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PROPERTIES OF THE PHOSPHOLIPASE D FROM PEANUT SEEDS

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SUMMARY

1. Phospholipase D hydrolyzed phosphatidyl glycerol in the absence of ether, but the rate of hydrolysis was accelerated considerably when ether was added.

2. Although very dilute aqueous dispersions of lecithin (1 μM) or ultrasonically irradiated lecithin in water were hydrolyzed by the enzyme in the absence of ether, addition of ether enhanced phospholipase D activity.

3. The enzyme cleaved the phospholipids bound to rat liver microsomes. Furthermore, microsomes favored the hydrolysis of aqueous dispersions of 0.8 mM lecithin in the absence of ether.

4. The phospholipids associated with serum β-lipoproteins were not attacked by phospholipase D; moreover, β-lipoproteins inhibited the hydrolysis of dilute aqueous dispersions of lecithin (approx. 0.1 μM).

5. Lecithin partitioned between ether and aqueous sodium acetate solutions, in the presence or absence of $CaCl_2$ (40 mM), at a ratio of 4 to 1.

6. The hydrolysis of lecithin by phospholipase D in an ether–water system was shown to occur in the aqueous phase.

7. Low concentrations (0.1–0.5 mM) of cetyltrimethylammonium bromide as well as the calcium salts of phosphatidic acid, phosphatidyl glycerol or phosphatidyl methanol activated the hydrolysis of lecithin in the biphasic ether–water system.

INTRODUCTION

A previous paper described the extraction, partial purification and some of the properties of a soluble phospholipase D (phosphatidyl choline–phosphatido hydrolase, EC 3.1.4.4) from dry peanut seeds[1]. It was shown there that the enzyme preparation did not contain phospholipase A activity. Both water transfer (hydrolysis) and alcohol transfer (transphosphatidylation) activities of this phospholipase D were shown and found to be similar to the catalytic activity of phospholipase D purified from cabbage leaves[2–5].

The phospholipase D obtained from carrot roots by KATES[6,19] was associated

with the plastids. In a biphasic system of water and ether this enzyme acted on that portion of the substrate which partitioned into the ether phase.

In addition to lecithin and cardiolipin[1], the present work shows that phosphatidyl glycerol is being hydrolyzed by the peanut phospholipase D. Attempts were made to clarify the mode of the cleavage of lecithin by this enzyme in a biphasic system of water and ether. Because it was shown that phosphatidic acid affects the reaction of lecithin hydrolysis when the cabbage enzyme was used[3], we also studied the hydrolysis of lecithin by the peanut enzyme, in the presence of added calcium salts of phosphatidic acid, phosphatidyl glycerol and phosphatidyl methanol—all possible products of phospholipase D activity.

Because phospholipase D might well be useful in elucidating the role and function of phospholipids in intact membranes, lipoproteins and lipid–enzyme complexes, we studied the action of this enzyme on phospholipids of membranes and β-lipoproteins.

MATERIALS AND METHODS

The isolation and purification of phospholipase D from peanut seeds, the preparation of [^{3}H]choline lecithin and most of the methods used were described earlier[1]. The calcium salt of phosphatidic acid was prepared on a large scale from ovolecithin by the phospholipase D-catalyzed reaction[1]. The calcium salt of *sn*-3-phosphatidyl-1′-*rac*-glycerol was prepared from ovolecithin by the transphosphatidylation reaction catalyzed by phospholipase D (ref. 1). In the same manner phosphatidyl glycerol labeled with [1-^{14}C]palmitate was prepared from [1-^{14}C]palmitoyl lecithin obtained from rat liver (*cf.* ref. 1).

Phosphatidyl methanol (calcium salt) was prepared from ovolecithin by the phospholipase D-catalyzed transphosphatidylation as follows: 20 ml of water containing 1 mmole of sodium acetete, 0.8 mmole of $CaCl_2$ and 0.1 mmole of ovolecithin were adjusted to pH 5.7. 8 ml of methanol and 8 mg of partially purified peanut phospholipase D (Stage 2 in ref. 1) were added, and the mixture was incubated in a stoppered erlenmeyer flask for 4 h at 26° with continuous shaking. Most of the products thus formed precipitated on the walls of the vessel. The lipids were extracted according to BLIGH AND DYER[7]; the chloroform solution of the lipids was applied to a silicic acid column, and chromatography of the lipids was processed as outlined in ref. 1. The phosphatidyl methanol was eluted from the column with 6–15% (v/v) methanol in chloroform.

