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PURIFICATION AND PROPERTIES OF PHOSPHOLIPASE A_1 FROM RAT AND CALF BRAIN

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SUMMARY

A phospholipase has been purified about 50-fold from rat and calf brain, using separate procedures for each of these sources. The enzyme from both tissues hydrolyzed the 1 (or α') position of lecithin but not the 2 (or β) position of this substrate. It is therefore classified as a phospholipase A_1. In the presence of Triton X-100 the enzyme from rat brain hydrolyzed phosphatidyl ethanolamine 5 times slower and that from calf brain, 3 times slower than lecithin. If both Triton X-100 and sodium taurocholate were added to the reaction mixtures, phosphatidylethanolamine and phosphatidylcholine were hydrolyzed at about the same rates. Both preparations hydrolyzed lysolecithin at only 2–5% of the corresponding rate of hydrolysis of lecithin. The rat brain phospholipase had very low lipase activity, while the enzyme from calf brain hydrolyzed tripalmitin at about two thirds of the rate of hydrolysis of lecithin.

Phospholipase activity was stimulated by the addition of the nonionic detergent, Triton X-100, or the anionic detergent, sodium taurocholate, calcium ions had no effect on the reaction. The pH optimum of the reaction was 4.0 and the K_m 0.8 mM. The reaction was competitively inhibited by fatty acid; the K_i, using palmitate, was 0.07 mM, *i.e.* 11 times lower than the K_m.

INTRODUCTION

Phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) hydrolyzes one ester bond of phosphatidylcholine or phosphatidylethanolamine. The products of this reaction are fatty acid and monoacyl phosphoglyceride. The most extensively investigated phospholipases are those present in snake venoms. They hydrolyze the β-position of the phospholipids, releasing 1-acyl-glycerophosphorylcholine or 1-acyl-glycerophosphorylethanolamine (reviewed by VAN DEENEN AND DE HAAS[1], and by LANDS[2]). Phospholipase A activity has also been observed in several mammalian tissues (reviewed by VAN DEENEN AND DE HAAS[3]), and evidence has been obtained that these tissues might have two enzymes, with different specificities. One ("phospholipase A_2" (ref. 3)) is similar, in its positional specificity, to the enzyme from snake venom and hydrolyzes the ester bond in the 2-position of the glycerophosphatides. The second

("phospholipase A_1") removes the fatty acid from the 1-position. Indications for the presence of the two reactions were obtained, by showing that tissues have both 1-acylglycerophosphorylcholine and 2-acylglycerophosphorylcholine[4-7]. More direct evidence for the presence of these two enzymes was obtained by VAN DEN BOSCH AND VAN DEENEN[8] and by SCHERPHOF, WAITE AND VAN DEENEN[9]. The latter authors have shown that the two respective enzymes have a different subcellular location in rat liver. While the mitochondria show mostly phospholipase A_2, the microsomes have mostly phospholipase A_1 activity. These data were further elaborated by WAITE AND VAN DEENEN[10], but the respective enzymes were not extracted from the subcellular fractions and have not been purified. VAN DEN BOSCH *et al.*[11] have shown that pancreatic tissue has a heat-labile phospholipase A_1. However, DE HAAS, SARDA AND ROGER[12] and DE HAAS AND SLOTBOOM (personal communication) have shown that this activity is, most probably, due to the action of pancreatic lipase and not to that of a specific phospholipase. VOGEL AND BIERMAN[13] have shown that after injection of heparin, an enzyme which hydrolyzes lecithin to 2-acyl-glycero-3-phosphorylcholine is found in serum. However these authors have not ascertained whether this activity, which is optimal at pH 9.6, is performed by a specific phospholipase A_1 or is due to the plasma lipase.

ROITMAN AND GATT[14] have isolated an enzyme from rat brain which hydrolyzes sphingomyelin to ceramide (*N*-acyl sphingosine) and phosphorylcholine. This preparation also hydrolyzed lecithin and was considered to be a phospholipase C (EC 3.1.4.3) acting, similarly, on both phosphatides. Later, BARENHOLZ, ROITMAN AND GATT[15], separated this preparation into two separate enzymes. One, a sphingomyelinase, has a phospholipase C-like activity; it splits off the phosphorylcholine moiety of sphingomyelin but has no action on lecithin. The second is a phospholipase A, which splits one fatty acid off lecithin, but which has no action on sphingomyelin. When the positional specificity of the latter enzyme was tested, it was identified as a phospholipase A_1 (ref. 16). It hydrolyzed only the 1-position of lecithin but did not attack the 2-position of this substrate. The present paper describes, in detail, the isolation and properties of this enzyme. It also provides further evidence to support the definition of the enzyme as a phospholipase A_1.

