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ISOLATION OF A PHOSPHOLIPASE A FROM *AGKISTRODON PISCIVORUS* VENOM

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SUMMARY

1. A protein with phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) activity has been isolated from *Agkistrodon piscivorus* venom by a combination of isoelectric precipitation and boiling, hydroxylapatite column chromatography and preparative acrylamide electrophoresis.

2. Sedimentation equilibrium and gel filtration studies indicate the isolated phospholipase A has a molecular weight of about 14 000 ± 500.

3. Activity assays using hydroxamate formation indicate the enzyme can hydrolyze an acyl ester linkage of either L-α-phosphatidyl choline (synthetic, dipalmitoyl) or of phosphatidyl ethanolamine although the rate and extent of each reaction is different.

4. The *Agkistrodon piscivorus* phospholipase A is active in the presence of ether and sodium deoxycholate but Ca^{2+}, classically considered an absolute requirement for venom phospholipase A activity, does not appear to be necessary.

INTRODUCTION

Phospholipase A (phosphatide acyl-hydrolase, EC 3.1.1.4) catalyzes the hydrolysis of diacyl phosphoglycerides into their monoacyl analogues with the release of free fatty acids from the β position. SLOTTA AND FRAENKEL-CONRAT[1] and SLOTTA[2] reported the first purification of a phospholipase A from snake venom. The protein, which they crystallized from the venom of *Crotalus terrificus terrificus*, was named crotoxin. In 1955, NEUMAN[3], using amberlite column chromatography, separated crotoxin into a phospholipase A fraction and a fraction having neurotoxic activity. Since that time many workers have attempted to purify phospholipase A from various snake venoms using $(NH_4)_2SO_4$ fractionation[4], elution after paper electrophoresis[5,6], zone electrophoresis on starch columns[7,8], starch gel electrophoresis[9,10], ion-exchange chromatography[11–14], gel filtration[15] and density gradient electrophoresis[16]. Their degree of success has varied almost as widely as the methods used.

In 1968, CURRIE *et al.*[17] crystallized two phospholipase A components associated with Indian cobra *(Naja naja)* venom toxin. Recently, WELLS AND HANAHAN[18] reported the isolation of two highly purified proteins with phospholipase A activity from the venom of *Crotalus adamanteus*. WU AND TINKER[19] reported the purification

of a phospholipase A_2 from *Crotalus atrox* venom and BRAGANCA *et al*[20]. have purified a *N. naja* phosphilipase A. This paper reports the isolation and characterization of a highly purified protein with phospholipase A activity from the venom of *Agkistrodon piscivorus* possessing charge and molecular weight properties which differ from those previously reported for other purified venom phospholipases A.

MATERIALS AND METHODS

Pooled samples of *A. piscivorus* venom were obtained from animals maintained in a serpentarium in our laboratory (courtesy of Dr. Carl Gans, Biology Department, SUNY at Buffalo) and were used fresh (storage for very brief periods, when necessary, was in ice).

Hydroxylapatite was purchased either as Bio Gel HT or as Bio Gel HTP from Bio Rad Laboratories.

Non-enzymic protein molecular weight markers used for molecular weight determination by gel filtration were purchased from Mann Research Laboratories, Inc.

Reagents used in acrylamide gel electrophoresis were purchased from the following sources: Cyanogum 41 Gelling Agent and ammonium persulfate from EC Apparatus Corp.; *N,N,N',N'*-tetramethylethylenediamine from Matheson Coleman and Bell.

L-α-Phosphatidyl choline (synthetic, dipalmitoyl) and phosphatidyl ethanolamine used as substrates in activity assay were purchased from General Biochemical Co. Substrates were checked for purity in the following two-dimensional thin-layer chromatographic system: first direction, chloroform–methanol–water (65:25:4, by vol.); second direction, 1-butanol–acetic acid–water (60:20:20, by vol.).

All other chemicals were obtained commercially and were of A.R. quality.