Under these experimental conditions nearly all of the lecithin was converted to phosphatidyl methanol and phosphatidic acid. The yield of the phosphatidyl methanol was about 90% (based on the initial lecithin concentration), and this agrees with results obtained by LENNARZ *et al.*[8], who prepared various phosphatidyl alcohols from ovolecithin using cabbage phospholipase D under experimental conditions similar to those described above.

The product was tentatively identified as phosphatidyl methanol on the basis of the following:

(1) An aliquot of the reaction mixture was extracted according to BLIGH AND DYER[7], and the lipid fraction chromatographed on thin-layer chromatoplates using the mixture of chloroform–methanol–NH_4OH (70:30:2, by vol.) for development.

The plate was then sprayed with the acid molybdate spray of DITTMER AND LESTER[9]. The only spots on the plate which gave a positive color reaction were those of phosphatidic acid, traces of lecithin and the newly formed phospholipid. No other compounds were detected on the plate following partial charring with conc. H_2SO_4.

(2) The purified phospholipid which was eluted from the silicic acid column revealed one spot on thin-layer chromatoplates, developed with the solvent mixture mentioned above, having an R_F value of 0.65 compared to 0.3 for lecithin, 0.41 for phosphatidyl glycerol and 0.03 for phosphatidic acid. These R_F values correspond to the values obtained by BARTELS AND VAN DEENEN[10].

(3) The pure phospholipid gave on thin-layer chromatoplates a positive color reaction with the phosphorus-detecting spray reagent of DITTMER AND LESTER[9]. All the other spray reagents such as ninhydrin, Dragendorf and fuchsin–H_2SO_3 gave negative results. In view of the ability of phospholipase D to catalyze the transphosphatidylation of the phosphatidyl moiety from lecithin to an alcohol, it seems reasonable to assume that the product obtained in the presence of methanol could well be phosphatidyl methanol.

Cetyltrimethylammonium bromide (Cetavlon) was a product of British Drug Houses, Poole, Dorset. Ether (analytical grade solvent) was obtained from Mallinckrodt, St. Louis, Mo. It was washed with water to remove traces of alcohol and then dried. Other portions of ether were saturated with water by shaking in a separatory funnel.

The β-lipoproteins of bovine serum albumin, designated Cohn Fractions III_0 and IV_1 (see ref. 11) were purchased as lyophilized powders from Nutritional Biochemicals Co., Cleveland, Ohio. Rat liver microsomes and rat β-lipoprotein were prepared as described previously[12].

Phospholipase D activity was assayed by determining the [^{3}H]choline released from tritium-labeled lecithin[1]. Most of the experiments were done with an enzyme preparation purified at "Stage 2" (see ref. 1).

The hydrolysis of *sn*-3-phosphatidyl-1′-*rac*-glycerol (having [1-^{14}C]palmitate at the C-1 position of the phosphatidyl moiety) by phospholipase D was done as follows: 0.4 μmole (7700 counts/min) of the substrate were dispersed in 1 ml of water containing 40 μmoles of $CaCl_2$, 50 μmoles of sodium acetate at pH 5.7 and 1 mg of enzymatic protein, in stoppered tubes which were constantly shaken in a water bath for 4 h at 26°. 1 ml ether per ml aqueous solution was sometimes added. At the end of the incubation the lipids were extracted[1] and chromatographed on thin-layer chromatographic plates coated with silica gel H (Merck and Co., Inc., Rahway, N.J.), with a mixture of chloroform–methanol–NH_4OH (70:30:2, by vol.) as solvent. Areas corresponding to phosphatidic acid and phosphatidyl glycerol were scraped into counting vials and counted in a Tri-Carb spectrometer (Packard Instrument Co., Inc.) using an ethanol–toluene scintillation mixture with the addition of 1 ml 10% ethanolic Triton X-100 per vial[1]. To convert the counts/min to μmoles of phosphatidyl glycerol, standard samples of labeled substrate were chromatographed on the same plate and a scaling factor calculated from the ratio of counts/min of the phosphatidyl glycerol spot to the counts/min added.