EXPERIMENTAL PROCEDURE

Substrates

Egg and liver lecithin and phosphatidyl ethanolamine were isolated by standard procedures.

Phosphatidyl choline and phosphatidylethanolamine labeled predominantly in the 1-position were prepared as follows. 0.5 ml of an aqueous solution containing 1 μmole of neutralized [1-^{14}C]palmitic acid (36 μC/μmole) and 0.4 mg of bovine serum albumin was injected into the tail vein of a 100-g rat. After 1 h the animal was sacrificed and the liver excised. The lipids were extracted according to the procedure of FOLCH, LEES AND SLOANE-STANLEY[17] and were then chromatographed on a column of "Unisil" silicic acid, using a discontinuous gradient of increasing concentrations of methanol in chloroform. The lecithin, which had a specific activity of about 50 000 disint./min per μmole, was used directly. The fraction containing phosphatidylethanolamine was contaminated with lecithin; it was therefore chromatographed on thin-layer plates

of silica gel and the pure cephalin was eluted from the plate. Hydrolysis with *Crotalus adamanteus* toxin[18] showed that at least 85% of the radioactivity of lecithin and about 95% of that of phosphatidylethanolamine were present in the 1-position. In many experiments this lecithin was employed. In others 1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylcholine was prepared by deacylation of the above lecithin with Crotalus toxin[18] and reacylation of the 2-position with non-radioactive oleic acid[19].

Lecithin labeled with both ^{32}P and [9,10-$^{3}H_2$]oleic acid in the 2-position was synthesized as described by GATT, BARENHOLZ AND ROITMAN[16].

1-[9,10-$^{3}H_2$]stearoyl-2-stearoyl-3-glycero-phosphorylcholine; 1-[9,10-$^{3}H_2$]stearoyl-2-[1-^{14}C]oleoyl-glycero-3-phosphorylcholine; 1-[9,10-$^{3}H_2$]stearoyl-2-[1-^{14}C]stearoyl-3-glycerophosphorylcholine; dioleoyl lecithin and dilinoleoyl lecithin were generous gifts of Drs. VAN DEENEN, VAN DEN BOSCH AND DE HAAS.

1-acyl-2-[1-^{14}C]oleoyl-glycero-3-phosphorylcholine was prepared from 1-acyl-glycero-3-phosphorylcholine by acylation with [1-^{14}C]oleic acid, essentially by the method of ROBERTSON AND LANDS[19] except that the microsomes *plus* supernatant were used as source of enzyme. Glycerol tri-[1-^{14}C]palmitate was purchased from The Radiochemical Centre, Amersham. 1-[1-^{14}C]palmitoyl-glycero-3-phosphorylcholine was obtained from the ^{14}C-labeled liver lecithin by deacylation with Crotalus toxin[18]. Non-radioactive 1-acyl-glycero-3-phosphorylcholine was similarly prepared from egg and liver lecithin.

Triton X-100 was obtained from Rohm and Haas; Unisil-silicic acid of 300–325 mesh from Clarkson Chemical Co. Sodium taurocholate from Calbiochem.

Assay of reaction mixtures

Incubation mixtures containing lecithin (0.2 ml) were terminated by the addition of 2 ml of "Dole's reagent" (400 ml of isopropylalcohol, 100 ml of heptane and 10 ml of 0.5 M H_2SO_4) (ref. 20) followed by 1.2 ml of heptane and 1 ml of water. After separation of the phases, the heptane layer was transferred to a second tube and 2 ml of heptane followed by 100 mg of silicic acid (Mallinckrodt) were added. The tube was thoroughly mixed, centrifuged and the liquid was decanted into a counting vial. 10 ml of scintillation fluid (5 g of 2.5-diphenyloxazole and 130 mg of 1,4-bis-(4-methyl-5-phenyloxazolyl-2)-benzene in 1 l of toluene) was added and the radioactivity was determined in a liquid scintillation counter. When phosphatidylethanolamine was used, the heptane layer was evaporated, chromatographed on thin-layer silica gel plates in ether–methanol–acetic acid (98:2:0.2, by vol.). The fatty acid spots were scraped off, extracted with mixtures of chloroform and methanol and counted. When tripalmitin was used, the heptane layer was treated with 0.1 M NaOH in 50% ethanol. The upper phase was discarded, the ethanolic layer was acidified, the fatty acid was extracted with heptane and counted.

For gas–liquid chromatography, the fatty acids were methylated with distilled diazomethane in ether. The methyl esters were chromatographed on ethyleneglycol succinate polyester in a Packard gas–liquid chromatogram.

RESULTS

Purification of the enzymes

Rat, calf or bovine brain was used for the purification of the phospholipase A_1.

