Phospholipase B activity of a purified phospholipase A from *Vipera palestinae* venom

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Abstract Phospholipase was isolated (in two fractions) from *Vipera palestinae* venom and it was shown to possess phospholipase A activity (hydrolyzing diacyl-*sn*-glycerophosphorylcholines, e.g., lecithin, in the 2-position) as well as lysophospholipase (phospholipase B) activity (hydrolyzing 1-monoacyl-*sn*-glycerophosphorylcholines, e.g., lysolecithin, yielding free fatty acid and glycerophosphorylcholine). Each of the two purified enzyme fractions was homogeneous as judged by electrophoresis on acrylamide gel and by immunodiffusion and immunoelectrophoresis, and both had essentially equal activities. The ratio of the specific activity, at various purification stages, to the specific activity of the whole venom was the same for A activity (substrate lecithin) as for B activity (substrate lysolecithin). The enzyme has a molecular weight of 16,000, six S-S bridges, and no free thiol groups. At pH 7, dimerization was observed in the ultracentrifuge. A dissociation constant of about 10^{-5} M was estimated. The amino acid composition for both fractions (140 amino acid residues) was found to be essentially the same. The A activity had a pH optimum at 9; B activity was low at this pH but increased steadily beyond pH 10.5. For the hydrolysis of lysolecithin the Lineweaver-Burk plot was found to be linear, giving $K_m = 1.1$ mM and $k_{cat} = 0.55$ sec^{-1} at 37°C and pH 10. 2-Deoxylysolecithin was also hydrolyzed by the enzyme at pH 10, with $k_{cat} = 0.01$ sec^{-1} (zero-order kinetics in the range 0.5–2.5 mM). For lecithin these constants could not be determined, but at 0.25 mM substrate the hydrolysis rate (at pH 9) of lecithin was about 1000 times the hydrolysis rate of lysolecithin (at pH 10).

Supplementary key words lecithin · lysolecithin · 2-deoxylysolecithin · lysophospholipase · micellar structure

PHOSPHOLIPASES, the enzymes that release fatty acids from glycerophosphatides, are classified into two kinds, A and B, according to whether they act on diacyl or monoacyl substrates, respectively.

Phospholipases of type A (EC 3.1.1.4) catalyze the hydrolysis of one ester bond in 1,2-diacyl-*sn*-glycero-3-phosphatide, forming a lyso derivative and releasing a molecule of free fatty acid. These enzymes have positional specificity, those hydrolyzing the 1- (or α-) position being designated A$_1$, and those hydrolyzing the 2- (or β-) position, A$_2$. The snake venom phospholipases of type A investigated so far are believed to be A$_2$ enzymes. Recently, several studies on enzymes from mammalian tissues (1–5) have shown the presence of either A$_1$ or A$_2$ phospholipases or both.

Phospholipases of type B, or lysophospholipases (EC 3.1.1.5), catalyze the hydrolysis of monoacyl phosphatides with the release of free fatty acid and the formation of glycerophosphoryl derivatives. Van den Bosch et al. (6) described a lysophospholipase from rat liver that catalyzes the hydrolysis of both 1-monoacyl-*sn*-glycero-3-phosphatide and 2-monoacyl-*sn*-glycero-3-phosphatide. Another phospholipase B having no positional stereospecific activity was found recently by Rao and Subrahmanyam (7) in the mosquito *Culex pipiens fatigans*. Phospholipase B activity of snake, bee, and scorpion venoms has been reported by Doery and Pearson (8) and by Mohamed, Kamel, and Ayobe (9). These authors studied the hydrolytic action of whole or boiled venoms on lysolecithin prepared from lecithin by enzymatic hydrolysis with snake venom, i.e., the 1-monoacyllyso derivative. Van Deenen and de Haas (10) found, using synthetic substrates, that whole *Crotalus adamanteus* venom catalyzes the hydrolysis of both 1,2-diacyl-*sn*-glycero-3-phosphatide and 2-monoacyl-*sn*-glycero-3-phosphatide (the β-acyllyso derivative),

whereas the 1-mono-acyl-*sn*-glycero-3-phosphatide (the α-acyllyso derivative) was not susceptible to the venom.

In the present study it is demonstrated that the purified phospholipase A from *Vipera palestinae* venom exhibits, under appropriate conditions, B activity as well. Moreover, it is shown that this enzyme also hydrolyzes 2-deoxylysolecithin (palmitoyl propanediol-(1,3)-phosphorylcholine) (11), which lacks the hydroxyl group in the 2-position.

EXPERIMENTAL PROCEDURE

Vipera palestinae venom (obtained from the Department of Zoology, Tel Aviv University) was collected and lyophilized. The dried venom was stored at −20°C until used. *Crotalas adamanteus* venom was purchased from L. Light and Co., Ltd., Colnbrook, England.

Purification of the phospholipase

Chromatographic techniques. All procedures for purification of the enzyme were conducted at 4°C. DEAE-cellulose (Whatman) was washed with acid and alkali as recommended by the manufacturer, equilibrated with phosphate buffer, pH 8.1, at the appropriate ionic strength, and then packed into a column. Sephadex G-50 (Pharmacia, Uppsala, Sweden) was allowed to swell in water for 24 hr, packed into a column under a pressure head of 50 cm of water, and equilibrated overnight with 0.1 M ammonium bicarbonate, pH 7.1, at a flow rate of 10 ml/hr.

Protein concentrations were estimated by the method of Lowry et al. (12), using bovine serum albumin as a standard. The absorbance was read at 750 nm.

Electrophoresis

Disc electrophoresis in polyacrylamide gel (Eastman Organic Chemicals, Rochester, N.Y.) was performed using the method of Davis (13). The separation was carried out in a Shandon disc electrophoresis apparatus (Shandon Scientific Co., Ltd., London, England) in 5 × 50 mm gels (15% acrylamide in Tris–glycine buffer, pH 8.3). The amount of protein applied was 20–100 μg in 100 μl of buffer solution containing one drop of glycerol and 5 μl of bromophenol blue (0.05% in water). Electrophoresis was carried out at 4°C, 2.5 mA per tube, until the dye reached the bottom of the tube. Proteins were stained with 1% amido black in 7% acetic acid for 30 min and washed with the latter acid containing the anion exchange resin, Dowex AG 1-X8 (220–400 mesh, Bio-Rad).

