Partial Purification and Characterization of the Phospholipase A_2 from Rat Liver Mitochondria*

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ABSTRACT: Phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) was purified 160-fold from rat liver mitochondria by precipitating the proteins with ammoniacal acetone, extracting the precipitated proteins with potassium chloride solution, fractionating the extract by ammonium sulfate precipitation, and separating the enzyme by gel filtration through Sephadex G-200. The purified enzyme-catalyzed production of equal amounts of unsaturated fatty acid and 1-acylglycerophosphorylethanolamine from phospholipids which indicates that the enzyme is specific for the 2 position and is not contaminated with a lysophospholipase. Phosphatidylethanolamine was most rapidly hydrolyzed at pH 9.5, less at pH 7.4. In contrast phosphatidylserine was hydrolyzed more extensively at pH 7.4 than pH 9.5. Phosphatidylcholine, phosphatidic acid, phosphatidylinositol, and cardiolipin were less extensively hydrolyzed. Mixtures of phosphatidylethanolamine and cardiolipin were hydrolyzed more nearly completely than was either compound alone at pH 7.4 but not at 9.5. Both hexadecyltrimethylammonium chloride and di-*n*-octadecyl phosphate stimulated hydrolysis of phosphatidylethanolamine at pH 7.4. Hexadecyltrimethylammonium chloride in low concentrations stimulated and di-*n*-octadecyl phosphate inhibited hydrolysis at pH 9.5. Monoacylglycerophosphorylethanolamine inhibited the reaction 50–75%. The rate at which fatty acids were removed from phosphatidylethanolamine (in decreasing order) was oleic, linoleic, linolenic, and arachidonic acids. This was uninfluenced by the nature of the acid in the 1 position. Hydrolysis of phosphatidylethanolamine was first order only during the first 3–5 min.

A phospholipase A in the mitochondria of rat liver was first demonstrated in 1965 (Scherphof and van Deenen; Rossi *et al.*, 1965). Tracer amounts of phosphatidylethanolamine were hydrolyzed to a greater extent than phosphatidylcholine, and the pH optimum for the enzyme was pH 7.5 (Scherphof and van Deenen, 1965). Bjørnstad (1966) reported that the mitochondrial phospholipase A hydrolyzed endogenous phospholipids optimally at pH 8.5–9.0, and he found that almost half of the fatty acids produced from the endogenous lipids were unsaturated. Scherphof *et al.* (1966) demonstrated that the phospholipase A in mitochondria, designated phospholipase A_2, hydrolyzes unsaturated fatty acids at the 2 position of phosphatidylethanolamine preferentially. Unless Ca^{2+} or fatty acid is added, there is a lag period in the reaction (Waite and van Deenen, 1967; Waite *et al.*, 1969a). The outer membrane of the mitochondria contains most of the phospholipase A_2 (Nachbaur and Vignais, 1968; Waite, 1969). Waite *et al.* (1969b) demonstrated that phospholipase A_2 activity is related to mitochondrial swelling, direct experimental support of the hypothesis of Wojtczak and Lehninger (1961) that phospholipid metabolism is related to morphologic changes of the mitochondrion.

We describe here a procedure for partially purifying phospholipase A from rat liver mitochondria, and present data on the characteristics of the reaction (as a function of time, substrate, and protein concentration), on substrate specificity, and on the effect of amphipathic ions on the reaction.

Materials and Methods

Materials. We synthesized 2-[1-^{14}C]linoleoyl- and 1-[9-10-^{3}H]palmitoyl-2-[1-^{14}C]linoleoylphosphatidylethanolamine (Waite and van Deenen, 1967). Phosphatidic acid and phosphatidylinositol (both dioleoyl) were purchased from Serdary Laboratories, London, Ontario, and cardiolipin (beef heart) from General Biochemicals, Chagrin Falls, Ohio. We prepared phosphatidylcholine, phosphatidylethanolamine, and monoacylphosphatidylethanolamine from rat liver, as before (Waite and van Deenen, 1967). Dioleoylglycerophosphorylethanolamine was the generous gift of Dr. G. L. Scherphof, Utrecht, The Netherlands. All phospholipids were purified by thin-layer chromatography before use.

Linoleic acid was obtained from Applied Science Laboratories, Inc., State College, Pa., di-*n*-octadecyl phosphate from Aldrich Chemical Co., Milwaukee, Wis., and hexadecyltrimethylammonium chloride from Eastman Organic Chemicals, Rochester, N. Y.

Method of Purification. Six to ten rat livers were homogenized in 0.25 M sucrose and the mitochondria isolated (Waite and van Deenen, 1967). The mitochondrial pellet was resuspended in a volume of 0.05 M glycine-NaOH (pH 9.5) equal to twice the original weight of the livers. This suspension was then slowly added to 10.5 volumes of ice-cold acetone containing 0.0125 ml of concentrated NH_4OH/100 ml. The mixture was stirred for 5 min and then centrifuged (25,000*g* for 10 min). The pellet was resuspended in 0.5 M KCl–0.05 M glycine-NaOH (pH 9.5) equal in volume to the mitochondrial suspension, and was dialyzed overnight against two changes of the buffer. The dialysand was centrifuged at 20,000*g* for 20 min, and the supernatant fraction was precipitated by adding solid $(NH_4)_2SO_4$ to 30–95% saturation. The precipitated proteins were resuspended in 10 ml of the glycine buffer, were dialyzed 2–3 hr against the same buffer, and were then filtered through a 2.5 × 90 cm column of Sephadex G-200. Over 90% of the phospholipase A activity emerged with the void volume. The preparation was used at this stage of purification.