With rat brain, the procedure employed for the purification of sphingomyelinase[15] was essentially followed. With calf brain, extracts of acetone-treated tissue powders were used as starting material. Both tissues yielded preparations of phospholipase A_1 free of phospholipase A_2.

Purification of rat brain phospholipase (Table I)

Whole, adult rat brains were homogenized with 9 vol. of a mixture of 0.25 M sucrose and 10^{-3} M EDTA (pH 7). The homogenate was centrifuged for 10 min at 800 × *g*, the supernatant was decanted and further centrifuged for 10 min at 25 000 × *g*. The sediment ("particles") was suspended in sucrose-EDTA (4 ml per g of brain),

TABLE I

PURIFICATION OF RAT BRAIN PHOSPHOLIPASE A_1

Incubation mixtures, in volumes of 1 ml each, contained 150 μmoles of sodium acetate buffer (pH 4.2); 1.25 mg of Triton X-100; 1.1 μmoles of 1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylcholine (about 50 000 disint./min per μmole) and appropriate amounts of enzyme fraction. After 1 h at 37°, the reaction was terminated, the fatty acid was isolated according to EXPERIMENTAL PROCEDURE and its radioactivity determined. One unit is defined as that amount of enzyme catalyzing the hydrolysis of 1 mμmole of substrate per h.

Fraction	*Volume (ml)*	*Protein*		*Activity*		*Specific activity (units/mg)*	*Purification (fold)*
		Total (mg)	*Recovery %*	*Total (units)*	*Recovery %*		
Homogenate	165	1910		58 800		31	
Particles	76	700	36.6	28 600	48.7	41	1.3
Cholate extract	63	56	2.9	16 900	28.8	301	9.7
pH 4.2 supernatant	63	10	0.5	16 700	28.4	1670	54.0

subjected to sonic disintegration in a Raytheon sonic oscillator for 8 min at 10 kcycles/sec, and centrifuged for 20 min at 25 000 × *g*. The sediment was suspended in a solution of 0.5% sodium cholate in sucrose-EDTA (1.5 ml per g of brain), stirred for 1 h at 4°, and centrifuged for 20 min at 35 000 × *g*. The supernatant was retained and the precipitate again extracted and centrifuged as above. The two supernatants were combined and dialyzed against two changes of 0.02 M Tris buffer (pH 7.4). The contents of the dialysis bag were centrifuged for 10 min at 25 000 × *g* and the supernatant ("cholate extract") was collected and stored at −20°. The amount needed for one day's work was dialyzed for 1 h against 0.03 M sodium acetate buffer (pH 4.2). The contents of the dialysis bag were centrifuged and the precipitate discarded. The supernatant had about a 50-fold higher specific activity than the homogenate.

Purification of calf brain phospholipase (Table II)

100-g portions of whole calf brain were blended with 400 ml of acetone, previously cooled to −20°. After centrifuging, at −10°, for 10 min at 10 000 × *g*, the sediment was again blended and centrifuged as before. The dehydrated and defatted powder was air-dried for 1 h and then dried, at 4°, in a vacuum dessicator over P_2O_5 and paraffin oil.

33 g of dried powder were blended, at 4°, for 30 sec with 400 ml of 0.04 M sodium phosphate buffer (pH 7.4) and the mixture was left overnight at 4°. It was then cen-

TABLE II

PURIFICATION OF CALF BRAIN PHOSPHOLIPASE A_1

Incubation mixtures, in volumes of 0.2 ml, each contained 30 μmoles of sodium acetate buffer (pH 4.2); 0.25 mg of Triton X-100; 0.22 μmoles of 1-[1-^{14}C]palmitoyl-2-acylglycero-3-phosphorylcholine and appropriate amounts of enzyme fractions. After 1 h at 37°, the fatty acid was isolated and its radioactivity determined.

Fraction	*Volume (ml)*	*Protein*		*Activity*		*Specific activity (units/mg)*	*Purification (fold)*
		Total (mg)	*Recovery %*	*Total (units)*	*Recovery %*		
Extract	610	6000		65 000		10.8	
pH 3.8 supernatant	710	1350	22.5	64 000	99	34	3.1
Ammonium sulfate supernatant (50–60%)	27	185	3.1	30 000	48	162	15.0
Supernatant after calcium phosphate	35	133	2.2	25 400	39	190	17.6
Second ammonium sulfate (0–70%)	7.5	80	1.3	16 000	25	200	18.5
Sephadex G-150 effluent (fractions 52–58)	24.5	10.6	0.16	5 330	8	500	46

trifuged for 10 min at 25 000 × *g*, and the sediment was blended with 300 ml of the same buffer. After 1 h at 4°, the mixture was centrifuged and the sediment again extracted for 1 h and centrifuged as before. All extracts were combined and adjusted, at 0°, to pH 3.8 with 1 M phosphoric acid (44 ml). After centrifuging for 20 min at 25 000 × *g*, the supernatant was adjusted to pH 8.3 with 1 M NaOH (64 ml).

