BBA 55496

ON THE SPECIFICITY OF RAT-LIVER LYSOPHOSPHOLIPASE

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(Received July 1st, 1968)

SUMMARY

1. A study on the specificity of rat-liver lysophospholipase activity (EC 3.1.1.5) revealed that both 1-acyl-*sn*-glycero-3-phosphorylcholine and 2-acyl-*sn*-glycero-3-phosphorylcholine are deacylated. From both positional isomers the unsaturated analogs appeared to be degraded at higher rates.

2. Circumstantial evidence is presented indicating that 2-acyl-*sn*-glycero-3-phosphorylcholine can be attacked directly by this lysophospholipase activity without a prior migration of the fatty acyl constituent.

3. Compounds lacking the free hydroxyl group present in lysophosphatidylcholines, *e.g.* acyl-ethylene glycolphosphorylcholine and 1-acyl-propane diol-3-phosphorylcholine, also fall in the enzyme's range of specificity.

4. Mono-acyl derivatives of *sn*-glycero-1-phosphorylcholine, *sn*-glycero-2-phosphorylcholine, as well as *sn*-glycero-3-phosphorylcholine, were found to be degraded.

5. Inhibition of lysophospholipase activity by various agents exhibited the same effect on the deacylation of both 1-acyl- and 2-acyl-*sn*-glycero-3-phosphorylcholine.

6. The degradation of mono-acyl-phosphatidylcholine appeared to be strongly inhibited in the presence of phosphatidylcholine.

INTRODUCTION

Phosphoglycerides in animal tissues are catabolized *via* the corresponding mono-acyl derivatives. An active lysophospholipase in rat liver, preventing the accumulation of these lysophosphoglycerides has been described[1-3]. DAWSON[1] found that the enzyme attacked both mono-acyl phosphatidylcholine and mono-acyl phosphatidylethanolamine, each being a competitive inhibitor for the breakdown of the other compound. These conclusions were drawn from the results of experiments done with lysophosphoglycerides prepared by the action of snake-venom phospholi-

Abbreviations: 1-(or 2-)acyl-3-*sn*-GPC, 1-(or 2-)acyl-*sn*-glycero-3-phosphorylcholine.

pase A and consequently having the 1-acyl-*sn*-glycero-3-phosphorylcholine or -ethanolamine structure (I, in Scheme 1).

In recent reports from this laboratory[4–7], as well as from several other research groups[8–11], there has been presented evidence for the occurrence of enzymes in animal tissues, which hydrolyze fatty acids located in the 1-position of phosphoglycerides, thus producing 2-acyl lysophosphoglycerides (Scheme 1, Reaction 2).

In view of the finding that the lysophosphatidylcholine fraction of rat liver is composed of both 1-acyl- and 2-acyl-isomers[5], it became of interest to investigate whether both lysophosphatidylcholine structures could be degraded further by enzymes present in rat liver. This report deals with the results obtained with soluble fractions from rat liver using synthetic 1-acyl- and 2-acyl-lysophosphatidylcholine both containing a labelled fatty acid constituent.

METHODS

Synthesis of substrates

1-[9,10-$^3H_2$]Stearoyl-sn-glycero-3-phosphorylcholine. 3-*sn*-Dioleoyl phosphatidylcholine was synthesized from the $CdCl_2$ adduct of *sn*-glycero-3-phosphorylcholine and oleoylchloride[12]. The pure phosphatidylcholine was converted into 1-oleoyl-*sn*-glycero-3-phosphorylcholine by treatment with snake-venom phospholipase A. This compound was then treated catalytically in the presence of Adams' catalyst (the Radiochemical Centre, Amersham, Great Britain) to yield 1-[9,10-$^3H_2$]stearoyl-*sn*-glycero-3-phosphorylcholine. The compound used for the enzymatic assay had a specific activity of $1.26 \cdot 10^9$ disint./min per mmole.

1-[1-^{14}C]Palmitoyl-sn-glycero-3-phosphorylcholine. Di-[1-^{14}C]palmitoyl-3-*sn*-phosphatidylcholine was synthesized in a small scale preparation of the established procedure[12]. [1-^{14}C]Palmitoylchloride (425 mg; 0.5 mC) was added in dry chloroform to 256 mg of the $CdCl_2$ adduct of glycerophosphorylcholine. The mixture was stirred vigorously in the presence of small glass beads whilst adding dropwise 0.2 ml of pyridine in dry chloroform. After a 4-h reaction, the mixture was deionized by passing through an ion-exchange column containing equal amounts of Amberlite IRC-50 (H^+) and IRA-45 (OH^-). After further purifications by means of silica column chromatography, 110 mg of pure di-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine was obtained. A 40-mg amount of this compound was emulsified by ultrasonication into 4 ml of a 0.1 M borate buffer with a $CaCl_2$ concentration of $5 \cdot 10^{-3}$ M. Following the addition of 3 mg of *Crotalus adamanteus* venom and 4 ml of ether, the mixture was shaken at room temperature until the enzymatic degradation was virtually complete. Purification of the reaction mixture by column chromatography yielded 26 mg of pure 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine (specific activity $4.3 \cdot 10^8$ disint./min per mmole).