Aqueous lecithin dispersions were irradiated ultrasonically for 5–15 min at 0–2° under N_2. A Branson sonifier (Branson Instruments, Inc.) with an output of 6 mA was used.

The partitioning of lecithin between ether and water was done in the presence of 50 mM sodium acetate (pH 5.7) as follows: 1 ml of an aqueous dispersion of 1–5 μmoles of [^{3}H]choline-labeled lecithin was diluted with ovolecithin to obtain a specific radioactivity of 10000 counts/min per μmole. After the addition of 1 ml of ether, the stoppered tube was agitated on a Vortex mixer for 5–10 min, then allowed to stand at room temperature for 15 minutes and finally centrifuged at 2500 rev./min. Aliquots from the aqueous phase were carefully withdrawn for counting. In another set of experiments ovolecithin was partitioned between ether and an aqueous dispersion of the lecithin in 50 mM sodium acetate and 40 mM $CaCl_2$. The concentrations of the lecithin used were from 0.5 to 5 mM. The tubes were shaken for 0.5, 1, 2.5 and 5 min, allowed to stand at room temperature for an additional 5–10 min, then centrifuged at 2500 rev./min for 10 min. Aliquots from the ethereal phase were withdrawn for analysis of organic phosphorus[1].

The concentrations of phospholipids are expressed in mM or μmoles/ml of phospholipid-phosphorus.

RESULTS

Hydrolysis of phosphatidyl glycerol

In a previous report we demonstrated that peanut phospholipase D catalyzed the hydrolysis of lecithin in the presence of ether and of cardiolipin in the absence of ether, but did not attack sphingomyelin. We have now extended our studies on the specificity of the enzyme and have shown that, when 0.4 μmole/ml of [^{14}C]palmitate-labeled phosphatidyl glycerol was incubated with 1 mg/ml of phospholipase D in the absence of ether, 0.16 μmole (40%) of this substrate was hydrolyzed in 4 h. At the same time equivalent amounts of phosphatidic acid were formed. When 1 ml of ether was added to 1 ml of the reaction mixture, it increased considerably the hydrolysis of phosphatidyl glycerol, *i.e.* 0.348 μmole (87%) of phosphatidyl glycerol was hydrolyzed.

Effect of sonication and ether on the rate of lecithin hydrolysis

Aquous dispersions of lecithin (5 mM) which had not been subjected to ultrasonic irradiation were not attacked by phospholipase D in the absence of ether. Addition of 0.25 mM calcium phosphatidate initiated hydrolysis at a slow rate of 0.0044 μmole/min. The reaction leveled off after 120 min, with an overall breakdown of lecithin of only 7.5%.

It was previously observed that ultrasonically treated aqueous dispersions of lecithin were not hydrolyzed by the enzyme[1]. However, we have repeated these experiments, and it was shown that phospholipase D released [^{3}H]choline from ultrasonically treated lecithin at a rate comparable to, or even higher than, the rate obtained with untreated lecithin in the biphasic ether–water system.

Because ultrasonically treated lecithin dispersions were cleaved by the enzyme in the absence of ether, it was assumed that the size of the lecithin aggregates determines the rate of the hydrolysis of the substrate. We therefore dispersed lecithin in 50 mM sodium acetate at concentrations as low as 1 μM with a Vortex mixer and incubated it with phospholipase D in the absence of ether. Although 12% of the counts in the lecithin were recovered as free [^{3}H]choline after 10 min of incubation, the

TABLE I

HYDROLYSIS OF VERY LOW CONCENTRATIONS OF LABELED LECITHIN IN THE ABSENCE OF ETHER

0.5 mg of enzymatic protein in 1 ml of water which contained 40 μmoles $CaCl_2$ and 50 μmoles of sodium acetate at pH 5.7, incubated with [^{3}H]choline lecithin (100000 counts/min, approx. 1 mμmole) at 26°, is designated a complete system. The reaction was done in the absence of ether except for System 4.

