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HYDROLYSIS OF SYNTHETIC MIXED-ACID PHOSPHATIDES BY PHOSPHOLIPASE A FROM HUMAN PANCREAS

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SUMMARY

An investigation was made into the action of a human pancreatic phospholipase A on various synthetic phosphatides. L-α-Phosphatidyl ethanolamines were readily hydrolysed in an aqueous system by this enzyme. Synthetic lecithins, however, were not attacked in an appreciable rate by the mammalian phospholipase A, contrary to the action of the enzymes from animal poisons. Addition of deoxycholate[1] or synthetic phosphatidic acid greatly enhanced the degradation of synthetic lecithins by the pancreatic phospholipase A.

The site of action of the human pancreatic phospholipase A was determined with the aid of mixed-acid lecithins and phosphatidyl ethanolamines containing in different molecular positions combinations of stearic and lauric acid, stearic and oleic acid, palmitic and linolenic acid, respectively. The enzyme was demonstrated to liberate always the β-esterified fatty acids while the γ-attached fatty acid constituents were recovered in the produced lyso compounds. Hence the mode of action of this phospholipase A from human pancreas is identical to the action of the corresponding phospholipase from animal poisons so far investigated.

INTRODUCTION

Recently MAGEE *et al.*[1] purified phospholipase A (phosphatide acyl-hydrolase, E.C. 3.1.1.4) from human pancreas. It is unknown which fatty acid ester bond of the phospholipid molecule is catalytically hydrolysed by this enzyme. In the last few years phospholipase A from snake venom *(Crotalus adamantheus)* has been demonstrated, contrary to earlier views, to act on the β-ester position only[2-6]. It cannot be precluded that the mode of action of phospholipase A from other biological origins might differ from the snake-venom enzyme by acting on γ-ester or on both γ- and β-ester positions of the phosphoglyceride molecule. For this reason an investigation was made on the site of action of the pancreatic phospholipase A, using various synthetic phospholipid substrates, containing within each molecule two different fatty acid constituents.

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MATERIALS

Phospholipase A

Preparations of phospholipase A, obtained in purified form from post-mortem human pancreas according to the methods described by MAGEE *et al.*[1], were kindly provided by Dr. W. L. MAGEE and Professor R. H. S. THOMPSON. The heat-treated phospholipase A referred to as fraction C in the original paper, was a white solid and a 30-fold purified product.

A comparison was made between the rate of hydrolysis of the synthetic phospholipids by pancreatic phospholipase A and this enzyme present in venom of *Crotalus adamanteus* (L. Light and Co., Colnbrook, England) and bee venom (Bienentrockengift, standardisiert, Fluka A.G., Buchs, Switzerland).

Synthetic mixed-acid phosphatides

A *de novo* synthesis of lecithins containing two different fatty acid constituents was reported by DE HAAS AND VAN DEENEN[7]; preparations of (γ-stearoyl-β-oleoyl)-L-α-lecithin, (γ-oleoyl-β-stearoyl)-L-α-lecithin, (γ-stearoyl-β-lauroyl)-L-α-lecithin and (γ-lauroyl-β-stearoyl)-DL-α-lecithin described in the original paper, were used in the present study. Specimens of kephalins, (γ-stearoyl-β-oleoyl)-L-α-phosphatidyl ethanolamine and (γ-oleoyl-β-stearoyl)-L-α-phosphatidyl ethanolamine were prepared fully synthetically as described by DE HAAS AND VAN DEENEN[7], as well as by an alternative procedure of DAEMEN *et al.*[8]. Two structurally isomeric L-α-phosphatidyl ethanolamines containing one poly-unsaturated fatty acid in different positions *viz.* (γ-linolenoyl-β-palmitoyl)-L-α-phosphatidyl ethanolamine and (γ-palmitoyl-β-linolenoyl) L-α-phosphatidyl ethanolamine were synthesized according to comparable methods[9].