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TABLE I: Purification of Phospholipase A_2 Achieved at Each Step of Preparation.[a]

Fraction	Protein (mg)	Phospholipase Activity			
		Sp Act.	Total Act.	-Fold Increase	Recov of Original Act. (%)
Mitochondria	2180.0	0.75	1640		
Mitochondria + KCl		1.75	3820		
Extract from mitochondria precipitated with acetone–NH_3	475.0	6.4	3040	3.7	80
$(NH_4)_2SO_4$ precipitate	294.0	8.8	2590	5.0	68
Sephadex filtrate	9.6	275.0	2640	160.0	69

[a] Purification and percentage recovery of the enzyme after each step is expressed in terms of the assay of the phospholipase A_2 in the presence of 0.5 M KCl. Specific activity is nanomoles [^{14}C]linoleic acid released per minute per milligram of protein. Total activity is the specific activity multiplied by the milligrams of protein.

Method of Assay. Hydrolysis of [^{14}C]phosphatidylethanolamine was measured in mixtures that contained (unless otherwise stated) 100 μM [^{14}C]phosphatidylethanolamine (15,000 cpm), 2 mM $CaCl_2$, 100 mM glycine-NaOH (pH 9.5), and enough enzyme to catalyze hydrolysis of 5–20 nmoles of [^{14}C]phosphatidylethanolamine. The total volume was 1.0 ml; incubation was for 5 min in the case of lipid-free enzyme preparations, 15 min for mitochondria. All lipids were added as sonicated suspensions in water. The reaction products, extracted by the method of Bligh and Dyer (1959), were separated by thin-layer chromatography, first in a chloroform–petroleum ether–acetic acid system (65:35:2, v/v), then in a chloroform–methanol–H_2O system (65:35:4, v/v). The silicic acid containing the radioactive compounds (detected by exposure to I_2 vapor) was scraped into scintillation vials containing Omnifluor scintillation mixture and a thixotropic gel.

The ^{3}H,^{14}C-labeled substrate was used in the same manner except all amounts were increased fourfold. The products were eluted from the silicic acid with methanol and counted without the thixotropic gel (Waite and van Deenen, 1967). Protein was determined by the method of Lowry *et al.* (1951).

Nonradioactive substrates were hydrolyzed in the same manner as the [^{14}C]substrate, except all amounts were increased fivefold, so that 500 nmoles of substrate was used. After incubating 30 min, we added 200 nmoles of pentadecanoic acid (the internal standard) and 0.2 ml of glacial acetic acid to stop the reaction. The fatty acids released were extracted with pentane and separated from other lipids by thin-layer chromatography in an ether–petroleum ether–formic acid system (25:75:1.5, v/v). After the fatty acids were eluted from the silicic acid with chloroform, and the chloroform was evaporated in a stream of nitrogen, methyl esters of the fatty acids were made by use of methanol–H_2SO_4 (Rogozinski, 1964). These esters were analyzed with a Barber-Coleman gas chromatograph fitted with a 6-ft. column packed with ethylene glycol succinate on Anakrom ABS, 90–100 mesh (Analabs, Inc., Hamden, Conn.). The column was operated at 190° with a nitrogen flow of 75 ml/min; the esters were detected with a hydrogen-flame detector. Monoacylglycerophosphorylethanolamine was extracted (Bligh and Dyer, 1959), isolated by thin-layer chromatography with use of a chloroform–methanol–NH_4OH–H_2O system (65:35:3:2, v/v), and eluted from the silicic acid with methanol. Methanol–H_2SO_4 was used to transesterify the fatty acids on the monoacylglycerophosphorylethanolamine. A standard mixture of fatty acids treated with methanol–H_2SO_4 showed no preferential loss of any of the fatty acids.

Results

Enzyme Purification. Table I shows the increase in specific activity and recovery of the phospholipase A_2 during the various stages of purification. Since the phospholipase A_2 in the mitochondria is more active in the presence of KCl (Figures 1 and 2), 100% activity was taken as that activity observed in the mitochondria when the assays were run in KCl.

The ammoniacal acetone was found to be the best solvent for removing lipid and for recovering the enzyme. No phospholipids (phosphorus extractable with lipid solvents) were found in the soluble fraction obtained from the mitochondria precipitated in 90% ammoniacal acetone, the "soluble" fraction being the proteins not sedimenting at 25,000*g* for 20 min.