System	*Time of incubation (min)*	*[^{3}H]Choline (counts/min)*
1. Complete	0	0
2. Complete	10	12000
3. Complete	20	23200
4. Complete + 1 ml ether	10	64400
5. *Minus* enzyme	20	48

addition of 1 ml of ether increased the hydrolysis of the substrate to 64% in the same period of time (Table I).

Phospholipase D and natural lipoproteins

The experiments described in Fig. 1 show that phospholipase D splits the phospholipids of rat liver microsomes. The disappearance of the spots of lecithin and phosphatidyl ethanol amine on the thin-layer chromatographic plates was accompanied by an accumulation of phosphatidic acid. It was also noticed that the microsomal-bound sphingomyelin disappeared, despite the failure in previous attempts to hydrolyze pure sphingomyelin[1]. We did not attempt to identify the products of sphingomyelin hydrolysis. However, in results not shown, phospholipase D did not cleave the phospholipids bound to bovine serum β-lipoproteins.

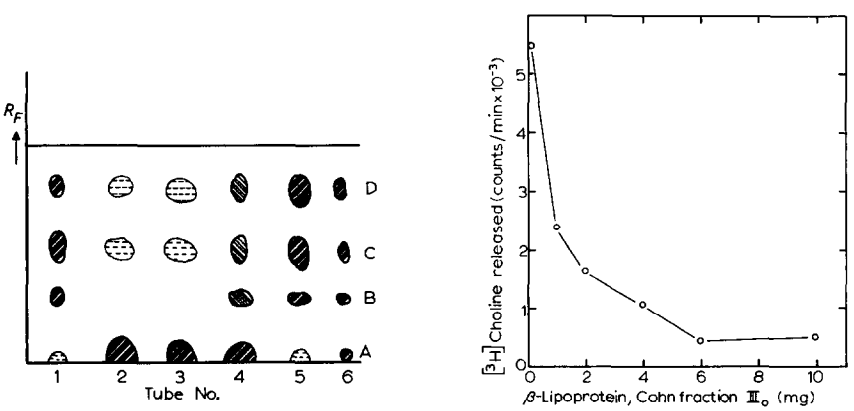


Fig. 1. Thin-layer chromatography of the products of the hydrolysis of phospholipids bound to rat liver microsomes by phospholipase D. 3–4 mg of rat liver microsomes were incubated at 26° for 30 min in 1 ml of water containing 50 μmoles of sodium acetate at pH 5.7, 40 μmoles of $CaCl_2$ and 0.85 mg enzymatic protein. The extracted lipids[1] were chromatographed on thin-layer chromatoplates with chloroform–methanol–NH_4OH (70:30:2, by vol.) as solvent, and visualized by spraying with acid molybdate[11]. The standard mixture of: A, phosphatidic acid; B, sphingomyelin; C, lecithin; D, phosphatidyl ethanolamine was run on the same plate. Tube 1, zero time with ether; Tubes 2 and 3, 30 min incubation with ether; Tube 4, 30 min incubation without ether; Tube 5, zero time without ether; Tube 6, phospholipid standards.

Fig. 2. Effect of β-lipoproteins on the hydrolysis of [^{3}H]choline lecithin by phospholipase D. An assay mixture of 1 ml water contained 50 μmoles sodium acetate at pH 5.7, 40 μmoles $CaCl_2$, 0.29 mg protein of phospholipase D and approx. 1 mμmole [^{3}H]choline lecithin with 10000 counts/min. Incubation was done at 26° for 15 min without ether.

TABLE II

EFFECT OF RAT LIVER MICROSOMES ON PHOSPHOLIPASE D ACTIVITY

Phospholipase D at a concentration of 0.6 mg protein was incubated with 4 mg of lyophilized rat liver microsomes for different periods of time (Expt. A), or for 60 min with increasing amounts of microsomes (Expt. B) in a final volume of 1 ml water containing 40 μmoles $CaCl_2$, 50 μmoles sodium acetate (pH 5.7) and 0.8 μmole [^{3}H]choline lecithin (18000 counts/min). Incubation was done at 26° in the absence of ether.