METHODS

Determination of the mode of action of pancreatic phospholipase A

Enzymic hydrolysis: A borate-buffered reaction system (0.10 M, pH 7.0) was used in most of the experiments reported. Final pH adjustments were made with a glass electrode. The synthetic lecithins were emulsified in buffer to a concentration of 20 mg/ml. A solution of sodium deoxycholate in water (50 mg/ml) was added, so that 0.6 ml of the resulting emulsion contained 10 mg of lecithin and 5 mg of sodium deoxycholate. To this emulsion 0.5 ml was added of the enzyme solution (5 mg/ml) in buffer. The incubation was carried out under shaking at 37°. The phosphatidyl ethanolamines were treated in a similar way, omitting, however, the addition of deoxycholate.

The enzymic hydrolysis was controlled at 30-min intervals by making qualitative examinations of the degree of breakdown with the aid of thin-layer chromatography. Small samples of the incubation medium were introduced on microscopic slides which had been covered with a layer of silica (Merck A. G. silica for thin-layer chromatography) by immersing them in an emulsion of silica in chloroform according to the method of PEIFER[10]. The thin-layer chromatograms were developed and stained as described previously for macroslides[11]. This method allowed clear-cut in-

formation to be obtained about the degree of breakdown in a time span of about 15 min. The enzymic hydrolysis was in most cases continued until a complete or maximal breakdown of the substrates could be detected.

At the end of the incubation the complete reaction mixture was subjected to freeze-drying.

Separation of hydrolysis products: The lyophilized material was extracted with 1 ml of pure chloroform; a sample of 0.3 ml was applied in a streak of 3–5 cm to Whatman paper (3 MM) impregnated with silicic acid. The paper chromatograms were developed in a carbon dioxide atmosphere with diisobutylketone–acetic acid–water (40 : 25 : 5, v/v) according to MARINETTI *et al.*[12]. Localization of the spots was carried out by staining parts of the paper chromatograms, serving as references, with rhodamine 6G reagent, the tricomplex staining procedure[13], the reagent of HANES AND ISHERWOOD[14] and in the case of kephalins also with ninhydrin. The corresponding zones on the paper chromatograms consisting of the liberated fatty acids and formed lysophosphatides were cut out and used for analysis of the fatty acids.

Determination of fatty acids: The papers were cut into small pieces and refluxed with 25 ml of a 3% solution of hydrochloric acid in methanol for 2 h in an atmosphere of nitrogen. Subsequently 25 ml of pentane (Merck A.G., reagent grade) were added and the liquid, after separating from the paper, transferred into a separation funnel. The solution was shaken with 25 ml of water and the separated water layer treated with two 25-ml vols. of pentane, which were added to the original organic solvent phase. The combined pentane fractions were again shaken three times with 25 ml of water in order to remove the remaining hydrochloric acid. After drying over anhydrous sodium sulphate, the solution was concentrated under an atmosphere of nitrogen. The samples of fatty acid methyl esters thus obtained have been analysed by gas–liquid chromatography, using a polyethylene glycoladipate column and argon ionization as the method of detection.

The chromatograms of the enzymically freed fatty acid fractions sometimes showed small peaks, corresponding to fatty acids, which were not present in the synthetic phosphatides as such. This difficulty could be overcome by using highly purified solvents in all manipulations involved and by pre-washing the silica papers used in the chromatographic procedure with pure chloroform.

Estimation of the degree of enzymic hydrolysis: Although for the determination of the mode of action of the enzyme the hydrolysis experiments were stopped after a time sufficient for a practically complete breakdown as indicated qualitatively by thin-layer chromatography, additional determinations on the degree of breakdown were made on a larger scale. Samples containing an amount of 50 μg lipid phosphorus were subjected to chromatography on silica-impregnated paper and the amounts of lyso compounds produced and diacyl-phosphatides remaining were estimated, using methods described previously[15]. In a number of experiments pentadecanoic acid or margaric acid were added as internal standards to the enzymic hydrolysates in order to determine the degree of hydrolysis from the gas–liquid chromatograms of the liberated fatty acid fractions. Both latter methods were also employed for the quantitative comparison of the rate of hydrolysis of the synthetic substrates by phospholipase A preparations from different origins.

