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PURIFICATION AND PROPERTIES OF PHOSPHOLIPASE A FROM PORCINE PANCREAS

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SUMMARY

1 Freshly prepared homogenates of pig pancreatic tissue contain a small amount of phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4), during autolysis, however, a considerable rise in lipolytic activity occurs

2 By use of heat treatment, $(NH_4)_2SO_4$ precipitation and chromatographic procedures, the enzyme has been purified about 200 times and characterized by chemical and enzymatic procedures

3 The protein, which has a molecular weight of about 13 800 ± 500 appears to consist of one single polypeptide chain terminating in alanine (NH_2) and cystine (COOH), and cross-linked intramolecularly by 7 disulphide bridges.

4 The enzyme acts stereospecifically on all common types of 3-*sn*-phosphoglycerides, hydrolysing exclusively fatty acid ester bonds at the glycerol-C-2 position, regardless of chain length or degree of unsaturation In contrast to the snake venom phospholipase A, the pancreatic enzyme shows a marked preference for anionic phospholipids such as phosphatidic acid, cardiolipin and phosphatidyl glycerol

INTRODUCTION

Pancreatic phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) has been the subject of considerable research in the past. Pancreatic tissue of ox[1], man[2], horse[3] and rat[3,4] has been used as enzyme source and occasionally the enzyme has been partially purified by virtue of its remarkable heat stability. The great variety of assay techniques and the various starting materials used (autolysed tissue, pancreatic juices, pancreatin, *etc*) undoubtedly have contributed to some of the conflicting results reported

The first purification study using fresh ox pancreas was undertaken by RIMON AND SHAPIRO[1] who were unable to extract an active enzyme from homogenates of fresh pancreas They showed that activity appeared after storage of the frozen pancreas for a period of at least 2 weeks and that this activity was confined to the insoluble particles of the homogenate MAGEE *et al.*[2], who used fresh *post-mortem* human pancreas, could not detect any differences in phospholipase A activity between fresh and frozen

stored tissue, but they confirmed the water-insoluble nature of the enzyme. Special treatments were required before the enzyme became fully water soluble The latter enzyme preparation was shown[5] to possess a mode of action similar to that of snake venom phospholipase A[6], though considerable differences in reaction rates were found. The high specificity of (phospho)lipases make them invaluable tools, not only for analytical and preparative purposes, but also for inducing selective alterations in biological systems Moreover, the affinity of lipolytic enzymes to lipids might provide us with a potentially useful model system to investigate lipid–protein interactions Such studies require the availability of highly purified enzyme preparations and a thorough knowledge about their substrate requirements and mode of action In the present study, we describe the isolation and purification of phospholipase A from pig pancreas and compare its substrate requirements with those of the corresponding enzyme, present in crude snake venom.

EXPERIMENTAL

Materials

Porcine pancreas was obtained from the municipal slaughterhouse, usually within 30 min after the death of the animal The tissue, after being freed of most of the fat and connective tissue, was stored frozen at −20° DEAE-cellulose (chromedia) and CM-cellulose (Powder CM 70) obtained from Whatman were washed and prepared in columns according to the procedure of PETERSON AND SOBER[7]. Sephadex media (bead form) obtained from Pharmacia, Uppsala, were prepared for use according to the instructions of the manufacturer

Diisopropylfluorophosphate was purchased from Koch-Light, England and obtained through the courtesy of Dr R A OOSTERBAAN, Medisch Biologisch Laboratorium T N O , Rijswijk, The Netherlands 2,4-Dinitrofluorobenzene and sodium deoxycholate were obtained from E Merck, Darmstadt, Germany. Phenylisothiocyanate was purchased from Eastman Kodak *N*-Dinitrophenylamino acids and phenylthiohydantoins of amino acids were products from Sigma, St Louis, Mo , U.S.A Anhydrous hydrazine was prepared from its monohydrate (Schuchardt, Munchen) according to the procedure of BRAUNITZER[8]

Aqueous suspensions of bovine carboxypeptidase A and of porcine carboxypeptidase B were obtained from Sigma, St Louis, U S A and freed from amino acids by washing and dialysis, respectively. Lyophilized bee venom, bovine pancreatic ribonuclease A, pepsin, pancreatic α-amylase, bovine trypsin (3 × cryst from trypsinogen) and bovine α-chymotrypsin (3 × cryst) were products of Fluka (puriss) Highly purified pancreatic lipase was prepared according to the methods of DESNUELLE and co-workers[9] and finally "chromatographed" on Sephadex G-75 to remove possible traces of phospholipase A. Synthetic phospholipids were prepared as described previously[10].

METHODS

Protein concentration was routinely determined by the method of LOWRY *et al.*[11], using as standard crystalline bovine serum albumin.

Phospholipase A activity was determined by potentiometric titration with 0 1 M

standardized NaOH on the TTT-1-Radiometer autotitrator at 40° and pH 8.0 as described first for lipase assay by DESNUELLE, CONSTANTIN AND BALDY[12] As substrate, an aqueous emulsion of egg yolk was used in the presence of sodium deoxycholate and Ca^{2+}

Column chromatography on ion exchangers and Sephadex was performed at 4°. The absorbance of the column eluents was continually monitored and recorded at 254 mμ or 280 mμ with a LKB-8300 A Uvicord II.