2-[9,10-$^3H_2$]Stearoyl-sn-glycero-3-phosphorylcholine. This compound was prepared by a chemical acylation of 1-*O*-benzyl-*sn*-glycero-3-phosphorylcholine with [9,10-$^3H_2$]stearoylchloride as described in detail by SLOTBOOM, DE HAAS AND VAN DEENEN[13]. Treatment of the resulting compound with snake-venom phospholipase A released over 95% of the [^{3}H]stearate indicating that almost completely pure 1-*O*-benzyl-2-[9,10-$^3H_2$]stearoyl-*sn*-glycero-3-phosphorylcholine was obtained. The protecting benzyl group was removed by hydrogenolysis in absolute ethanol yielding

2-[9,10-$^3H_2$]stearoyl-*sn*-glycero-3-phosphorylcholine (specific radioactivity = $2.22 \cdot 10^9$ disint./min per mmole).

2-[1-^{14}C]Oleoyl-sn-glycero-3-phosphorylcholine. This compound was prepared by the action of highly purified lipase *(Rhizopus arrhizus)* on 1-acyl-2-[1-^{14}C]oleoyl-*sn*-glycero-3-phosphorylcholine, as described by SLOTBOOM[14]. Purification of the 2-[1-^{14}C]oleoyl-3-*sn*-GPC was carried out on a Sephadex LH-20 column with chloroform–methanol (1:1, by vol.) as the elution solvent system. 91% of the purified compound was shown to be degraded by snake-venom phospholipase A_2 (specific radioactivity = $7.3 \cdot 10^8$ disint./min per mmole).

[9,10-$^3H_2$]Stearoyl-ethyleneglycol-phosphorylcholine. Ethylene glycol was acylated with [9,10-$^3H_2$]stearoylchloride (molar ratio 1 to 0.9). The resulting product was shown by thin-layer chromatography to be mainly mono-stearoyl glycol with a small contamination of di-stearoyl glycol. This mixture was treated with 2-bromoethyl-phosphoric acid dichloride in the presence of triethylamine in dry chloroform according to the procedure of HIRT AND BERCHTOLD[15]. The trimethylamine group was incorporated by treatment of the [9,10-$^3H_2$]stearoyl-glycol-phosphoryl-ethyl-bromo-ester with trimethylamine at 60° for 40 h in a Carius tube. Pure [9,10-$^3H_2$]stearoyl-glycol-phosphorylcholine was obtained from the reaction mixture by silica column chromatography. The purified product had a specific activity of $4.24 \cdot 10^9$ disint. per min per mmole.

Non-labelled substrates

A series of 1-acyl-*sn*-glycero-3-phosphorylcholines containing either decanoate, myristate, oleate or linoleate as the acyl constituent was synthesized as described in detail for 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphorylcholine. The synthesis of other isomeric mono-acyl phosphatidylcholines, including 3-stearoyl-*sn*-glycero-1-phosphorylcholine, 2-stearoyl-*sn*-glycero-1-phosphorylcholine, 1-stearoyl-*sn*-glycero-2-phosphorylcholine and 3-stearoyl-*sn*-glycero-2-phosphorylcholine have been described by DE HAAS AND VAN DEENEN[16]. 1-Palmitoylpropane diol-3-phosphorylcholine was a generous gift of Dr. H. EIBL, Max Planck Institut für Immunbiologie, Freiburg, W. Germany.

Determination of lysolecithin structure

To establish the structure of the 1-acyl- and 2-acyl-*sn*-glycero-3-phosphorylcholine substrates, use was made of a method developed[16] and previously applied in this laboratory[5]. The method is based on the fact that only 2-acyl-*sn*-glycero-3-phosphorylcholine appeared to be susceptible to the action of phospholipase A_2. The enzyme was prepared by heat treatment (5 min at 100°) of *Crotalus adamanteus* venom in 0.01 M acetate buffer (pH 4.5), at a concentration of 10 mg/ml. After centrifugation for 10 min at $27500 \times g$, the clear supernatant was brought to pH 7.0 and used for degradation experiments.