The solution was placed in crushed ice and fractionated with solid ammonium sulfate; the fraction which sedimented between 50–60% saturation had the highest specific activity. It was taken up in 0.02 M sodium phosphate buffer (pH 7.4), and dialyzed against the same buffer. It was then treated with calcium phosphate gel (3 mg per mg protein). After 30 min at 0°, the mixture was centrifuged for 10 min at 25 000×*g* and the supernatant was retained. The sediment was washed, twice, with 0.004 M sodium phosphate buffer (pH 7.4) (0.25 ml per each ml of the solution prior to treatment with calcium phosphate); all three supernatants were combined and adjusted with solid ammonium sulfate to 70% saturation. The sediment thus obtained was taken up in 0.02 M sodium phosphate buffer (pH 7.4) and dialyzed against the same buffer. After centrifugation, the supernatant was subjected to gel filtration on a column of Sephadex G-150, as follows.

5.8 ml of enzyme (62 mg) were adsorbed on a column of Sephadex G-150 (20 cm × 2.3 cm) previously equilibrated with 5 mM sodium phosphate buffer (pH 7.4). The protein was eluted, at 4°, with the same buffer; and collected in fractions of 3.5 ml. Fig. 1 shows the elution pattern. About 50% of the total protein was eluted, devoid of enzymatic activity, prior to elution of the enzyme. A 2–4-fold increase of the specific activity was obtained in this step, resulting in a total purification of about 50-fold.

The enzyme was not adsorbed by calcium phosphate gel even at a ratio of 10 mg per 1 mg protein. However, as shown in Table II, the adsorption step, followed by reprecipitation with ammonium sulfate and dialysis, resulted in a relatively large loss of protein, but only small additional purification. The use of calcium phosphate might, therefore, be omitted and the supernatant after the first fractionation with ammonium sulfate subjected to gel filtration through Sephadex.

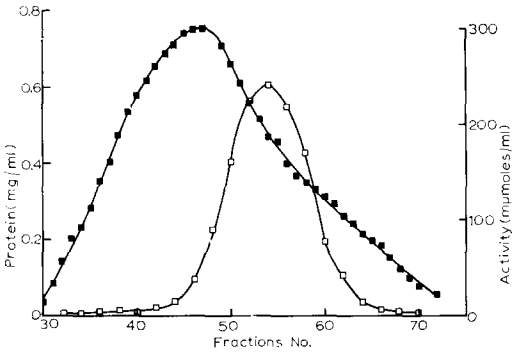

Fig. 1. Gel filtration of calf brain phospholipase A_1 on a column of Sephadex G-150. For details see text. ■—■, protein; □—□, activity.

The enzyme could also be prepared from acetone powders of bovine brain. The initial specific activity of the extract was somewhat lower (about 7 units/mg). However, after fractionation with ammonium sulfate, treatment with calcium phosphate gel and refractionation with ammonium sulfate, preparations were obtained with a specific activity in excess of 200 mμmoles/mg per h.

Properties of the enzyme

Stability. The cholate extract of rat brain was stable for at least 8 months at −20°. However, a supernatant obtained by adjusting the pH of this extract of 3.5 was unstable and lost 75% of its activity when stored at −20° for 10 days. The purified enzyme from calf brain was stable for at least 8 months, when stored at −20°. When heated without substrate, the rat brain enzyme was stable for 10 min at temperatures up to 58°, but rapidly lost its activity between 60 and 65°.

Effect of detergents

Hydrolysis of lecithin by the enzymes from both rat or calf brain was stimulated about 5-fold by the addition of the non-ionic detergent Triton X-100 (optimum at about 1 mg/ml of reaction mixture) (Fig. 4), or the anionic detergent, sodium taurocholate (2.5 mg/ml of reaction mixture). Mixtures of Triton X-100 and taurocholate resulted in higher activities than those obtained with each of these detergents, alone. However, addition of sodium cholate to reaction mixtures having optimal concentrations of Triton X-100, inhibited the reaction; this might be due to precipitation of cholic acid at the low pH of the reaction. Lecithin dispersions which were subjected to sonic oscillation at 10–20 kcycles/sec, still required addition of these detergents for enzymatic activity. The cationic detergent, Cetavlon (cetyl trimethylammonium-bromide), was strongly inhibitory, either when added alone or in combination with Triton X-100. Addition of 0.5 mg/ml Cetavlon to reaction mixtures having 1 mg/ml of Triton X-100, completely inhibited the hydrolysis of lecithin.