Unless the proteins precipitated by ammoniacal acetone were extracted with the buffered 0.5 M solution of KCl, most phospholipase A_2 remained in the pellet (Figure 1). Higher concentrations of KCl did not remove more of the phospholipase A_2, although some was still detected in the particulate fraction. Solutions of NaCl, KNO_3, K_2CO_3, and K_2SO_4 were equally effective; corresponding molar concentrations of glycine–NaOH (pH 9.5) were not. Addition of KCl did not cause an appreciable change in the amount of protein solubilized (about 25%). Figure 2 shows that KCl, added to the assay mixture, inhibits the activity of the enzyme freed from the particulate fraction, but stimulates the activity of the enzyme still associated with the particulate fraction which had not been extracted with KCl.

When the solubilized enzyme was precipitated with $(NH_4)_2SO_4$ (35–95% saturated) to concentrate the solution, the enzyme had only slightly higher specific activity. Phospholipase A_2 begins to precipitate when the solution is 35% saturated, but is not entirely precipitated until the solution is 95% saturated with $(NH_4)_2SO_4$.

The phospholipase A_2 was purified 30-fold by gel filtration through Sephadex G-200 (Table I). More than 90% of the

TABLE II: Hydrolysis of 1-[9,10-^{3}H]Palmitoyl-2-[1-^{14}C]linoleoylglycerophosphorylethanolamine by Phospholipase A_2 in Various Stages of Purity.[a]

Fraction	Monoacylglycerophosphoryl-ethanolamine (nmoles)			Fatty Acid (nmoles)			Glycerophosphoryl-ethanolamine	
	^{3}H	^{14}C	^{3}H/^{14}C	^{3}H	^{14}C	^{3}H/^{14}C	nmoles	%
Mitochondria	26.2	6.8	3.8	9.0	36.8	0.24	2.4	6
Mitochondria + KCl	36.0	2.2	16.4	13.4	60.0	0.22	12.4	22
Extract from mitochondria precipitated with acetone–NH_3	32.2	1.4	23.0	6.0	46.6	0.13	5.2	12
Sephadex filtrate	47.0	2.2	21.3	4.4	49.6	0.09		

[a] The reaction mixtures contained 2.1 mg of mitochondrial protein, 0.76 mg of solubilized protein, and 0.112 mg of the filtrate protein; the incubation time was 10 min. There was 9% less monoacylglycerophosphorylethanolamine recovered than free fatty acid. The values presented here have been corrected for this difference. The amount of glycerophosphorylethanolamine produced was calculated as the difference between the nanomoles of fatty acid and monoacylglycerophosphorylethanolamine recovered, divided by two. The per cent glycerophosphorylethanolamine is the percentage of monoacylglycerophosphorylethanolamine hydrolyzed to glycerophosphorylethanolamine.

phospholipase A_2 emerged from the column with the void volume. Although clear when placed on the column, the enzyme preparation was slightly turbid when it emerged. Gel filtration in the presence of 0.5 M KCl did not change the elution behavior.

At this stage the enzyme preparation lost all activity in 3–4 days when stored at 0°; freezing and thawing accelerated inactivation. For most experiments, we used the enzyme the same day it was prepared, but occasionally it was used 1–2 days later, which accounts for the lower specific activity.

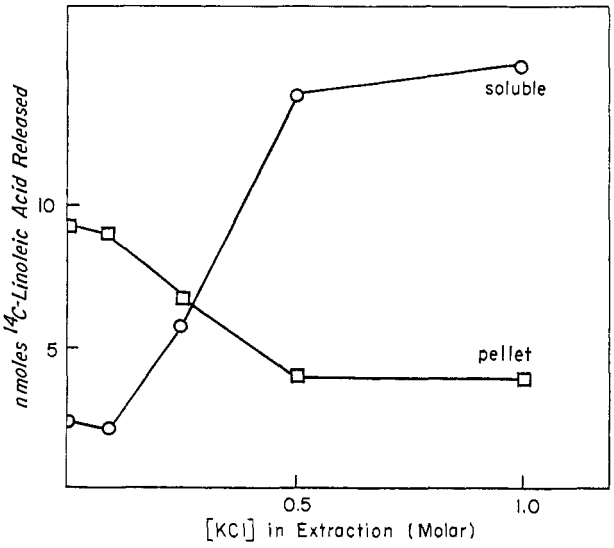

FIGURE 1: Relation between enzyme activity and concentration of KCl used to extract the enzyme. Mitochondria precipitated with acetone and NH_4OH were extracted with 0.05 M glycine (pH 9.5) equal in volume to the mitochondria before precipitation. The mixture was then centrifuged and the pellet again resuspended in 0.05 M glycine. A 2.0 M KCl solution was added to aliquots for the resuspended pellet to give the indicated final concentrations, the mixture stirred for 5 min at 0° and then centrifuged 25,000*g* for 10 min. The pellet was resuspended in 0.05 M glycine equal in volume to that of the mixture before centrifugation. All fractions were dialyzed 3 hr against 0.05 M glycine (pH 9.5). Equal volumes of each fraction were then assayed as described in Experimental Section.