Lysophospholipase source and assay of enzymatic activities

Freshly prepared 10% homogenates (w/v) of rat liver in 0.25 M sucrose were centrifuged for 15 min at $15000 \times g$ followed by a second centrifugation at $120000 \times g$ for 1 h in a Spinco L2-65 ultracentrifuge. The supernatant obtained in this manner was used as the enzyme source. Protein determinations were done according to the method of LOWRY *et al.*[17].

Appropriate amounts of phospholipid substrates were emulsified in 0.1 M Tris–HCl buffer (pH 7.2), in 0.125 M KCl by ultrasonic vibration. After incubation with the soluble rat-liver fraction at 37° for the indicated times, the lipids were extracted according to the procedure of BLIGH AND DYER[18]. Phosphatidylcholine, lysophosphatidylcholine and fatty acids were separated by thin-layer chromatography (solvent system: chloroform–methanol–water (65:35:4, by vol.)), whereafter, the radioactive compounds were eluted from the silicic acid with chloroform–methanol (20:80, by vol.) and used for radioactivity measurements in a Tricarb liquid scintillation spectrometer. When the release of only the acyl residues from the substrates was to be measured, the incubation mixture was extracted according to a modified Dole procedure as described by IBRAHIM[19]. Aliquots of the heptane layer were used for radioactivity determinations of the free fatty acid fractions. In control experiments, both assays gave exactly the same results, at least for saturated acyl constituents. When rates of hydrolysis were to be determined it was established that the extent of degradation was linear with time and enzyme concentration.

RESULTS AND DISCUSSION

The lysophospholipase activity of rat liver was found to be 12.6 and 82%, respectively, in the mitochondrial, microsomal and soluble protein fractions obtained by differential centrifugation of a 10% rat-liver homogenate in 0.25 M sucrose. This localization is in agreement with the observations made by other investigators on rat-liver lysophospholipase[20,21]. However, WINKLER[22] found about 70% of the total lysophospholipase activity of bovine adrenal medulla to be particle-bound. A similar distribution was found by KHAN AND HODGSON[23] for the lysophospholipase activity of *Musca domestica*. In view of the recovery of the bulk of the lysophospholipase from rat liver in the particle-free supernatant fraction, further experiments were carried out with the supernatant fraction only.

This subcellular fraction is also known to contain both phospholipase[24] and lipase[25] enzymes that could possibly attack lysophosphatidylcholines. However, even when tested on their usual substrates, phosphatidylcholine and triglyceride, respect-

TABLE I

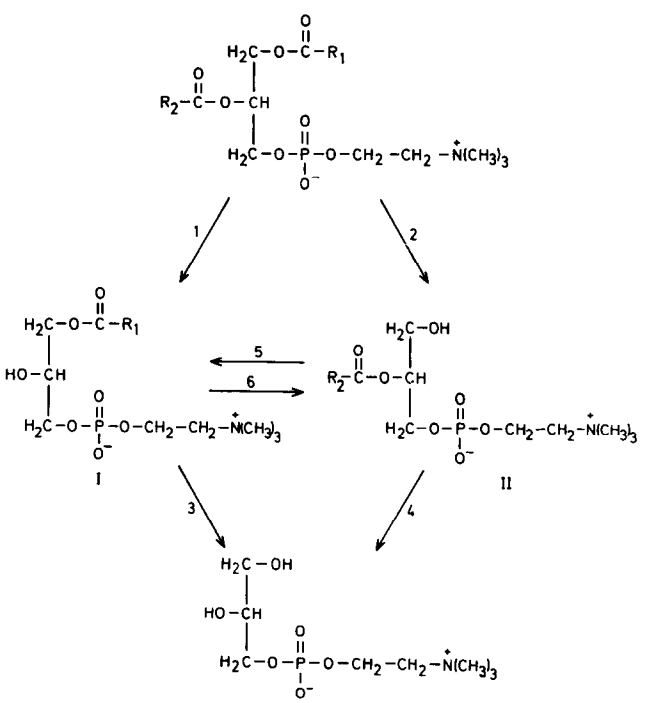
HYDROLYSIS OF PHOSPHOLIPIDS BY RAT-LIVER SUPERNATANT

20 mμmoles of the indicated substrates were incubated for 15 min at 37° with 100 mg of rat-liver supernatant protein in 0.7 ml of 0.02 M Tris–HCl buffer (pH 7.2). Hydrolyses of non-radioactive substrates were determined by phosphorous determinations on incubation mixtures at a 50-fold increased scale. The mean results of at least 3 experiments after correction for non-enzymic hydrolysis, are given. GPC, glycero-3-phosphorylcholine moiety; PC, phosphorylcholine moiety.

