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THE SUBSTRATE SPECIFICITY OF PHOSPHOLIPASE A

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SUMMARY

Investigations on variously modified analogues of phospholipids elucidated the following substrate characteristics for phospholipase A (*Crotalus adamanteus*).

1. Within the class of α-phosphoglycerides L-isomers are readily hydrolysed, while D-α-phospholipids appeared not to be attacked.

2. L-α-Lecithins containing fatty acids with greatly varying chain length are susceptible to phospholipase A action; however, certain water-soluble short-chain compounds are hydrolysed at a very slow rate only.

3. Aside from effects on the surface charge of the phosphoglyceride micelles the nature of the polar headgroup esterified to the phosphoryl moiety turned out not to form any prerequisite, and its presence even appeared to be dispensable.

4. Contrary to a blocking of the amino function, protection of the hydroxyl function of phosphatidylethanolamine caused an inactivation of the substrate properties.

5. Both, a γ-benzyl-β-acyl-α-phosphoglyceride and a β-acyl-lyso derivative, were hydrolysed, whereas the corresponding structural isomers carrying the fatty acid in γ-position appeared not to be susceptible to phospholipase A action.

6. Glycol analogues were demonstrated to exhibit substrate activity.

7. Phospholipase A catalyses the hydrolysis of a symmetric β-lecithin into an optical-active lysolecithin.

8. An isolated specimen of cardiolipin was hydrolysed, whereas sphingomyelin resisted phospholipase A action.

INTRODUCTION

Using synthetic phospholipids of the mixed-acid type as substrates for phospholipase A (phosphatide-acyl hydrolase, EC 3.1.1.4) it was demonstrated that the enzyme present in snake-venom[1-3] (*Crotalus adamanteus*) and human pancreas[4] specifically catalyses the hydrolysis of the β-fatty ester linkage of L-α-phosphoglycerides. A similar conclusion has been reached by other investigators on account of principally different approaches[5-7].

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The established positional specificity of phospholipase A still requires an explanation in terms of a defined substrate–enzyme interaction, allowing only the β-ester position of the L-α-phospholipid molecule to be susceptible to the hydrolytic action. For these reasons the substrate specificity of phospholipase A has been verified on a number of synthetic compounds.

MATERIALS AND METHODS

Synthetic substrates

The preparation of a series of ten L-α-lecithins containing two identical fatty acids has been reported[8]. The fatty acid chain length of these compounds (Table III, formula Ia, $R_1 = R_2$) varied from 2 to 24 carbon atoms. Mixed-acid lecithins (I, R_1 is unequal to R_2) were prepared by a synthesis *de novo*[9] and included both L- and D-isomers (Ia and Ib respectively). Methods for the synthesis of mixed-acid phosphatidylethanolamines[3,9–11] (II), phosphatidylserine[12] (III), phosphatidic acid[13] (IV), and phosphatidylethanolamine analogues[3] carrying various protecting groups (VI and VII) have been published as well. The synthesis of two structurally isomeric (γ,β)-acyl-benzyl-α-glycerylphosphorylethanolamines (VIII and IX) and two isomeric lysophospholipids, *viz.* β-acyl-lysophosphatidylethanolamine (X) and γ-acyl-lysophosphatidylethanolamine (XI), was carried out recently by SLOTBOOM[14]. Several glycol analogues (XII) have been prepared according to methods applied for the synthesis of the corresponding phosphoglycerides (compare *e.g.* DAEMEN *et al.*[10] and DE HAAS AND VAN DEENEN[12]). A β-lecithin (XIII) was prepared, starting from α,γ-distearine according to the methods outlined by HIRT AND BERCHTOLD[15].

Determination of the action of phospholipase A

Most of the enzymic experiments were carried out by incubating a 0.02 M emulsion (or solution) of the substrate to be assayed in a 0.1 M borate buffer (pH 7.0), calcium acetate $2.5 \cdot 10^{-3}$ M. In a number of experiments the 2,4,6-collidine-buffered system described by MAGEE AND THOMPSON[16] was used. If necessary the emulsions were prepared by ultrasonic vibration and in some experiments the substrate was emulsified together with half its weight of deoxycholate. To 1 ml of the medium a weighed amount of 1–4 mg of lyophilized venom from *Crotalus adamanteus* was added, and the mixture was incubated under shaking at 37°. Control experiments without snake venom added were always performed. The proceeding of the enzymic hydrolysis was qualitatively examined by making at various intervals thin-layer chromatograms, using the microslide procedure of PEIFER[17]. After a given incubation period quantitative determinations of the degree of degradation were carried out by estimating the amount of the produced phosphorus-containing hydrolysis product and the substrate eventually remaining. The compounds present in the incubation mixture therefore were separated by paper chromatography. In the case of lipid-soluble substrates use was made of silica-impregnated paper with the solvent system introduced by MARINETTI *et al.*[18], while the hydrolysates of water-soluble compounds were subjected to chromatography on Whatman paper No. 1 with either butanol – ethanol – water (5:5:2, v/v) or propanol – ammonia – water (6:3:1, v/v) as developer. Details about the various techniques involved were reported previously[3,4].

RESULTS

Stereospecificity of phospholipase A

On account of experiments performed on synthetic DL-α-lecithin LONG AND PENNY[19] already concluded that phospholipase A acts on L-α-isomers only. The stereospecificity of phospholipase A was confirmed by comparing the substrate activity of two lecithin antipodes, both containing an identical fatty acid composition. Contrary to the L-α-isomer the synthetic D-α-lecithin was not hydrolysed upon incubation with phospholipase A (Table I). Also after a prolongation of the incubation time

TABLE I

ACTION OF PHOSPHOLIPASE A ON ENANTIOMERIC PHOSPHOLIPIDS

Incubation time, 20 h.