Amino acid analysis was performed by the method of SPACKMAN, STEIN AND MOORE[13] on a Technicon amino acid analyzer. Acid hydrolysis was carried out in 6 M HCl *in vacuo* at 110° for periods of 24 h and 48 h. *Norleucine* was incorporated as an internal standard[14]. The tryptophan to tyrosine ratio was determined spectrophotometrically in 0.1 M NaOH, following the procedure of BENCZE AND SCHMID[15] The half-cystine content of the enzyme was also measured by the method of GOA[16]

Amino-terminal analysis The N-terminal amino acid was determined and quantitated by the dinitrophenylation technique[17], and by formation of the phenylthiohydantoin derivative[18,19].

Carboxyl-terminal analysis. The release of carboxyl-terminal residues by bovine carboxypeptidase A or B was measured at pH 7 0–8 0 at 37°. After conversion of the freed amino acids into the corresponding DNP- derivatives, the separated amino acids were quantitated by the method of LEVY[20] Carboxyl-terminal amino acids were also determined by the hydrazinolysis method of AKABORI, OHNO AND SARITA[21] as modified by BRAUNITZER[8] Separation of the hydrazides formed and the carboxyl-terminal amino acids was performed as described by LAY AND POLGLASE[22]

Determination of sulphydryl groups. The SH– content of the protein was determined spectrophotometrically by titration with *p*-chloromercuribenzoate as described by BOYER[23] and BENESCH AND BENESCH[24].

Electrophoresis Starch-gel electrophoresis was carried out on microscopic slides covered with 12% starch in 0.05 M univalent buffer solution of pH 2–9 Samples were subjected to electrophoresis for 2–4 h at 5 V/cm.

Disc electrophoresis was carried out on polyacrylamide as described by PEACOCK, BUNTING AND QUEEN[25] Protein staining was done with a 0.2% solution of Amido Schwarz in 7% acetic acid

Determination of molecular weight The molecular weight of the protein was determined by Sephadex G-75 "chromatography" as described by WHITAKER[26] and by ultrasedimentation.

Sedimentation analysis was carried out in a Spinco model E ultracentrifuge at 20–22° with the Schlieren optical system The samples were sedimented in a synthetic boundary cell at 59 780 rev /min

RESULTS AND DISCUSSION

A. Assay

As substrate, an aqueous emulsion of egg yolk was used, prepared by homogenizing one egg yolk in 100 ml of water. Per assay, 10 ml of the yolk emulsion was diluted to 30 ml, the final concentration of sodium deoxycholate and $CaCl_2$ being $2.7 \cdot 10^{-3}$ M and $6 \cdot 10^{-3}$ M, respectively. Enzymically released fatty acids were automatically titrated at pH 8 0 and 40° Under these assay conditions, the reaction followed es-

sentially zero-order kinetics and a linear relationship was found between the velocity of the enzymic breakdown and the amount of enzyme used The curves obtained usually were linear for at least 3–4 min reaction time. Activity is expressed as the uptake of alkali in μequiv/min. Specific activity is given by the number of μequiv of alkali, consumed per min, per mg of protein. Initially, pure egg lecithin was used as substrate, but this assay system possessed a rather low sensitivity and required too large amounts of purified phospholipid. In accordance with the findings of CONDREA, DE VRIES AND MAGER[27] and DAWSON[28], natural lipoprotein complexes, such as egg yolk emulsions, were found to give 5–10 times higher activity in the assay and large amounts of substrate could be obtained without any purification being required. Moreover, under the experimental conditions of the assay, *i.e.* in the presence of sodium deoxycholate and Ca^{2+}, other pancreatic enzymes such as pure ribonuclease, amylase, trypsin, chymotrypsin, lipase and carboxypeptidases A and B, appeared to be inactive or to possess such a low specific activity toward egg yolk lipoprotein that their contribution in the phospholipase A assay could be neglected Pancreatic cholesterol esterase and lysolecithinase(s), which probably might also use egg yolk lipoproteins as substrate, are known to be inhibited at least partially by sodium deoxycholate[2] and they are also known to be destroyed during the heat treatment[29]

In the above assay system the enzyme has a rather broad pH optimum between 7 9 and 8 4 and no definite temperature optimum has been determined, between 27° and 52° activity increases, but above 45° the characteristics become less linear Increasing the amount of EDTA added to the assay system caused a progressive diminution of enzyme activity, resulting in complete inhibition at an EDTA concen-