General properties of the enzyme

Hydrolysis of lecithin, using either the rat or calf brain enzymes had a pH optimum of 4.0, with either phosphate-citrate or acetate buffers (Fig. 2), and a K_m

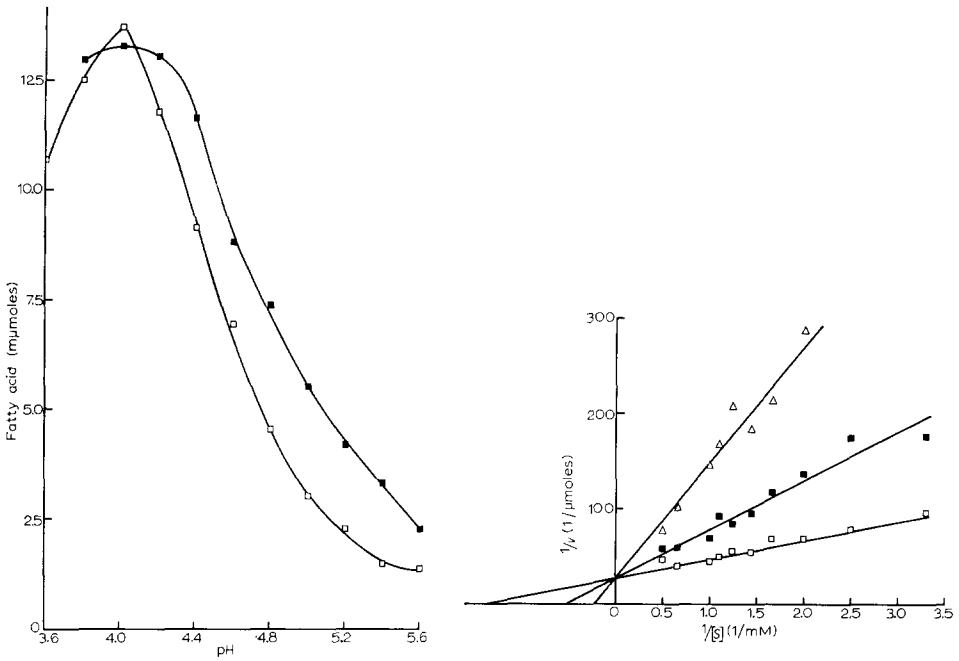

Fig. 2. Dependence of the rate of hydrolysis on pH. Incubation mixtures, in volumes of 0.2 ml each, contained 30 μmoles of acetate or phosphate-citrate buffers; 0.15 mg of Triton X-100; 0.1 μmoles of 1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylcholine and 10 μg of rat brain enzyme. After 90 min at 37°, the fatty acid was isolated as described in EXPERIMENTAL PROCEDURE and its radioactivity determined. □—□, phosphate-citrate buffer; ■—■, acetate buffer.

Fig. 3. Dependence of the rate of hydrolysis on substrate and fatty acid concentrations. Incubation mixtures, in volumes of 0.2 ml each, contained 30 μmoles of sodium acetate buffer (pH 4.2); 0.25 mg of Triton X-100; varying concentrations of 1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylcholine and 240 μg of calf brain enzyme (40–50% ammonium sulfate fraction). After 1 h at 37°, the fatty acid was isolated and its radioactivity determined. □—□, no palmitate; ■—■, $1.5 \cdot 10^{-4}$ M palmitate; △—△, $4 \cdot 10^{-4}$ M palmitate.

of $8 \cdot 10^{-4}$ M (Fig. 3). The enzyme did not require metal ions; addition of calcium ions did not increase the reaction rate and EDTA did not decrease it. The dependence on the length of incubation is shown in Fig. 4. Without Triton X-100, the reaction rate was linear for 2 h. With Triton X-100, higher, but less linear rates were obtained. The deviation from linearity depended on the degree of hydrolysis and is probably due to a progressive inhibition of the hydrolysis of lecithin by the products of the reaction as discussed in the following paragraph.

Inhibition by the products of the reaction

As shown in Fig. 4, reaction rates were not linear with the time of incubation. It was suspected that the products of the reaction might be inhibitory and decrease the reaction rate as they accumulated. When fatty acid or lysolecithin (1-acylglycerophosphorylcholine) was added to the reaction mixtures, each of these compounds inhibited the hydrolysis of lecithin. However, the degree of inhibition by fatty acid was much more pronounced. Thus, at equimolar amounts of substrate and inhibitor, 80% inhibition was obtained with palmitate, but practically none with lysolecithin;