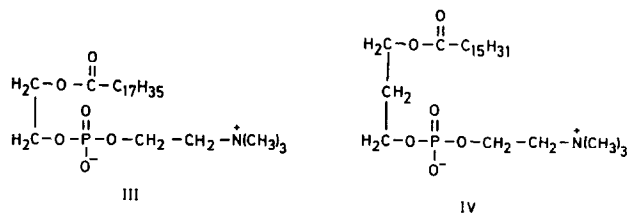
Substrate	*mμmoles hydrolyzed*	*Substrate*	*mμmoles hydrolyzed*
1-[9,10-$^3H_2$]Stearoyl-GPC	6.8	2-[9,10-$^3H_2$]Stearoyl-GPC	3.2
1-[1-^{14}C]Palmitoyl-GPC	7.1	2-[1-^{14}C]Oleoyl-GPC	4.5
1-Decanoyl-GPC	3.1	Lauroyl-ethylene glycol-PC	3.4
1-Myristoyl-GPC	15.0	[9,10-$^3H_2$]Stearoyl-ethylene glycol-PC	3.6
1-Oleoyl-GPC	9.5		
1-Linoleoyl-GPC	13.8	Di-[1-^{14}C]palmitoyl-GPC	0.00
1-Palmitoyl-propane diol-3-PC	6.3	1-Acyl-2-[1-^{14}C]Linoleoyl-GPC	0.37
		1-Benzyl-2-[9,10-$^3H_2$]stearoyl-GPC	0.08
		Tri-[1-^{14}C]oleoyl glycerol	0.04

ively the activity of these lipolytic enzymes under the conditions of the assay was very low compared to the rate of hydrolysis of the isomeric mono-acyl phosphatidylcholines; this result is therefore attributed to lysophospholipase activity (*cf.* Table I). In experiments to further characterize the specificity of the lysophospholipase from rat liver, it was found that the nature of the acyl constituent to be released, was of some importance with respect to the rate of hydrolysis. Although 1-stearoyl- and 1-palmitoyl-*sn*-glycero-3-phosphorylcholines were degraded at essentially the same rates: the unsaturated 1-oleoyl- and 1-linoleoyl-*sn*-glycero-3-phosphorylcholines appeared to be hydrolyzed somewhat faster. The results presented in Table I clearly indicate that diacyl phosphoglycerides and other substrates having 2 hydrophobic regions in their molecular structure, *e.g.* 1-benzyl-2-[9,10-$^3H_2$]stearoyl-*sn*-glycero-3-phosphorylcholine, are attacked at a much lower rate or not at all by enzymes in the rat-liver supernatant. On the other hand, compounds bearing only one fatty acyl group are readily hydrolyzed. The presence of a free hydroxyl group in the molecule is no prerequisite for enzymatic cleavage of the ester bond by lysophospholipase, as indicated by the results obtained with 1-palmitoyl-propane diol-3-phosphorylcholine.

The observed rates of de-acylation of 1-acyl-3-*sn*-GPC and 2-acyl-3-*sn*-GPC do not allow any definite conclusions with regard to whether 2-acyl-3-*sn*-GPC is directly attacked by the lysophospholipase (Scheme 1, Reaction 4). Since 2-acyl-3-*sn*-GPC is hydrolyzed at a somewhat lower rate than the isomeric 1-acyl-3-*sn*-GPC, a degradating mechanism for 2-acyl-3-*sn*-GPC involving a migration of the acyl residue to the 1 position, followed by a hydrolysis cannot be excluded (Reactions 5



Scheme 1. Pathway for the hydrolysis of acyl constituents from diacyl phosphatidylcholine, 1-acyl-3-*sn*-GPC (I) and 2-acyl-3-*sn*-GPC (II). R_1 and R_2 = fatty acyl residue.



III IV

Scheme 2. Structural formulas of stearoyl-ethylene glycollphosphorylcholine (III); 1- palmitoyl-propane diol-phosphorylcholine (IV).

and 3, respectively). Taking into account the known lability of 2-acyl-3-*sn*-GPC[13,26], a contribution of the latter mechanism can, to some extent, be expected. However, it has to be pointed out that the acyl-ethylene glycol-phosphorylcholine derivatives, although lacking the possibility of acyl migration, are degraded at a rate resembling that of 2-acyl-3-*sn*-GPC. The main difference between the 'glycol lecithin' (Scheme 2, III) and the 2-acyl-3-*sn*-GPC (Scheme 1, II) is that the former lacks the free hydroxyl group present in the latter. This hydroxyl group is not necessary for hydrolysis by lysophospholipase (compare 1-palmitoyl-glycero-3-phosphorylcholine (Scheme 1, I) and 1-palmitoyl-propane diol-3-phosphorylcholine (Scheme 2, IV). Hence the major pathway in the de-acylation of 2-acyl-3-*sn*-GPC is very likely to be a direct attack on this compound by a lysophospholipase (Scheme 1, Reaction 4).