Immunization procedure

Two white New Zealand rabbits, each weighing about 3 kg, were injected intradermally in several places on the back and in each foot pad with 1 ml of an emulsion prepared from equal volumes of *Vipera palestinae* whole venom in saline (6 mg/ml) and complete Freund's adjuvant. After 10 days the animals were given an antigen booster intramuscularly (1 mg of *V. palestinae* whole venom in complete Freund's adjuvant). This inoculation was repeated twice, at 4-wk intervals. 1 wk after each booster injection, the animals were bled by cardiac puncture and the sera were tested for precipitating antibodies. The antibody content of the immune sera was estimated by absorbance measurements at 280 nm after dissolving the washed specific precipitates in 0.1 N NaOH.

Immunological techniques

Immunodiffusion experiments were performed as described by Ouchterlony (14) in petri dishes with 1% Difco agar in 0.025 M Veronal buffer, pH 8.2. The concentrations of the whole venom and the partially purified and the purified phospholipases were 6 mg/ml, 1.5 mg/ml, and 0.5 mg/ml, respectively. The wells were filled with 0.01 ml of the appropriate samples, and diffusion was allowed to proceed for 2–3 days at 4°C in a moist chamber. After extensive washing with 0.9% NaCl solution for 3 days, the plates were placed for 5 min in a solution of 1% amido black in 7% acetic acid and then rinsed in three changes of the latter solvent. For immunoelectrophoresis studies, glass slides (76 × 25 mm) were coated with 3.5 ml of 1.5% agar solution in 0.025 M Veronal buffer, pH 9.0. *V. palestinae* whole venom, partially purified phospholipase, and purified phospholipase (10 mg/ml, 1.5 mg/ml, and 0.5 mg/ml, respectively) were placed in the holes and electrophoresis was performed for 120 min at 140 V and 4°C. The concentration of the tray buffer was 0.05 M. After completion of the electrophoresis, anti-whole *V. palestinae* serum was placed in the main trough and the slides were developed for 48 hr at 4°C in a moist atmosphere. Washing and staining were performed as described for immunodiffusion.

Molecular weight determinations

The molecular weight determinations of the phospholipases were carried out by dodecyl sulfate–polyacrylamide gel electrophoresis, as described by Weber and Osborn (15), using RNase, lysozyme, performic acid-oxidized hemoglobin, trypsin, chymotrypsinogen A, pepsin, and ovalbumin as molecular weight standards.

Equilibrium ultracentrifugation was carried out at 22,000 rpm at 20°C in a Beckman model E ultracentrifuge equipped with an Epon double-sector cell (1.2-cm optical path, 3-mm column height). Data were collected with the photoelectric scanner and multiplexer accessory after 48 hr. No change in the scan was observed after an additional 10 hr. The enzyme solution contained 0.5 mg of protein/ml in 0.1 M phosphate buffer, pH 7.0, 0.2 M NaCl. A $\bar{v}$ of 0.72 was used in calculations.

Amino acid analyses

Amino acid analyses were performed with samples of 0.2 mg of protein in a Beckman Spinco automatic amino acid analyzer, model 120B. Samples were hydrolyzed in vacuo in 6 N HCl at 110°C for 22, 48, and 72 hr. Separate samples were oxidized with performic acid prior to hydrolysis (16) and used for the estimation of cystine as cysteic acid (17). Tryptophan was analyzed colorimetrically according to Spies and Chambers (18).

Thiol group estimation

Free thiol groups of purified phospholipases in 1% SDS were determined with 5,5'-dithiobis(2-nitrobenzoic acid) according to the method of Ellman (19). The absorbance was read at 412 nm.

Assay of phospholipase A activity

For specific activity determinations at the various purification stages of the enzyme, the pH-stat method (TTA₃ Radiometer autotitrator type SBR2C/SBU 1) was employed, using dilute egg yolk (1:20, with saline) as substrate. Liberation of acid was measured at pH 8.0 and 37°C by titration with 0.005 N NaOH under a constant stream of argon. One unit of activity was defined as the amount of enzyme that liberates 1 μmole of free fatty acid/min under the above conditions. This was permissible since the activity of a given sample was found to be proportional to its concentration in the assay mixture, indicating that the substrate was present in large excess.

Substrates

Rat liver homogenate was extracted with chloroform–methanol 2:1 as described by Entenman (20), and lecithin was isolated from the phospholipid mixture by chromatography on alumina according to Rhodes and Lea (21). The lecithin was further purified by TLC using silica gel H without binder (E. Merck A.G., Darmstadt); the plates were developed in chloroform–methanol–water 65:25:4. On each plate (20 × 20 cm, 0.5 mm thick) about 4 mg of lipid could be separated. The band containing the lecithin (detected by iodine vapor) was scraped off and eluted with several 5-ml portions of chloroform–methanol 1:9 (22). The successive portions were filtered through a fine-pore sintered glass filter, and the combined filtrates were evaporated to dryness in a rotary evaporator at 25°C under reduced pressure; the residue was dissolved in chloroform–methanol 2:1.

Lysolecithin was prepared enzymatically from lecithin using various phospholipase A preparations but mainly VP enzyme. The reaction mixture (1 ml) containing lecithin (10 mM), ammonium acetate buffer, pH 9.0 (0.1 M), and CaCl₂ (0.5 mM) was sonicated in an MSE sonicator, with cooling in an ice bath, until it was homogeneous (about 1 min). Following this procedure, the VP enzyme (45 μg protein/ml) and ether (10% v/v) were added and the mixture was incubated for 2 hr at 37°C. 3 ml of methanol was then added, and the mixture was evaporated to dryness with nitrogen; the residue was extracted with 1 ml of chloroform–methanol 2:1. The lysolecithin was separated from the fatty acids produced in the reaction and from any residual unhydrolyzed lecithin by TLC using the developing solvent system described for the separation of lecithin. The pure lysolecithin band was eluted with chloroform–methanol–water 66:33:1 (2 × 5 ml) and chloroform–methanol–water 5:10:1 (2 × 5 ml).

2-Deoxylysolecithin (palmitoyl propanediol-(1,3)-phosphorylcholine) (11) and palmitoyl hexanediol-(1,6)-phosphorylcholine (11) were a gift from Dr. H. Eibl, Göttingen.