Expt.	*Phospholipase D (mg protein)*	*Microsomes (mg)*	*Time (min)*	*[^{3}H]Choline (μmoles)*
A	0.6	4	30	0.22
	0.6	4	60	0.51
	0.6	4	90	0.77
	—	4	60	0
B	0.6	1	60	0.084
	0.6	2	60	0.182
	0.6	4	60	0.330

TABLE III

EFFECT OF β-LIPOPROTEIN ON LECITHIN HYDROLYSIS BY PHOSPHOLIPASE D

[^{3}H]Choline lecithin at a concentration of approx. 0.1 mμmole (10000 counts/min) was incubated with bovine serum β-lipoprotein (Cohn Fraction III_0) or rat serum β-lipoprotein (density < 1.063) in a final volume of 1 ml water containing 50 μmoles sodium acetate (pH 5.7), 40 μmoles $CaCl_2$ and 0.29 mg enzymatic protein. The mixtures were incubated at 26° and shaken in the absence of ether. Additions were as mentioned in the table.

System	*[^{3}H]Choline (counts/min)*
Control *minus* β-lipoproteins	5500
β-Lipoprotein (Fraction III_0), 2 mg	1700
β-Lipoprotein (rat serum), 2.5 mg	590

When rat liver microsomes were added to an incubation mixture of phospholipase D and tritium-labeled lecithin in the absence of ether, they promoted the hydrolysis of the substrate (Table II). Under these experimental conditions the microsomes did not contain phospholipase D activity. In contrast, bovine serum β-lipoproteins (Cohn Fractions III_0 and IV_1) and rat serum β-lipoprotein (density <1.063), in the absence of ether, inhibited the hydrolysis of low concentrations (0.1 μM) of lecithin (Table III and Fig. 2). The inhibition was not due to the dilution of the labeled lecithin with the lipoprotein-bound phospholipids, as could qualitatively be ascertained by the thin-layer chromatographic determination of a decreased formation of phosphatidic acid.

Partition of lecithin between ether and aqueous salts solutions

The partitioning of lecithin between ether and an aqueous solution of 50 mM sodium acetate, under the conditions described in MATERIALS AND METHODS, was 10–20% in the aqueous phase and 80–90% in the ether phase. The initial amounts of lecithin employed were 0.5–5 μmoles per 1 ml of the aqueous solution. It was also observed that the addition of 40 mM $CaCl_2$ to the sodium acetate solutions did not considerably affect the partitioning values. Mixing the aqueous lecithin dispersions with ether for 0.5, 1, 2.5 or 5 min, before allowing the tubes to stand for 15 min at room temperature and then centrifuging, gave the same values for the partition coefficient indicating that equilibrium had been reached.

The site of enzymatic hydrolysis

KATES[6,19] demonstrated that the hydrolysis of lecithin catalyzed by phospholipase D from carrot plastids occurs in the ether phase of an assay system similar to that used in these experiments. Under the conditions conducive to optimal lecithin hydrolysis, most of the substrate is partitioned to the ether phase, yet the peanut phospholipase D differs from the carrot enzyme.

A dispersion of lecithin in a solution of buffer, $CaCl_2$ and enzyme was shaken with ether and the phases separated immediately by centrifugation. The separated phases were each transferred to another tube and incubated for 15 min; also, in order to reconstitute the original conditions, ether was sometimes added to the separated aqueous phase and buffer and $CaCl_2$ to the separated ethereal phase (but no enzyme) and then incubated for 15 min. It was observed that following incubation choline was released only from the lecithin present in the aqueous phase obtained from the original biphasic system (Table IV). These results indicate that the enzyme acted on that portion