Isoelectric precipitation and boiling. Whole, fresh *A. piscivorus* venom was made 0.25 M with respect to potassium phosphate by addition of 1 M KH_2PO_4–K_2HPO_4 buffer (pH 7.4). The pH of the venom solution was then lowered to 5.9 by the dropwise addition of 1 M phosphoric acid and the precipitate removed by centrifugation at 15 000 rev./min, for 10 min (Servall, Model RC II, SS34 rotor). The clear yellow supernatant was heated, with stirring, in a boiling-water bath for 5 min. A large flocculent precipitate resulted which was removed by centrifugation as before. The resulting supernatant was extensively dialyzed first against glass-distilled water and then against 0.01 M KH_2PO_4/K_2HPO_4 buffer (pH 6.8).

Hydroxylapatite column chromatography. Either Bio Gel HTP or Bio Gel HT, after hydration and/or extensive equilibration with 0.01 M KH_2PO_4–K_2HPO_4 buffer (pH 6.8) were passed as a slurry into a column 2 cm × 25 cm. Dialyzed, boiled venom supernatant (not less than 1 but not more than 5 mg of protein per ml bed volume) was placed on the column. The column was washed first with 0.01 M KH_2PO_4–K_2HPO_4 (pH 6.8) and then with 0.1 M KH_2PO_4–K_2HPO_4 (pH 6.8) until no more protein was eluted at each concentration of buffer as determined by absorbancy at 280 mμ. Finally, the column was washed with 0.15 M KH_2PO_4–K_2HPO_4 (pH 6.8) to elute the active fraction. The active fraction was pooled, dialyzed extensively against deionized distilled water and concentrated by dialysis against solid sucrose.

Preparative acrylamide gel electrophoresis. A preparative acrylamide gel electrophoresis apparatus (Canalco) was used with column PD 2/70 for all runs. A simplified

acrylamide gel system, described by TOMBS *et al.*[21], was modified as follows: 5% acrylamide gels, 3.5 cm in height, were used with a continuous Tris–glycine buffer system (pH 8.9) (E-C Apparatus Corp., Technical Bulletin No. 146). The buffer contained: Tris 138.2 g; glycine, 21.0 g; water to 4 l.

Not more than 1 ml (10–15 mg protein) of the concentrated fraction, from hydroxylapatite chromatography, was applied to the top of the gel. A constant current (Canalco electrophoresis constant rate source, Model 1400) of 10 mA during the 1st h of the run and 15 mA for the next 3 h was maintained. Small amounts of inactive ultraviolet absorbing material were eluted during the first 2 h and the active fraction was eluted during the 3rd h.

Phospholipase activity assay. Activity was monitored by a modification[22] of the hydroxamate assay for phospholipase A activity developed by BROWN AND BOWLES[23]. Reaction mixtures containing phosphatidyl choline, sodium deoxycholate, Tris–HCl buffer and diethyl ether were preincubated in screw-capped test tubes at 37° for 5 min. Whole venom or purified phospholipase A was added and the solution was incubated at 37° for the desired time interval. Remaining reagents were added in the usual order.

In rate studies larger aliquots of phosphatidyl choline were dried in 25-ml glass-stoppered erlenmeyers and resuspended with proportionately larger amounts of deoxycholate, buffer and ether. At designated times 1.3-ml aliquots were pipetted into screw-capped test tubes into which 2.0 ml of ethanol had previously been pipetted. Remaining reagents were added in the usual order.

Controls (zero time) had ethanol and working hydroxylamine added before phospholipase A. Controls (no phospholipase A) were determined as for the standard curve for each set of experiments.