Conditions for enzymatic hydrolysis

The lipid substrates were prepared by drying the chloroform–methanol solutions of the phospholipid in a stream of nitrogen. Lecithin and lysolecithin were generally used as 0.25 mM solutions in 0.1 M ammonium acetate buffer, pH 8.5 and 10, respectively, containing CaCl₂ (0.5 mM). The lecithin-containing systems were sonicated, and ether (10% v/v) was added prior to incubation with the enzyme. With lysolecithin this treatment was omitted because 0.25–2.5 mM solutions were completely clear. The enzyme was then added and the mixture (1 ml) was incubated at 37°C for a given time. After incubation 3 ml of methanol was added, the mixture was evaporated with nitrogen, and the residue was reextracted with chloroform–methanol 2:1. One-dimensional chromatography was performed using as the developing system chloroform–methanol–10% ammonia 65:35:7.5. With this solvent system, the glycerophosphorylcholine remains at the application point, while the R_F values of the lysolecithin and lecithin spots are 0.18 and 0.35, respectively. The percentage hydrolysis in each aliquot was calculated from the phosphorus content (23) of the lecithin-, lysolecithin-, and glycerophosphorylcholine-containing spots. To examine the lipolytic or esterolytic activity of the purified phospholipase on tri- and monoglycerides or their fatty acid esters, the following procedure was adopted. The substrates at concentrations of 10 mM were sonicated in an ice bath for 2–3 min, with an MSE 20 kc/sec ultrasonic disintegrator, in the presence of 0.1% Triton X-100 and 1% bovine albumin (fatty acid poor). The incubation was carried out for 1 hr at 37°C, in solutions of 5 mM Tris–HCl, pH 7.3, or 5 mM glycine–NaOH, pH 9.8, containing 5 mM CaCl₂. The concentration of the enzyme was 20 μg/ml. The lipolytic activity was evaluated by estimation, as described below, of the released free fatty acids.
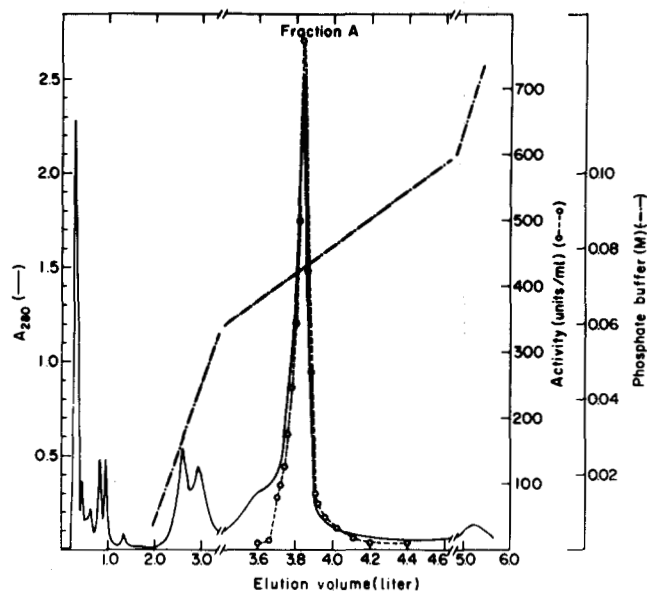
FIG. 1. Separation of whole VP venom on the first DEAE-cellulose column. Fraction A contains phospholipase activity. Conditions as described in Experimental Procedure. Buffer concentrations (—·—) are experimental values, determined conductometrically in the effluent.
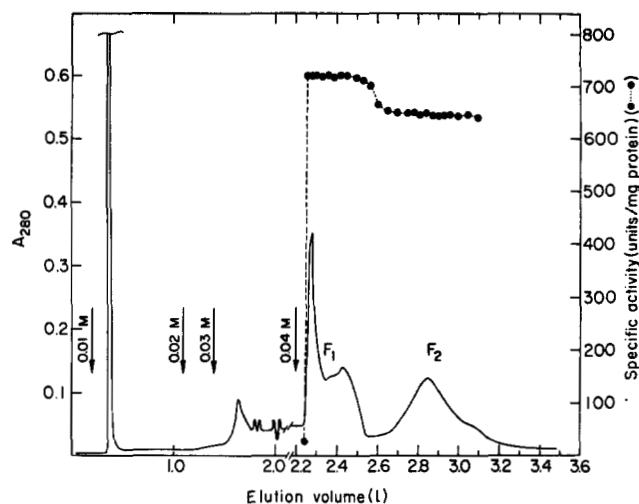

FIG. 2. Further purification of the phospholipase fraction on a second DEAE-cellulose column. Conditions as described in Experimental Procedure. Fractions F_1 and F_2 contain phospholipase activity.

The spontaneous hydrolysis of lysolecithin was about 2–2.5%/hr below pH 10.2; at pH 10.5 it was about 10%. All experiments were run with appropriate blanks.

Identification of the reaction products of lysolecithin hydrolysis

Free fatty acids produced in the enzymatic reaction were estimated by the method of Dole and Meinertz (24). The disappearance of the carboxylic acid ester bond was followed by the hydroxamic acid reaction (25). Glycerophosphorylcholine, which was separated chromatographically from the residual lysolecithin as described above, was eluted from the application point by methanol–water 1:1. The GPC was identified by two-dimensional ascending TLC by comparison with commercial GPC (liberated from the GPC–cadmium chloride complex as described in the section Chemicals). The two solvent systems used were n-butanol–acetic acid–water 60:20:20 and propanol–water 64:36 (R_F about 0.3). For estimation of the choline moiety, the GPC was hydrolyzed with 1 N HCl for 20 min at 110°C in a sealed evacuated ampoule. The free choline formed was estimated by means of the potassium triiodide reagent as described by Appleton et al. (26).

Chemicals

Glycerophosphorylcholine was prepared from its cadmium chloride complex, type II (Sigma Chemical Co., St. Louis, Mo.). The cadmium was removed by dissolving the complex in water (to a concentration of

20 mg/ml) and mixing with the cation exchanger Chelex R 100, Dowex A (Bio-Rad). The Chelex was first conditioned with 0.5 M HCl, washed with water to pH 7.0, and then reconditioned with sodium acetate. The absence of cadmium ions was verified using the dithizone method (27).

Palmitic and oleic acids, glyceryl tributyrate, and EDTA were purchased from E. Merck, A.G., and sodium deoxycholate from Difco Laboratories, Detroit, Mich. Glyceryl monostearate, glyceryl-1-monooleate and 1-monopropionate, ethyl and methyl oleates, and ethyl myristate were purchased from Eastman Organic Chemicals, and bovine albumin, free fatty acid poor, from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.

Buffers

Phosphate buffers were prepared by mixing equimolar solutions of Na_2HPO_4 and NaH_2PO_4 in appropriate proportions to obtain the desired pH. The molarity of these buffers refers, therefore, to phosphorus. Ammonium bicarbonate buffer was prepared by passing CO_2 into a solution of 0.1 M NH_4HCO_3 until the desired pH was obtained.