Positional Specificity of the Enzyme. The mitochondrial preparations were shown to have some phospholipase A_1 and (or) lysophospholipase activity in addition to the phospholipase A_2 activity (Waite and van Deenen, 1967). ^{3}H,^{14}C-labeled phosphatidylethanolamine was used as substrate for the phospholipase A_2 to determine how much of these enzymes was present at different stages of purification (Table II). When mitochondria were used, some [2-^{14}C]monoacylglycerophosphorylethanolamine was produced, and 6% of the monoacylglycerophosphorylethanolamine was hydrolyzed further to glycerophosphorylethanolamine. If KCl was added to the assay mixture less [^{14}C]monoacylglycerophosphorylethanolamine was recovered, but more glycerophosphorylethanolamine. The soluble fraction, obtained from the ammoniacal acetone precipitated mitochondria, appeared to be as specific for the 2 position as is

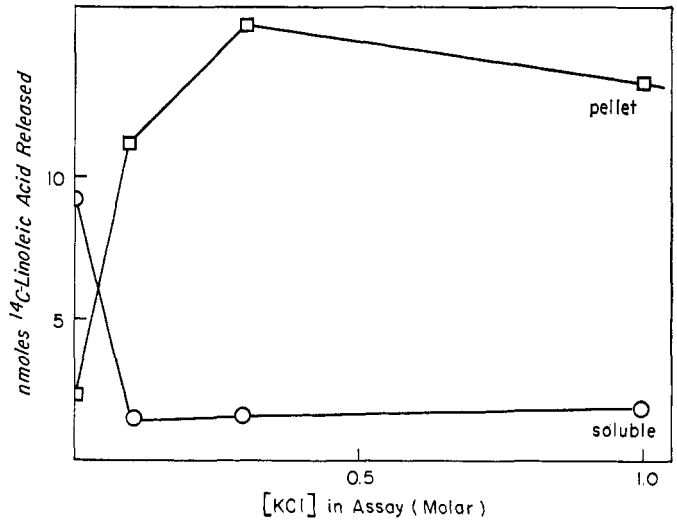

FIGURE 2: Relation between enzyme activity and concentration of KCl in the reaction mixture. Phospholipase A_2 activity in the dialyzed soluble fraction obtained from a 0.5 M KCl extract and in the particulate fraction resuspended in 0.05 M glycine (pH 9.5) which had not been extracted were assayed with the indicated concentrations of KCl.

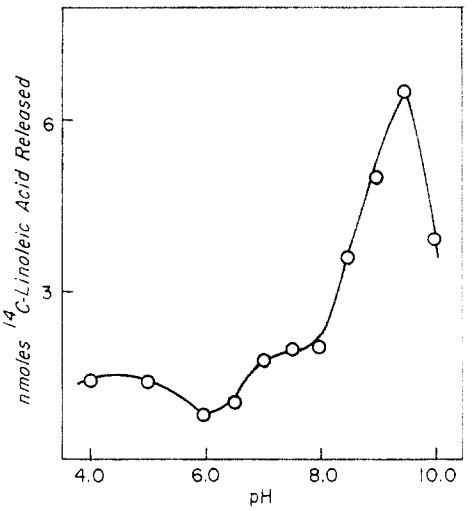

FIGURE 3: Relation of phospholipase A_2 activity and pH of the reaction mixture. The pH of the reaction mixture was maintained with 0.10 M buffer (4.0–6.0, sodium acetate; 6.0–8.5 Tris–maleate; 8.5–10.0 glycine–NaOH). The amount of enzyme from Sephadex filtration was 15 μg of protein and the incubation time was 5 min.

Crotalus adamanteus venom phospholipase: the $^3H/^{14}C$ ratio of the products with the soluble fraction was 23.0 for monoacylglycerophosphorylethanolamine and 0.13 for the fatty acid, as compared to 15.6 and 0.15, respectively, with the venom. Nonetheless, the soluble fraction hydrolyzed 12% of the monoacylglycerophosphorylethanolamine to glycerophosphorylethanolamine. The activity that gave rise to glycerophosphorylethanolamine was lost on gel filtration. These values were corrected for the difference (9%) between the amount of fatty acid and monoacylglycerophosphorylethanolamine recovered when the venom phospholipase A_2 was used. This difference is attributed to the small amount of [^{3}H]palmitic acid in the 2 position and [^{14}C]linoleic acid in the 1-position (shown by $^3H/^{14}C$ ratios), or to the loss of some monoacylglycerophosphorylethanolamine when the lipid was extracted from the silicic acid.