Molecular weight determination by gel filtration. The method used was based on that described by ANDREWS[24]. Sephadex G-75 beads, 40–120 μ, were hydrated and extensively equilibrated with 0.05 M Tris–HCl buffer (pH 7.6, containing 0.1 M NaCl), and allowed to settle under their own weight in a 1 cm × 75 cm glass column. Non-enzymic protein molecular weight markers in groups of two or three (about 4 mg protein) or 2 mg of the purified venom enzyme were applied to the column. Protein elution was monitored by absorbance at 280 mμ and elution volumes, V_e, (volume for each protein which corresponded to the maximum concentration as determined from the elution profile) were determined.

Sedimentation equilibrium studies. Sedimentation equilibrium studies were carried out in an analytical ultracentrifuge (Spinco, Model E) with interference optics. The changes in concentration throughout the cell were followed by the procedure of DERECHIN[25].

The molecular weight was calculated using the Svedberg equation (with the usual symbolism):

$$M = \frac{RT}{(1-\bar{v}\varrho)\,\omega^2} \frac{\mathrm{d}\ln c}{\mathrm{d}r^2}$$

The density of the solution employed, determined by pycnometry in a bath maintained at 20 ± 0.01°, was found to be 1.0036 g/ml. The concentration of the protein in g/100 ml at any level of the cell (plot) was calculated from the relationship 42.65

TABLE I

PURIFICATION OF PHOSPHOLIPASE A FROM *A. piscivorus* VENOM

Unit of enzyme activity: amount of enzyme which hydrolyzes 1 μmole of L-α-phosphatidyl choline (synthetic, dipalmitoyl) in 1 min. Molecular weight L-α-phosphatidyl choline (synthetic, dipalmitoyl) taken as 740.

Fraction	*Total activity (units)**	*Specific activity (units/mg protein)*	*Yield (%)*
Whole venom	5500	5	100
After boiling	4800	10	87
After hydroxylapatite	717	22	13
After acrylamide electrophoresis			
Band A	263	63	5
Band C	14	9	0.3

* Activity monitored by modified hydroxamate assay described in METHODS.

fringes = 10 mg/ml based on a calibration of the instrument with bovine serum albumin.

Protein measurement. Protein concentration was measured by the method of LOWRY *et al.*[26] using crystalline bovine serum albumin as the standard.

RESULTS

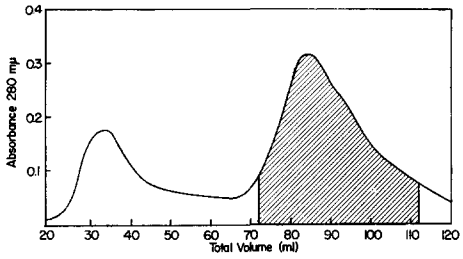
Purification procedure

After initial attempts to fractionate the venom with $(NH_4)_2SO_4$ fractionation, gel filtration using Sephadex G-75 and ion-exchange chromatography on DEAE- and CM-cellulose each yielded products having no higher specific activity and as many or more protein components than boiled dialyzed venom, isoelectric precipitation and heat inactivation were chosen for the starting treatment of crude venom.

Isoelectric precipitation and boiling. The phospholipase A activity of *A. piscivorus* venom was stable when subjected first to an isoelectric precipitation at pH 5.9 and then to a boiling water bath for 5 min (Table I). Isoelectric precipitation served to remove all particulate material converting the initially slightly turbid venom into a clear yellow solution. Boiling served to remove large amounts of contaminating protein and improved the stability of phospholipase A activity. Whole venom turned brown and gave off an odor of putrifaction, on storage at 2°, while the boiled fraction remained stable at 2° for several months.

Hydroxylapatite column chromatography. Elution of the fraction obtained after boiling from hydroxylapatite with 0.15 M KH_2PO_4–K_2HPO_4 (pH 6.8) results in the protein profile seen in Fig. 1. The first peak to be eluted has no activity. The second peak (lined area) shows phospholipase A activity and gel electrophoresis showed that it was composed of two protein bands both of which migrated toward the cathode (Fig. 3, Tubes 3).