RESULTS AND DISCUSSION

Purification of the enzyme

Lyophilized venom, 1 g dissolved in 4 ml of 0.005 M phosphate buffer, pH 8.1, was dialyzed at 4°C overnight against the same buffer.

Step 1. After dialysis the material was loaded onto a column of DEAE-cellulose (3 × 45 cm) equilibrated

with the above buffer. Elution was first carried out with the starting buffer, at a rate of 60 ml/hr, and fractions of 20 ml were collected. After the unadsorbed material had come off, an exponential gradient elution was started using 2.5 l of 0.005 M phosphate buffer, pH 6.8, in the mixing vessel and 2.5 l of 0.2 M phosphate buffer, pH 6.8, in the reservoir. A typical elution pattern is shown in Fig. 1. The phospholipase activity, eluted at about 0.074 M (6.8 mmho, 3.7–4.0 l), was collected, concentrated, and dialyzed for 24 hr against three changes of 2 l each of distilled water. During dialysis a fine precipitate appeared; this was removed by centrifugation. After dialysis the solution was lyophilized.

Step 2. The product of step 1 was dissolved in 20 ml of 0.001 M phosphate buffer, pH 8.1, and loaded onto a second DEAE-cellulose column (1.5 × 50 cm) equilibrated with the last-mentioned buffer. Elution was first carried out with 0.005 M phosphate buffer, pH 6.8, followed by a stepwise elution with 0.01 M, 0.02 M, 0.03 M, and finally 0.04 M phosphate buffer, pH 6.8, at a flow rate of about 40 ml/hr. Fractions of 10 ml were collected. The elution pattern of this column, showing separation into three fractions, is seen in Fig. 2. The first fraction eluted (350–420 ml) did not contain any phospholipase activity, but both fractions F_1 (2260–2550 ml) and F_2 (2710–3300 ml) were active against lecithin and, under appropriate conditions, lysolecithin. Fractions F_1 and F_2 were pooled separately, dialyzed against water, and freeze-dried.

Step 3. Each fraction was dissolved in 1 ml of 0.1 M ammonium bicarbonate, pH 7.1, loaded onto a Sephadex G-50 column (1.50 × 185 cm), equilibrated with the same solvent, and eluted at a flow rate of 10 ml/hr. Fractions of 5 ml were collected. Typical elution patterns are shown in Fig. 3. This step did not increase the specific activity of the enzyme, measured as phospholipase A activity.
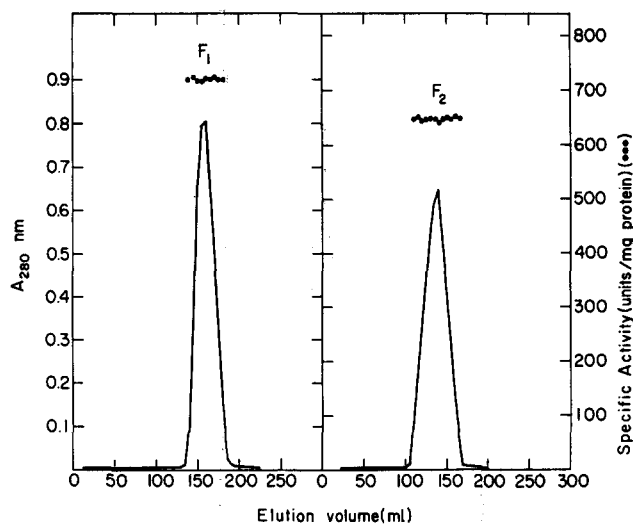

FIG. 3. Gel filtration of phospholipase F_1 and F_2 on Sephadex G-50. Conditions as described in Experimental Procedure.

The purification of the enzyme is summarized in Table 1 and Fig. 4.

Criteria of purity

As can be seen from Figs. 2 and 3, the specific phospholipase A activities of the fractions F_1 and F_2 emerging from the second DEAE-cellulose column and from the Sephadex G-50 column indicate the homogeneity of each of the above fractions. This is consistent with the results obtained by acrylamide gel electrophoresis, as shown in Fig. 4 (bands 3, 4, 5, and 6). In double-diffusion antigen–antibody reactions (Fig. 5) a single continuous precipitin line was formed between the F_1 and F_2 fractions, indicating their immunological identity. With the whole venom and with partially purified phospholipase, several precipitin lines were obtained. A single, distinct arc was also obtained when either of the

TABLE 1. Summary of purification of phospholipase from *Vipera palestinae* venom

Fraction from:	Protein	Total Activity	Specific Activity	Yield	Ratio to Specific Activity of Whole Venom
	mg	*units[a]*	*units/mg protein*	*%*	
Lyophilized venom	1,000	130,000	130	100	1
DEAE-cellulose, first column	120	60,600	505	46.6	3.88
DEAE-cellulose, second column					
F_1	30.2	21,700	720	16.7	5.54
F_2	24	15,600	650	12.0	5.0
Sephadex G-50 column					
F_1	18.9	13,600	720	10.5	5.54
F_2	17.1	11,100	650	8.6	5.0

[a] One unit of activity was defined as the amount of enzyme that liberates 1 μmole of free fatty acid/min, using egg yolk emulsion as substrate under the conditions described in Experimental Procedure (Assay of phospholipase A activity).
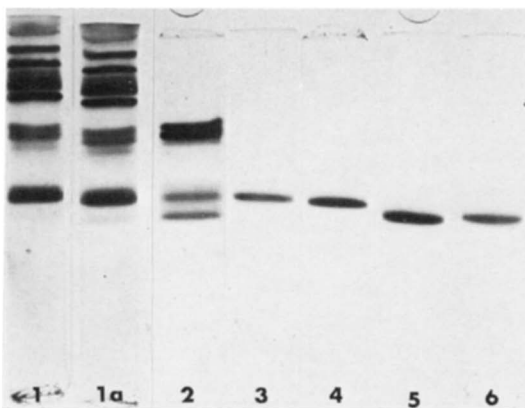
FIG. 4. Analytical acrylamide gel electrophoresis showing purification achieved at each step of the fractionation of phospholipase from VP venom. *1*, whole VP venom; *1a*, same, after storage for 4 days in phosphate buffer, pH 8, 4°C; *2*, active fraction from first DEAE-cellulose column; *3*, fraction F_1 from second DEAE-cellulose column; *4*, fraction F_1 after gel filtration on Sephadex G-50; *5*, fraction F_2 from second DEAE-cellulose column; *6*, fraction F_2 after gel filtration on Sephadex G-50. Electrophoresis conditions are described in Experimental Procedure.

fractions F_1 or F_2 was studied by immunoelectrophoresis (Fig. 6), while numerous arcs were obtained with the whole venom and at least four arcs were obtained with a partially purified fraction.