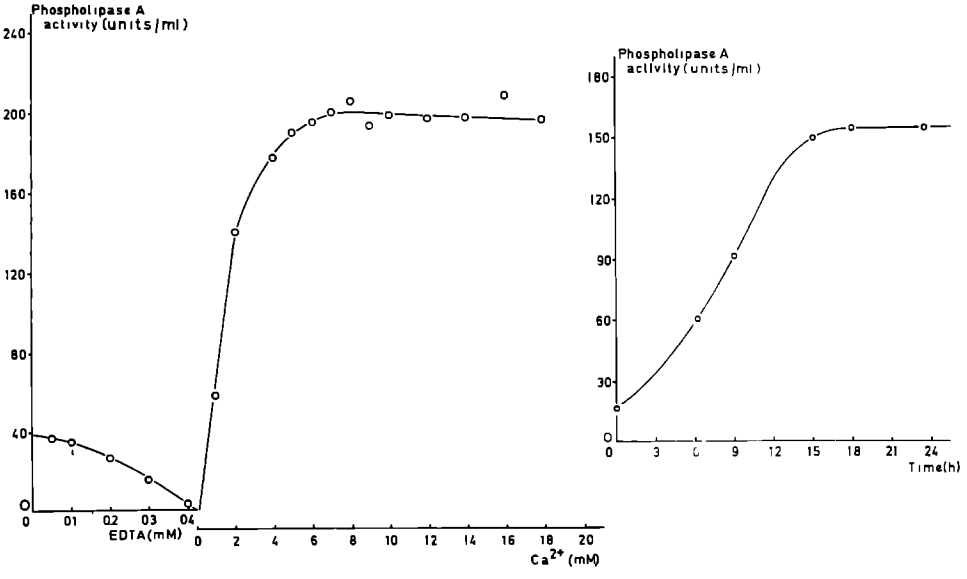


Fig 1 Influence of EDTA and Ca^{2+} upon phospholipase A activity Further details are given in the text

Fig 2 Increase in lipolytic activity of a pancreatic homogenate upon storage at 22°. Further details are given in the text

tration of about 0.4 mM. Subsequent supply of Ca^{2+} fully restored the initial enzyme activity at a Ca^{2+} concentration of $6 \cdot 10^{-3}$ M (see Fig 1) RIMON AND SHAPIRO[1] also found Ca^{2+} to be required for the action of ox pancreatic phospholipase A, whereas MAGEE *et al* [2] reported an inhibitory effect of Ca^{2+} on human pancreatic phospholipase A in assay systems, containing deoxycholate Under our assay conditions, the porcine enzyme appeared to be highly stimulated by the simultaneous presence of Ca^{2+} and deoxycholate These contradictory results on the influence of Ca^{2+} and deoxycholate might be caused by the properties of the different enzymes investigated. However, the differences in the rather complicated assay procedures used are believed to be of greater significance Occasionally, the specificity of the assay procedure was checked by incubation of the enzyme preparation with synthetic mixed-acid lecithins Quantitation by thin-layer chromatography showed that a good correlation exists between the amount of alkali consumed in the assay and the degree of conversion of lecithin into 1-acyl-*sn*-glycero-3-phosphorylcholine

TABLE I

PURIFICATION OF PHOSPHOLIPASE A

500 g of the defatted pancreas were homogenized for 3 min in a MSE-Atomix with 1500 ml of 0 15 M NaCl and stored for 20 h at 22°

Step	*Total activity (10^3 units)*	*Specific activity*	*Yield (%)*
1 Crude homogenate after storage	256	4	100
2 Heat treatment at pH 4 0	205	—	80
3 Removal of precipitated material and floating fat by centrifugation and filtration	175	7	69
4 Dialysis	193	42	75
5 0 40–0 60-Satd $(NH_4)_2SO_4$ precipitate	150	158	59
6 Sephadex G-50 percolation	135	350	53
7 DEAE-cellulose	88	550	34
8 CM-cellulose	79	850	31

B. Purification procedure (see Table I)

500 g of defatted porcine pancreas were homogenized for 3 min in a MSE Atomix with 1500 ml of 0 15 M NaCl and the homogenate was stored at 22–23° for 12–24 h in the presence of some toluene. As shown in Fig 2 enzymic activity rose with time of autolysis and after reaching its constant maximum value, the homogenate was brought to pH 4 0 with conc HCl and heated at 70° for 3 min.

The mixture, after being rapidly cooled to 0°, was freed from insoluble material by centrifugation at 4000 rev /min for 30 min in an IEC International centrifuge, model PR2 at 0° and the floating fat layer was removed by filtration at 4°

The clear yellow solution was dialyzed overnight against running tap water, cleared by centrifugation and subsequently subjected to fractional precipitation with solid $(NH_4)_2SO_4$ at pH 7 0 and 0° After stirring for 1 h, the precipitate at 0 40 satn was removed by centrifugation at 4000 rev./min for 30 min and the supernatant fluid was brought to 0 6 satn of $(NH_4)_2SO_4$. After centrifugation for 1 h at 0°, the precipitate was

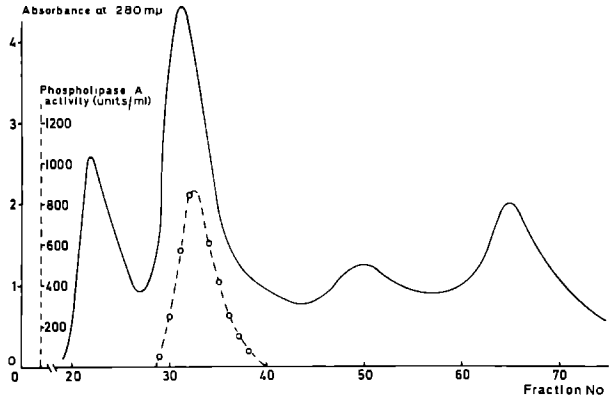


Fig 3 Elution pattern of phospholipase A from a Sephadex G-50 column (70 cm × 3 cm) with 0 75 M NaCl ———, absorbance at 280 mμ, - - - - -, enzyme units/ml