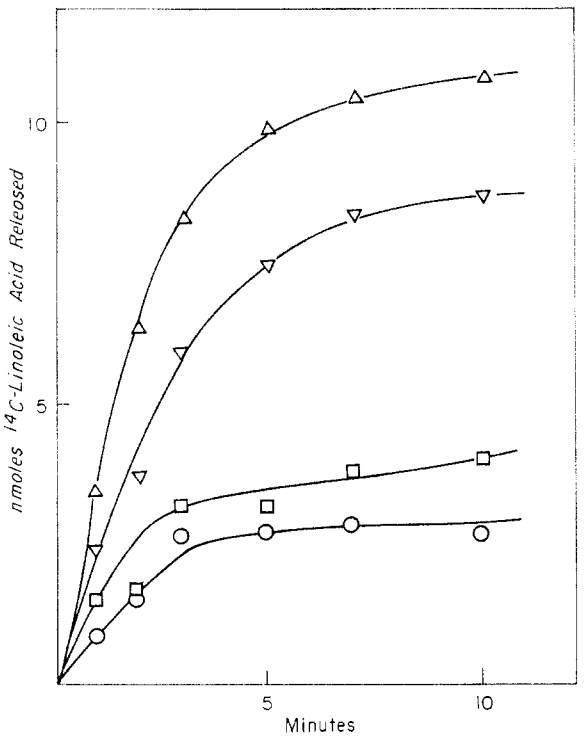

FIGURE 4: The phospholipase A_2 activity as a function of time. Phospholipase A_2 assays were run for the indicated times using 3.75 μg (○), 7.5 μg (□), 15 μg (▽), and 22.5 μg (△) filtrate protein.

TABLE III: Phospholipase A_2 Specificity.[a]

Expt	Substrate	Fatty Acid Released (nmoles) pH 7.4	pH 9.5
1	Phosphatidylserine	74	57
	Cardiolipin	24	37
	Phosphatidic acid		30
	Phosphatidylinositol		16
	Phosphatidylcholine		52
	Phosphatidylethanolamine	78	213
2	Cardiolipin	14	22
	Phosphatidylethanolamine	19	62
	Cardiolipin with phosphatidylethanolamine	50	76
	Dioleoylphosphatidylethanolamine		90

[a] The fatty acids released from the indicated substrates were estimated by gas–liquid chromatography. Protein content was 0.60 mg (expt 1) and 0.38 mg (expt 2), incubation time, 10 min and substrate concentration, 0.10 mM, except when cardiolipin and phosphatidylethanolamine were used together, the concentration of each was 0.05 mM. All values were corrected for nonenzymatic hydrolysis. No fatty acids were detected when the enzyme was incubated without substrate.

Assay Conditions. Optimal conditions for the assay of the phospholipase A were determined. Figure 3 shows the phospholipase A activity in relation to pH. Optimal activity is found at pH 9.5; the activity was much less if the pH was changed 0.5 pH unit either way. Intact mitochondria had a similar activity–pH relationship (Waite *et al.*, 1969a,b). The activity in the acid pH range cannot be attributed to the lysosomal phospholipase; because of its smaller molecular size (R. C. Franson and M. Waite, unpublished data) it would not appear in the void volume of the Sephadex filtrate.

Hydrolysis of phosphatidylethanolamine was directly proportional to time for only the first 3 min of the incubation (Figure 4). Hydrolysis continued for as long as 30 min, but at a slower rate. In some experiments the reaction was linear with time for 5–6 min, possibly because of variation in the liposome size of the substrate.

Substrate concentration was varied to determine the concentration for optimal activity by adding different amounts of an ultrasonic suspension to the reaction mixture. Little increase in hydrolysis occurred at substrate concentrations greater than 75–100 μM, so a concentration of 100 μM phosphatidylethanolamine was used routinely in the assays. Phospholipase A_2 activity also increased with increasing amounts of protein. The specific activity was about 25% greater with 10–20 μg of protein than with 30–100 μg. Gen-

TABLE IV: Hydrolysis of Various Phosphatidylethanolamines by Phospholipase A_2's from Two Sources.[a]

Type of Fatty Acid	Fatty Acid Released (%)		Fatty Acid Composition of the Monoacylglycerophosphorylethanolamine Formed (%)	
	By Mitochondrial Phospholipase A_2	By *Crotalus adamanteus* Phospholipase A_2	By Mitochondrial Phospholipase A_2	By *Crotalus adamanteus* Phospholipase A_2
Palmitic	0	0	29	29
Palmitoleic	0	1	2	3
Stearic	0	0	45	44
Oleic	26	19	14	14
Linoleic	54	49	5	6
Linolenic	5	5	0	0
Arachidonic	15	25	4	4

[a] The fatty acid composition was from the reaction described in expt 1 of Table III, and each fatty acid is expressed as the percentage of the total fatty acid found. Conditions for the reactions that include the enzyme from *Crotalus adamanteus* were such that there was more than 90% hydrolysis. These data are representative of those obtained in five experiments.