It should be noted that the appearance of two separate enzyme fractions, F_1 and F_2, in step 2 of the purification does not necessarily mean that two different enzymes were originally present in the whole venom. One of the two fractions (F_2) may be due to an artifact, e.g., spontaneous hydrolysis (during the purification procedure) of a side-chain amide group of glutamine or asparagine, in about half of the enzyme molecules. The difference
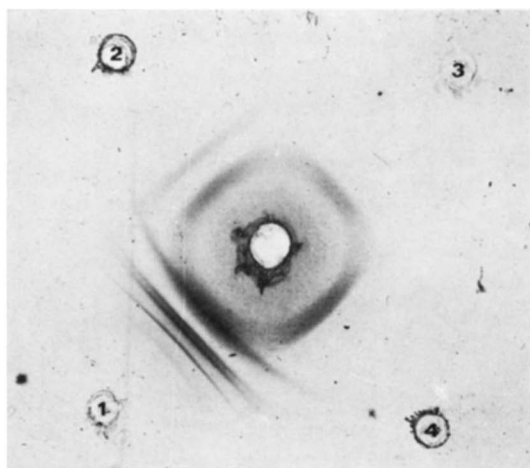

FIG. 5. Agar gel double diffusion. *1*, whole VP venom; *2*, active fraction from first DEAE-cellulose column; *3* and *4*, phospholipase fractions F_1 and F_2, respectively, at final purification stage (after Sephadex G-50 gel filtration). The antibody against the whole venom was placed in the center well. For details see Experimental Procedure.
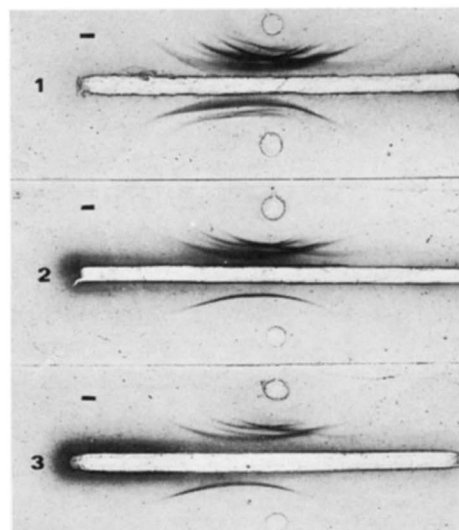
FIG. 6. Analytical immunoelectrophoresis. Upper wells, *1*, *2*, and *3*, whole VP venom. Lower wells: *1*, active fraction from first DEAE-cellulose column; *2* and *3*, phospholipase fractions F_1 and F_2, respectively, at final purification stage (after Sephadex G-50 gel filtration). Trough, in all plates: rabbit anti-whole VP venom.

in electrophoretic behavior of F_1 and F_2 may be accounted for by a difference in one electrical charge per molecule. This is supported by the fact that fraction F_2 did not appear in the acrylamide electrophoresis of the whole venom, but appeared gradually on storage in phosphate buffer, pH 8 (see Fig. 4).

Molecular weight determination

The two phospholipase fractions F_1 and F_2 showed practically the same molecular weights of about 16,000 when estimated by SDS–acrylamide gel electrophoresis (Fig. 7).

Wells and Hanahan (28) isolated two phospholipase A_2 fractions from *Crotalus adamanteus* venom, both having identical amino acid composition and molecular weight (30,000). Later, Wells (29) showed that these fractions are dimers of (inactive) identical subunits (MW 15,000) by gel filtration in 6 M guanidine–0.1 M mercaptoethanol. Salach et al. (30) isolated a number of isoenzymes from *Naja naja* (molecular weights in the range 8500–20,200) and from *V. russellii* (MW: 15,900; 23,800) but found no evidence of subunit structure.

In view of the above, equilibrium ultracentrifugal analyses were carried out with the VP enzyme (F_1) at pH 7.0, where phospholipase A activity is observed. The result obtained was very similar to that reported by Wells (29) for *C. adamanteus* venom at pH 3.75. The log OD vs. r^2 plot (Fig. 8) was curved, indicating a monomer–dimer equilibrium. Indeed, the slope at the meniscus (OD = 0.018, c = 15 µg/ml) corresponded roughly to an $\overline{MW}_{wt}$ of 15,000, whereas the slope near the bottom of the cell (OD = 1.2, c = 1 mg/ml) was twice as large.
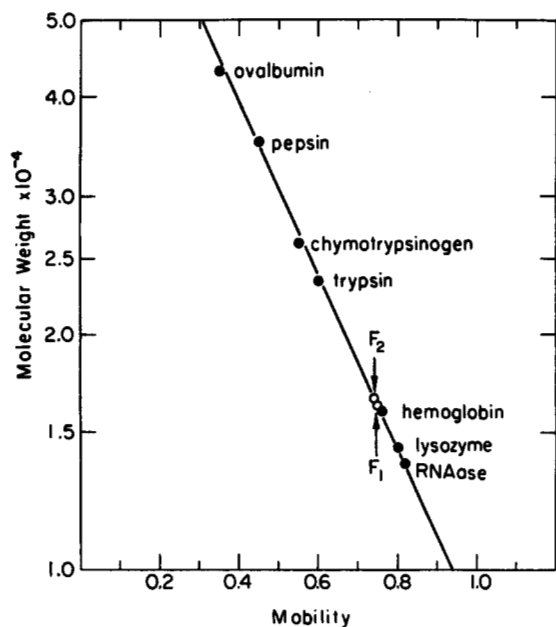
FIG. 7. Semilog plot of molecular weights against distance of migration on SDS–acrylamide gel. The arrows indicate the mobility of phospholipase fractions F_1 and F_2. The interpolated MW, for both fractions, is about 16,000.

FIG. 8. Log OD vs. r^2 plot at sedimentation equilibrium (22,000 rpm and 20°C) at pH 7.0. Initial OD: 0.64 ($c = 0.5$ mg/ml). The arrow indicates the OD on the curve at which its slope is 5/3 the slope at the meniscus.

Moreover, from the shape of the plot one can get an estimate of the dissociation constant, K_{diss}, of the dimer at pH 7.0. K_{diss} is equal to the dimer concentration at which monomer and dimer molarities are equal, i.e., where the weight average MW equals $(1 + 2^2)/(1 + 2) = $ 5/3 the molecular weight of the monomer. This point can be found on the curve (5/3 the slope at the meniscus); it corresponds to an OD of 0.15, or a concentration of 0.13 mg/ml (8×10^{-6} M). Hence, $K_{diss} \sim 10^{-5}$ M at pH 7. The dissociation behavior as a function of pH merits further investigation, but it seems clear that at a concentration of 10^{-2} µg/ml, used in the lecithin assays (see Fig. 9), which is five orders of magnitude below the K_{diss}, the VP phospholipase is practically fully dissociated into monomers of an MW of 16,000.