Preparative acrylamide electrophoresis. Fig. 2 shows the elution pattern obtained when preparative acrylamide electrophoresis of the concentrated active fraction from hydroxylapatite was performed. Analytical acrylamide electrophoresis and



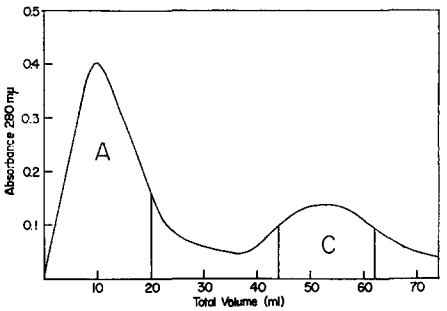
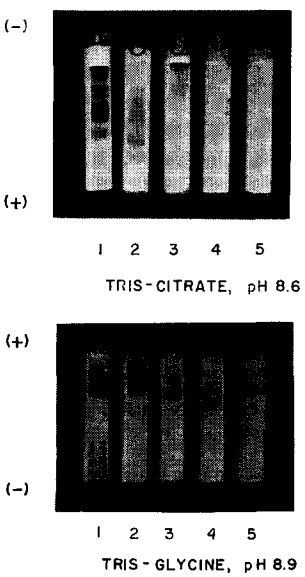


Fig. 1. Protein profile of eluate from hydroxylapatite column after elution of the venom fraction obtained after boiling with 0.15 M KH_2PO_4–K_2HPO_4 (pH 6.8). The second peak, represented by the lined area, shows phospholipase A activity.

Fig. 2. Typical protein profile obtained after preparative acrylamide electrophoresis of the active fraction eluted from hydroxylapatite. Electrophoretic conditions described under METHODS.

activity assay was performed on eluate fraction before pooling to insure the best possible separation.

Analytical acrylamide electrophoresis was performed at each stage of the purification procedure (Fig. 3). The upper gels, made in Tris–citrate buffer (pH 8.6) were run toward the anode. The lower five gels made in Tris–glycine buffer (pH 8.9) were run toward the cathode. The two patterns marked (1) were obtained with whole, fresh venom. The patterns marked (2) show the protein bands remaining after iso-



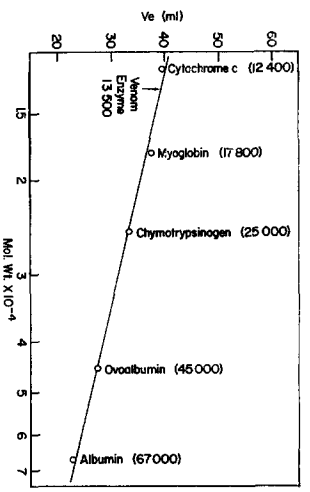


Fig. 3. Analytical acrylamide electrophoresis showing purification achieved at each step of the fractionation of phospholipase A from *A. piscivorus* venom. Electrophoresis was carried out at 4° for 1 h. Gels were stained with Amido schwarz 10B.

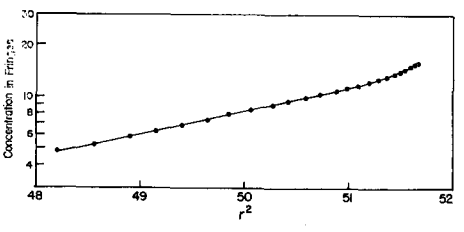
Fig. 4. Estimation of molecular weight of *A. piscivorus* phospholipase A by gel filtration on Sephadex G-75. Elution volume (V_e) plotted as a function of the logarithm of molecular weight. Conditions of the experiment described in METHODS.

electric precipitation and boiling. The tubes marked (3) show that the active fraction from hydroxylapatite has only two protein bands. Tubes marked (4) and (5) show the electrophoretic pattern of the proteins in Bands A and C, respectively, after preparative acrylamide electrophoresis. The protein in each of these Tubes 4 and 5 migrates toward the cathode as a single homogeneous band, with the protein in Band A appearing more positively charged since it migrates further toward the cathode. When the activities of the protein in Band A and the protein in Band C were tested on synthetic L-α-phosphatidyl choline (dipalmitoyl) it was apparent that the specific activity of the protein in Band A was about 7-fold higher (Table I). The protein in Band A was used for all further enzymatic studies.