dissolved in the minimal amount of 0 75 M NaCl and the slightly turbid solution was cleared by centrifugation at 20 000 rev /min for 15 min in a Sorvall superspeed centrifuge, model RC 2-B The supernatant fluid was "chromatographed" at 4° over Sephadex G-50 using 0 75 M NaCl as eluent A typical elution pattern is given in Fig 3 The enzymically active fractions were combined and freed of salt by exhaustive dialysis, first against distilled water and subsequently against 0 005 M Tris buffer (pH 8.0) The dialysed solution was cleared by centrifugation, vacuum-concentrated by ultrafiltration and chromatographed on DEAE-cellulose at 4° The DEAE-cellulose was equilibrated against 0 005 M of Tris buffer (pH 8.0) and, after application of the sample to the column, cationic proteins were eluted with the same buffer. After appearance of the breakthrough peak, the remaining proteins were eluted by a linear gradient developed by use of 0 3 M NaCl in 0 005 M Tris buffer (pH 8 0) (see Fig 4) The effluent

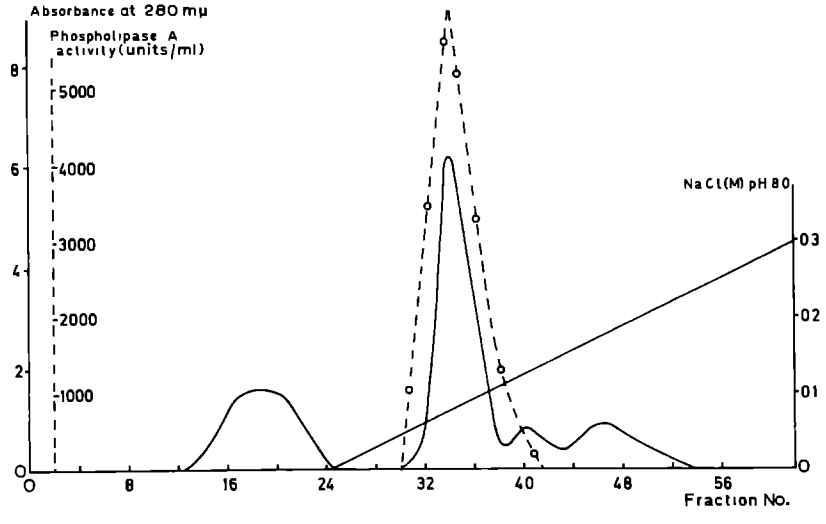


Fig 4 Elution pattern of phospholipase A from a DEAE-cellulose column (50 cm × 2 5 cm) ———, absorbance at 280 mμ, - - - - -, enzyme units/ml

fractions containing phospholipase A were combined, freed of salts by dialysis at 4° against 0 005 M acetate buffer (pH 6.0) and vacuum-concentrated by ultrafiltration Rechromatography was performed on CM-cellulose equilibrated against the same buffer. The column was washed free of unabsorbed protein and a linear NaCl gradient between 0 and 0 4 M was developed in 0.005 M acetate buffer (see Fig. 5). The enzymically active fractions which possessed a constant specific activity were combined, exhaustively dialysed at 4° against distilled water and lyophilized.

The yield at each purification step and the degree of purification is summarized in Table I.

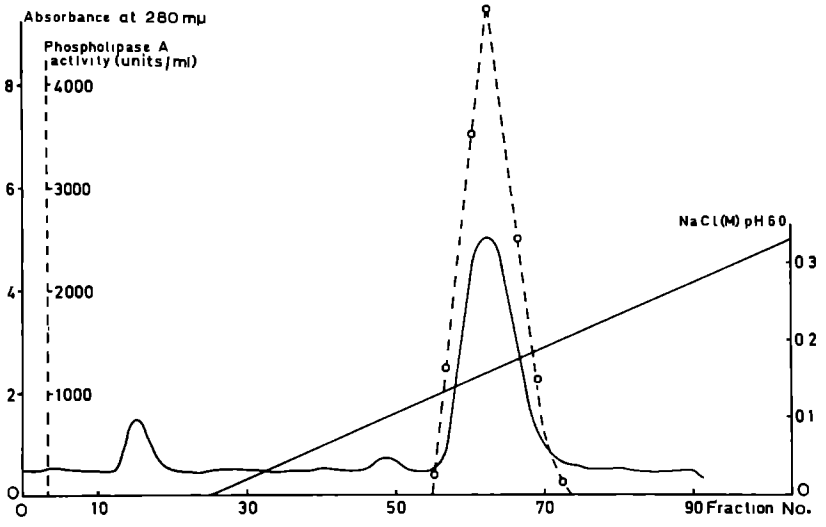


Fig 5 Elution pattern of phospholipase A from a CM-cellulose column (50 cm × 2 5 cm) ———, absorbance at 280 mμ, - - - - -, enzyme units/ml