Amino acid analyses of the fractions F_1 and F_2 are given in Table 2. The two sets of results are almost identical. No free thiol groups were found in either case. The calculated molecular weights of the two fractions based on amino acid analyses (using SDS–acrylamide gel electrophoresis results for order of magnitude) were 15,736 for F_1 and 15,665 for F_2, the difference being that between a lysine and a glycine residue. These differences may be significant, but they are close to the experimental error of the method. Amide nitrogen was not determined.

Characterization of the enzymatic activity

In addition to their main phospholipase A activity, the purified enzyme fractions exhibited pronounced phospholipase B activity, producing glycerophosphoryl-
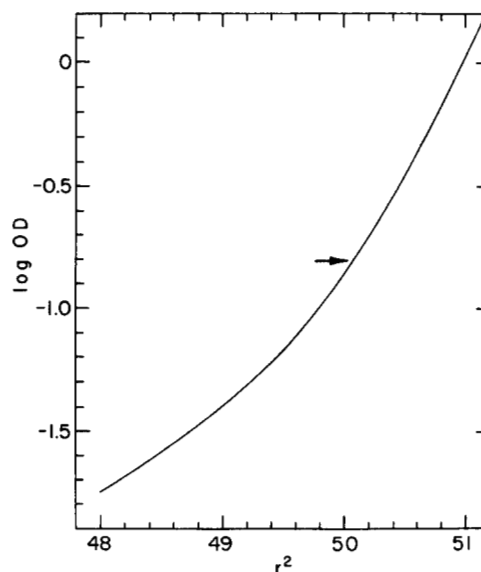
choline from lysolecithin[3] under appropriate conditions of pH and reactant concentration. This "B activity" was observed whether the lysolecithin serving as substrate was prepared by the action of *Vipera palestinae* or of *Crotalus adamanteus* venom (Table 3). It is of interest to note that *C. adamanteus* venom was also able to hydrolyze the various lysolecithin preparations extensively when the appropriate conditions for phospholipase B activity were applied (Table 3). The finding by van Deenen and de Haas (10) that *C. adamanteus* venom does not hydrolyze 1-monoacyl-sn-glycero-3-phosphatidylethanolamine may have been due to the limited pH range tested.

In order to ascertain that the hydrolysis of lysolecithin observed is indeed due to an attack by the enzyme on the 1-acyl moiety, the possibility of base-catalyzed acyl migration (from carbon 1 to carbon 2) had to be eliminated. If this migration proceeds slowly as compared with the enzyme action, it must be the rate-determining step. The finding that hydrolysis rate is proportional to enzyme concentration (see Table 4) seems to eliminate this possibility. A similar conclusion can be drawn from experiments in which the substrate was preincubated for 30 min at pH 10. No changes in rates were observed.

There still remains the rather remote possibility that acyl migration is much faster than enzymatic hydrolysis (e.g., half-life less than a few minutes). For this reason a

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[3] Lysolecithin prepared by *Crotalus adamanteus* venom was shown by van Deenen and de Haas (10) to be 1-monoacyl-sn-glycero-3-phosphorylcholine. The lysolecithin used in most of the present study (prepared by the action of the purified phospholipase from *Vipera palestinae* venom) can be assumed to have the same structure.

TABLE 2. Amino acid composition of *Vipera palestinae* phospholipases

	F_1		F_2		F_1	F_2
	moles of amino acid residue per:				*nearest integer*	
	1 mole Asp	*18 moles Asp*	*1 mole Asp*	*18 moles Asp*		
Lysine	0.57	10.2	0.50	9.0	10	9
Histidine	0.15	2.7	0.14	2.5	3	3
Arginine	0.18	3.2	0.18	3.3	3	3
Aspartic acid	1.00	18.0	1.00	18.0	18	18
Threonine	0.40	7.2	0.38	6.9	7	7
Serine	0.39	7.0	0.40	7.2	7	7
Glutamic acid	0.65	11.7	0.64	11.6	12	12
Proline	0.23	4.1	0.21	3.7	4	4
Glycine	0.79	14.2	0.84	15.2	14	15
Alanine	0.38	6.9	0.38	6.8	7	7
Half-cystine	0.67	12.1	0.68	12.3	12	12
Valine	0.41	7.3	0.40	7.2	7	7
Methionine	0.22	4.0	0.21	3.8	4	4
Isoleucine	0.23	4.2	0.21	3.7	4	4
Leucine	0.48	8.7	0.48	8.6	9	9
Tyrosine	0.51	9.2	0.49	8.8	9	9
Phenylalanine	0.44	8.0	0.43	7.8	8	8
Tryptophan		2.0		2.0	2	2
Total					140	140

substrate analog for which this migration is impossible, i.e., 2-deoxylysolecithin, was tested. Indeed, at pH 10, this compound is hydrolyzed by the enzyme, yielding free fatty acid and α-phosphorylcholine-propanediol-(1,3). Under the same conditions the corresponding hexanediol-(1,6) derivative was resistant to the action of the enzyme.

It is of interest that 2-deoxylysolecithin is also digested by *C. adamanteus* whole venom at pH 10.

Identification of the products of enzymatic hydrolysis of lysolecithin

In a representative experiment, the amount of free fatty acids released from 3.5 μmoles of lysolecithin by the purified VP enzyme was 2.8 μmoles, and 2.8 μmoles of the carboxylic acid ester bonds disappeared. The reaction mixture was found to contain neither inorganic phosphorus nor free choline. The glycerophosphorylcholine formed in the reaction was separated from the residual lysolecithin (0.7 μmole) by TLC (as described in Experimental Procedure), and after elution from the plate and hydrolysis, yielded 2.4 μmoles of organic phos-

phorus and 2.5 μmoles of choline. On two-dimensional chromatography, the unhydrolyzed spot behaved identically with commercial GPC (see Experimental Procedure) and did not separate from a mixture with the authentic sample.

pH profile of phospholipase A and B activities

The effect of pH on the rate of lecithin and lysolecithin hydrolysis by VP phospholipase is shown in Fig. 9. (All the kinetic studies were performed with the purified fraction F_1.) Whereas the hydrolysis of lecithin had an optimum at pH 9.0, the hydrolysis of lysolecithin remained rather low until pH 8.5 and then increased beyond pH 10.5 without showing a maximum. Above pH 10.5, marked spontaneous splitting of the substrate occurred.