Table I summarizes the steps in the purification procedure together with the degree of purification achieved. Crude *A. piscivorus* venom appears to exhibit a low phospholipase A activity, *i.e.*, 5 units/mg protein when compared to that of crude *C. adamanteus* venom, *i.e.*, 100 units/mg protein[18] but is equal in activity to *C. atrox* venom[19]. The total purification achieved is 12.5-fold compared to 14-fold for *C. adamanteus*[18] and 35-fold for *C. atrox* venom[19]. However, any attempt to correlate the results of several recently reported purifications of phospholipase A (refs. 18, 19, 27) should also consider variations in electrophoretic patterns due to storage conditions[28], variations in the conditions under which activity was assayed[19] and varied exposure to preparative procedures[19,28]. In addition several workers[16,19,29,30] have shown that in organic environment, *e.g.* chloroform and/or ether, both the rate and extent of phospholipase A hydrolysis is increased while in highly aqueous environments (such as that employed with *A. piscivorus* phospholipase A) it is decreased unless the substrate is highly dispersed[19]. Similar initial phospholipase A activities and yields were obtained in eight preparations using different pools of fresh venom, the data presented in Table I are representative.

Molecular weight of the enzyme

The results obtained by the gel filtration method are given in Fig. 4 which shows a plot of the elution volume (V_e) obtained for the standards and for *A. piscivorus*



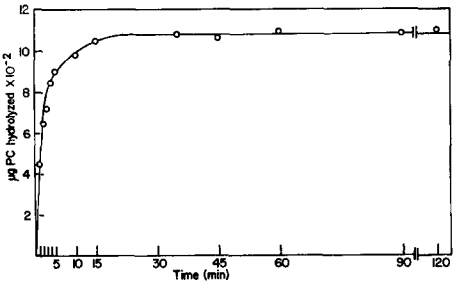


Fig. 5. Sedimentation equilibrium plot for *A. piscivorus* phospholipase A. A 0.23% solution of phospholipase A in 0.02 M glycine – NaOH buffer (pH 8.0) containing 0.1 M NaCl (ionic strength, 0.1) was centrifuged at 17 976 rev./min for 48 h at 3°.

Fig. 6. Time-course of hydrolysis of synthetic L-α-phosphatidyl choline (dipalmitoyl) (PC) by *A. piscivorus* phospholipase A. The reaction was carried out using the modified hydroxamate assay described in METHODS. The reaction mixture contained: 1.0 ml 0.05 M Tris–HCl buffer (pH 7.4); 0.3 ml 0.034% sodium deoxycholate; 0.1 ml ether; 1500 µg phosphatidyl choline and 6 µg phospholipase A. Complete hydrolysis = 1500 µg phosphatidyl choline.

phospholipase A against the logarithm of the molecular weight. The molecular weight of *A. piscivorus* phospholipase A was estimated to be 13 500 ± 250.

Sedimentation equilibrium analysis on extensively dialyzed solutions of *A. piscivorus* phospholipase A were carried out. Fig. 5 shows a plot of the concentration of *A. piscivorus* phospholipase A (expressed in fringes) as a function of the square of the distance from the center of rotation. The plot is linear over almost the entire length indicating that the *A. piscivorus* phospholipase A is homogeneous. The slight upward curvature near the end of the plot indicates the presence of trace amounts (less than 5%) of higher-molecular-weight material at the bottom of the cell. This material could be either a denatured form of *A. piscivorus* phospholipase A or a small amount of a higher-molecular-weight contaminant.