C Comments on the purification procedure

Autolysis period Homogenates of fresh pig pancreas appeared to exhibit a very low phospholipase A activity. RIMON AND SHAPIRO[1] previously reported that in ox pancreas phospholipase A activity could be demonstrated only after storage of the tissue at —12° for at least 2 weeks Although we were unable to detect differences in phospholipase A content between homogenates of fresh or frozen-stored pig pancreas, upon autolysis of homogenates at room temperature a large increase in activity was found, finally reaching a constant value (see Fig. 2) This rise in enzyme activity might be caused by autolytic solubilization of the enzyme However, even after prolonged autolysis, it turned out that, upon low-speed centrifugation, most of the enzymic activity was found in the precipitate whereas only 25–30% of the lipolytic activity became solubilized Moreover, the final high level of enzymic activity obtained by autolysis at room temperature could be reached much more quickly at 4° by the addition of a small amount of crystalline trypsin to the fresh homogenates. The nature of this activation is further discussed in the accompanying paper.

Heat treatment Heat stability experiments, carried out for 1–10 min between

pH values of 2 and 8, showed that most of the lipolytic activity can be recovered by heating for 3 min at pH 4.0 and 70°.

Centrifugation and filtration. Denatured proteins and other insoluble material were removed by low-speed centrifugation and the floating fat layer separated by filtration of the supernatant fluid at 0–4° The filtration process has to be carried out at low temperature to avoid big losses of enzyme activity caused by adsorption of the enzyme to dispersed fatty material. It is very important to carry out the centrifugation and filtration at pH 4.0 in order to avoid recombination between the enzyme and the lipids present and to keep the enzyme water soluble

Initially, after the heat treatment at pH 4.0, we brought the pH of the homogenate to 7.0 before centrifugation It was thus found, in accordance with the reports of RIMON AND SHAPIRO[1] and MAGEE *et al*[2], that, upon centrifugation, all enzymic activity was bound to the insoluble material Treatment with sodium deoxycholate was required to solubilize the enzyme If this solution, after fractional $(NH_4)_2SO_4$ precipitation, was put on a Sephadex G-200 column, it appeared that the enzyme was eluted in two peaks, one at the void volume of the column, indicating a very high molecular weight, whereas the other enzymically active peak was considerably retarded A comparable behaviour was previously reported by SARDA *et al*[30] for pancreatic lipase and these authors formulated the hypothesis that the so-called "rapid lipase" fraction, which is eluted at the void volume of Sephadex G-200, consisted of lipase associated with endogenous lipids Apparently, the low molecular weight protein (mol wt of pancreatic lipase is about 35 000) is strongly adsorbed to lipid and is eluted from Sephadex G-200 in a position corresponding to the particle size of the lipid aggregates In accordance with these facts, we found, upon analysis of the lipids present in our "rapid phospholipase A" fraction, considerable amounts of monoacyl phosphoglycerides, free fatty acids and some cholesterol esters As could be expected, repeated *n*-butanol extractions of "rapid phospholipase A", according to the procedure of MORTON[31], shifted the elution volume of the enzyme to that of the slow-moving phospholipase A, whereas addition of synthetic 1-acyl-*sn*-glycero-3-phosphorylcholine to slow phospholipase A fractions restored the elution volume of the enzyme to the breakthrough volume found for "rapid phospholipase A" Although the conversion of "slow phospholipase A" into the rapid form by addition of synthetic lysolecithin is accompanied by a 6-fold rise in specific activity of the enzyme, indicating a rather specific adsorption of phospholipase A to the lipid micelles, this procedure was not further investigated as a possible route of purification. The final purification of the enzyme on ion-exchange columns required lipid-free proteins which makes *n*-butanol extraction of the lipids unavoidable. In contrast to the formation of "rapid phospholipase A" from the slow form *plus* lysolecithin, which proceeds in good yield, the splitting of the complex could be effected only by repeated *n*-butanol treatment finally giving a low yield of lipid-free enzyme. Preference was therefore given to the experimental conditions of low pH, described above when apparently no reformation of "rapid phospholipase A" occurs.

Dialysis. As may be clear from the specific activity shown in Table I, the dialysis procedure is very effective in removing low molecular weight peptides probably originating from the autolysis period. The rise in total activity regularly encountered after dialysis can not easily be explained

Sephadex chromatography. Although chromatography of the 0.40–0.60 satd

$(NH_4)_2SO_4$ fraction on Sephadex G-50 gave a 2-fold purification of the enzyme in a high yield, this step, especially in larger-scale preparations, had to be omitted because of the rather low capacity of Sephadex columns. Usually in these cases DEAE-cellulose chromatography had to be carried out twice to get an end-product of the same purity.