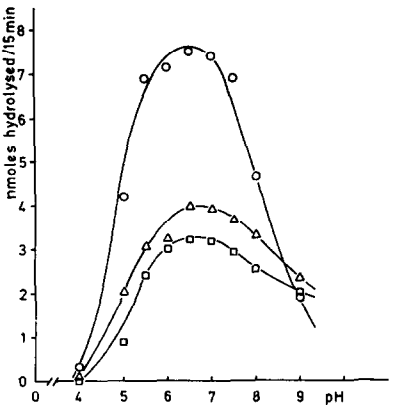
The finding that acyl-ethylene glycol-phosphorylcholine can be degraded by an enzyme present in rat-liver supernatant contrasts with the results of ERBLAND AND MARINETTI[21], who found stearoyl-ethylene glycol-phosphorylcholine to be inactive as a substrate for lysophospholipase in rat-liver supernatant, though the compound was found to inhibit considerably 1-acyl-3-*sn*-GPC hydrolysis. This inhibition can probably be attributed to a competition between the glycol lecithin and the mono-acyl phosphatidylcholine for the lysophospholipase (*cf.* Table II). The fact that these authors investigated the metabolism of ^{32}P-labelled 1-acyl-3-*sn*-GPC

TABLE II

INHIBITION OF MONO-ACYL PHOSPHATIDYLCHOLINE HYDROLYSIS BY GLYCOL LECITHIN

20 mμmoles of radioactive substrates were incubated for 15 min at 37° with 100 μg of rat liver supernatant protein and varying amounts of either glycol lecithin or non-labelled 1-stearoyl-glycero-3-phosphorylcholine.

Substrate	*Addition*		*mμmoles radioactive substrate hydrolysed*	*% inhibition*
	Compound	*Amount (mμmoles)*		
1-[1-^{14}C]Palmitoyl-GPC	Lauroyl glycol lecithin	—	4.8	—
		25	4.0	17
		50	3.5	27
2-[9,10-$^{3}H_2$]Stearoyl-GPC		—	2.3	—
		25	1.8	22
		50	1.7	26
1-[1-^{14}C]Palmitoyl-GPC	1-Stearoyl-GPC	—	4.5	—
		20	3.8	16
		40	2.8	38
2-[9,10-$^{3}H_2$]Stearoyl-GPC		—	2.5	—
		20	1.8	28
		40	1.4	44



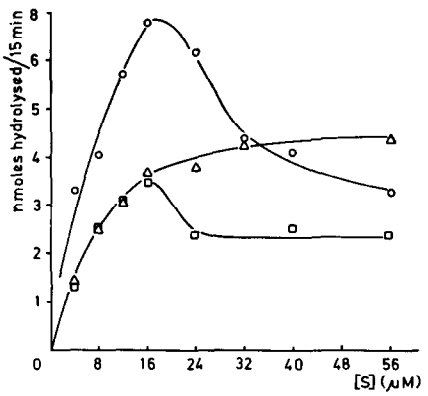


Fig. 1. Influence of pH on the deacylation of substrates. Reaction mixtures contained 20 mμmoles of the indicated substrate, 30 μmoles of Tris–maleate buffer of varying pH values and 100 μg of rat-liver supernatant protein in a final volume of 0.7 ml. The mixtures were incubated for 15 min at 37°. ○——○, 1-acyl-3-*sn*-GPC; □——□, 2-acyl-3-*sn*-GPC; △——△, ethylene glycol-lecithin.

Fig. 2. Effect of substrate concentration on lysophospholipase. Varying amounts of the indicated substrates were incubated with 100 μg of protein and 20 μmoles of Tris–HCl buffer (pH 7.2) in a final volume of 1.0 ml for 15 min at 37°. ○——○, 1-acyl-3-*sn*-GPC; □——□, 2-acyl-3-*sn*-GPC; △——△, ethylene glycol lecithin.

in the presence of unlabelled stearoyl-ethylene glycol-phosphorylcholine may be the reason why they did not detect the breakdown of glycol lecithin.

Recently, phospholipid derivatives of ethylene glycol and 1,3-propanediol have been shown to occur naturally in heart and liver tissue[27] and the observation that these compounds can be hydrolyzed by rat-liver lysophospholipase may be of some importance in relation to their metabolism.