Compound	*Degree of hydrolysis (%)*
(γ-stearoyl-β-oleoyl)-L-α-Lecithin	96
(γ-stearoyl-β-oleoyl)-D-α-Lecithin	0
(γ-stearoyl-β-lauroyl)-L-α-Lecithin	100
(γ-stearoyl-β-lauroyl)-DL-α-Lecithin	53
(γ-palmitoyl-β-linolenoyl)-L-α-Phosphatidylethanolamine	100
(γ-linolenoyl-β-palmitoyl)-L-α-Phosphatidylethanolamine	100
(γ-oleoyl-β-palmitoyl)-L-α-Phosphatidylserine	100
(γ-stearoyl-β-lauroyl)-DL-α-Phosphatidylserine	48

up to 72 h no indications were obtained for an enzymic degradation of this compound. Consequently, preparations of DL-α-phosphoglycerides were found to be hydrolysed to an extent of about 50 % (Table I).

The stereospecific action of phospholipase A towards symmetric β-lecithins will be discussed separately.

Effects of the nature of the substrate fatty acid constituents

Notable differences are to be noted in the rate of hydrolysis of synthetic lecithins containing saturated fatty acids of varying chain length (Fig. 1). A maximal rate of breakdown by phospholipase A revealed L-α-(didecanoyl)-lecithin, a compound which furnished fair and stable emulsions. The uncomplete degradation of some long-chain homologues, under the conditions used, probably is due to inadequate emulsification. After ultrasonic irradiation a complete breakdown of *e.g.* L-α-(distearoyl)-lecithin could be accomplished after an appropriate incubation time. Generally, all long-chain compounds, rendering satisfactory emulsions, turned out to be hydrolysed by phospholipase A into their lyso derivatives.

The results obtained with the short-chain homologues deserve further consideration. As regards L-α-(diheptanoyl)-lecithin, exhibiting both lipid- and water-soluble characteristics, a fair rate of breakdown was to be noted. However, shortening of the chain length of the fatty acid constituents appeared to be accompanied by a significant decrease of the substrate activity. This is demonstrated by the two water-soluble substances, *viz.* L-α-(dibutyryl)-lecithin and L-α-(diacetyl)-lecithin, showing a very

limited breakdown when compared with the C_{10}-homologue (Fig. 1). Incubation of the C_4-compound together with an emulsion of the C_{10}-homologue did not result into any appreciable acceleration of the hydrolysis of the former compound.

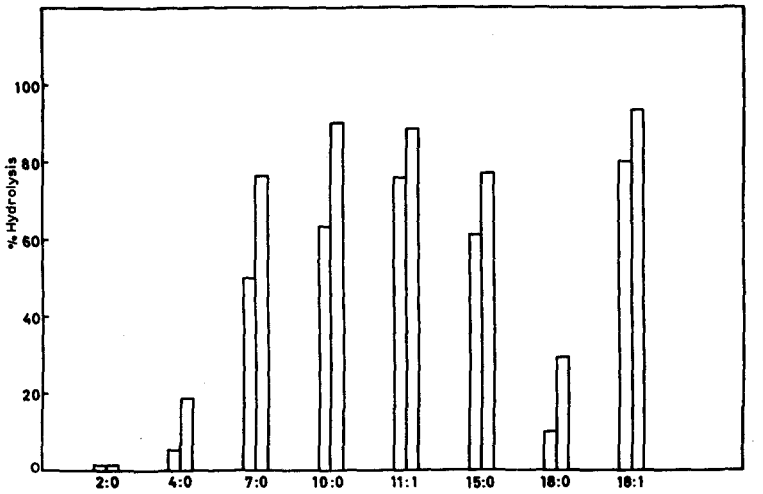


Fig. 1. Hydrolysis of a series of L-α-lecithins by snake-venom phospholipase A. Percentages of hydrolysis are given after incubation for 1 h (left bar) and 3 h (right bar) of a 0.02 M solution or emulsion of the lecithin in a borate-buffered system containing 1 mg of *Crotalus adamanteus* venom per ml medium. The individual lecithins are indicated by means of their fatty acid components, *e.g.* 10:0 stands for L-α-(didecanoyl)-lecithin and 18:1 for L-α-(dioleoyl)-lecithin.

Although the studied short-chain compounds are in principle susceptible to the action of phospholipase A and further progress of their breakdown could be obtained by extension of the incubation time or increase of the venom concentration, there is an undeniable difference between the rate of hydrolysis of water-soluble and lipid-soluble substrates. The results suggested that this difference to some extent might be attributed to variations between the abilities of both types of homologues to form a lipid interface. Measurements of the effects of these lecithins on the interfacial tension of the chloroform–water system according to expectation revealed notable differences between the C_2- and C_4-homologues on one side and the C_7- and higher homologues on the other (Fig. 2). This coincidence in shifts between the substrate properties and interfacial behaviour of the studied lecithins supports the view that the susceptibility to phospholipase A is to some extent dependent on their ability to attain a certain orientation. In this respect it is of interest to refer also to the interposition revealed by the C_7-homologue in studies on monomolecular layers of the lecithins under consideration. At the air–water interface the C_{10}-lecithin was found to form a stable film, while the C_7-homologue gave a layer which faded away upon increasing the pressure[20].