TABLE I

DEGRADATION OF SYNTHETIC PHOSPHATIDES BY PHOSPHOLIPASE A FROM DIFFERENT ORIGINS

Emulsions of 15 mg of phosphatide together with 1 mg of enzyme preparation in 1.5 ml of borate buffer (pH 7) were incubated 3 h at 37°. Determinations of the degree of hydrolysis were made by a gas-chromatographic estimation of the amount of released fatty acid (see METHODS).

Substrate	Hydrolysis (%) by phospholipase A from		
	Human pancreas	Bee venom	Crotalus adamanteus venom
(γ-Stearoyl-β-lauroyl)-L-α-lecithin	4	80	45
(γ-Oleoyl-β-stearoyl)-L-α-lecithin	10	92	80
(γ-Stearoyl-β-oleoyl)-L-α-phosphatidyl ethanolamine	80	95	85
(γ-Oleoyl-β-stearoyl)-L-α-phosphatidyl ethanolamine	90	99	85

RESULTS

In contrast with experiments performed on phospholipase A from snake and bee venom, synthetic lecithins emulsified in a borate-buffered system were not attacked at an appreciable rate by the human pancreatic phospholipase A (Table I). In keeping with previous observation made by MAGEE *et al.*[1] on ovolecithin, the syn-

TABLE II

DETERMINATION OF THE MODE OF ACTION OF PHOSPHOLIPASE A FROM HUMAN PANCREAS BY HYDROLYSIS OF SYNTHETIC MIXED-ACID PHOSPHATIDES

The lecithins and phosphatidyl ethanolamines were incubated with the enzyme for 6 and 4.5 h, with and without the addition of deoxycholate respectively, under conditions described in the METHODS section. The degree of hydrolysis was measured by determination of the amounts of produced lyso compound and of unhydrolysed substrate. The fatty acid composition of the freed fatty acid fraction and lyso compounds, previously separated by silica paper chromatography, were determined by gas liquid chromatography (compare METHODS section).

Substrates	Degree of hydrolysis (%)	Fatty acid composition of hydrolysis products	
		Liberated fatty acids	Lyso compounds
(γ-Stearoyl-β-oleoyl)-L α-lecithin	96	2.8% stearic acid 97.2% oleic acid	100% stearic acid 0% oleic acid
(γ-Oleoyl-β-stearoyl)-L-α-lecithin	95	100% stearic acid 0% oleic acid	1% stearic acid 99% oleic acid
(γ-Stearoyl-β-lauroyl)-L-α-lecithin	79	0.8% stearic acid 99.2% lauric acid	100% stearic acid 0% lauric acid
(γ-Lauroyl-β-stearoyl)-DL-α-lecithin	48	98% stearic acid 2% lauric acid	0% stearic acid 100% lauric acid
(γ-Stearoyl-β-oleoyl)-L-α-phosphatidyl ethanolamine	100	5% stearic acid 95% oleic acid	99% stearic acid 1% oleic acid
(γ-Oleoyl-β-stearoyl)-L-α-phosphatidyl ethanolamine	100	98% stearic acid 2% oleic acid	0% stearic acid 100% oleic acid
(γ-Palmitoyl-β-linolenoyl)-L-α-phosphatidyl ethanolamine	100	3% palmitic acid 97% linolenic acid	99.5% palmitic acid 0.5% linolenic acid
(γ-Linolenoyl-β-palmitoyl)-L-α-phosphatidyl ethanolamine	100	100% palmitic acid 0% linolenic acid	2% palmitic acid 98% linolenic acid

thetic lecithins were, however, readily hydrolysed by the pancreatic phospholipase A in the presence of deoxycholate (compare Table II). Thin-layer chromatography and quantitative determinations performed by chromatography on silica-impregnated paper showed that the investigated pancreatic preparations catalysed the hydrolysis of the synthetic lecithins into lysolecithins and free fatty acids only. In accordance with earlier reports[4], no detectable lysophospholipase (phospholipase B) activity was exhibited by the enzyme preparations.