Varying the pH of the medium has a very similar effect on the extent of hydrolysis of both 2-stearoyl-3-*sn*-GPC and stearoyl-ethylene glycol-phosphoryl-choline (Fig. 1). Various additional influences on the lysophospholipase activity also

TABLE III

EFFECT OF INHIBITORS ON LYSOPHOSPHOLIPASE ACTIVITY

The incubation mixture consisted of 200 μmoles of either 1-[9,10-$^3H_2$]stearoyl-3-*sn*-GPC, 2-[9,10-$^3H_2$]stearoyl-3-*sn*-GPC or [9,10-$^3H_2$]stearoyl-ethylene glycol-phosphorylcholine, 5 μmoles of Tris–HCl buffer (pH 7.2), 1.0 mg of rat-liver supernatant protein and the indicated amounts of the additions used in a final volume of 0.5 ml. Reactions were stopped after 30 min incubation at 37°. Mean values of 2 experiments are given.

Addition	*Amount (μmoles)*	*% hydrolysis of substrate*		
		1-Acyl-GPC	*2-Acyl-GPC*	*Acyl-glycol-PC*
None	–	63	37	51
EDTA	5	61	37	48
Glutathion	5	59	37	51
KCN	5	57	37	50
$HgCl_2$	5	3	2	1
PCMB	0.005	50	31	44
1-Acyl-GPC	0.4	29	17	22
	0.8	17	12	13
Heat treatment	10 min 70°	4	2	1

exhibit very similar effects on the hydrolysis of both isomeric mono-acyl phosphatidylcholines and the ethylene glycol lecithin (Table III). Although these results do not permit a conclusion as to whether all 3 substrates are degraded by the same enzyme, some properties of the lysophospholipase become apparent. The enzyme activity appears not to require bivalent metal ions. In accordance with the results of other investigators[1,21,28], the lysophospholipase activity was found to be only very slightly inhibited by cyanide and PCMB. Enzymatic activity was almost completely destroyed by heat treatment (10 min at 70°) both at pH 4 and pH 7. Addition of 1-acyl-3-*sn*-GPC prior to heat treatment did not protect the enzyme against inactivation. It can be concluded from these inhibition experiments that the enzyme degrading 1-acyl-3-*sn*-GPC has properties similar to that degrading 2-acyl-3-*sn*-GPC. If there are two of these enzymes, their physicochemical features also show great similarities. In this respect, it is worthwhile noting that during chromatography of an $(NH_4)_2SO_4$ fraction of the rat-liver supernatant on Sephadex G-200, the ratio of breakdown of 1-acyl- and 2-acyl-3-*sn*-GPC remained constant in all tubes that contained lysophospholipase activity. This ratio was still identical with that of the original homogenate, but the specific activity had increased about 15-fold.

The observation that the degradation of 1-[9,10-$^3H_2$]stearoyl-3-*sn*-GPC and 2-[9,10-$^3H_2$]stearoyl-3-*sn*-GPC is equally inhibited by the addition of non-labelled 1-stearoyl-3-*sn*-GPC (Tables II and III), provides further evidence that 2-acyl-3-*sn*-GPC can be attacked directly by a lysophospholipase. If, in the incubation containing 2-[^{3}H]acyl-3-*sn*-GPC, the deacylation were to proceed *via* a migration to the 1-acyl isomer (Reaction 5), a much lower 1-[^{3}H]acyl-3-*sn*-GPC concentration would be expected in this tube. This can be deduced from control experiments which show that the lysophosphatidylcholine remaining after incubation of 2-[^{3}H]acyl-3-*sn*-GPC with the supernatant fraction, is still composed of over 80% of the 2-acyl isomer. Hence, in these tubes the presumed radioactive 1-[9,10-$^3H_2$]stearoyl-3-*sn*-GPC formed by the migration from the 2-acyl structure, would become much more diluted and consequently its degradation much more restricted by the inhibition of non-labelled 1-acyl-3-*sn*-GPC. The fact that an equal inhibition was found for the hydrolysis of 1-acyl- and 2-acyl-3-*sn*-GPC, endorses the view that the degradation of the 2-acyl isomer can proceed without a prior migration of its acyl constituent to the 1-position. From this point of view, it is also comprehensible that the effect of 1-stearoyl-3-*sn*-GPC on the hydrolysis of both 2-stearoyl-3-*sn*-GPC and stearoyl-ethylene glycol-phosphorylcholine is the same.