ROHOLT AND SCHLAMOWITZ[21] reported that the water-soluble C_6-homologue, *viz.* L-α-(dihexanoyl)-lecithin, can act as substrate for phospholipase A, also when monomers are the chief, if not the exclusive species of this lecithin in solution. Our results on the breakdown of *e.g.* the C_4-homologue at concentrations below the critical micelle concentration are in agreement with this observation. Although ROHOLT AND SCHLAMOWITZ do not arrive at any definite conclusion at this point, it

has been suggested to us that the report of these investigators does interfere with our view that the fatty acid chain length of the substrates, contributing to a certain molecular orientation, plays a part in attaining a substrate–phospholipase A complex. However, various experimental data of ROHOLT AND SCHLAMOWITZ being in excellent agreement with our observations do endorse our view; *e.g.* L-α-(dioctanoyl)-lecithin which forms an emulsion with water was observed by these investigators to be attacked 70 times more rapidly than the water-soluble C_6-analogue.

In their fundamental investigations BANGHAM AND DAWSON[22] studied the action of phospholipase B on unimolecular films of lecithins, thereby elucidating several

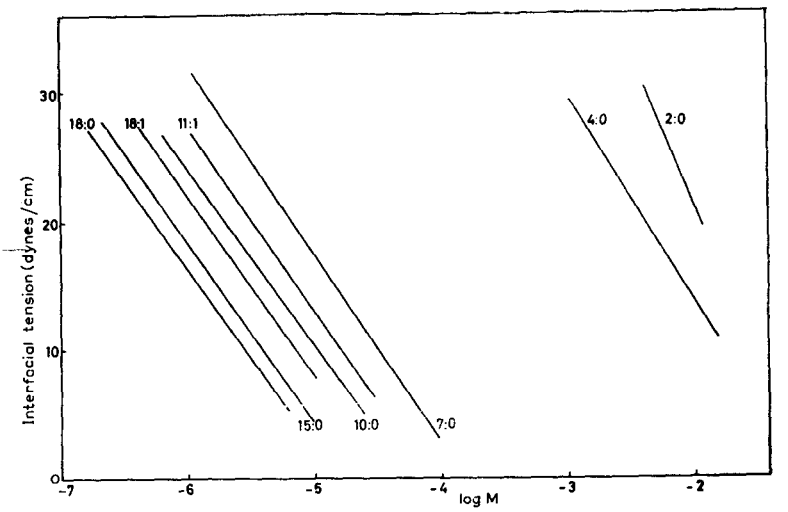


Fig. 2. Effects of L-α-lecithins on the interfacial tension of a chloroform–water system. For abbreviations compare Fig. 1.

features of the substrate enzyme interaction at the lipid–water interface. The results of these elegant investigations suggested that this enzyme can penetrate low-pressure films of lecithins. It will be of interest to subject various lecithin homologues to comparable studies with phospholipase A.

In summary, the obtained results suggest that the action of phospholipase A is greatly promoted when the nature of the fatty acid constituents favours a certain molecular orientation of the substrate molecules. It cannot be precluded that thereby the fatty acyl chain(s) is directly involved in attaining a substrate–phospholipase A complex by interaction with a hydrophobic region of the active site of the enzyme.

Effects of the nature of the substrate headgroup

No fundamental differences exist with respect to the enzymic hydrolysis of lecithins and phosphatidylethanolamines (Table I). As reported previously[4] the rate of hydrolyses of both types of phospholipids may differ, this also depending on the origin of the enzyme. Next to choline and ethanolamine phospholipids also a *N,N*-dimethylethanolamine carrying substrate analogue turned out to be susceptible to the enzymic action as will be dealt with later in this paper. In the borate-buffered system no appreciable hydrolysis of phosphatidylserine was observed. However, the

synthetic serine phosphoglycerides were attacked in a 2,4,6-collidine–ether system[16] (Table I), this in agreement with the observations of RATHBONE *et al.*[23] on isolated specimens of this phospholipid type. Previous studies[4,24] already indicated that phosphatidic acid can be hydrolysed into the lyso derivative and fatty acid by phospholipase A preparations from mammalian pancreas. As regards the snake-venom phospholipase A no appreciable hydrolysis of the synthetic phosphatidic acid could be observed in the systems used. As established by DAWSON AND BANGHAM[22,25] phospholipases require the substrate-micelles to have a certain zeta potential before activity commences. In a recent study we outlined that phospholipase A also obeys

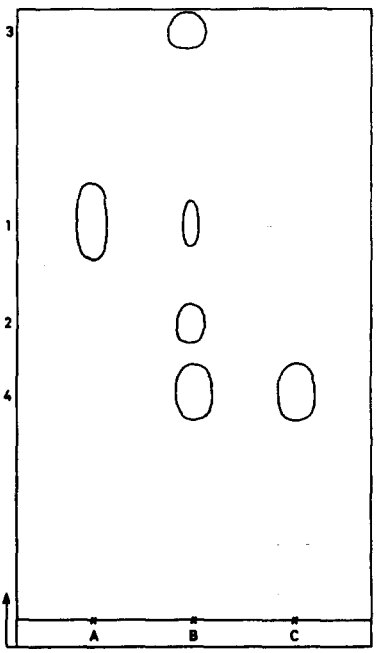


Fig. 3. Paper chromatographic demonstration of the action of snake-venom phospholipase A on phosphatidic acid. Paper chromatograms on silica-impregnated paper were developed according to MARINETTI *et al.*[18] after incubation of: A, 10 mg (γ-oleoyl-β-palmitoyl)-L-α-phosphatidic acid in 1 ml of borate buffer containing 1 mg of snake venom for 16 h at 37°; B, 5 mg of the phosphatidic acid emulsified together with 5 mg of pentadecanoylcholine and 1 mg of snake venom under identical conditions; C, 5 mg of pentadecanoylcholine with 1 mg of snake venom. 1, (γ-oleoyl-β-palmitoyl)-L-α-phosphatidic acid; 2, lysophosphatidic acid; 3, liberated fatty acid; 4, pentadecanoylcholine.