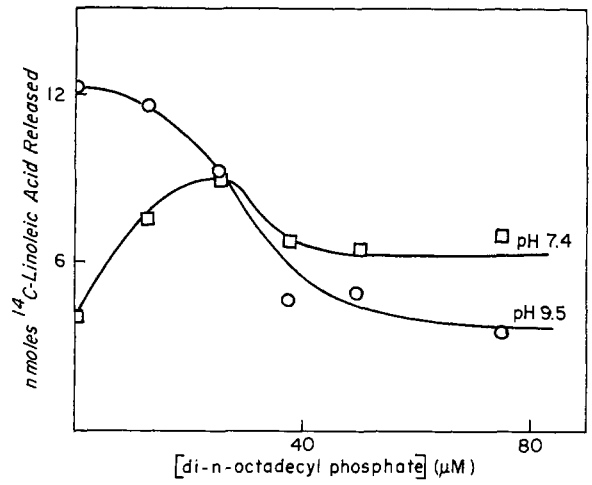

FIGURE 5: The effect of increasing concentrations of di-*n*-octadecyl phosphate on phospholipase A_2 activity. Ultrasonic suspension of di-*n*-octadecyl phosphate were added to the reaction mixture to give the designated concentrations. The lipids were allowed to interact for 15 min before the reaction was initiated by the addition of enzyme. The reaction mixture contained 22.5 μg of protein and was incubated 5 min.

erally, we used 40–80 μg of protein in assays to minimize experimental errors encountered with use of less protein.

Substrate Specificity and pH Effects. Table III presents data on the fatty acids released from phospholipids, as determined by gas–liquid chromatography. Of the six phospholipids used, the most fatty acids were released from phosphatidylethanolamine at pH 9.5. At pH 7.4, however, phosphatidylserine, an acidic phospholipid that contains nitrogen, was hydrolyzed to the same extent as phosphatidylethanolamine (expt 1). Also, hydrolysis of phosphatidylserine at pH 7.4 was slightly more complete than at pH 9.5. The reverse was true for another acidic phospholipid, cardiolipin. Phosphatidylserine was more fully hydrolyzed than the nonnitrogenous phospholipids. Although not shown in these data, about 1.5 times as much fatty acid was released from phosphatidylcholine at pH 9.5 as at pH 7.4. Liposomes prepared with mixtures of phosphatidylethanolamine and cardiolipin were hydrolyzed more completely at pH 7.4 than were liposomes of these phospholipids prepared separately (expt 2; 50 *vs.* 33 nmoles released). Hydrolysis of the mixed liposomes at pH 9.5 was less complete than that of the liposomes of the individual phospholipids (76 *vs.* 84 nmoles released). The nature of the fatty acids in the phospholipid also influenced the hydrolysis: dioleoylphosphatidylethanolamine was more efficiently hydrolyzed than was phosphatidylethanolamine isolated from rat livers (90 *vs.* 62 nmoles of fatty acid released).

Oleic acid comprised 26% of the fatty acids hydrolyzed from phosphatidylethanolamine by the mitochondrial phospholipase A_2, although it made up only 19% of the fatty acids in the 2 position, as shown by hydrolysis of the substrate by the phospholipase A_2 from *Crotalus adamanteus* venom (Table IV). Conversely, only 15% of fatty acids recovered was arachidonic acid, even though it comprised 25% of the total in the 2 position. Linoleic and linolenic acid were removed to extents intermediate to those of oleic and arachidonic acids. We conclude that the phospholipase preferentially catalyzes hydrolysis of those fatty acids in the 2 position that have the fewest double bonds. The fatty acid in the 1 position did not influence the hydrolysis; the same fatty acid composition was found for monoacylglycerophosphorylethanolamine as was found by use of snake venom phospholipase A.

Effect of Adding an Anionic or a Cationic Lipid. The data obtained with different substrates (Table III) suggest that the ratio of phospholipase A activity at pH 7.4 to that at pH 9.5 varies according to the charge on the substrate liposome. This effect was studied with phosphatidylethanolamine in combination with two ionic lipids that are not substrates; di-*n*-octadecyl phosphate, an anionic lipid, doubled the activity

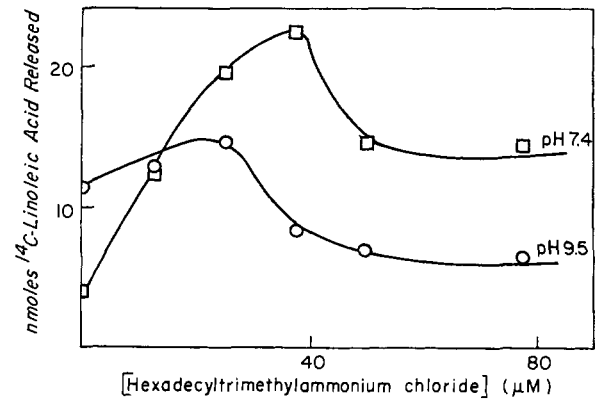

FIGURE 6: The effect of increasing concentrations of hexadecyltrimethylammonium chloride on phospholipase A_2 activity. The assays were the same as those described in the legend of Figure 5 except hexadecyltrimethylammonium chloride replaced di-*n*-octadecyl phosphate.

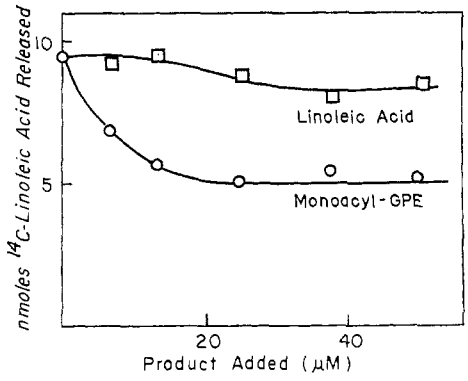

FIGURE 7: The effect of increasing concentrations of monoacylglycerophosphorylethanolamine and linoleic acid on phospholipase A_2 activity. Ultrasonic suspensions of monoacylglycerophosphorylethanolamine (monoacyl-GPE) (○) and linoleic acid (□) were added to give the indicated concentrations. The incubation time was 5 min and each reaction mixture contained 32 μg of protein.