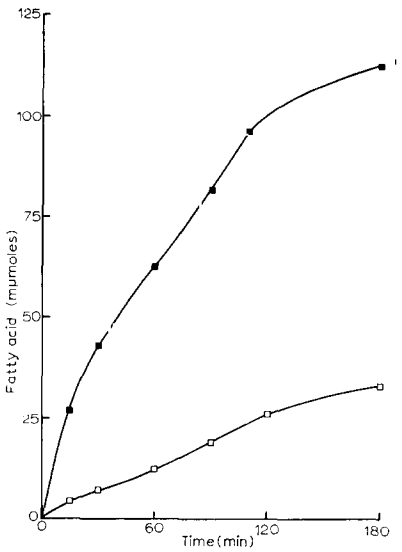

Fig. 4. Dependence of the rate of hydrolysis on incubation time and on Triton X-100. Incubation mixtures, in volumes of 0.2 ml each, contained 30 μmoles of sodium acetate (pH 4.2); 0.46 μmoles of 1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylcholine and 0.36 mg of calf brain enzyme (50–65% ammonium sulfate fraction). At the specified times, the reaction was terminated and fatty acid isolated. □—□, no Triton X-100; ■—■, with 0.25 mg Triton X-100.

50% inhibition was obtained at about 0.15 mM palmitic acid and 3 mM lysolecithin. Linoleic acid had a very similar effect to that of palmitic acid. (It should be stressed that 1-acylglycerophosphorylcholine is not a product of the reaction; 2-acylglycerophosphorylcholine, the direct product was not available in pure form and was not tested.)

Fig. 3 shows that the inhibition by palmitate is competitive. The K_i, $7 \cdot 10^{-5}$ M, is 11 times less than the K_m.

Trials were made to increase the reaction rates and make them more linear with time by adding serum albumin to the reaction mixtures. However, this compound had no effect at pH 4.2 or 5, using up to 2 mg albumin per 0.2 ml of reaction mixture; above 2 mg, inhibition ensued. Calcium ions were tried next but had no effect on the rates of hydrolysis at the low pH of the reaction. Trials to extract the fatty acid formed, during incubation, by covering the reaction mixtures with ether (in the presence or absence of calcium ions), heptane or paraffin oil, did not significantly increase the reaction rates.

Substrate specificity

Table III summarizes the rates of hydrolysis of phosphatidyl choline, labeled in the 1 or 2 position, of phosphatidyl ethanolamine, of lysolecithin labeled in the 1-position and of tripalmitin, by the enzymes from rat and bovine brain. The rat brain phospholipase catalyzed only negligible or very low hydrolysis of the 2 position of lecithin, of the 1-position of lysolecithin or of tripalmitin. Phosphatidyl ethanolamine was hydrolyzed, but at only 20% of the corresponding rate of phosphatidyl choline. The bovine brain enzyme had very low activity toward the 2-position of lecithin or

TABLE III

SUBSTRATE SPECIFICITY OF THE ENZYMES

Incubation mixtures, in volumes of 0.2 ml each, contained 30 μmoles of acetate buffer (pH 4.2); 0.25 mg of Triton X-100; 0.23 μmoles of substrate and 32 μg of rat brain enzyme supernatant (obtained after dialysis against acetate buffer, pH 4.2) or 110 μg of bovine brain enzyme (a fraction obtained between 50–60% saturation with ammonium sulfate). After 1 h at 37°, the reaction was terminated, fatty acid was isolated and its radioactivity determined.

Substrate	*Fatty acid released*	
	Rat brain phospholipase (mμmoles)	*Bovine brain phospholipase (mμmoles)*
1-[1-^{14}C]palmitoyl-2-acyl-glycero-3 phosphorylcholine	19.50	21.30
1-acyl-2-[1-^{14}C]oleyl-glycero-3-phosphorylcholine	0.04	0.25
1-[1-^{14}C]palmitoyl-2-acyl-glycero-3-phosphorylethanolamine	4.25	7.20
1-[1-^{14}C]palmitoyl-glycero-3-phosphorylcholine	0.45	1.20
Glycerol tri-[1-^{14}C]palmitate	1.10	14.85

the 1-position of lysolecithin. It however hydrolyzed phosphatidyl ethanolamine at one third and tripalmitin at two thirds of the corresponding rate of phosphatidyl choline. It therefore seems to be of lower purity than the enzyme from rat brain, still being contaminated with lipase activity. To ensure that the above results are not artifacts, due to the effect of the concentration of the detergent used, these experiments were repeated in the absence and in the presence of several concentrations of Triton X-100. Although some differences in the relative rates of hydrolysis of the various substrates were observed at different concentrations of detergent, the general pattern, as shown in Table III was not altered significantly.

Evidence for the positional specificity of the enzyme

(1) Egg lecithin was incubated with the enzyme from rat brain and the reaction was terminated by the method of FOLCH, LEES AND SLOANE-STANLEY[17]. The phases were separated, the lower phase was evaporated and the residue chromatographed on a column of Unisil, using a discontinuous gradient of increasing concentrations of methanol in chloroform. The fraction containing the fatty acids, released by the enzyme, was partitioned in the biphasic system of DOLE[20]. The heptane phase was collected, shaken with 0.1 M NaOH in 50% ethanol and, after acidification, the free fatty acids were reextracted into heptane. This solvent was evaporated, the fatty acids were dissolved in ether and methylated with distilled diazomethane in ether.