to such a rule[4]. Therefore one may speculate that the resistance of phosphatidic acid to snake-venom phospholipase A may be due to the notable negative surface charge of the substrate micelles being unfavourable for the interaction with the enzyme. Consequently, reduction of this charge by adding an appropriately charged lipophylic substance might favour the enzymic breakdown of phosphatidic acid. When emulsified together with lecithin, indeed a small but definite degradation of phosphatidic acid by snake-venom phospholipase A resulted. This was demonstrated by the appearance of a spot of both a lysophosphatidic acid and free fatty acid on the paper chromatogram. Lecithin cannot be considered to be the most favourable activator because of its rapid degradation by phospholipase A and its limited effect on the reduction of the charge of the micelles. Therefore experiments were carried out with pentadecanoylcholine[8]. The addition of this choline ester, being positively charged and not hydrolysed by the snake-venom preparation, significantly enhanced the breakdown of phosphatidic acid by phospholipase A (Fig. 3).

Recently we reported[26] that a specimen of phosphatidylglycerol (V) was hydrolysed by snake-venom phospholipase A into its lyso derivative as well.

The combined results show that the presence of a certain type of headgroup does not form a strict requirement for phospholipase A, and this enzyme can even develop its action towards a substrate not having any polar group esterified to the phosphoryl moiety.

Effects of blocking the hydroxyl and amino function of the phosphoryl nitrogenous moiety

Inasmuch as the charged groups of the phospholipid molecule are likely to play a part in the primary interaction with the binding center of the enzyme, it is of interest to investigate the effects of a blockade of the ionizable groups on the substrate

TABLE II

ACTION OF PHOSPHOLIPASE A ON PHOSPHOLIPID ANALOGUES

Incubation time, 20 h.

Compound	*Percentage of hydrolysis*
Lithium-γ-palmitoyl-β-linolenoyl-L-α-glycerylphosphoryl-2′-oxyethylphthalimide (VI)	100
Benzyl-γ-palmitoyl-β-linolenoyl-L-α-glycerylphosphoryl-2-oxyethylphthalimide (VII)	0
γ-Benzyl-β-stearoyl-DL-α-glycerylphosphorylethanolamine (VIII)	49
γ-Stearoyl-β-benzyl-DL-α-glycerylphosphorylethanolamine (IX)	0
β-Stearoyl-DL-α-glycerylphosphorylethanolamine (X)	20–40 *
γ-Stearoyl-DL-α-glycerylphosphorylethanolamine (XI)	0
Lauroyl-glycol-phosphorylcholine (XIIa)	44
Lauroyl-glycol-phosphoryl-(*N*-dimethyl)-ethanolamine (XIIb)	61
Lauroyl-glycol-phosphorylethanolamine (XIIc)	29
Lauroyl-glycol-phosphorylserine (XIId)	< 5
α,γ-Distearoyl-β-glycerylphosphorylcholine (XIII)	95

* No accurate data available.

activity. The afore-mentioned experiments already demonstrated that a polar headgroup esterified to the phosphoryl group is dispensable in the substrate molecule. In agreement with this observation synthetic intermediates in the synthesis of phosphatidylethanolamine having the amino group protected by a phthaloyl group, *viz.* γ-β-diacyl-L-α-glycerylphosphoryl-2′-oxyethylphthalimide (VI), appeared to be completely hydrolysed into the corresponding lyso derivative (Table II). Hence, in contrast to previous suggestions[19] it can be definitely concluded that a nitrogenous moiety in the substrate is not involved in the interaction with phospholipase A. On the other hand those synthetic analogues having in addition the hydroxyl function of the phosphoryl group protected *e.g.* benzyl γ,β-diacyl-L-α-glycerylphosphoryl-2′-oxyethylphthalimide (VII) turned out to be not attacked by phospholipase A (Table II). This finding strongly suggests the ionizable hydroxyl function of the substrates to play part in the interaction with the active site of phospholipase A.

Action of phospholipase A on substrates containing one fatty acid residue

Phospholipase A catalyses the degradation of different types of L-α-phosphoglycerides into the corresponding lyso compound and a free fatty acid by selective hydrolysis of the β-ester linkage. The question can be forwarded whether the presence

of a second apolar constituent, either a fatty acid or an aldehydogenic chain, presents a strict stipulation for substrate activity to this enzyme. Fresh experiments, carried out with various types of synthetic compounds, showed that this condition has not to be fulfilled (Table II).

A DL-α-phosphatidylethanolamine analogue, carrying in γ-position a benzylether group and a fatty acid in β-ester position (VIII), was demonstrated to be susceptible to the action of phospholipase A (Table II); addition of deoxycholate was found to promote this process. Since the investigated compound consisted of a mixture of D- and L-isomers a complete breakdown could not be expected. The structural isomer (IX) was found to resist any action of phospholipase A under similar experimental conditions. Thus, a positional interchange of the ether and ester linkages resulted into an inactivation of the substrate properties. This was found to be true also in the case of two structurally isomeric lyso compounds. β-Acyl-DL-α-glycerylphosphorylethanolamine emulsified together with deoxycholate was found to be (slowly) hydrolysed by phospholipase A under the production of glycerylphosphorylethanolamine. For obvious reasons a complete breakdown of this DL compound could not be achieved. The studied γ-acyl isomer (XI), when incubated with phospholipase A under similar conditions, turned out not to be hydrolysed.

