg electron transport particle protein) NADH oxidase was inhibited almost completely within the first 15 min of incubation. The loss of activity (Table VII) was also reflected in the inhibition of ubiquinone-2 and

TABLE VII

EFFECT OF LOW LEVELS OF PHOSPHOLIPASE A TREATMENT OF ELECTRON TRANSPORT PARTICLES ON NADH DEHYDROGENASE SYSTEM

0.05 *μg* phospholipase A per mg electron transport particle protein. Specific activity is expressed as *μ*moles NADH oxidized per min per mg protein.

Electron acceptors	*Control electron transport particle*	*Control electron transport particle + piericidin*	*NADH dehydrogenase activity*			
			15 min	*30 min*	*45 min*	*60 min*
Ferricyanide	14.5		14.5	14.2	14.1	14.2
Duroquinone	0.207	0.017	0.052	0.034	0.034	0.027
Ubiquinone-2	0.241	0.026	0.060	0.043	0.034	0.034
Juglone	0.431	0.354	0.388	0.397	0.405	0.414

duroquinone reduction. On the other hand, piericidin A-insensitive juglone reductase activity increased slightly and ferricyanide reductase remained essentially constant. Inhibition of the succinoxidase system following phospholipase A digestion was much less than inhibition of the NADH oxidase system. Also very little change was observed in cytochrome oxidase activity during the digestion period (60 min).

Since phospholipase A digestion of electron transport particles produces compounds which could inhibit the activities of NADH dehydrogenase, the extent of the inhibition due to incubation of electron transport particles with fatty acids and lysophospholipids was investigated. Fig. 1 shows the effect of oleate concentration on NADH dehydrogenase activities. Oleate was added to the electron transport particles in the cold (0°) and assayed immediately. Even under the conditions of short incubation in the cold, reduction of cytochrome *c*, ubiquinone-2, and duroquinone by NADH was found to be inhibited almost completely at 1.25 μmoles oleate per mg electron transport particle protein. Ferricyanide activity was not affected whereas juglone activity was activated approx. 50 %. At a concentration of oleate (0.35 μmole oleate per mg electron transport particle protein) approximately equivalent to fatty acid released during the digestion of electron transport particles with phospholipase A, reduction of cytochrome *c*, ubiquinone-2, and duroquinone by NADH was only partially inhibited. Since the phospholipase A digestions were done at 30° for 1 h or more, it is possible that heat could influence the degree of fatty acid inhibition. Figs. 2–4 show the effect of heat treatment (30°) *versus* incubation in the cold (0°) on cytochrome *c*, ubiquinone-2, and duroquinone activities of oleate-treated electron transport particles. At 0.35 μmole oleate per mg electron transport particle protein, the inhibition of all activities was partial in the cold and remained almost the same during the course of the incubation. Incubation at 30° with oleate caused further inhibition of activity than incubation in the cold. The inhibition was progressive and almost complete after 60 min incubation. Heat treatment without oleate caused little change in activity.

FLEISCHER and co-workers[28,29] and MACHINIST AND SINGER[12] both have reported that ubiquinone and cytochrome *c* reduction can be restored by bovine serum albumin washing and addition of phospholipid only after brief incubation (3–10 min) with phospholipase A. They have reported that longer incubations with phospholipase A caused irreversible inactivation. In light of the discovery that heat markedly influences the degree of fatty acid inhibition of NADH dehydrogenase activities, the reversibility of oleate inhibition when electron transport particles are incubated at 0 and at 30° was investigated. Table VIII gives the effect of bovine serum albumin washing on oleate-treated electron transport particles. Activities of oleate-treated electron transport particles incubated in the cold were restored almost completely. On the other hand, activities of electron transport particles treated with oleate at 30° were recovered to only 50 % of the original electron transport particle activities. Washing of electron transport particles which was subjected only to heat treatment caused little change in all activities. Thus heat treatment appears to cause a marked irreversibility when NADH dehydrogenase activities are inhibited by fatty acids.