The synthetic L-α-phosphatidyl ethanolamines proved highly susceptible to the action of the pancreatic phospholipase A, and in contrast to the lecithins did not require the presence of deoxycholate in order to obtain a ready and complete enzymic breakdown (Table I). With a view to previous results obtained on the activation of phospholipase C (*C. welchii* α-toxin) action towards phosphatidyl ethanolamines by addition of lecithins[11,16], it was considered of interest to investigate whether reversely action of the pancreatic phospholipase A towards lecithins could be enhanced by the presence of phosphatidyl ethanolamines. Only a weak activation appeared to occur, as was shown by paper-chromatographic determination of the decrease of lecithin and the increase of lysolecithin in enzymic hydrolysates containing various propor-

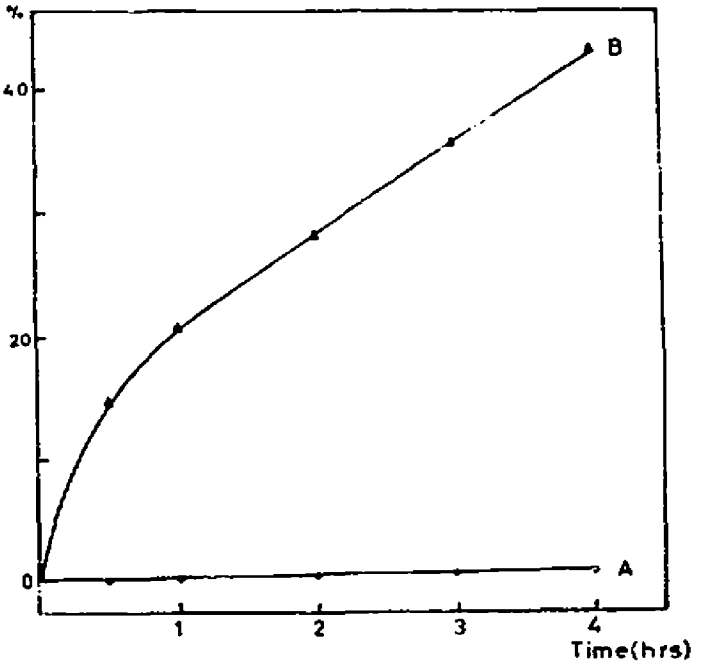


Fig. 1. Effect of the addition of phosphatidic acid on the rate of hydrolysis of a synthetic lecithin by human pancreatic phospholipase A. Ordinate: percentage of hydrolysis of lecithin, as determined by estimation of the disappearance of lecithin and the production of lysolecithin after a paper-chromatographical separation. Abscissa: time of incubation. Lower curve (A): incubation of 5 mg of (γ-oleoyl-β-stearoyl)-L-α-lecithin in 1 ml of borate buffer with 3 mg of human pancreatic phospholipase A. Upper curve (B): incubation of 5 mg of (γ-oleoyl-β-stearoyl)-L-α-lecithin, emulsified together with 5 mg of (γ-oleoyl-β-palmitoyl)-L-α-phosphatidic acid in 1 ml of borate buffer with 3 mg of human pancreatic phospholipase. (In the latter experiments a separation was achieved on silica-impregnated paper, with decreasing R_F value, of phosphatidic acid, lecithin, lysophosphatidic acid and lysolecithin, respectively.)

tions of a synthetic lecithin and a phosphatidyl ethanolamine, emulsified together. However, the more negatively charged phosphatidic acid, *viz.* a synthetic specimen of (γ-oleoyl-β-palmitoyl)-L-α-phosphatidic acid* brought about a notable increase in the degree of hydrolysis of a synthetic lecithin by the pancreatic enzyme (Fig. 1).