Further support for the view, that only one fatty acid ester linkage located at a certain position is required to permit the action of phospholipase A, was furnished by experiments using glycol analogues (XII). The glycol derivatives, containing choline (XIIa) or *N,N*-dimethylethanolamine (XIIb), were clearly attacked by phospholipase A (Table II). The hydrolysis products, *viz.* glycylphosphorylcholine and glycylphosphoryldimethylethanolamine, have been identified by a paper chromatographical comparison with the products obtained after a weak alcaline saponification of XIIa and XIIb respectively. Furthermore the enzymically liberated fatty acids were analysed by gas–liquid chromatography. Contrary to the afore-mentioned glycol derivatives, analogues composed with ethanolamine (XIIc) and serine (XIId) were water insoluble and rather difficult to disperse. A limited breakdown of XIIc was observed, whereas the serine derivative, also in the collidine-buffered system, was hardly attacked. Nevertheless, the combined results allow to conclude that in principle a hydrolysis of the available fatty acid ester bond of glycol-phospholipids is catalysed by phospholipase A.

It is noteworthy, that the compounds carrying only one fatty acid constituent, and which were susceptible to phospholipase A action, generally were found to be hydrolysed at a rather slow rate.

Action of phospholipase A on β-lecithin

Previous reports[19,27] suggested that a symmetric β-lecithin, having the phosphorylcholine moiety attached to the 2-position of glycerol, is not attacked by phospholipase A. This statement now was doubted, since related compounds carrying a fatty acid esterified at a position adjacent to the alcohol–phosphoryl linkage turned out to be susceptible to the hydrolytic action of this enzyme. Inasmuch as in the earlier studies carried out in both laboratories the distearoyl-β-lecithin was studied, this negative result probably might be attributed to the fact that inadequate emulsions have been used. Indeed (α,γ-distearoyl)-β-lecithin, when emulsified in a borate buffer by sonic vibration, was found to be hydrolysed completely by an

appropriate amount of snake venom (4 mg of venom to 10 mg of substrate) after a 20-h incubation (Table II). Only one equivalent of fatty acid was demonstrated to be released with the concomitant formation of stearoyl-β-glycerylphosphorylcholine. Also after prolongation of the incubation time no detectable quantities of glycerylphosphorylcholine were formed, thus indicating that the enzymically formed lyso-β-lecithin did not act as substrate for phospholipase A.

Taking into consideration the prediction of OGSTON[28], an asymmetric breakdown of the symmetric β-lecithin resulting in the formation of an optically active γ-stearoyl-β-glycerylphosphorylcholine had to be expected. After degradation of the β-lecithin by snake-venom phospholipase A the lyso derivative was isolated and shown to exhibit an optical activity, $[\alpha]_{578}^{20} = +1.45$ (*c*, 10 in chloroform – methanol (9:1, v/v)).

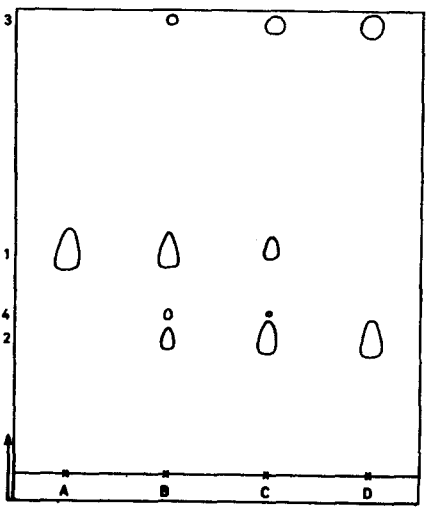


Fig. 4. Thin-layer chromatographic demonstration of the action of snake-venom phospholipase A. Thin-layers were prepared according to the microslide procedure of PEIFER[17] with silica acid (Kieselgel G nach Stahl, Merck). The chromatograms were developed with the solvent system described by MARINETTI *et al.*[18] for 15 min at room temperature. The compounds were detected by heating the dried slides at 150° after spraying with a 10% solution of sulfuric acid. A, Control of cardiolipin; B, Thin-layer chromatogram after incubation of 5 mg of cardiolipin with 2 mg of snake venom in 0.5 ml of borate buffer for 5 h at 37°; C, Like B, but an equal volume of ether was added to the incubation mixture; D, Like C, but incubation was continued for 16 h. 1, cardiolipin; 2, lyso derivative of cardiolipin; 3, liberated fatty acid; 4, unidentified compound.

Cardiolipin and sphingomyelin

Whereas all other compounds investigated in the present study were synthetically prepared, the action of phospholipase A on cardiolipin and sphingomyelin was verified on isolated preparations of these phospholipid classes.

Cardiolipin (XIII) was obtained from the "Rijks Instituut voor de Volksgezondheid" (Utrecht, The Netherlands) and was prepared according to the well-known procedure outlined by Pangborn*. Upon examination with thin-layer chromatography this preparation of cardiolipin revealed only one spot (Fig. 4). Although cardiolipin was attacked by phospholipase A in the aq. borate-buffered system its breakdown proceeded rather slow. However, addition of an equal volume of ether greatly accelerated the hydrolysis process, resulting in a complete breakdown of cardiolipin after a 16-h incubation period (Fig. 4). Quantitative determinations of the fatty acid ester bonds revealed that at the final stage of the enzyme reaction 49% of the originally present fatty acid ester bonds were remaining. This observation was confirmed by a determination of the relative amount of fatty acids liberated by phospholipase A action. For this purpose a weighed amount of margaric acid was added as an

* World Health Organization, Monograph Series No. 6.

internal standard to the incubation mixture. After complete hydrolysis the products were separated on silica-impregnated paper and the proportion of liberated fatty acids to margaric acid present in the spot of fatty acids was determined by gas–liquid chromatography (for details of the techniques compare refs. 3 and 4). Comparison of these results with those of fatty acid analysis performed on intact cardiolipin to which an identical amount of margaric acid was added again demonstrated that 50 % ($\pm$5 %) of the fatty acid constituents was liberated by the action of phospholipase A. Thus, this preliminary investigation strongly suggests that half of the fatty acid ester bonds present in cardiolipin is hydrolysed by this enzyme. Considering the probable structure of this phospholipid (XIII) it will be of interest to investigate whether an intermediate product containing 0.75 equiv of the original fatty acids is primarily produced during the action of phospholipase A. The possibility of this reaction sequence to some extent is supported by the presence of a not yet identified spot on the thin-layer chromatograms (Fig. 4) but certainly more detailed investigations are required.