Lysophospholipids also represent a possible factor in the inhibition of electron transport activities as a result of phospholipase A digestions. When electron transport particles were incubated with lysophosphatidylethanolamine at a concentration equivalent to the average value of lysophosphatide produced during phospholipase A

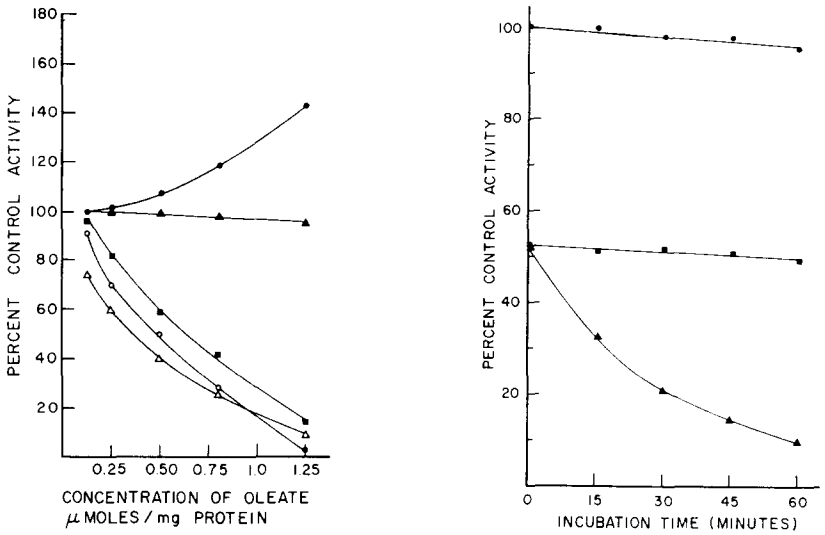

Fig. 1. Effect of oleate concentration on quinone, ferricyanide, and cytochrome *c* activities of electron transport particles. Oleate was added to the electron transport particles in the cold (0°) and assayed immediately. ●—●, juglone activity; ▲—▲, ferricyanide activity; ■—■, ubiquinone-2 activity; ○—○, duroquinone activity; △—△, cytochrome *c* activity.

Fig. 2. Effect of heat and cold treatment on oleate inhibition of cytochrome *c* reductase activity. Electron transport particles were treated with 0.35 μmole oleate per mg protein. ●—●, heat (30°) treatment only; ■—■, cold (0°) + oleate treatment; ▲—▲, heat (30°) + oleate treatment.

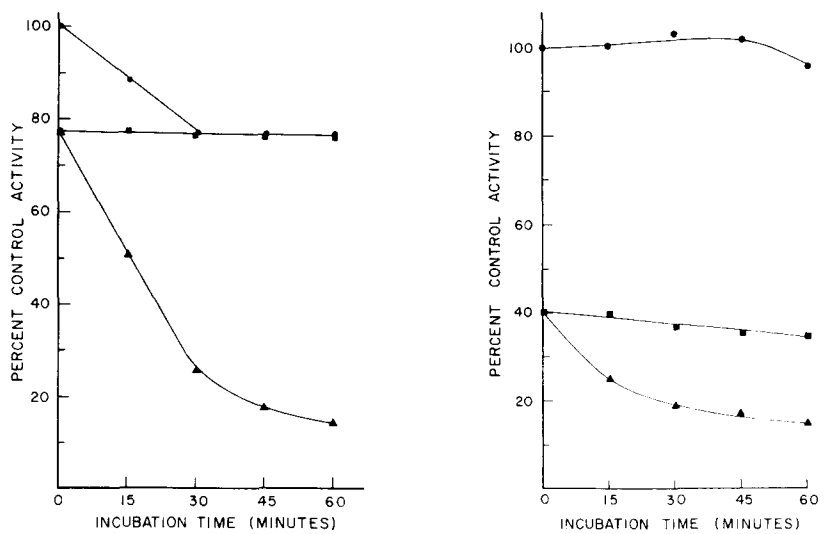

Fig. 3. Effect of heat and cold treatment on oleate inhibition of ubiquinone-2 activity of electron transport particles. Electron transport particles were treated with 0.35 μmole oleate per mg protein. ●—●, heat (30°) treatment only; ▲—▲, heat (30°) + oleate treatment; ■—■, cold (0°) + oleate treatment.

Fig. 4. Effect of heat and cold treatment on oleate inhibition of duroquinone activity of electron transport particles. Electron transport particles were treated with 0.35 μmole oleate per mg protein. ●—●, heat (30°) treatment only; ■—■, cold (0°) + oleate treatment; ▲—▲, heat (30°) + oleate treatment.