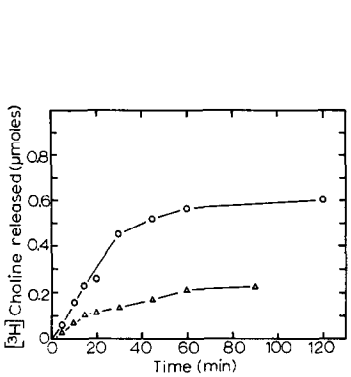
TABLE IV

THE NATURE OF LECITHIN HYDROLYSIS IN A BIPHASIC WATER–ETHER SYSTEM

5 or 10 μmoles of [^{3}H]choline lecithin (2000 counts/min per μmole) were dispersed in 1 ml of water containing 40 μmoles $CaCl_2$, 50 μmoles sodium acetate at pH 5.7 and 0.5 mg enzymatic protein in a stoppered tube. 1 ml of ether was added, the tubes shaken rapidly (less than 15 sec) and then centrifuged at 2500 rev./min. Expts. I–IV described below were then followed and each phase analyzed for [^{3}H]choline (*cf.* ref. 1): Expt. I. Each phase was incubated separately at 26° for 2, 15 or 30 min. Expt. II. The phases were separated and recombined immediately, then incubated for 15 min at 26°. Expt. III. The phases were separated, each was incubated separately for 15 min, then recombined and further incubated for 15 min at 26°. Expt. IV. The phases were separated and transferred each to another tube. A fresh portion of ether was added to the aqueous phase, and a fresh solution of buffer and $CaCl_2$ was added to the ethereal phase. Each of these systems was now incubated for 15 min at 26°. No [^{3}H]choline was formed in that system which contained the original ethereal phase.

Expt.	*Initial lecithin (μmoles)*	*Incubation time (min)*	*[^{3}H]Choline formed (μmoles) in*		
			Ether	*Water*	*Recombined*
I	5	2	0	0.29	—
	5	30	0	0.64	—
	10	15	0	0.625	—
II	10	15	—	—	0.67
III	10	15+15	—	—	0.915
IV	10	15	0	0.38	—

of the lecithin that partitioned to the aqueous phase in the short mixing period and apparently no enzyme partitioned to the original ethereal phase which contains most of the lecithin. It thus seems that the rate of lecithin hydrolysis in the biphasic system is governed by the continuous diffusion of the substrate from the ethereal phase to the aqueous phase which contains the water-soluble enzyme. To check this possibility we compared the hydrolysis of an aqueous dispersion of lecithin (1 mM) in a biphasic ether–water system in tubes that were constantly shaken and tubes that were only shaken for very short periods (less than 15 sec) and then allowed to stand at 26°. The rate of the lecithin hydrolysis in the system not shaken was 0.007 μmole/min and that obtained when the tubes were shaken was 0.0116 μmole/min. Furthermore, in the former case the reaction stopped after 90 min with 23% hydrolysis, whereas in the latter case the reaction proceeded to 60% hydrolysis (Fig. 3).



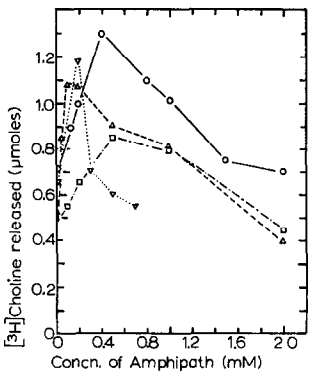


Fig. 3. The effect of shaking on the time-course of hydrolysis of lecithin in an ether–water system. A volume of 1 ml water containing 50 μmoles sodium acetate, 40 μmoles $CaCl_2$, 0.5 mg protein of phospholipase D and 1 μmole of [^{3}H]choline lecithin was mixed with 1 ml of ether. Incubation was done at 26° without shaking (△) or with constant shaking (○).

Fig. 4. Effect of cetyltrimethylammonium bromide or the calcium salts of phosphatidic acid, phosphatidyl glycerol or phosphatidyl methanol on the enzymatic hydrolysis of [^{3}H]choline lecithin. 4 μmoles of lecithin were dispersed in 1 ml of water containing buffer, $CaCl_2$ and enzyme and ether as in Fig. 3 and shaken at 26° for 15 min with the following compounds: ○, cetyltrimethylammonium bromide; ▽, calcium phosphatidate; △, calcium phosphatidyl glycerol; □, calcium phosphatidyl methanol.