Determinations of the site of action of the phospholipase A were made by estimating the liberated fatty acid and the fatty acid constituents of the produced lyso compound by gas–liquid chromatography, after the hydrolysis of the substrate had proceeded as completely as attainable. This was achieved in the case of the lecithins by addition of deoxycholate to the incubation mixture. The results of a typical experiment are reproduced in Fig. 2, showing the paper-chromatographic behaviour of a synthetic lecithin and its hydrolysis products, together with the gas–liquid chromatograms of the fatty acid methylesters obtained from the concerning spots.

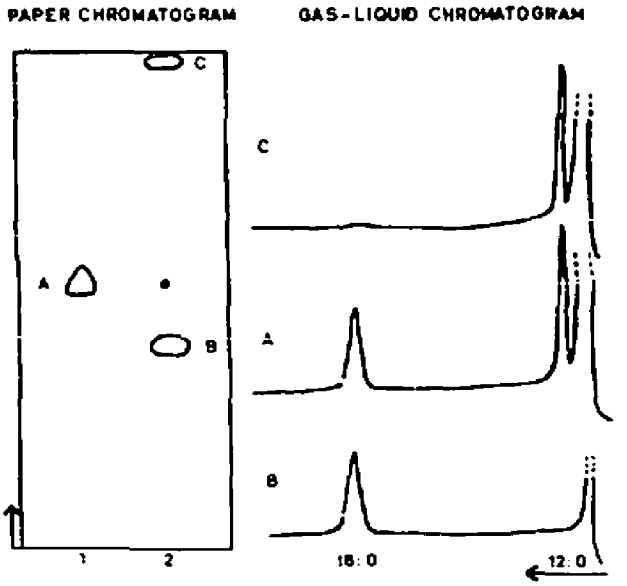


Fig. 2. Demonstration of the mode of action of human pancreatic phospholipase A. On the left silica paper chromatogram of (1) (γ-stearoyl-β-lauroyl)-L-α-lecithin (spot A), and (2) the hydrolysis products obtained after a breakdown of the phosphatide producing the lysolecithin (spot B) and free fatty acid (C). On the right gas–liquid chromatograms of the fatty acid methylesters obtained from the corresponding spots on the paper chromatograms. A, Gas–liquid chromatogram of (γ-stearoyl-β-lauroyl)-L-α-lecithin showing equimolar amounts of stearic acid (18 : 0) and lauric acid (12 : 0); B, Gas–liquid chromatogram of the produced lyso compound demonstrating stearic acid to be the fatty acid constituent; C, Gas–liquid chromatogram indicating the enzymically released fatty acid to be lauric acid.

Apparently, from the compound studied, *viz.* (γ-stearoyl-β-lauroyl)-L-α-lecithin, the human pancreatic phospholipase A liberates the β-esterified fatty acid, while the γ-fatty acid remains within the lysolecithin molecule. This turned out to be true also for the hydrolysis of the other mixed-acid phosphatides, as demonstrated by the results compiled in Table II. Within the class of lecithins, two structural isomers

* Synthesis to be published.

containing oleic acid and stearic acid in reversed position liberated different fatty acids, while the respective lysolecithins consequently turned out to contain also different fatty acid constituents. The results of the breakdown of the synthetic lecithins carrying stearic acid and lauric acid consistently indicated the β-specificity of this enzyme. It has to be noted that the hydrolysis of the DL-compound was less than 50%, even when the incubation period was prolonged up to 24 h. This may be due to a stereospecificity of the pancreatic enzyme for L-α-isomers. Phospholipase A from snake venom was demonstrated not to act on synthetic D-α-lecithins[5].