TABLE VIII

EFFECT OF BOVINE SERUM ALBUMIN WASHING ON CYTOCHROME *c*, UBIQUINONE-2, AND DUROQUINONE ACTIVITIES OF OLEATE-TREATED ELECTRON TRANSPORT PARTICLES

Treatment	*Specific activity (μmoles NADH per min per mg protein)*		
	Cytochrome c	*Ubiquinone-2*	*Duroquinone*
Heat-treated electron transport particles (30°)	0.585	0.208	0.200
Heat-treated electron transport particles + bovine serum albumin*	0.575	0.213	0.205
Heat-treated electron transport particles + oleate**	0.031	0.045	0.019
Heat-treated electron transport particles + oleate + bovine serum albumin	0.310	0.108	0.094
Cold-treated electron transport particles (0°) + oleate	0.249	0.097	0.162
Cold-treated electron transport particles + oleate + bovine serum albumin	0.505	0.188	0.148

* Bovine serum albumin concn., 1%.
** Oleate concn., 0.35 μmole per mg electron transport particles.

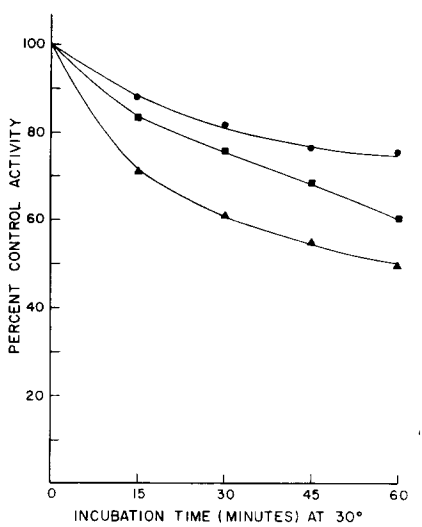

Fig. 5. Effect of incubation of lysophosphatidylethanolamine on ubiquinone-2, duroquinone, and cytochrome *c* activities of electron transport particles. Electron transport particles were treated with 0.5 μmole lysophosphatidylethanolamine per mg protein at 30°. ●—●, ubiquinone-2 activity; ▲—▲, duroquinone activity; ■—■, cytochrome *c* activity.

digestion of electron transport particles (0.5 μmole per mg electron transport particle protein), the rates of reduction of cytochrome *c*, ubiquinone-2, and duroquinone were diminished. However, unlike fatty acid inhibition, the level of inhibition by lysophosphatidylethanolamine did not exceed 50% (Fig. 5).

Due to the inhibitory nature of the products of phospholipase A digestion on NADH dehydrogenase activities, treatment of electron transport particles with phospholipase C was investigated. When electron transport particles were incubated with phospholipase C (0.05 mg phospholipase C per mg electron transport particle protein), ubiquinone-2 and duroquinone activities were found to be inhibited to different degrees (Table IX). Whereas ubiquinone-2 activity was inhibited 40 %, duroquinone activity declines 75 %. Piericidin A-insensitive NADH–juglone reductase activity showed slight activation. NADH–ferricyanide reductase activity was unaffected as in the case of the phospholipase A treatment. Electron transport of the succinate system was almost completely blocked as indicated by the almost complete inhibition of succinate–cytochrome *c* reductase activity. Succinate–DCIP reductase activity remained unaffected during this digestion.

TABLE IX

EFFECT OF PHOSPHOLIPASE C TREATMENT ON RESPIRATORY CHAIN-LINKED ENZYME SYSTEMS

Digested with 52 μg phospholipase C per mg electron transport particle protein, 30° for 80 min, 0.06 M Tris·HCl buffer (pH 7.4). Succinate–DCIP reductase specific activity expressed as mM succinate per min per mg enzyme protein and succinate–cytochrome *c* reductase expressed as μmoles cytochrome *c* per min per mg enzyme protein.

	Specific activity at different intervals					
	Control electron transport particle	*Control electron transport particle + piericidin*	*20 min*	*40 min*	*60 min*	*80 min*
NADH–ferricyanide reductase	11.5		11.5	11.2	11.1	11.2
NADH–juglone reductase	0.423	0.310	0.336	0.336	0.345	0.345
NADH–duroquinone reductase	0.233	0.016	0.091	0.069	0.060	0.060
NADH–ubiquinone-2 reductase	0.172	—	0.112	0.100	0.100	0.095
Succinate–DCIP reductase	0.14	—	0.14	0.14	0.14	0.14
Succinate–cytochrome *c* reductase	0.69	—	0.31	0.18	0.05	0.05

When phospholipase C was tested for its specificity of attack on mitochondrial lipids, it was found that this enzyme acted mainly on lecithin and was practically inactive with phosphatidylethanolamine and cardiolipin under the conditions used for the digestion of electron transport particles. Electron transport particles were treated with 0.05 mg phospholipase C per mg electron transport particle protein, approx. 90 % of the lecithin and 10 % of the phosphatidylethanolamine was cleaved in 60 min. At this concentration of enzyme, cardiolipin was not hydrolyzed.