Since the partial specific volume of *A. piscivorus* phospholipase A was not known, calculations of the molecular weight were made for three assumed values of $\bar{v}$: *i.e.* $\bar{v} = 0.70$, M_{app} 13 361; $\bar{v} = 0.72$, M_{app} 14 326; and $\bar{v} = 0.74$, M_{app} 15 514 ($\bar{v}_{aa}$ for *C. adamanteus* phospholipase A 0.718 (ref. 18)).

Enzyme activity studies

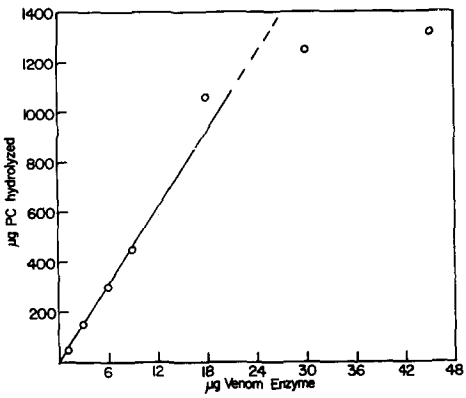
Fig. 6 illustrates a time-course of hydrolysis of synthetic phosphatidyl choline by *A. piscivorus* phospholipase A. It is apparent that although the reaction proceeds very rapidly, 50% of the reaction being complete in the first 2 min, it does not go to completion even after 120 min incubation time. Thin-layer chromatographic analyses performed at each of the time points illustrated in Fig. 6 also showed that although the size of the phosphatidyl choline spot decreased while the sizes of lysophosphatidyl choline and palmitic acid spots increased during the course of the reaction, the substrate spot was still visible after 120 min incubation.

The reaction rate of this phospholipase A appears to increase linearily with enzyme concentrations at low concentrations, however, even at high enzyme concentrations, the reaction does not go to completion (Fig. 7).

An attempt was made to determine if incomplete hydrolysis was due to an inhibition of the enzyme during the course of the reaction or unavailability of the residual substrate. If hydrolysis of phosphatidyl choline by phospholipase A was allowed to proceed until the rate approached zero (15 min) and another aliquot of phospholipase A was added about 10% of the original hydrolysis occurred over the next 15-min period (Fig. 8).

In a similar experiment, an aliquot of substrate was added after the hydrolysis of phosphatidyl choline by phospholipase A had proceeded for 15 min. The hydrolysis during the next 15 min was equal to that in the first 15-min interval (Fig. 9), indicating that the phospholipase A initially present had not become inactivated.

Phospholipase A from several venoms has been shown to be activated by the presence of Ca^{2+} in the reaction mixture[31–33]. Table II shows that when Ca^{2+} (added as $CaCl_2$ in the range 0.05–2.0 mM) was added to reaction mixtures containing *A. piscivorus* phospholipase A no stimulation occurred and instead the enzyme appeared to be inhibited. Although these results cannot be considered to be conclusive, they are supported by the results of neutron activation analysis of the synthetic phosphatidyl choline solution[34]. The substrate solution contribution of Cd^{2+}, which also activates phospholipase A (ref. 19), and which is a possible contaminant in synthetically prepared phosphatidyl choline, is less than 3 nM in the reaction mixtures used.



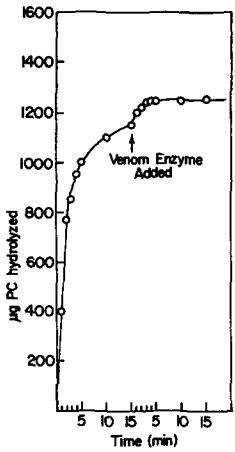
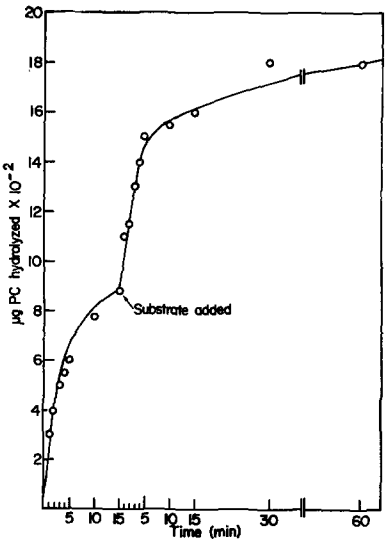