From the possible isomers of mono-acyl phosphatidylcholines, those derived from glycero-3-phosphate appear to be degraded to greater extents than the derivatives of glycero-1-phosphate. The lysophospholipase, unlike the phospholipase A_2 from snake venom, exhibits no stereospecific action. The enzyme has a rather broad range of specificity, including mono-acyl phosphatidylcholines having their phosphorylcholine moieties attached to the 2 position (Table IV).

A study of the influence of substrate concentration on the rate of hydrolysis showed an inhibition of enzymatic activity at high concentrations of mono-acyl phosphatidylcholines. Both 1-acyl- and 2-acyl-*sn*-glycero-3-phosphorylcholines were maximally degraded at concentrations of about 16 μM with the particular enzyme level used (Fig. 2). When the substrates were mixed in an equimolar ratio, maximum hydrolysis was obtained at a concentration of about 8 μM for each of the mono-acyl

TABLE IV

HYDROLYSIS OF STEREOCHEMICAL ISOMERS OF MONO-ACYL PHOSPHATIDYLCHOLINES

1000 mμmoles of each of the indicated substrates were incubated for 15 min with 5 mg of supernatant protein in a final volume of 1 ml. Reactions were stopped by addition of 3 vol. of chloroform–methanol (1:2, by vol.). Decrease in mono-acyl phosphatidylcholines was measured by means of phosphorous determinations on the lysophosphatidylcholine spots after thin-layer chromatography of known aliquots of total lipid extracts.

Substrate	*mμmoles hydrolyzed/15 min*
1-Stearoyl-3-*sn*-GPC	405
3-Stearoyl-1-*sn*-GPC	210
2-Stearoyl-3-*sn*-GPC	191
2-Stearoyl-1-*sn*-GPC	90
1-Stearoyl-2-*sn*-GPC	200
3-Stearoyl-2-*sn*-GPC	105

phosphatidylcholines. Although the glycol lecithin alone did not reveal an inhibition, a similar inhibitory effect could be observed when 1-acyl-3-*sn*-GPC and glycol lecithin were mixed. Also in this case, the substrate concentration *versus* enzymatic activity curve showed a maximum at 8–10 μM of each substrate at the given enzyme concentration. Further experiments demonstrated that the concentration of mono-acyl phosphatidylcholine causing inhibition was highly dependent on the protein concentration (Fig. 3). The fact that this inhibition appears to be dependent on the substrate to protein ratio may help to explain the differences in the substrate concentration curves for lysophospholipases that have been obtained by various investigators using 1-acyl-3-*sn*-GPC as substrate. Both DAWSON[1] AND SHAPIRO[29] found

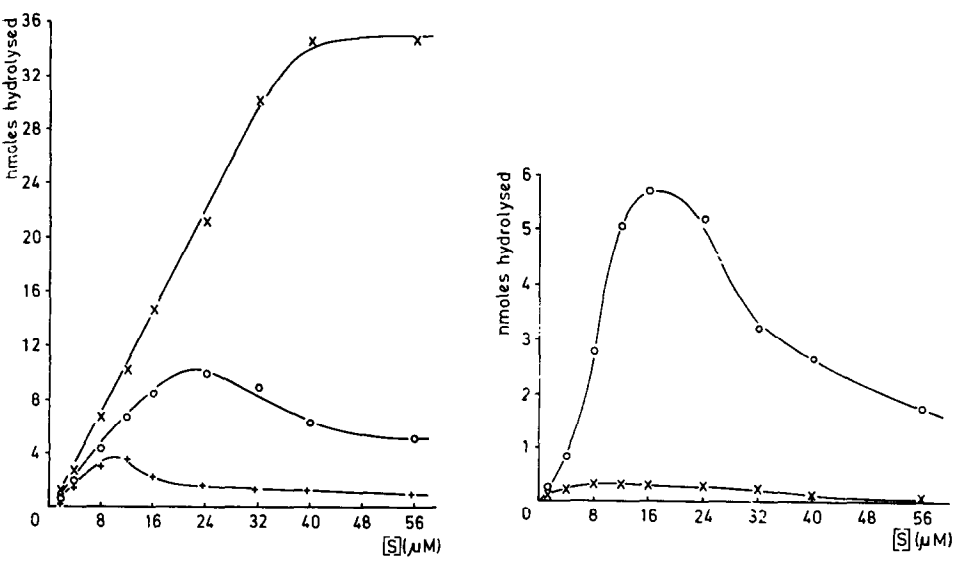


Fig. 3. Effect of substrate concentration on various levels of lysophospholipase. The incubation mixture consisted of varying amounts of 1-acyl-3-*sn*-GPC, 20 μmoles of Tris–HCl buffer (pH 7.2) and either 500 μg (×——×), 167 μg (○——○) or 56 μg (+——+) of supernatant protein in a final volume of 1.0 ml.