DISCUSSION

Relative specificities of attack by phospholipase A on lipids of beef-heart mitochondria recorded here clearly show that cardiolipin is least labile to this enzyme and is attacked only under relatively drastic conditions. The slightly greater specificity for phosphatidylethanolamine in comparison to lecithin, observed by us, is in agreement with the observations of GALLAI-HATCHARD AND GRAY[25] during their studies on the action of *N. naja* venom phospholipase A on red cell membranes. From the correlation between cardiolipin hydrolysis by phospholipase A and release of membrane-

bound respiratory chain-linked NADH dehydrogenase in soluble form, cardiolipin breakdown appears to be an essential prerequisite for release of the NADH dehydrogenase. Using regulated levels of phospholipase A, we have been able to show that hydrolysis of almost the entire phosphatidylethanolamine and lecithin does not cause release of the NADH dehydrogenase. Since about 80 % of the maximum possible lysophosphatides and fatty acids are formed during this digestion, the hypothesis that fatty acids and lysophosphatides act as solubilizing agents of the NADH dehydrogenase or other membrane proteins can be discarded. This conclusion is further supported by the finding that externally added fatty acid and lysophosphatide to intact membrane do not solubilize the enzyme.

The inability of *C. adamanteus* venom to bring about the solubilization of NADH dehydrogenase may be attributed to its inactive nature towards beef-heart mitochondria cardiolipin. Since the venom causes breakdown of lecithin and phosphatidylethanolamine only, and does not extract membrane-bound NADH dehydrogenase from electron transport particles, the specific role of cardiolipin cleavage in the release of NADH dehydrogenase is further asserted. *C. adamanteus* venom not only shows an inability to hydrolyze cardiolipin but also differs from *N. naja* venom in its specificity of attack on phosphatidylethanolamine and lecithin. Whereas *N. naja* venom acts on phosphatidylethanolamine at faster rates than on lecithin, the rates of hydrolysis of lecithin and phosphatidylethanolamine by *C. adamanteus* venom are almost equal. This suggests the possibility that phospholipase A of these venoms consists of varying proportions of isoenzymes (*cf.* ref. 8) which differ in their substrate specificity for individual phospholipids. Recently DE HAAS *et al.*[26] have isolated phospholipase A from porcine pancreas which shows a greater specificity towards acidic phospholipids (*e.g.* cardiolipin) than neutral phospholipids. This also suggests that selective specificity of phospholipases from different sources may be due to the presence of isoenzymes.

The apparent lack of linear correlation between phospholipase A activity of different fractions of *N. naja* and their extraction power for NADH dehydrogenase of the respiratory chain of cardiac mitochondria observed by earlier investigators might be due to the use of lecithin as a substrate in the assay system for phospholipase A activity. Since hydrolysis of membrane lecithin by phospholipase A does not bear any correlation with extraction of NADH dehydrogenase, a linear correlation between conventional phospholipase A activity and NADH dehydrogenase extraction capabilities of different fractions of *N. naja* venom would not be obtained. Moreover, according to SALACH *et al.*[8] *N. naja* venom phospholipase A consists of six isoenzymes which vary considerably in their phospholipase A activity and also in their solubilization capacity. No linear correlation between these two functions is observed in these isoenzymes. It may be possible that those isoenzymes which have more specificity for beef-heart cardiolipin are better extracting agents for NADH dehydrogenase of the respiratory chain of cardiac mitochondria, although they seem to be poorer in phospholipase A activity determined by using lecithin in the assay system.