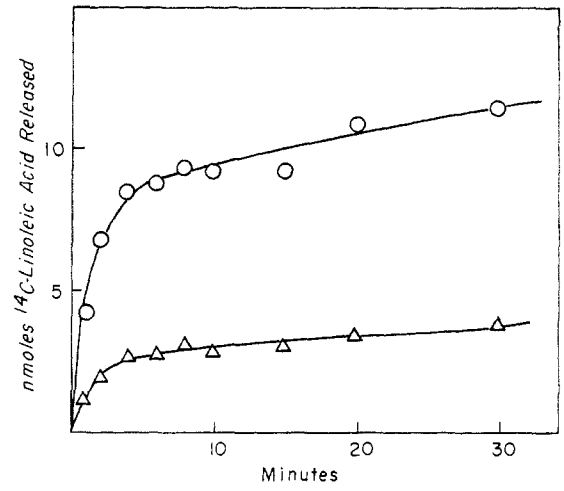

FIGURE 9: The effect of monoacylglycerophosphorylethanolamine on phospholipase A_2 activity at different incubation times. The conditions and additions were the same as those described in Figure 8 except 100 μM [^{14}C]phosphatidylethanolamine was used with 25 μM (△) or no (○) monoacylglycerophosphorylethanolamine.

at pH 7.4 but inhibited it at pH 9.5 (Figure 5); hexadecyltrimethylammonium chloride, a cationic lipid, stimulated phosphatidylethanolamine hydrolysis fivefold at pH 7.4 (Figure 6). A 40 μM concentration of hexadecyltrimethylammonium chloride is required for maximum stimulation, however, a concentration that is inhibitory at pH 9.5.

Inhibition by the Product. Linoleic acid added to the assay mixture causes little change in the hydrolytic activity whereas monoacylglycerophosphorylethanolamine inhibits maximally at a concentration of 20 μM (about one-fifth the concentration of substrate present) (Figure 7). There was inhibition at all substrate concentrations (Figure 8) and all incubation times (Figure 9) in the presence of both suboptimal (12.5 μM) and optimal (25 μM) concentrations of monoacylglycerophosphorylethanolamine.

Discussion

The mitochondria must be extracted with organic solvents to remove lipids before the enzyme can be characterized. This is not necessary with other phospholipases purified thus far, for example, that from *Crotalus adamanteus* (Wells and Hanahan, 1969), *Crotalus atrox* (Wu and Tinker, 1969), or pork pancreas (De Haas *et al.*, 1968). Most of the phospholipase A precipitated with ammoniacal acetone was associated with insoluble proteins, probably owing to protein–protein interactions of the sort that can be disrupted with salts. This effect of salt did not depend on the kind of anion or cation, although glycine, a zwitterion, was not effective.

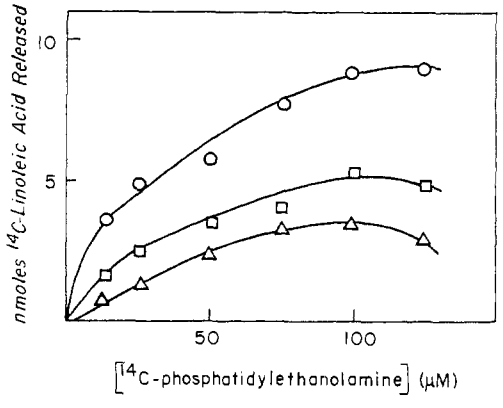

FIGURE 8: The effect of monoacylglycerophosphorylethanolamine on phospholipase A_2 activity at increasing concentrations of substrate. The reaction mixture contained the designated concentrations of [^{14}C]phosphatidylethanolamine with 25 μM (△), 12.5 (□), or no (○) monoacylglycerophosphorylethanolamine. The amount of enzyme was 32 μg of protein and the incubation time was 5 min.

The phospholipase A_2 appears to be in an aggregated form, judging from its behavior on Sephadex G-200 and its hazy appearance after gel filtration. No lipid was found in this preparation either by phosphorus determination of a chloroform extract of the enzyme or by iodine visualization of a chromatogram of the chloroform extract. If the enzyme were in the monomeric form, the molecular weight of the enzyme would be 2×10^5 or greater. In contrast, the molecular weights of other phospholipases A range from 13.8×10^3 for the enzyme from pork pancreas (De Haas *et al.*, 1968) to about 30×10^3 for the *Crotalus adamanteus* venom enzymes (Saito and Hanahan, 1962). Since KCl did not change the characteristics of the enzyme emerging from the Sephadex column it evidently does not disaggregate the enzyme.