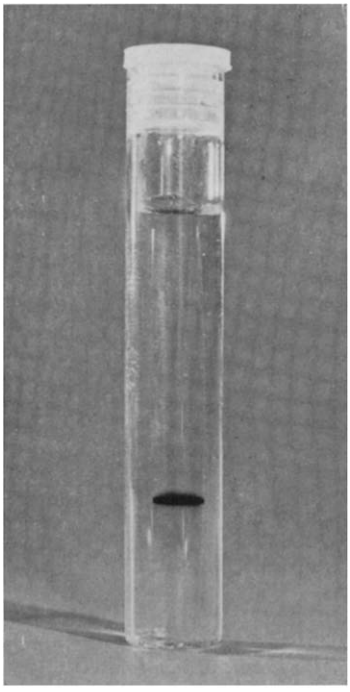


Fig 6 Disc-gel electrophoresis of purified phospholipase A The protein was subjected to polyacrylamide electrophoresis in 0 38 M Tris buffer (pH 8 15) under the standard conditions described in detail by ORNSTEIN AND DAVIES[32] (80–100 V/cm at 20° for 2 h, 5 mA/tube)

D Properties of phospholipase A

Electrophoretic analysis Electrophoresis was carried out in starch gel as well as by the disc technique on polyacrylamide[25,32] Using buffer systems of various pH values (pH 4.5–9) the purified protein revealed only one band, (see Fig 6). A plot of moving distance against pH under standardized conditions of voltage, ionic strength, time and current, showed the isoelectric point of the protein to be 7 4.

Amino acid composition The amino acid composition of the protein is given in Table II Half-cystine was also determined by the method of GOA[16] (14 residues per molecule of phospholipase A, assuming a mol wt of 14 000). A most striking feature of the amino acid composition appears to be the relatively high amount of aspartic acid (*plus* asparagine) and the very high content of half-cystine *p*-Chloromercuribenzoate titrations at pH 4.6 and 7 0, even in the presence of 8 M urea or 5 M guanidine · HCl, showed the absence of free SH– groups It may be concluded, therefore, that the phospholipase A molecule is cross-linked by 7 disulphide bonds and devoid of cysteine sulphydryl groups.

TABLE II

AMINO ACID COMPOSITION OF PHOSPHOLIPASE A

The results given are the average recoveries of two analyses after hydrolysis of phospholipase A for 24 and 48 h

Amino acid	*g residue per 100 g protein*	*Nearest integer per 14 000 g*	*Nearest integer × min mol wt*	*Integral number × mol wt of residue*
Asp*	19 08	23	13 869	2647 024
Thr	4 57	7	15 470	707 728
Ser	6 52	10	13 300	870 780
Glu*	6 62	7	13 650	903 798
Pro	4 46	6	15 486	582 684
Gly	2 46	6	13 920	342.312
Ala	4 05	8	14 000	568 624
Val	1 55	2	13 300	198 260
Cys	9 52	14	14 980	1444 016
Met	1 76	2	14 900	262 392
Ile	3 92	5	14 400	565 780
Leu	5 76	7	13 720	792 092
Tyr	8 70	8	14 960	1305 360
Phe	5 27	5	13 950	730 810
Lys	8 04	9	13 860	1153 548
His	3 13	3	13 140	411 420
Arg	4 58	4	13 640	624 752
Trp**	—	2	—	372 412
Total		128	14 150	14483 792

* As no amide content has been determined these values represent the sum of Asp + Asn and Glu + Gln

** Determined spectrophotometrically according to the method of BENCZE AND SCHMID[15]

Molecular weight. The molecular weight of the protein was calculated from (a) sedimentation analysis, (b) Sephadex filtration[26] and (c) the amino acid composition.

(a) The sedimentation pattern observed in the ultracentrifugation at the various concentrations analysed showed only one symmetrical peak The extrapolated value at zero concentration for $s_{20,w}$ was 2 17 S and the extrapolated value for the diffusion constant, $D_{20,w}$, was $1\ 36 \cdot 10^{-6}$ cm^2·sec. The partial specific volume ($\bar{v}$) calculated[33] from the known amino acid composition was 0 71 cm$^3 \cdot$g^{-1} and the calculated molecular weight is therefore 13 500 ± 5%

(b) Fig 7 gives the calibration graph of the elution volume of standard proteins against the logarithm of their molecular weight from a Sephadex G-75 column. The elution volume of phospholipase A ($v/v_0 \times 100 = 244 \pm 3$) indicates a molecular weight of 13 900 ± 450.

(c) The molecular weight of phospholipase A, calculated from the amino acid composition, is 14 150 (Table II).

Terminal amino acids Pancreatic phospholipase A was found to possess only one amino-terminal amino acid, alanine. Quantitation of the amino acid by the dinitrophenylation technique[17] and by the Edman degradation[18,19] yielded 0 85 mole and

TABLE III

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

Substrate	*Degree of hydrolysis (%)*	*Fatty acid composition of hydrolysis products**	
		Liberated fatty acids	*Lyso compound*
2-Stearoyl-3-oleoyl-*sn*-glycero-1-phosphorylcholine	0		
rac-1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine	50	97% oleic acid 3% palmitic acid	100% palmitic acid 0% oleic acid
1-Oleoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine	100	100% stearic acid 0% oleic acid	96% oleic acid 4% stearic acid
1-Oleoyl-2-butyryl-*sn*-glycero-3-phosphorylcholine	100	100% butyric acid 0% oleic acid	100% oleic acid 0% butyric acid
1-Butyryl-2-oleoyl-*sn*-glycero-3-phosphorylcholine	100	97% oleic acid 3% butyric acid	97% butyric acid 3% oleic acid
1-Oleoyl-2-isolauroyl-*sn*-glycero-3-phosphoryl ethanolamine	100	100% isolauric acid 0% oleic acid	100% oleic acid 0% isolauric acid
1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoryl ethanolamine	100	100% oleic acid 0% palmitic acid	100% palmitic acid 0% oleic acid
1-Palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphoryl ethanolamine	100	97% linoleic acid 3% palmitic acid	100% palmitic acid 0% linoleic acid
1-Oleoyl-2-isolauryl-*sn*-glycero-3-phosphoryl-*rac*-1′-*sn*-glycerol	100	95% isolauric acid 5% oleic acid	98% oleic acid 2% isolauric acid