Effect of cetyltrimethylammonium bromide, phosphatidic acid, phosphatidyl glycerol and phosphatidyl methanol on hydrolysis or transphosphatidylation of lecithin

Fig. 4. shows the effect of additives on the hydrolysis of lecithin by phospholipase D. The substances, except the positively charged cetyltrimethylammonium bromide, are the products of phospholipase D activity. All these added compounds, at a concentration range of 0.1–2 mM, enhanced the activity of the enzyme with an optimum at 0.1–0.5 mM.

In another experiment it was also shown that the enhancing effect of calcium phosphatidate was not limited to the hydrolysis reaction but could also be demonstrated in the enzyme-catalyzed transphosphatidylation to methanol. Fig. 5 shows that a concentration of 0.8 mM of calcium phosphatidate caused a considerable increase in the release of [^{3}H]choline from lecithin with a concomitant increase in the formation of phosphatidyl methanol, qualitatively determined on thin-layer chromatography (results not shown). No reaction occurred in the absence of Ca^{2+} even if sodium phosphatidate was added. It therefore seems that the activating effect is due to calcium phosphatidate.

DISCUSSION

Our results show that ether does stimulate considerably the hydrolysis of phosphatidyl glycerol by phospholipase D from peanuts. This extends our previous observations made on the hydrolysis of lecithin[1] and confirms those made by KATES[6,19] that phospholipase D isolated from cabbage or spinach can display broad specificity. Phospholipase D catalyzes the splitting of choline, ethanolamine or serine from their respective phosphoglycerides when the medium is saturated with ether; the enzyme released these bases from a mixture of soybean phospholipids in the absence of ether[6,19]. Ether does not seem to be required with all phospholipids. For instance, DAVIDSON

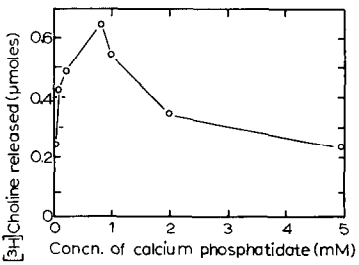


Fig. 5. Effect of calcium phosphatidate on the enzyme-catalyzed transphosphatidylation of [^{3}H]-choline lecithin to methanol. The conditions of the reaction were similar to those given in Fig. 4, except that ether was replaced by 0.2 ml methanol per ml water.

AND LONG[17] have indicated that ether is inhibitory for the hydrolysis of lysolecithin. We have shown, in a previous report, the same is true for the cleavage of diphosphatidyl glycerol (cardiolipin)[1]. Thus, although ether activates the hydrolysis of certain substrates, it does not have to be present, provided that the substrate is properly dispersed as in the case of ultrasonically irradiated dispersions of lecithin or very low concentrations of lecithin (10 nM; *cf.* Table I) or is dispersed by detergents (*cf.* ref. 1).

The phospholipids present in rat liver microsomes are also cleaved by the enzyme in the absence of ether. The hydrolysis of sphingomyelin bound to the microsomes by phospholipase D, as shown in the present study, is probably promoted by the presence of other phospholipids producing the proper conditions for cleavage, which could not be obtained when pure sphingomyelin was present alone.

The hydrolysis of aqueous dispersions of lecithin by phospholipase D also occurs when ether is replaced by rat liver microsomes (Table II). It is known that liver microsomes contain phospholipase A activity. It could be reasoned that the microsomes exert their stimulatory effect by the formation of lysolecithin, which could be related to the enhancing effect of the microsomes on the hydrolysis of lecithin by phospholipase D. However, the data available show that rat liver microsomal phospholipase A does not hydrolyze lecithin[13,14].