The 160-fold purified phospholipase was most active on phosphatidylethanolamine at pH 9.5, with lower activity at pH 7.4, as was the case for intact mitochondria (Waite *et al.*, 1969a). It is possible that the hydrolysis at pH 9.5 and at pH 7.4 is catalyzed by two distinct enzymes. If this were the case these enzymes would have been purified to the same extent by these procedures. On the other hand, the enzyme which catalyzed the formation of [2-^{14}C]acylglycerophosphorylethanolamine and glycerophosphorylethanolamine from the ^{3}H,^{14}C-labeled substrate were lost during purification. Attempts are being made to determine the possible contribution of a contaminating enzyme to the phospholipase A activity observed at pH 7.4.

Scherphof and van Deenen (1965) and Bjørnstad (1966) reported the optimal activity of phospholipase A to be at pH values different than those reported here. Our findings make it reasonable to attribute the differences to variations in the lipid composition of the substrate. For example, more fatty acid was released from a mixture of cardiolipin and

phosphatidylethanolamine by the purified enzyme at pH 7.4 than was released from the two separately.

There are at least two explanations for the effects of added lipid on hydrolysis of phosphatidylethanolamine at the two pH values. First, it is possible that binding of the enzyme to the substrate is influenced by charge characteristics (ζ potential), as is *Crotalus adamanteus* venom phospholipase A (van Deenen and De Haas, 1963) and *Clostridium perfringens* phospholipase C (Bangham and Dawson, 1962). Optimal activity was found when the liposome was anionic, *e.g.*, at pH 7.4 (phosphatidylethanolamine with no net charge) with di-*n*-octadecyl phosphate to supply anions or at pH 9.5 (phosphatidylethanolamine with net negative charge) without added di-*n*-octadecyl phosphate. Extreme negative charge decreased activity, as shown with phosphatidylethanolamine in the presence of increasing concentrations of di-*n*-octadecyl phosphate at pH 9.5. Except at low concentrations hexadecyltrimethylammonium chloride inhibits at pH 9.5, possibly because of a large drop in the negative charge of the liposome. The fact that phosphatidylcholine (zwitterionic) is a poor substrate both at pH 7.4 and 9.5 supports the possibility that the enzyme–liposome interaction is favored by a net negative charge on the liposome. The effect of charge does not account completely for these observations: acidic phospholipids that do not contain nitrogen (cardiolipin, phosphatidic acid, and phosphatidylinositol) are poorly hydrolyzed and, moreover, hexadecyltrimethylammonium chloride stimulates activity at pH 7.4, even though the liposome would have a net positive charge at this pH.

Since further explanation is obviously needed, we suggest that the charge of the lipids in the liposome influences the packing of molecules in the liposome, which in turn influences the hydrolysis (Dawson, 1963). Lipids with no net charge could pack in the liposome too tightly for the enzyme to have access to the ester bonds. This might explain why both di-*n*-octadecyl phosphate and hexadecyltrimethylammonium choride stimulate hydrolysis of phosphatidylethanolamine at pH 7.4.

We have not considered the effect of Ca^{2+}, since there was an absolute requirement for Ca^{2+}, which indicates that it influences more than the ζ potential of the liposome. Bangham and Dawson (1962) showed that Ca^{2+}-activated phospholipase C catalyzes hydrolysis even when the Ca^{2+} had not altered the net charge on the substrate.

Our gas–liquid chromatographic analysis of the products showed that the enzyme hydrolyzes the 2-acyl moiety with the fewest double bonds. Oleic acid is one of the principal acids replaced in the liver phospholipids when rats deficient in essential fatty acids are fed a corn oil diet. This replacement is related to the mitochondrial phospholipase A_2 activity (Waite and van Golde, 1968). Our results do not agree with those of Bjørnstad (1966), who reported that 57% of the fatty acids released from endogenous phospholipids were saturated. It is not possible from Bjørnstad's data, however, to ascribe the origin of the fatty acids; possibly some were liberated from nonphospholipid esters, such as glycerides. Alternatively, the composition he found for the fatty acids possibly reflects the actual activity of the phospholipase A in the mitochondria, but the specificity was influenced drastically by the lipid composition of the reaction mixture.

The hydrolysis was linear only for 3–5 min, then slowed to about 5 to 10% of the original rate. We suggest that the enzyme binds to the liposome and rapidly catalyzes the hydrolysis, resulting in reorganization of the liposome because the physical–chemical nature of lipids (monoacylglycerophosphorylethanolamine and fatty acid *vs.* phosphatidylethanolamine) has changed which may limit the rate of the reaction. Since monoacylglycerophosphorylethanolamine inhibited hydrolysis, it appears that this compound must be removed from the enzyme site by the reorganization of the liposome before hydrolysis can proceed. This is analogous to the inhibition of *Naja naja* phospholipase A_2 by free fatty acid (Dawson, 1963). Fatty acids did not influence the activity of the purified mitochondrial phospholipase A_2, in contrast to their effect on the enzyme in intact mitochondria (Waite *et al.*, 1969a). This suggests that the fatty acid activates the enzyme within the mitochondrial membrane only.

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