Sphingomyelin (XV) is known to resist the action of snake-venom phospholipase A. Also in our hands no breakdown of sphingomyelin was obtained under conditions suitable for the hydrolysis of L-α-phosphoglycerides. In the sphingomyelin molecule the fatty acid constituent is bound through an amide linkage. Thus, it may be that phospholipase A, contrary to several other enzymes does not combine esterase and peptidase activity. On the other hand it cannot be beforehand precluded that another factor causes the substrate inactivity of this sphingolipid and fresh investigations using synthetic sphingomyelin and lecithin analogues are required to elucidate this problem.

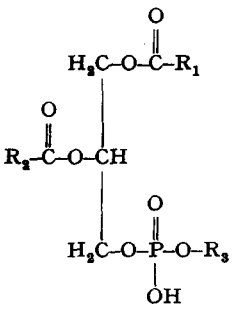
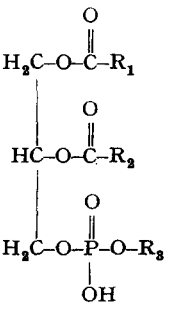
DISCUSSION

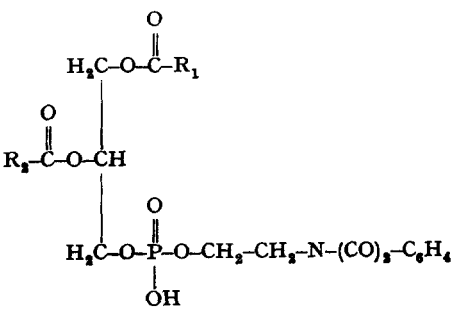
Since the major aspiration of the present study is the elucidation of the requirements in chemical structure of phospholipids to serve as substrates for phospholipase A, the major results obtained have been summarized in Table III. Since D-α-lecithins (Ib), in contrast to the L-α-isomers (Ia), are not susceptible to the action of this enzyme, it appears that a certain steric position of the β-acyl chain of the substrate is essential for the hydrolysis of the corresponding ester linkage. In principle the hydrolysis of L-α-lecithins, containing fatty acids with greatly varying chain length, is catalysed by phospholipase A. However, the rate of hydrolysis of water-soluble homologues *e.g.* L-α-(diacetyl)-lecithin and L-α-(dibutyryl)-lecithin is significantly diminished when compared with long-chain compounds, thus suggesting the apolar acyl chain to play a part in the mechanism of the enzymic reaction. The resemblance in variations of the interfacial properties and the rate of breakdown between various lecithins suggested that the ability of the phospholipid molecule to attain a certain molecular orientation is important for the interaction with phospholipase A. Perhaps an acyl chain is directly concerned in the attachment of the substrate to the enzyme by an interaction with a hydrophobic region of the active center of the enzyme.

Leaving out of consideration the effects of the surface charge of the substrate micelles, it can be stated that phospholipase A does not oblige the substrate to contain a certain headgroup esterified to the phosphoryl moiety. Phospholipids having choline (Ia), ethanolamine (II), serine (III), and glycerol (V) esterified to the diacyl-L-α-glycerophosphoric acid unit, as well as the latter compound (IV) itself are hydrolysed

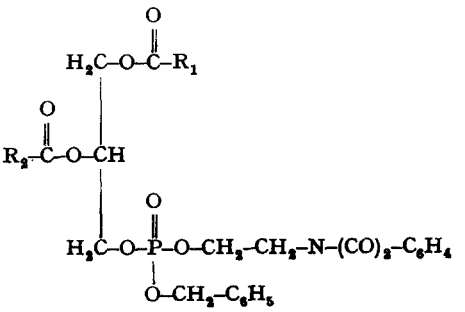
TABLE III

SUBSTRATE SPECIFICITY OF PHOSPHOLIPASE A (*Crotalus adamanteus*)

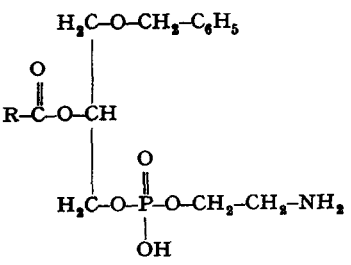
Hydrolysis	*No hydrolysis*
	
Ia $R_3 = CH_2-CH_2-N^+(CH_3)_3OH^-$	Ib $R_3 = CH_2-CH_2-N^+(CH_3)_3OH^-$
II $R_3 = CH_2-CH_2-NH_2$	
III $R_3 = CH_2-CH(NH_2)-COOH$	
IV $R_3 = H$	
V $R_3 = CH_2-CHOH-CH_2OH$	