Fig. 4. Hydrolysis of 1-acyl-*sn*-glycero-3-phosphorylcholine by lysophospholipase. The incubation mixture contained varying amounts of 1-palmitoyl-3-*sn*-GPC, either free (○——○) or incorporated in a liposomal structure (×——×).

TABLE V

THE EFFECT OF REACTION PRODUCTS ON LYSOPHOSPHOLIPASE

The incubation mixture contained varying amounts of 1-palmitoyl-3-*sn*-GPC, 200 μg of protein, 20 μmoles of Tris–HCl buffer (pH 7.2) and, when indicated, 10 mμmoles of palmitate or glyceryl phosphorylcholine in a final volume of 1.0 ml of palmitate or glyceryl phosphorylcholine in a final volume of 1.0 ml.

mμmoles 1-palmitoyl-3-sn-GPC	*mμmoles substrate hydrolysed*		
	Addition		
	None	*Palmitate*	*Glyceryl phosphorylcholine*
1.3	0.26	0.23	0.33
4	0.84	1.09	1.37
8	2.82	2.84	2.54
12	5.06	5.05	4.40
16	5.72	5.37	5.95

the lysophospholipase of the liver and pancreas, respectively, to be inhibited by high substrate concentrations. On the other hand, MARPLES AND THOMPSON[28], as well as ERBLAND AND MARINETTI[21], found an increase in lysophospholipase activity with increased 1-acyl-3-*sn*-GPC concentrations until saturation of the enzyme was reached.

The results shown in Table V indicate that inhibition of lysophospholipase activity is due to the substrate itself and not to the reaction products formed. Inhibition of lysophospholipase became apparent when about 6 mμmoles of monoacyl phosphatidylcholines were hydrolyzed (*cf.* Fig. 2). No inhibition was observed even when 10 mμmoles of either palmitic acid or glycerolphosphorylcholine were added (Table V).

In an attempt to change the micellar structure of lysophosphatidylcholine, this compound was incorporated into the liposomal structure[30] by shaking with glass beads a mixture of 1-[1-^{14}C]palmitoyl-3-*sn*-GPC and dioleoyl phosphatidylcholine (3 mole % lysophosphatidylcholine) and 0.02 M Tris–HCl buffer (pH 7.2) in 0.125 M KCl. This treatment results in a dramatic decrease in susceptibility of the substrate to lysophospholipase (Fig. 4). The hydrolysis could not be enhanced by increasing the lysolecithin concentration in the liposome-producing system up to 20 moles %.

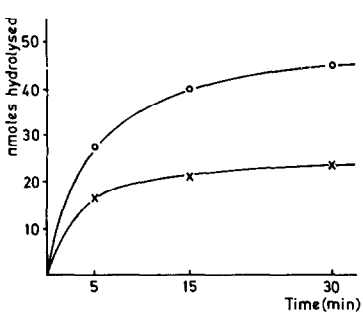


Fig. 5. Hydrolysis of phosphatidylcholines by snake venom phospholipase A. The reaction mixtures contained 125 mμmoles of phosphatidylcholine (90 mole % dioleoyl-GPC and 10 mole % 1-acyl-2-[1-^{14}C]linoleoyl-GPC) and 100 μg snake venom *(Crotalus adamanteus)* in 1.0 ml 0.02 M Tris–HCl (pH 7.2) in 0.125 M KCl with $[Ca^{2+}] = 5 \cdot 10^{-4}$ M. ○——○, sonicated; ×——×, non-sonicated.

Also, sonication of the liposomes to give smaller particles did not enhance the degradation of 1-acyl-3-*sn*-GPC. On the other hand, liposomes of dioleoyl phosphatidylcholine containing 1-acyl-2-[1-^{14}C]linoleoyl-*sn*-glycero-3-phosphorylcholine were readily attacked by snake venom phospholipase A, indicating that liposomes form a suitable structure for attack by the appropriate lipolytic enzymes (Fig. 5). Treatment of the liposomes with ultrasonic vibration increased the extent of the hydrolysis by snake-venom phospholipase A about 2-fold, which could be expected after increasing the total surface area; see also KATES, MADELEY AND BEARE[31]. Although other explanations can be given for the inhibitory effect of lecithin on lysophospholipase, it is our working hypothesis that the association of lysolecithin with lecithin makes the substrate inaccessible to the lysophospholipase. It will be of interest to elucidate which factors determine the difference in specificity and physicochemical behaviour between lysophospholipase and phospholipase A.

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