The fractions containing lysolecithin were collected from the Unisil column and hydrolyzed with 1 M NaOH in 90% ethanol. After acidification, the fatty acids were extracted with heptane; the heptane was shaken with ethanolic NaOH, and the phases were separated. The lower phase was acidified, the fatty acids were extracted and methylated as above. A suitable zero time was run and subjected to the procedure described above. The methyl esters were chromatographed on a Packard gas–liquid chromatogram, using ethylene glycol succinate polyester on Gas Chrom-P as the stationary phase. The results are summarized in Table IV. The fatty acids released by the phospholipase were about 95% saturated (minor components were not included in the calculation of the percent composition) while those of the second product of the

TABLE IV

FATTY ACID COMPOSITION OF THE PRODUCTS OF HYDROLYSIS OF LECITHIN BY RAT BRAIN PHOSPHOLIPASE A_1

For details, see text.

Fatty acid	*Fatty acids released by enzyme (%)*	*Fatty acids of remaining lysolecithin (%)*
$C_{16:0}$	75	10
$C_{16:1}$	—	2.5
$C_{18:0}$	19	2.2
$C_{18:1}$	5	66
$C_{18:2}$	0.5	19.5

reaction, lysolecithin, were about 90% unsaturated (the presence of about 10% of palmitic acid might perhaps be due to some contamination with lecithin).

(2) Lecithin, labeled with both ^{32}P and tritiated oleic acid in the β-position, was prepared and hydrolyzed by the rat brain phospholipase[16]. The $^{3}H/^{32}P$ ratio of the lysolecithin obtained by enzymatic hydrolysis was very close to that of the substrate lecithin. By comparison, lysolecithin isolated from the reaction mixture of this lecithin, hydrolyzed with the venom of *Crotalus adamanteus* had a 200 times lower $^{3}H/^{32}P$ ratio[16]. Since the venom phospholipase removes the 2-position of lecithin, this implies that the brain enzyme hydrolyzes the 1-position of this substrate.

(3) [1-^{3}H]stearoyl-2-stearoyl-glycero-3-phosphorylcholine was incubated with the enzyme from rat brain, the hydrolysis products were separated on thin-layer silica gel plates, eluted with methanol and counted. The ratio of radioactivities of the fatty acids to lysolecithin was 47:1 and 120:1 in two separate experiments. The possibility was considered that calcium ions might stimulate a phospholipase A_2 activity, hydrolyzing the 2-position and thus affecting this ratio. A third experiment was therefore conducted, in which 5 mM $CaCl_2$ was added to the incubation mixture. This, however, had no effect, the ratio of radioactivities of the fatty acid to lysolecithin was 100:1.

(4) A similar experiment was performed using 1-stearoyl-[2-^{14}C]stearoyl-glycero-3-phosphorylcholine, and fatty acid and lysolecithin were isolated. The radioactivity ratio of fatty acid to lysolecithin was 0.04.

(5) [1-^{3}H]stearoyl-[2-^{14}C]stearoyl-glycero-3-phosphorylcholine was incubated with the enzyme from rat brain and the products of hydrolysis were isolated as before. The $^{3}H/^{14}C$ ratio was 30:1 in the fatty acids and 0.04 in the lysolecithin. These results were not altered when this experiment was repeated in the presence of 5 mM $CaCl_2$.

The above evidence together with the data presented in Table III supply ample evidence that the brain phospholipase, described here, hydrolyses the 1-position of lecithin but not the 2-position of this substrate, nor the 1 or 2 position of lysolecithin. Since in these experiments all fatty acids, removed from the 1-position, were saturated, it is not known whether the enzyme will also hydrolyze a similar bond having an unsaturated fatty acid. To clarify this point, dioleoyl, dilinoleoyl and distearoyl phosphatidylethanolamine were incubated with the enzyme from rat brain. Since the first two compounds were not available with an isotopic label, the results were evaluated qualitatively. The products of the reaction were chromatographed on thin-

layer plates of silica gel and visualized by charring with sulfuric acid. An inspection of the plates showed that fatty acid and lysolecithin spots were obtained with all three substrates. The quantity of linoleoyl lysolecithin was however smaller than that of the oleoyl lysolecithin, and the latter was less than the stearoyl lysolecithin. This suggests that the enzyme can hydrolyze an ester bond having an unsaturated fatty acid, though perhaps at a lesser rate than a corresponding bond with a saturated fatty acid.