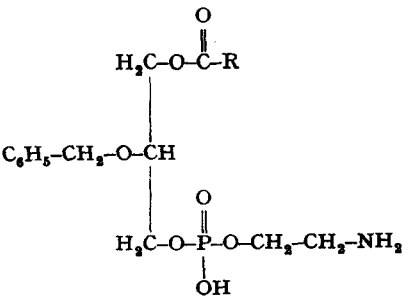
VI



VII

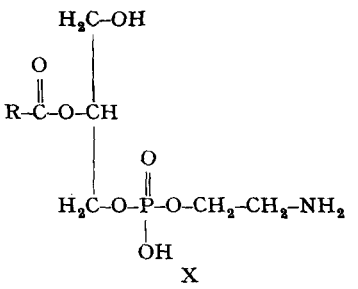
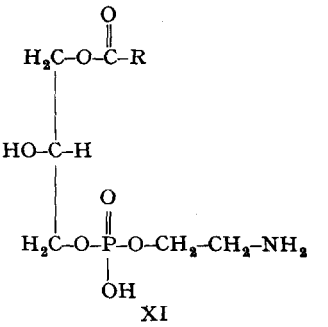
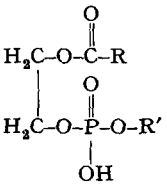
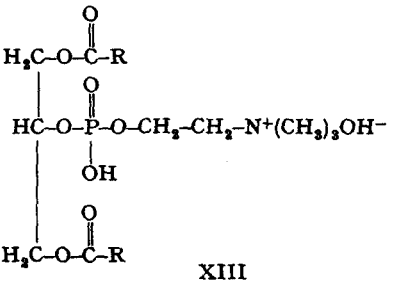
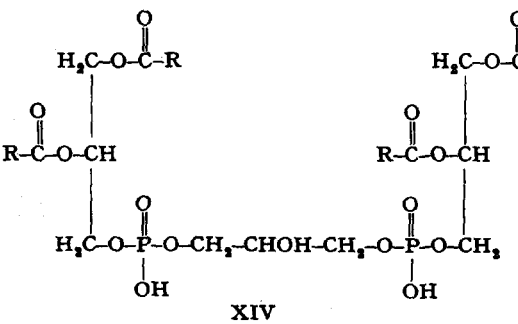
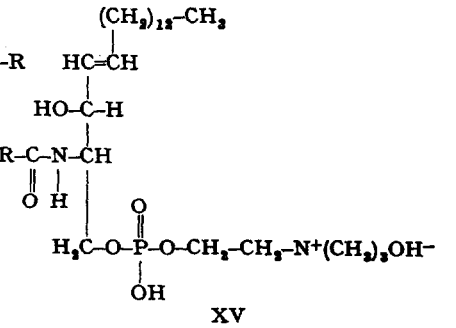


VIII



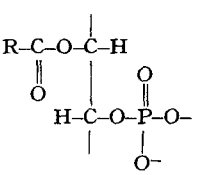
IX

(Continued Table III)

Hydrolysis	*No hydrolysis*
 X	 XI
 XIIa $R' = CH_2-CH_2-N^+(CH_3)_3OH^-$ XIIb $R' = CH_2-CH_2-N(CH_3)_2$ XIIc $R' = CH_2-CH_2-NH_2$ XIId $R' = CH_2-CH(NH_2)-COOH$	
 XIII	
 XIV	 XV

upon incubation with phospholipase A. These results are further supported by the observation that *N*-phthaloyl derivatives of L-α-phosphatidylethanolamine (VI) are subject to phospholipase A hydrolysis as well. However, protection of the free hydroxyl function of the phosphoryl group (VII) caused inactivation of the substrate properties, thus indicating that the presence of this negative group affords an essential condition for the substrate–phospholipase A interaction. It will be of interest to investigate whether the phosphoryl moiety can be superseded by another negatively charged group. Replacement of one fatty acid ester linkage by a benzylether function does not effect the substrate activity, at least when this substitution is made at the γ-position of the glycerol constituent. The apparent degradation of the γ-benzyl-β-acyl compound (VIII) is in accordance with the well established hydrolysis of plasmalogens[29]. However, when the β-fatty acid ester linkage is substituted by a benzylether no enzymic hydrolysis results. The inability of γ-acyl-β-benzyl-DL-α-glycerylphosphorylethanolamine (IX) to serve as substrate indicates the necessity of the fatty acid to be liberated to occupy a position adjacent to the phosphoryl–alcohol linkage. Actually, this observation again conclusively confirms our previous statements that the site of attack of phospholipase A to α-phosphoglycerides is restricted to the β-ester position. These conclusions are further substantiated by the fact that the synthetic β-acyl lyso compound, *viz.* β-stearoyl-DL-α-glycerylphosphorylethanolamine (X), is subject to the action of phospholipase A, whereas the structurally isomeric γ-stearoyl-DL-α-glycerylphosphorylethanolamine (XI) does resist the attack of this enzyme. Taking into account the β-specificity of phospholipase A it is not surprising that the latter synthetic compound does not exhibit substrate activity since a γ-acyl-lysophosphoglyceride has to be the enzymic breakdown product of γ,β-diacyl-L-α-phosphoglycerides.

Endorsing the view that only one fatty acid ester bond located adjacent to the phosphoryl–alcohol linkage is necessary for phospholipase A to develop its action, it was shown that glycol derivatives (XII) are able to act as substrates. Taking into consideration the behaviour of variously modified synthetic compounds the minimum structural requirements to serve as substrates for phospholipase A (*Crotalus adamanteus*) may be illustrated as follows:



When the carbon atom carrying the fatty acid ester linkage is asymmetric, only one antipode appears to possess a steric configuration favourable for interaction with phospholipase A. Furthermore, it is not necessary that the alcoholic carbon atom esterified to the phosphoryl group is a primary one; also a secondary carbon atom can function as is demonstrated by the breakdown of a synthetic β-lecithin (XIII). The asymmetric degradation of this class of compounds, resulting into the formation of an optically active lyso-β-lecithin, which resists further breakdown, reaffirms the steric course of phospholipase A-catalysed reactions.