The results of experiments with mixed-acid phosphatidyl ethanolamines serving as substrates for the pancreatic phospholipase A, confirmed and extended the information on the site of action of this enzyme. The two isomeric L-α-phosphatidyl ethanolamines composed of oleic acid and stearic acid, upon enzymic hydrolysis behaved like the corresponding L-α-lecithins. Furthermore, the positional specificity of the phospholipase A was demonstrated by the breakdown of two isomeric L-α-phosphatidyl ethanolamines esterified with linolenic acid and palmitic acid in different positions. Apparently the enzyme does not exhibit a preference for either a saturated fatty acid, a long-chain fatty acid or a poly-unsaturated fatty-acid constituent (Table II).

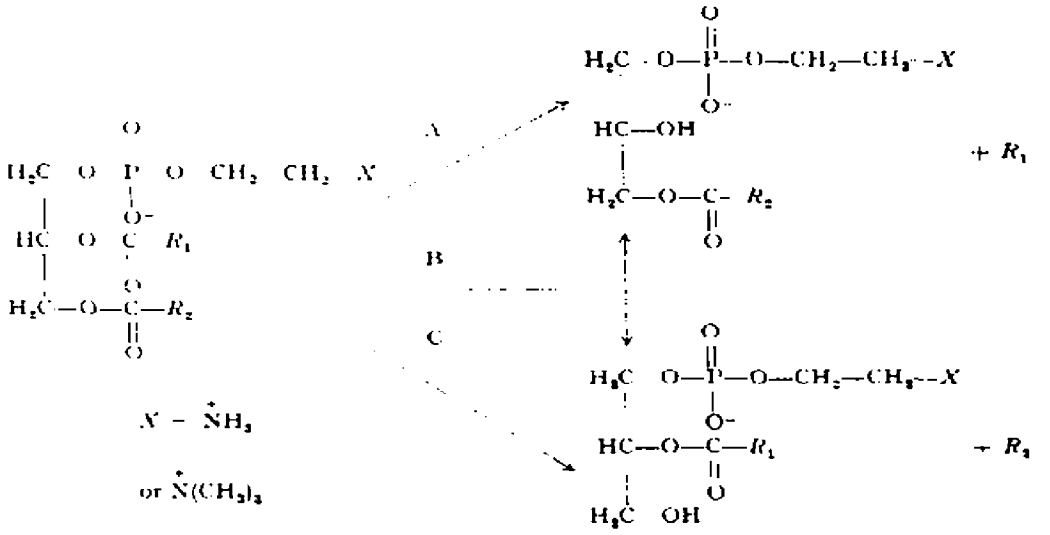
DISCUSSION

In keeping with the observations of MAGEE *et al.*[1] on ovolecithin, the phospholipase A of human pancreas does not attack the synthetic lecithins readily unless the substrate has been emulsified with sodium deoxycholate. Phosphatidic acid to some extent could replace deoxycholate. The synthetic phosphatidyl ethanolamine, when emulsified adequately, did not require the addition of any activator to obtain an optimal breakdown. These observations are in agreement with the reports of RIMON AND SHAPIRO[17] indicating that the rate of reaction by a phospholipase A from ox pancreas was much slower with lecithin than with other substrates *e.g.* phosphatidyl ethanolamine and phosphatidic acid. Probably both corresponding pancreatic enzymes exhibit a maximal activity towards negatively charged substrate micelles. In this respect it is noteworthy that these preparations of pancreatic phospholipase A showed optimal activity at pH 9.0 (MAGEE *et al.*[1]). The present experiments were carried out, however, in a medium pH 7.0 in order to prevent non-enzymic hydrolysis which could lead to erroneous assumptions regarding the site of attack of the enzyme. As regards the activating effect of deoxycholate on lecithin hydrolysis, various possibilities can be envisaged. As pointed out already by MAGEE *et al.*[1], the increase in activity produced with sodium deoxycholate rather than ether, suggests that the deoxycholate itself is capable of some solubilization of the enzyme, but the effect may also be due to solubilization of the substrate molecules. Actually, addition of sodium deoxycholate does clarify the emulsions of synthetic lecithins considerably. On the other hand the synthetic phosphatidyl ethanolamines, being more difficult to emulsify than the corresponding lecithins, were hydrolysed at a faster rate than the lecithins, even in the absence of deoxycholate. This finding indicates that the activating effect of deoxycholate may perhaps be explained on the basis of the work of DAWSON AND BANGHAM[18-21] on the activation of certain phospholipases. These authors showed that various enzymes of this class require the phosphatide micelles to have a certain minimum net zeta-potential before hydrolysis commenced; they