* The fatty acid composition of the free fatty acid fraction and lyso compound, previously separated by thin-layer chromatography, were determined by gas–liquid chromatography

0.35 mole of alanine, respectively, for 1 mole of phospholipase A Efforts to split off the carboxyl-terminal amino acid of the protein with DFP-treated carboxypeptidases A and B were unsuccessful even in the presence of denaturing agents like urea or guanidine·HCl or after performic acid oxidation of the protein However, hydrazinolysis of the oxidized protein according to the method of BRAUNITZER[8] revealed the presence of cysteic acid, indicating that cystine or cysteine occupies the C-terminal position As the enzyme appeared to be devoid of cysteine sulphydryl groups (see the section on amino acid composition), we tentatively conclude that cystine is the C-terminal amino acid of phospholipase A

Heat stability The low molecular weight and high degree of linkage by disulphide bonds are in accordance with the spherical shape of the molecule as determined in the ultracentrifuge, and undoubtedly contribute to the very high stability of the enzyme against heat and denaturing agents such as urea and guanidine·HCl. The enzyme resists boiling for 5 min at pH 4.0 and even after treatment for 1 h at 98° about 45% of the initial activity is still present. Upon storage of the enzyme in the presence of 8 M urea at 20° virtually no decrease in activity was observed after 22 h.

Inhibition of the enzyme A number of common metal ions were investigated for their inhibitory effect on phospholipase A activity As shown in Fig 8, relatively strong inhibitions were found only for Zn^{2+}, Cd^{2+} and Pb^{2+}. Under these conditions Cu^{2+}, Ag^{+}, Ba^{2+}, Hg^{2+}, F^{-} and CN^{-} at 10^{-3} M give less than 20% inhibition of enzyme activity. EDTA strongly inhibits through its ability to complex with Ca^{2+} (see Fig. 1)

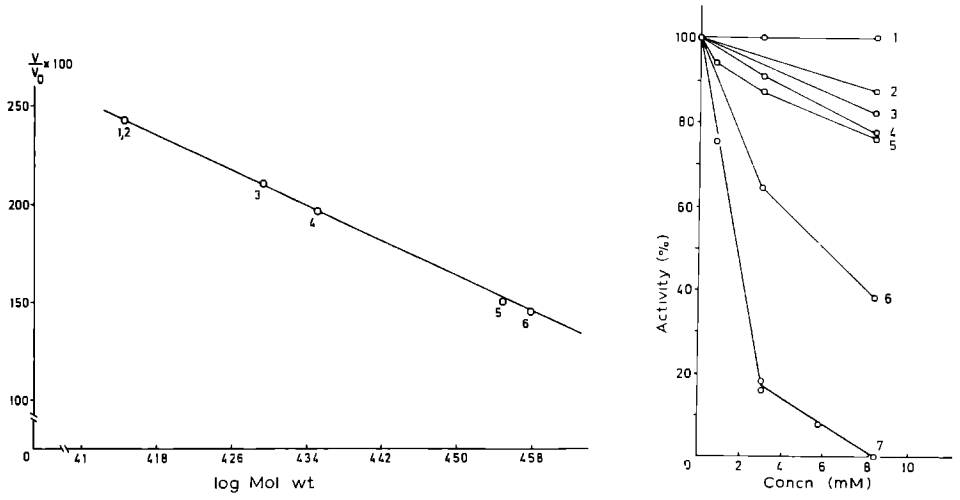


Fig 7 Diagram showing the relationship between the logarithm of the molecular weight and elution volume (V), through a Sephadex G-75 column (75 cm × 3 cm) with 0 75 M NaCl The dead volume (V_0) was determined with Dextran blue 2000 (1) Ribonuclease A, mol wt 13 700, (2) pancreatic phospholipase A, (3) bee venom phospholipase A, mol wt 19 700 (ref 42), (4) α-chymotrypsin, mol wt 22 500, (5) pepsin, mol wt 35 500, (6) porcine pancreatic lipase, mol wt 38 000 (ref 30)

Fig 8 Inhibition of phospholipase A activity by various ions Curve 1, F^-, CN^-, Fe^{2+}, Mg^{2+}, Cu^{2+}, Ag^+; Curve 2, Ba^{2+}, Curve 3, Hg^{2+}, Curve 4, UO_2^+, Curve 5, Mn^{2+}, Curve 6, Co^{2+}, Curve 7, Cd^{2+}, Zn^{2+}, Pb^{2+}

So far we have not been able to find experimental conditions under which diethyl-*p*-nitrophenyl phosphate (E 600) or DFP inhibits the enzyme

The enzyme exhibits a typical protein spectrum with a maximal absorbance at 280 mμ, a minimal absorbance at 253 mμ and a ratio of absorbances $A_{280\ m\mu}/A_{260\ m\mu} = 1.72$, ($A_{280\ m\mu}/A_{253\ m\mu} = 1.97$).