The observed susceptibility of naturally occurring cardiolipin (XIV) is in keeping with the proposed minimal substrate requirements of phospholipase A, although the

structure ascertained for this phospholipid[30] still requires confirmation by a chemical synthesis. As mentioned above the cause of the substrate inactivity of sphingomyelin (XV) requires further experimental approaches.

An adequate explanation of the steric and positional specific course of phospholipase A-catalysed reactions must accommodate all experimental facts obtained so far on the substrate (in)activity. The observations on the mode of action of this enzyme on the most active substrates, *viz.* the L-α-phosphoglycerides, make it reasonable to identify the specific action of this enzyme with a three-points interaction[28] or its geometric equivalent between this class of substrates and the active site of the enzyme. As a first approximation three groups attached to the asymmetric carbon atom of the L-α-phospholipid molecule have to be chosen, which groups may be involved in the combination with the enzyme. Because of the limited potential of the hydrogen atom attached to the β-carbon atom the three remaining structural elements are presumed to be involved in this process. Tentatively we identify within these elements the following functional groups to play a part in the interaction with phospholipase A.

1. The hydroxyl function of the phosphoryl moiety. To support this choice it may be recalled that blockade of this group caused inactivation of the substrate properties. The negatively charged group might be attracted by a "cationic site" of the enzyme or owing to the requirement of calcium ions it is even more likely that the hydroxyl group is linked via a calcium ion to the enzyme. Recently also DAWSON[31] concluded on account of experiments on the ether activation of phospholipase A action that a phospholipase A–calcium complex may link with anion sites (phosphate) on the surface of lecithin micelles.

As outlined in this paper a nitrogenous function is dispensable for substrate activity, thus ruling out an essential involvement of this group in the process under discussion.

2. The β-fatty acid ester linkage. An interaction of the ester linkage to be hydrolysed with a reactive locus of the enzyme seems most attractive. In current theories on the action of hydrolases *e.g.* acetylcholine esterase it is postulated that an esteratic site, carrying a nucleophilic group, interacts through a covalent bond with the electrophilic carbon of the carbonyl group from the substrate[32]. In another recent theory[33] the carbonyl function of the substrate is supposed to play a key-function also with respect to the final development of the action of hydrolases[34].

3. The oxygen–alkyl function connected to the γ-carbon atom. Inasmuch as L-α-phosphoglyceride analogues, which carry at the γ-position of the glycerol moiety an ether function, are readily attacked by phospholipase A, it appears not to be justified to presume a second carbonyl group to be involved. According to HEIN AND NIEMANN[34] not only point interactions have to be envisaged but the interaction may be extensive and involve large areas of a group. In the present case the apolar chain may play a part in the combination with the enzyme. The impaired breakdown of compounds containing only one acyl chain (at β-position) and of short-chain homologues perhaps is in favour of this speculation. With a view to the observed susceptibility of lysophospholipids and glycol derivatives it can be argued that this third point of interaction has to be replaced by a sterical factor. To facilitate this discussion, however, the most simple model, *viz.* a three-points interaction, will be considered only.

Presuming that the three indicated structural elements of the α-phosphoglyceride molecule combine with three loci of the asymmetric active site of phospholipase A, it is apparent that only one of the antipodes is in a position favourable for interaction, thereby accounting for the observed stereospecificity of the enzyme. Additionally, the asymmetry of the active centre of the enzyme, containing one esteratic site at a distinct location to the site combining with the hydroxyl group of the substrate, does allow the hydrolysis of the β-fatty acid from the L-α-phosphoglyceride molecule only. This model also accounts for the observed susceptibility of the L-isomers of VIII and X and the substrate inactivity of IX and XI as well. The glycol analogues (XII) carry two groups which according to our preliminary and simplified proposals may interact with phospholipase A. The enzymic susceptibility of these derivatives, not having an asymmetric centre, does not interfere with the afforded mechanism.

The observed breakdown of a symmetric β-lecithin (XIII) offers an opportunity to check this model of substrate–phospholipase A interaction, based on the general principles outlined by OGSTON[28]. The β-lecithin contains two fatty acid ester bonds located adjacent to the glyceryl–phosphoryl linkage, thus providing in its molecule twice the minimal structural requirements for phospholipase A established in the present study. Presuming a three-points combination of the β-lecithin with the asymmetric active centre of phospholipase A, according to the proposals made above, only one fatty acid ester bond will be cleaved and an asymmetric lyso-β-lecithin has to result. Actually, in the experiments carried out so far a breakdown of the symmetric β-lecithin into an optically active α-acyl-β-glycerylphosphorylcholine has been observed. The latter hydrolysis product turned out not to be susceptible to the action of phospholipase A. When compared with the glycol analogues (XII) and β-acyl-L-α-glycerylphosphorylethanolamine (X) this lyso-β-lecithin similarly contains two groups which might interact with the enzyme, but the latter compound possesses a defined sterical configuration being quite different from that of both former classes of compounds. It is realized that the requirements of a three-points interaction between substrate and enzyme are met equally well by other geometrical equivalents. With the aid of molecule models it can be envisioned that through its configuration the phospholipase A-produced lyso-β-lecithin may be subject to sterical hindrance when attempting to combine with the active site of the enzyme.

New experimental approaches are stimulated by the presented explanation and further investigations certainly are required to provide a more solid base for the understanding of the mechanism of the interaction of phospholipids with phospholipase A.

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