demonstrated that certain activators can bring about the required charge of the substrate micelles. Bearing in mind the observations that the human pancreatic phospholipase exhibits an optimal activity at pH 9.0 with ovolecithin as substrate, and that the more negatively charged phosphatidyl ethanolamines are hydrolysed at a faster rate than the lecithins, the necessity of adding deoxycholate for obtaining an optimal hydrolysis of the latter phosphatides may perhaps be due to a reduction of the charge of the lecithin micelles.

The tentative conclusion that the human pancreatic phospholipase A does require a net minimum negative charge of the substrate micelles before hydrolysis starts, is supported by the observation that addition of a phosphatidic acid accelerates the enzymic breakdown of lecithin. Inasmuch as the synthetic phosphatidic acid itself was rather difficult to emulsify, this effect is not to be attributed to an enhanced solubilization of the lecithin substrate.

As regards the mode of action of the phospholipase A from human pancreas, the nature of the liberated fatty acids and of the fatty acid constituents of the lyso compounds obtained by enzymic hydrolysis of the mixed-acid phosphatides demonstrated that the enzyme preferentially releases the fatty acid esterified onto the β-position of the phosphoglyceride molecule. The results described above have been independently confirmed by THOMPSON *et al.*[22]. Envisaging the various possibilities summarized in Reaction scheme 1, the results obtained allow the conclusion that this mammalian phospholipase A, like the snake- or bee-venom phospholipase A, acts according to pathway A.



Reaction scheme 1

Considering in detail the results compiled in Table II, it appears that to a very small extent the hydrolysis of the γ- or α'-fatty acid (pathway C) was also effected during the experiments. It has to be noticed that this small effect was encountered with various structural isomers, thus ruling out any preference for a certain fatty acid (pathway B). Since the investigated mixed-acid phosphatides included com-

pounds having in different molecular positions combinations of stearic and lauric acid, stearic and oleic acid, palmitic and linolenic acid, the hydrolysis results make clear that the action of the enzyme is not directed by chain length or degree of unsaturation of the fatty acid constituents. Inasmuch as the amounts of liberated γ-fatty acids are very limited, it is not believed likely that the studied enzymic preparation contained an active mono fatty acid releasing phospholipase (pathway C), having a mode of action differing from the major abundant enzyme. Small imperfections in the elaborate synthesis *de novo* of the mixed-acid phospholipids, together with inaccuracies of the analytical methods involved, probably may account for the greater part of the deviations noted. Furthermore, the results of experiments with the pancreatic phospholipase A, which shows only slight activity in a pure ethereal medium[1], may be influenced to a small degree by a non-enzymic hydrolysis occurring in an aqueous system.

The results leading to the conclusion that pancreatic phospholipase A is β-specific—although done on other types of phosphatides—are conflicting with the observations of RIMON AND SHAPIRO[17], who reported that the bovine pancreatic phospholipase A converts a phosphatidic acid into a β-acyl-α-glycerolphosphate. The structure of this hydrolysis product, however, was established by oxidative means, which method is now believed to give erroneous results[3,23]. The mode of action of the pancreatic phospholipase *A*, as determined by RIMON AND SHAPIRO, however, agreed with the opinion accepted at that time with respect to the site of attack of the snake-venom phospholipase A. This holds true for the present study, showing that the mode of action of the mammalian enzyme and that of the enzyme from non-mammalian poisons studied so far, are identical, but contrary to earlier views are catalyzing the hydrolysis of fatty acid present in β-ester linkage. This coincidence, of course, does not preclude the existence of other mono fatty acid releasing phospholipases with a different mode of action.

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