The alkaline range found for the phospholipase B activity of purified VP phospholipase A is similar to that reported for phospholipases B from other snake venoms (8), insects (7), and bacteria (31). On the other hand, phospholipases B from animal tissues (32) and molds

TABLE 3. Comparison of phospholipase B activities

Lysolecithin Prepared by:	Source	Concentration	Lysolecithin Hydrolyzd[a] pH 7	pH 10
		μg/ml	*%/hr*	
VP (purified)	VP (purified)	25	15.6	80
	CA (whole)	450	6.5	90
CA (whole)	VP (purified)	25	13.5	92
	CA (whole)	450	10	100

[a] Reaction mixture as described in Experimental Procedure.

TABLE 4. Effect of enzyme concentration on velocity of lysolecithin hydrolysis by *Vipera palestinae* phospholipase

Enzyme Concentration	Time of Incubation	Substrate Hydrolyzed[a]
μg/ml	*min*	*%*
0.5	60	26.2
1.0	30	25.0
2.0	15	27.1
4.0	7.5	25.4

[a] Reaction mixture as described in Experimental Procedure. The pH was 10.0.
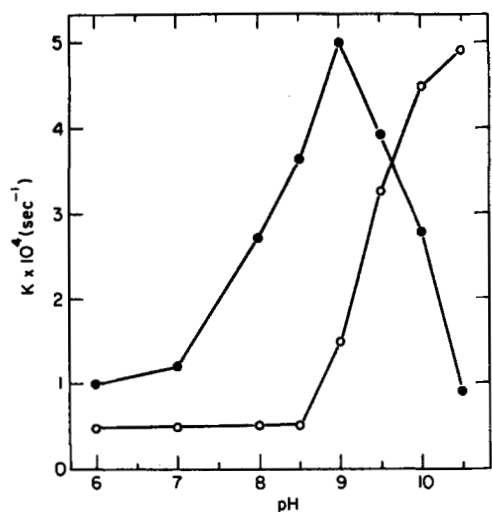
FIG. 9. Effect of pH on the pseudo first-order rate constants, k, of hydrolysis of lecithin (●) and lysolecithin (○) by phospholipase (F_1) of VP. The system contained 0.25 mM substrate in ammonium acetate buffer, 0.1 M, and 0.5 mM CaCl₂. Enzyme concentrations: ○, 10 μg/ml; ●, 10^{-2} μg/ml.
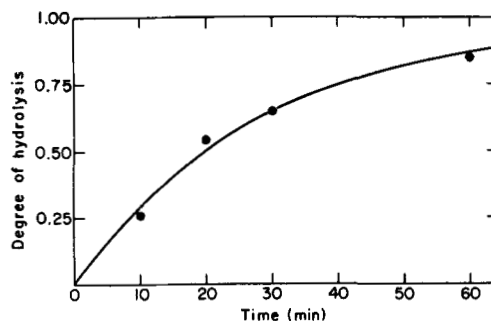

FIG. 10. Time course of lysolecithin hydrolysis by VP phospholipase (F_1) at pH 10. The substrate concentration was 0.25 mM. The system was as described in Experimental Procedure. The full line is a calculated first-order curve.

(33) exhibit a neutral or acidic pH optimum. The lysophospholipase found in rat brain by Leibovitz and Gatt (34) has a broad pH optimum range between 7.2 and 8.6.

Effect of enzyme and substrate concentration on lysolecithin hydrolysis

The time course of the enzymatic hydrolysis of lysolecithin by VP phospholipase at pH 10 is given in Fig. 10. Table 4 shows that the reaction velocity (at about 30% substrate hydrolysis) is proportional to the enzyme concentration.

The Lineweaver-Burk plot for the action of purified VP phospholipase on lysolecithin is shown in Fig. 11. The Michaelis-Menten constant and k_{cat} value found at pH 10 were $K_m = 1.1$ mM and $k_{cat} = 0.55$ sec^{-1}. (The k_{cat} value equals $V_{max}/[E]$ and is the first-order rate constant, in sec^{-1}, for the formation of the products from the enzyme–substrate complex; the enzyme concentration [E], in M, is based on the molecular weight of 16,000.) A K_m value of 0.1 mM was reported by Leibovitz and Gatt (34) for rat brain lysophospholipase acting upon 1-monoacyl-sn-glycero-3-phosphorylcholine, and a K_m value of 0.103 mM was found by Rao and Subrahmanyam (7) for the Culex pipiens fatigans enzyme acting upon the same substrate.

The enzymatic hydrolysis of deoxylysolecithin at pH 10 was found to be independent of substrate concentration (zero order) in the range 0.5–2.5 mM. The rate measured was 0.08 μmole/ml/hr at an enzyme concentration of 37.5 μg/ml. Since this rate represents V_{max}, the value of $k_{cat} = 1 \times 10^{-2}$ sec^{-1} could be calculated directly.

Effect of Ca²⁺, EDTA, deoxycholate, ether, and the reaction products

EDTA completely inhibited the hydrolysis of lysolecithin by the purified phospholipase. Ca²⁺ ions had a marked stimulatory effect when added to the EDTA-containing media, in sufficient excess. Sodium deoxycholate had a weak stimulatory effect in EDTA-containing media but acted as an inhibitor in Ca²⁺ ion-containing systems, probably by binding calcium. Ether (at a concentration of 10% v/v) had a slight inhibitory action in systems containing only the enzyme and lysolecithin.

These observations may be compared with related ones reported in the literature. Magee et al. (35) and Waite and van Deenen (3) found that sodium deoxycholate inhibits the lysophospholipase activity of human pancreas and rat liver. Several phospholipase B preparations, from ox pancreas (36), rat liver (6, 32), Penicillium
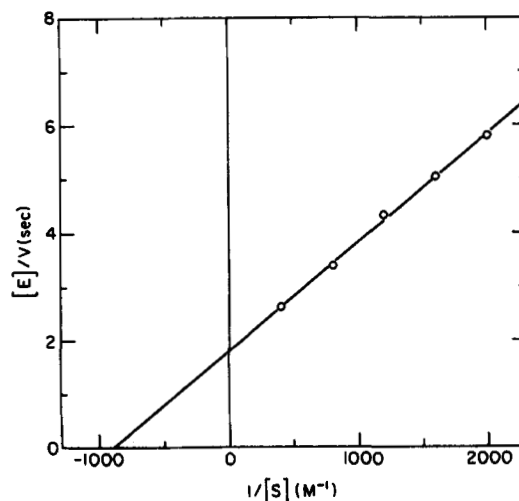

FIG. 11. Lineweaver-Burk plot for the hydrolysis of lysolecithin by purified phospholipase (F_1) at pH 10 and 37°C. Enzyme concentration, [E], 6 μg/ml or 3.75 × 10^{-7} M (based on MW of 16,000). The v values are initial rates in M/sec obtained from TLC plates of aliquots (phosphorus analysis). The reaction mixture contained 0.5 mM CaCl₂ and 0.1 M ammonium acetate buffer.