Substrate requirements and specificity of the enzyme With the exception of phosphatidyl inositol which was isolated from yeast[34], all other phospholipids investigated were obtained by *de novo* syntheses already reported[10,35–40]

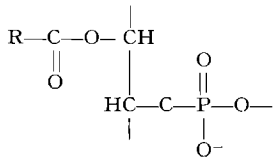
Table III gives the results of enzymatic hydrolysis of several synthetic phosphatides containing different fatty acids The incubation system contained 10 mg of phospholipid and 3 mg of sodium deoxycholate dispersed in 1 ml of 0 1 M borate buffer (pH 7 2) ($[Ca^{2+}] = 5\ 10^{-3}$ M) with the aid of a few drops of ether. The purified enzyme (specific activity about 800) was added in amounts of 50 μg and incubations were carried out at 25° for 0 5–2 h. The degree of breakdown was followed by thin-layer chromatography

Apparently, the porcine pancreatic phospholipase A liberates from 3-*sn*-phosphatidyl choline, 3-*sn*-phosphatidyl ethanolamine and 3-*sn*-phosphatidyl glycerol the fatty acid esterified at the 2-position, while the 1-bound fatty acid remains within the corresponding lysocompound. No preference of the enzyme for fatty acid chain length and saturation or unsaturation could be detected The enzyme acts highly stereospecifically The *sn*-glycero-1-phosphoryl isomers are not hydrolysed at all and

racemic substrates are broken down 50%. In this respect the porcine enzyme behaves very similarly to phospholipase A from snake venom[38]. Large differences between both enzymes were found, however, by studying the velocity of breakdown of various substrates. Whereas the snake venom phospholipase A slowly attacks very acidic phospholipids and becomes more active only upon addition of positively charged amphiphatic molecules[39], the reverse situation is found for the pancreatic enzymes. In accordance with our previous reports on the substrate requirements of phospholipase A from human pancreas[5], the porcine enzyme also attacks the neutral lecithin molecule rather slowly Addition of deoxycholate to the lecithin dispersion, which presumably makes the substrate more negatively charged, greatly enhanced the activity of the enzymes In support of this idea is the high susceptibility towards the enzyme of anionic phospholipids such as phosphatidic acid and its monomethyl ester, phosphatidyl glycerol, cardiolipin and, to a lesser degree, also phosphatidyl serine and phosphatidyl ethanolamine With these substrates, addition of deoxycholate though improving the quality of the dispersion, had either no stimulatory effect (phosphatidyl ethanolamine) or even inhibited the breakdown (phosphatidic acid, cardiolipin). It has to be kept in mind, however, that deoxycholate is a rather specific activator. It can not be replaced by other negatively charged detergents such as sodium dodecyl sulphate or sodium taurocholate and its stimulating effect on lecithin hydrolysis even depends on the type of lecithin used Although we found that all long-chain lecithins investigated so far are hydrolysed much more quickly in the presence of deoxycholate, it turned out that this detergent slightly inhibits the hydrolysis by pancreatic phospholipase A of 2-acyl-lysolecithins and lecithins containing butyric acid and oleic acid

Under incubation conditions which allow a complete hydrolysis of anionic phospholipids such as phosphatidic acid, phosphatidyl glycerol and cardiolipin (5 mg of phosphatide in 1 ml of 0 1 M Calcium borate buffer (pH 7.1) was incubated at 37° for 60 min with 2 μg of purified phospholipase A), no hydrolysis was observed with the following compounds: 2-acyl lysolecithin, glycol analogues of lecithin and phosphatidyl ethanolamine and lecithins belonging to the β-series in which the phosphoryl nitrogenous moiety is attached at the secondary glycerol –OH group. In principle, these lipids can be hydrolysed by pancreatic phospholipase A by using prolonged incubation periods and 10–100 times more enzyme These hydrolyses are still incomplete, however, and progress very slowly. Sphingomyelin fully resisted enzymic breakdown In accordance with the findings for snake venom phospholipase A, the pancreatic enzyme, too, requires for its activity at least one free phosphate ionisation. Blocking of this final phosphate –OH group, *e g.* in phosphorus triesters, makes the compound unacceptable as a substrate for the enzyme.

Summarizing these results, we may conclude that the mammalian enzyme and the phospholipase A from snake venom both require the same minimum structural elements to exert their action, that is, a fatty acid ester bond in a position adjacent to the alcohol–phosphate ester bond:



However, in contrast to the snake venom enzyme, porcine pancreatic phospholipase A preferentially attacks anionic phospholipids. This finding confirms earlier reports of RIMON AND SHAPIRO[1] and MAGEE *et al* [2], who investigate the enzyme derived from ox and human pancreas, respectively The phospholipase A from snake venom (*Crotalus adamanteus*) has been purified by SAITO AND HANAHAN[41]. Two proteins with enzymatic activity were obtained which were chromatographically separable and differed in their electrophoretic mobility and isoelectric points The proteins which exhibited a similar positional specificity—identical to that of the pancreatic phospholipase described here—had similar sedimentation constants The molecular weight was estimated to be in the range of 30 000–35 000, thus being at least twice that of the pancreatic enzyme

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