It has been reported[12] that brief incubations of phospholipase A cause inhibition of the NADH oxidase system and solubilize considerable amounts of proteins and lipids. During our investigations, when the level of phospholipase A used was enough to cause hydrolysis of about 70 % of the total lipids, only 2.6 % of the lipid and 5.7 % of the protein was solubilized. However, at higher concentrations of enzymes causing the breakdown of more than 90 % lipid, only 14 % of the lysophosphatides and 12 %

of the protein were in the supernatant. Lysophosphatides apparently are held to the membrane even after conversion of most of the phospholipids into lyso compounds.

In addition to the utilization of phospholipase A as a solubilizing agent, the enzyme has been used to show a direct role of phospholipid in mitochondrial electron transport[12, 28, 29]. In both cases the authors have noted that long-term digestions with phospholipase A cause irreversible inactivation of the NADH oxidase system. Our studies indicate that fatty acid released following the breakdown of phospholipid by phospholipase A is a potent inhibitor of the NADH dehydrogenase system. The level of inhibition of the phospholipid-dependent sites (*i.e.* NADH–ubiquinone-2, duroquinone, and cytochrome *c* reductases) by fatty acid is dependent on the concentration of fatty acid, temperature, and time of incubation. Two types of inhibition are evident. The incubation of fatty acid with mitochondrial particles in the cold causes the inhibition of electron transport at fatty acid levels 10-fold that present during phospholipase A treatment. The inhibition is not progressive with time of incubation and can be reversed by removal of the fatty acid. The other type of inhibition occurs when mitochondrial particles are incubated with fatty acids in the presence of heat. This type of inhibition is progressive with time of incubation and appears to be irreversible since removal of fatty acid by bovine serum albumin washing restored activity only partially. The inhibition occurs at temperatures used for phospholipase A treatments and at levels of fatty acid which are known to be produced during phospholipase A digestion. Lysophospholipids, on the other hand, only partially inhibit NADH dehydrogenase activities and are thus not as effective as fatty acid, but fatty acid and lysophospholipid together represent potent inhibitors of phospholipid-dependent enzyme systems. On the other hand, full reactivation of NADH–cytochrome *c* reductase activity in mitochondrial preparations extracted of phospholipid with phospholipase A has been accomplished by FLEISCHER AND FLEISCHER[28] using special conditions in which the incubation with phospholipase A is done in presence of bovine serum albumin to sequester fatty acids followed by washing out of the byproducts with bovine serum albumin.

The use of phospholipase C represents a possible alternative to phospholipase A when studying the role of phospholipid in membrane-bound enzyme systems. At the concentration of phospholipase C used in the present experiments with isolated phospholipid systems and membrane-bound systems, the enzyme was found to be much more active towards lecithin than phosphatidylethanolamine or cardiolipin which remained almost completely intact. It has been observed by DE HAAS *et al.*[27] and also by us during these studies that purified cardiolipin could be cleaved by phospholipase C at high concentrations of enzyme and long-term incubation. However, when electron transport particles were digested with high levels of phospholipase C (450 μg phospholipase C per mg electron transport particle protein) even after 3 h no breakdown of cardiolipin was observed. During these digestions almost all lecithin and about 15 % of the total phosphatidylethanolamine was cleaved and the supernatant obtained by centrifugation at 122000 $\times g$ was devoid of any NADH–ferricyanide activity which was almost quantitatively recovered in the pellet. Thus the availability of cardiolipin in electron transport particles to phospholipase C hydrolysis is less than with the purified cardiolipin.

Phospholipase C when used to study the role of phospholipids in the mitochondrial electron transport system shows different effects than phospholipase A. Our

data indicates that the NADH dehydrogenase system is more sensitive than the succinate system to the breakdown of phospholipids by phospholipase A. The succinate system responds to the breakdown of lecithin by phospholipase C by being completely inhibited whereas the NADH dehydrogenase system is only partially inhibited. Furthermore, the site of duroquinone activity in the NADH dehydrogenase system is inhibited to a greater degree than the site of ubiquinone activity when phospholipase C is used. Phospholipase A causes the complete inhibition of both NADH–duroquinone reductase and NADH–ubiquinone reductase. The difference between the two phospholipases may be due to the manner in which the phospholipid is hydrolyzed, the type of phospholipid hydrolyzed, and to the action of the by-products of the phospholipase digestion. FLEISCHER AND FLEISCHER[28] have observed that complete removal of both intact phospholipid and lysophospholipid is necessary before loss of activity of the succinate system is observed following phospholipase A treatment. This finding and our observation that phospholipase C treatment inhibits the succinate system would suggest a role for the charged region of the phospholipid in maintaining the function of the succinate system.