TABLE 5. Summary of specific activities of phospholipase fractions obtained in the course of the purification procedure towards lecithin and lysolecithin

Fraction from:	Lecithin Hydrolysis		Lysolecithin Hydrolysis	
	Specific Activity	Ratio to Specific Activity of Whole VP Venom	Specific Activity	Ratio to Specific Activity of Whole VP Venom
	$units/mg$ $protein$		$units^a/mg$ $protein$	
Lyophilized venom	130	1	0.23	1
DEAE-cellulose, first column	505	3.88	0.87	3.78
DEAE-cellulose, second column				
F_1	720	5.54	1.3	5.65
F_2	650	5.0	1.1	4.8
Sephadex G-50 column				
F_1	720	5.54	1.28	5.57
F_2	650	5.0	1.14	4.96

[a] One unit of activity was defined as the amount of enzyme that hydrolyzes 1 μmole of lysolecithin/min at pH 10.0. Conditions as described in Experimental Procedure.

notatum (33), mosquito (7), and certain bacteria (31), do not require Ca^{2+} ions for their activity. On the other hand, phospholipase B activities found in several snake venoms are known to be stimulated by Ca^{2+} and Mg^{2+} ions and inhibited by EDTA (8).

Glycerophosphorylcholine up to 5 mM had no influence on the rate of hydrolysis of lysolecithin by VP phospholipase. The effect of fatty acids could not be investigated because of the insolubility of their calcium salts.

Effect of storage and heating

Storage of the purified dried enzyme at $-20°C$ for several months did not change its lysophospholipase activity. Repeated thawing and freezing of aqueous solutions (concentration 0.5 mg/ml) caused some decrease in activity.

The phospholipase B activity of the purified enzyme was only slightly affected by boiling in a water bath at pH 5.5 for 15 min; about 10–15% of the activity was lost. This heat stability is unlike the behavior of similar enzymes from molds (33) or animal tissues (32), which are heat labile. On the other hand, stability to heat has been reported for phospholipase B from other snake venoms (8) and bacteria (31).

The effect of temperature on the B activity of the purified VP phospholipase was measured. At pH 9 (chosen in order to minimize spontaneous splitting of lysolecithin) and with 15 μg/ml enzyme and 0.25 mM substrate, the percentage hydrolysis at 37, 45, and 60°C was 33.3, 40.8, and 47.7, respectively. The phospholipase B of the *Culex pipiens fatigans* has recently been reported (7) to have a maximum activity at about 45°C.

Enzyme specificity

The finding that the purified VP phospholipase specifically hydrolyzes both lecithin and lysolecithin fits in with preliminary observations (37) that it is also active towards both phosphatidylethanolamine and its lysoproduct, obtained from the former by action of purified phospholipase. This suggests that VP phospholipase in general hydrolyzes both diacyl- and 1-monoacyl-*sn*-glycerophosphatides, though with quite different rates. This conclusion is supported by the finding that 2-deoxylysolecithin is also deacylated by the enzyme although at a still lower rate. On the other hand, the purified VP phospholipase shows no nonspecific esterase activity: it did not hydrolyze glyceryl tributyrate, glyceryl monooleate, and glyceryl 1-monopropionate. Also, methyl or ethyl oleate and ethylmyristate were not hydrolyzed.

The finding that the purified VP phospholipase exhibits both A and B activities could be interpreted in two ways: either the preparation contains two enzymes which are not separated by the purification procedure or there is indeed only one enzyme with a dual activity determined by the reaction conditions such as pH and enzyme concentration. Evidence for the second alternative is the homogeneity of the phospholipase preparations by all the criteria described in this study (e.g., the constancy of specific activity throughout a chromatographic peak, acrylamide electrophoresis, and immunoelectrophoresis); the parallelism between the behavior of the A and B activities (both require Ca^{2+} ions and are inhibited by EDTA, both are heat resistant); and, most convincingly, the fact that the ratio of the A and B activities at the various purification stages of the enzyme remains the same (Table 5).

The explanation for the large difference in the A and B activities of the purified phospholipase may lie, at least in part, in the difference between the physicochemical properties of lecithin and lysolecithin micelles in water (38). Whereas lecithin is known to form a laminar structure even at concentrations as low as 10^{-5} g/ml (about 0.012 mM), lysolecithin forms regular spherical micelles, with a critical concentration for micelle formation of 10^{-4} g/ml (about 0.16 mM). The micellar size and shape may play a dominant role in the binding of the enzyme to the substrate. It was shown by Sarda and Desnuelle (39) that pancreatic lipase does not act on dissolved substrates and is active only when adsorbed on an ester–water interface, apparently because the active site of the enzyme is then exposed. In a more recent study, Entressangles and Desnuelle (40) were able to prove that the activity of pancreatic lipase towards short-chain glycerides was considerably raised if the substrate was in the form of isotropic aggregated particles rather than dispersed molecules. Van Deenen and de Haas (10) found that the action of *C. adamanteus* phospholipase A on two water-soluble substrates, diacetyl- and dibutyryllecithin, was much less efficient than its action on the lipid-soluble C_{10} homolog. The fact that ether enhances phospholipase A activity and inhibits phospholipase B activity may also be related to the physicochemical properties of the substrates, since lecithin is ether-soluble whereas lysolecithin is not.

On the other hand, it seems probable that the observed differences in susceptibility are a reflection of the capability of the various substrates to interact with the active site of the enzyme so that the proper geometry for binding and catalysis is attained. Inspection of molecular models of the various substrates (made from CPK space-filling atom models) reveals that, by proper rotation of bonds, the ester carbonyl of the 1-acyl and the 2-acyl compounds can be brought into equal distance from the phosphorus atom. An interpretation would now be that the conformation of the molecule in the latter case (2-acyl) fits the active site better than in the former (1-acyl), explaining the general difference in rates. The fact that the "unfavorable" conformation is hydrolyzed better at high pH may be due to a relaxation of the enzyme structure at elevated pH, a known phenomenon. This may facilitate binding, but at the same time could drastically decrease catalytic efficiency.

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