Fig. 7. The effect of increasing concentrations of *A. piscivorus* phospholipase A on the hydrolysis of synthetic L-α-phosphatidyl choline (dipalmitoyl) (PC). Reaction conditions as described in Fig. 6; complete hydrolysis = 1500 μg phosphatidyl choline, total hydrolysis time, 5 min.

Fig. 8. The effect of an additional aliquot of *A. piscivorus* phospholipase A on the extent of hydrolysis. At zero time the reaction mixture was as described in Fig. 7, after 15 min reaction time an additional 6 μg phospholipase A were added. Complete hydrolysis = 1500 μg phosphatidyl choline (PC).



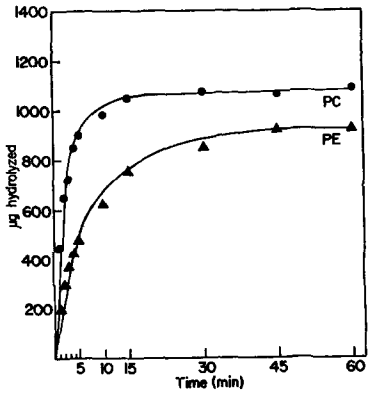


Fig. 9. The effect of an additional aliquot of substrate on the extent of hydrolysis. At zero time the reaction mixture was as described in Fig. 7, after 15-min reaction time an additional 1500 μg of phosphatidyl choline (PC) were added. Complete hydrolysis = 3000 μg phosphatidyl choline.

Fig. 10. Time-course of hydrolysis of phosphatidyl ethanolamine (PE) by *A. piscivorus* phospholipase A compared with time-course of hydrolysis of synthetic L-α-phosphatidyl choline (PC). The reaction conditions were as described in Fig. 7 except that ether was omitted when phosphatidyl ethanolamine was the substrate. Complete hydrolysis = 1500 μg phosphatidyl choline or phosphatidyl ethanolamine.

TABLE II

EFFECT OF Ca^{2+} ON THE ACTIVITY OF PHOSPHOLIPASE A

Reaction mixture: 1.0 ml 0.05 M Tris–HCl buffer (pH 7.4); 0.3 ml 0.034% sodium deoxycholate; 0.1 ml ether; 1500 μg synthetic L-α-phosphatidyl choline and 6 μg phospholipase A.

$CaCl_2$ conc. (mM)	*Phosphatidyl choline hydrolyzed (μg)**
—	1000
0.05	150
0.10	124
0.20	150
0.40	400
0.50	500
1.00	300
1.50	74
2.00	74

* Complete hydrolysis = 1500 μg; total hydrolysis time, 5 min.

Fig. 10 shows the time-course of hydrolysis with *A. piscivorus* phospholipase A when phosphatidyl ethanolamine replaces phosphatidyl choline as substrate. Phosphatidyl ethanolamine is less rapidly and completely hydrolyzed than phosphatidyl choline under these reaction conditions, and the reaction appears to consist of a rapid initial phase and a slower secondary phase.

DISCUSSION

Sedimentation equilibrium, molecular weight determination by gel filtrations and electrophoretic data indicate that a phospholipase A component of *A. piscivorus* venom has been isolated in relatively homogeneous form. Gel filtration experiments, suggest that the isolated phospholipase A has a molecular weight of 13 500 ± 250. Sedimentation equilibrium studies indicate a molecular weight range of 13 300–15 400 depending on the value of $\bar{v}$ chosen. Recently DE HAAS *et al.*[27] reported the isolation of a phospholipase A from porcine pancreas having a molecular weight of about 13 800 ± 500. From the known amino acid composition of this phospholipase A they calculated a partial specific volume of 0.71 cm^3/g. Using this value the calculated molecular weight of *A. piscivorus* phospholipase A is 13 844. The value obtained assuming a $\bar{v}$ of 0.72 cm^3/g (for *C. adamanteus*, 0.718) is 14 326 and is slightly larger than the value obtained by the gel filtration method. Thus the molecular weight of this enzyme is close to that found by DE HAAS *et al.*[27] for porcine phospholipase A, and the molecular weight of 14 500 ± 500 found by WU AND TINKER[19] for *C. atrox* phospholipase A.