ACKNOWLEDGEMENTS

Supported under grant AM04663 from the National Institute of Arthritis and Metabolic Diseases. F.J.R. was supported by training grant GM01195 and F.L.C. by Career Grant K6-21,839 from the Institute of General Medical Science.

REFERENCES

1 G. RENDINA AND T. P. SINGER, *J. Biol. Chem.*, 234 (1959) 1605.
2 R. L. RINGLER AND T. P. SINGER, *Biochim. Biophys. Acta*, 29 (1958) 661.
3 R. L. RINGLER, S. MINAKAMI AND T. P. SINGER, *J. Biol. Chem.*, 238 (1963) 801.
4 C. GREGOLIN AND T. P. SINGER, *Biochim. Biophys. Acta*, 67 (1963) 201.
5 B. FLEISCHER, A. CASU AND S. FLEISCHER, *Biochem. Biophys. Res. Commun.*, 24 (1966) 189.
6 T. E. KING, R. L. HOWARD, J. KETTMAN, JR., B. M. HEGDEKAR, M. KUBOYAMA, K. S. NICKEL AND E. A. POSSEHEL, in E. C. SLATER, *Flavins and Flavoproteins*, Elsevier, Amsterdam, 1966, p. 441.
7 T. CREMONA AND E. B. KEARNEY, *J. Biol. Chem.*, 239 (1964) 2328.
8 J. I. SALACH, P. TWRINI, J. HANBER, R. SENG, H. TISDELE AND T. P. SINGER, *Biochem. Biophys. Res. Commun.*, 33 (1968) 936.
9 G. P. BRIERLY, A. MEROLA AND S. FLEISCHER, *Biochim. Biophys. Acta*, 64 (1962) 218.
10 R. L. LESTER AND S. FLEISCHER, *Biochim. Biophys. Acta*, 47 (1961) 358.
11 S. FLEISCHER, S. H. KLOUWEN AND G. BRIERLEY, *J. Biol. Chem.*, 236 (1961) 2936.
12 J. M. MACHINIST AND T. P. SINGER, *J. Biol. Chem.*, 240 (1965) 3182.
13 H. LÖW AND I. VALLIN, *Biochim. Biophys. Acta*, 69 (1963) 361.
14 J. FOLCH, M. LEES AND G. H. SLOANE-STANLEY, *J. Biol. Chem.*, 226 (1957) 497.
15 G. ROUSER AND S. FLEISCHER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 385.
16 F. F. SUN AND F. L. CRANE, *Biochim. Biophys. Acta*, 172 (1969) 417.
17 B. SHAPIRO, *Biochem. J.*, 53 (1953) 663.
18 P. S. CHEN, JR., T. Y. TORIBARA AND H. WARNER, *Anal. Chem.*, 28 (1956) 1756.
19 T. YONETANI, *J. Biol. Chem.*, 236 (1961) 1680.
20 S. FLEISCHER, G. ROUSER, B. FLEISCHER, A. CASU AND G. KRITCHEVSKY, *J. Lipid Res.*, 8 (1967) 170.
21 S. MINAKAMI, R. L. RINGLER AND T. P. SINGER, *J. Biol. Chem.*, 237 (1962) 569.
22 H. D. TISDALE, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods of Enzymology*, Vol. 10, Academic Press, New York, 1967, p. 213.
23 S. TAKEMORI AND T. E. KING, *J. Biol. Chem.*, 239 (1964) 3546.
24 F. J. RUZICKA, Y. C. AWASTHI AND F. L. CRANE, *Federation Proc.*, 28 (1969) 1196.

25 J. GALLAI-HACHARD AND G. M. GRAY, *European J. Biochem.*, 4 (1968) 35.
26 G. H. DE HAAS, N. M. POSTEMA, W. NIEUWENHUIZEN AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 159 (1968) 103.
27 G. H. DE HAAS, P. P. M. BONSEN AND L. L. M. VAN DEENEN, *Biochim. Biophys. Acta*, 116 (1966) 114.
28 S. FLEISCHER AND B. FLEISCHER, in R. W. ESTABROOK AND M. E. PULLMAN, *Methods in Enzymology*, Academic Press, New York, 1967, p. 416.
29 S. FLEISCHER, A. CASU AND B. FLEISCHER, *Federation Proc.*, 23 (1964) 2305.