Phospholipase D has hitherto been tested mostly with purified individual phospholipids or mixtures of phospholipids. Earlier attempts to apply commercial cabbage phospholipase D preparations to human erythrocyte ghosts were unsuccessful[15] in hydrolyzing phospholipids of the membrane, but positive results were later obtained by BRUCKDORFER *et al.*[16]. These authors incubated rat erythrocyte ghosts with this commercial enzyme in a Tris–HCl buffer (pH 5.6) saturated with ether rather than in a biphasic ether–water system. They were also able to demonstrate the hydrolysis of human β-lipoprotein-bound lecithin by phospholipase D under similar experimental conditions[16]. It is therefore possible that this enzyme could be successfully employed to study the role and function of phospholipids associated with natural lipoproteins and membranes. Our results show, however, that serum β-lipoprotein has an inhibitory effect on the hydrolysis of very low concentrations of lecithin (0.1 μM). This inhibitory effect could result from an adsorption of the substrate to the lipoprotein which thus prevents its being accessible to the enzyme, corroborating our observations that peanut phospholipase D does not hydrolyze the phospholipids bound to β-lipoprotein.

Utilizing the water-soluble peanut phospholipase D in a water–ether mixture

in the presence of Ca^{2+}, we have shown that the enzyme catalyzes the hydrolysis of lecithin only in the aqueous phase of the biphasic water–ether system (Table IV). It may, therefore, be assumed that the enzyme which cleaves the diester bond of the polar head group of lecithin would rather have the polar group of the lecithin in the aqueous phase. From the fact that there was no activity found in the ether phase, it could be implied that the enzyme is insoluble in the ether even in the presence of lecithin. The partitioning of lecithin between ether and the aqueous phase containing 50 mM sodium acetate gave a constant K (ether–water) of approx. 4 at room temperature with dispersions of lecithin in the aqueous phase having initial concentrations up to 5 mM. BRUCKDORFER *et al.*[16] have shown that ether extracts only small amounts of lecithin or phosphatidic acid from an aqueous dispersion. However, their inclusion of salts (0.5 M NaCl or 0.1 M $CaCl_2$) or cholesterol increased the extractability of lecithin to a value similar to that found in the present study. The extraction by ether of phosphatidic acid from aqueous dispersions in the presence of cholesterol was very low[16]. It therefore seems that the rate of lecithin hydrolysis in a biphasic ether–water mixture is governed by the diffusion of the lecithin from the ethereal phase to the aqueous phase. Although the initial partitioning of lecithin between the ether and the aqueous phase shows that approx. 20% of the substrate is present in the aqueous phase, the results showing that more than 60% of the lecithin is hydrolyzed (in the aqueous phase) imply a continuous diffusion of the substrate from the ether phase to the aqueous phase as the reaction proceeds.

The activating effect of amphipathic compounds on the hydrolysis of lecithin by cabbage phospholipase D has been observed by DAWSON AND HEMINGTON[3] and with the peanut enzyme by HELLER *et al.*[1]. It has been pointed out by DAWSON AND HEMINGTON[3] that phosphatidate (probably as calcium salt) stimulated the hydrolysis of large lecithin particles (1–5 μ, which were obtained on shaking lecithin with an aqueous solution of buffer and $CaCl_2$) by phospholipase D. They could not, however, find any evidence that the phosphatidate produced an increased dispersion of the lecithin. Furthermore, electrophoretic mobility measurements of the mixed lecithin-phosphatidate in the presence of Ca^{2+} did not show any correlation with the activating effect of phosphatidate on the hydrolysis of lecithin[3]. The observations made recently by QUARLES AND DAWSON[18] indicate that amphipathic compounds cause a shift of the pH optimum of lecithin hydrolysis from 4.9 to 5.4–5.6 in the presence of sodium dodecyl sulfate and to 6.5–6.6 in the presence of phosphatidic acid.

Our studies confirm the observations of DAWSON AND HEMINGTON[3] on the activating effect of phosphatidic acid on the hydrolysis of lecithin (Fig. 4), even though our enzyme was obtained from a different source and the assay was done in the presence of ether. We also found that other amphipathic molecules such as phosphatidyl methanol or phosphatidyl glycerol gave a similar activation (Fig. 4).

Additional experiments are needed to show whether the rate of the hydrolysis of other phosphoglycerides in a biphasic ether–water system is also dependent on partitioning. Studies are also in progress on the effect of the products of the enzymatic activity (*i.e.* phosphatidic acid or phosphatidyl glycerol or phosphatidyl methanol) on the partitioning process which might shed light on the effect of the amphipathic compounds on the phospholipase D-catalyzed reactions.

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