In contrast to these findings, WELLS AND HANAHAN[18] have reported that two proteins with phospholipase A activity purified from *C. adamanteus* venom have molecular weights of about 30 000. In addition these proteins appear acidic in nature whereas the phospholipase A from *A. piscivorus* venom appears to be a basic protein. The significance of these variations in molecular weight and charge, in terms of the source of the enzyme or its reactivity is not immediately apparent.

Purified *A. piscivorus* phospholipase shows several properties classically attributed to phospholipase A. The enzyme has a high heat stability as no loss in activity occurs after 5 min in a boiling water bath (pH 5.9; 0.25 M KH_2PO_4–K_2HPO_4). Thin-layer chromatographic analysis using chromatographically pure synthetic phosphatidyl choline (dipalmitoyl) as the substrate shows that the purified enzyme, at pH 7.4, hydrolyzes a single ester linkage of the phosphatide liberating lysophosphatidyl choline and free palmitic acid. Hydrolysis of an acyl ester bond is also indicated by the decrease in hydroxamate formation upon incubation of the substrate with the isolated *A. piscivorus* enzyme. The purified phospholipase A also hydrolyzes phosphatidyl ethanolamine. It is active in the presence of both ether and sodium deoxycholate. Ca^{2+} does not appear to be necessary for activity, which may be because of the positive charge on the enzyme protein[18]. Indeed, the data for the addition of Ca^{2+} suggest that some minor component in the $CaCl_2$ solution might be an activator for the reaction.

The purified enzyme also has the ability to uncouple and/or inhibit mitochondrial respiration[35].

The nature of the venom protein in Band C, however, is not immediately apparent and the data presently available are insufficient for any conclusive statements to be made.

Purified *A. piscivorus* phospholipase A failed to hydrolyze the substrate completely under the reaction conditions used even in the presence of large amounts of enzyme. The experiments presented in Figs. 8 and 9 indicate incomplete hydrolysis cannot be attributed to an inactivation of the enzyme, since the addition of a second aliquot of the enzyme has little effect, while addition of a second aliquot of substrate does initiate further hydrolysis. These results suggest that the availability of the substrate limits the extent of hydrolysis. Support for this conclusion comes from the studies of VAN DEENEN AND DE HAAS[36], PERRIN AND SAUNDERS[37], GAMMACK *et al.*[38] and ATTWOOD *et al.*[39] who found that the fatty acid components of phosphatides determine not only the ability of the substrate to form micelles but also the shape of the micelles. The asymmetrical, elongated micelles are more susceptible to attack by hydrolytic enzymes than symmetrical micelles. HUGHES[40] showed that the surface potential of a unimolecular film of lysophosphatidyl choline was only three-fifths that of a phosphatidyl choline film. The above findings, along with those of WU AND TINKER[19] on the effect of dispersion of substrate, strongly suggest that incomplete hydrolysis observed with *A. piscivorus* phospholipase A results from differences in the physical state of the substrate during hydrolysis.

The bisphasic nature of the reaction seen when phosphatidyl ethanolamine replaces phosphatidyl choline as the substrate may result from a decrease in accessibility of reaction sites on the substrate micelles due to an accumulation of liberated fatty acids[16,29], since no ether could be added to the reaction mixture because marked turbidity developed in the presence of ether. Ultimate correlations on a molecular level will depend on the results of future kinetic and physico-chemical experiments.

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