DISCUSSION

The soluble enzyme described here is classified as a phospholipase A_1. This, according to the nomenclature proposed by VAN DEENEN and his co-workers, implies that the enzyme hydrolyzes the ester bond in the 1 (or α') position of lecithin and, phosphatidyl ethanolamine. The possible existence of a phospholipase A_1 in addition to phospholipase A_2 (specific for hydrolysis of the 2-position of the phosphoglycerides) has been discussed in the literature for several years. However, only very recently have WAITE AND VAN DEENEN[10] shown that a partial separation between these two enzymes can be obtained by subcellular fractionation of rat liver homogenates. These authors have not purified the respective enzymes, but have studied their properties in the intact subcellular fractions or in the same fractions after subjecting them to sonic irradiation. Upon comparing the properties of the purified enzymes from rat or calf brain with those of the liver preparation, several striking differences are noted. (1) While the phospholipase A_1 of liver is present in microsomes, the enzyme in brain is isolated from a particulate fraction sedimenting at about 15 000 $\times$ *g*. (2) The optimal pH of the liver enzyme is not given, however the incubations were performed at pH 7.4. At this pH the brain enzyme, whose optimal pH is 4, is inactive. (3) The liver enzyme hydrolyzes phosphatidylethanolamine at a higher rate than phosphatidylcholine; with the brain enzymes this order is reversed. (4) The liver preparation is still contaminated by phospholipase B; the brain enzyme is practically free of this contaminant. The first two differences suggest that the liver and brain enzymes are two distinct entities. The other two differences might disappear upon further purification of the enzymes from liver. It should be noted that both phospholipase A_1 preparations, from liver and brain, do not require calcium ions for activity.

The low optimal pH of the phospholipase from both rat and bovine brain suggests that it might be of lysosomal origin. BLASCHKO *et al.*[21] have recently reported on phospholipase activity in lysosomes isolated from bovine adrenal medulla. According to the method of preparation this enzyme[22], contained two phospholipase activities with pH optima at 4.0–4.2 and at pH 6.5, corresponding in mode of action to phospholipases A_1 and A_2, respectively. MELLORS AND TAPPEL[23], very recently, have described a phospholipase in rat liver lysosomes which hydrolyzes both fatty acids of phosphatidylcholine or phosphatidylethanolamine. In contrast to these three enzymes, VOGEL AND BIERMAN have shown that post-heparin serum has a phospholipase, whose pH optimum is about 9, and which hydrolyzes the 1-position of lecithin. The possibility that this enzyme might be identical with serum lipase has not been ruled out.

Under the assay conditions used, the brain enzyme does not hydrolyze the ester bond in the 2-position of lecithin. Furthermore it has only very low activity with lysolecithin (1-acyl-glycero-3-phosphorylcholine was tested directly (Table III)); that

2-acyl-glycero-3-phosphorylcholine is not hydrolyzed, as well, can be inferred from the experiments using doubly labeled lecithin (p. 313, evidence No. 5). These two properties should enable the use of this enzyme for preparation of 2-acyl-glycero-3-phosphorylcholine. However, since fatty acid, the product of hydrolysis strongly inhibits the reaction (the K_i is 11 times lower than the K_m), the hydrolysis of lecithin could not be taken to completion and required separation, by column or thin-layer chromatography, between the products and the unreacted substrate. It has been observed (LANDS AND DE HAAS, personal communications) that such isolation procedures result in a partial migration of the fatty acid in the 2-position of the 2-acyl-glycero-3-phosphorylcholine to the 1-position, yielding a mixture of the two isomers.

Preliminary experiments have suggested that brain tissue has a phospholipase A_2, as well. Experiments are in progress to purify this enzyme and compare its properties with those of the phospholipase A_1.

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1. In the experiment present in Table III, Triton X-100 was added to each reaction mixture. Under these conditions, phosphatidyl ethanolamine was hydrolyzed at a lower rate than phosphatidyl choline. It has now been found that if mixtures of Triton X-100 and sodium taurocholate are used, the rates of hydrolysis of lecithin, lysolecithin or tripalmitin are not significantly altered. However, under these conditions (optimal quantities are 1.5 mg Triton X-100 and 2 mg taurocholate per ml of reaction mixture) the rate of hydrolysis of phosphatidyl ethanolamine increases and is about equal to the rate of hydrolysis of lecithin.

2. As shown in Table III, 1-[1-^{14}C]palmitoyl-glycero-3-phosphorylcholine was a very poor substrate for the enzyme. A sample of 2-[9,10-^{3}H]stearoyl-glycero-3-phosphorylcholine, kindly supplied by Dr. A. J. Slotboom of Utrecht, has now been tested, it was hydrolyzed at a very slow rate, similar to that of 1-lysolecithin.

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