Phospholipase A_2 from *Crotalus atrox* Venom. I. Purification and Some Properties*

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ABSTRACT: Phospholipase A_2 (phosphatide acylhydrolase, EC 3.1.1.4) has been purified from *Crotalus atrox* (Western diamondback rattlesnake) venom using ammonium sulfate fractionation followed by Sephadex G-75 column chromatography. The active protein, appearing as one band on polyacrylamide gel disc electrophoresis at different pH values (4.5–8.4), is eluted from Sephadex G-75 with a constant specific activity 35 times that of the crude venom, and has no detectable protease, phosphodiesterase, or monoesterase activity. Using rat liver lecithin of known composition of fatty acids at the 1 and 2 positions, the positional specificity of the purified enzyme has been established by the exclusive release of 2-position fatty acids, which are largely unsaturated. The enzyme acts on ovolecithin in ethereal or chloroform solutions or in ultrasonicated aqueous dispersions with complete hydrolysis to lysolecithin plus fatty acids. Michaelis–Menten kinetics are apparently applicable in the ether and chloroform systems, the K_m values being 8.3 and 8.5 mM, respectively. The molecular weight, determined by Sephadex G-75 chromatography, appears to be in the range 14,500 ± 500. In contrast, the enzyme from the venom of the closely related snake *Crotalus adamanteus* has molecular weight about 30,000 (Saito, K., and Hanahan, D. J. (1962), *Biochemistry 1*, 521). Moreover, only one isozymic form appears to exist in *C. atrox* venom, while two isozymes are found in *C. adamanteus*. In ultrasonicated substrate dispersions, the enzyme has a broad pH optimum between 6.8 and 7.6 and a sharp temperature optimum at 46°. Against common belief that calcium ions alone can activate the enzyme, the purified phospholipase A_2 is activated to varying degrees by Ca^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+}, and Cd^{2+} but not by Hg^{2+}, Zn^{2+}, Cu^{2+}, or Ba^{2+} ions. At fixed Ca^{2+} ion concentration, increasing concentrations of Zn^{2+}, Cu^{2+}, and Ba^{2+} produce progressive inhibition, suggesting possible competition between these ions and calcium. The optimal calcium concentration for activity is 0.02 M.

The protective role of EDTA in venom solutions has been shown to be attributable to chelation of trace amounts of inhibitory divalent cations. Unlike phospholipase A_2 from other sources, the *C. atrox* enzyme is completely inactivated by diisopropylphosphoryl fluoride and iodoacetate. *O*-Methyliosourea also inactivates the enzyme; however *p*-mercuribenzoate and mercaptoethanol produce little inhibition. The purified phospholipase A_2 withstands heating to 80° for 30 min at pH 3.0, but similar treatment at pH 7.4 immediately inactivates it. Lyophilization also destroys the enzyme activity, which is slowly regained upon dissolution in aqueous buffer, after a lag phase of several hours.

The phospholipase A_2 (EC 3.1.1.4) of snake venoms catalyzes the hydrolysis of 1,2-diacyl-3-*sn*-glyceryl phosphatides, liberating the fatty acid from the 2 position (Hanahan *et al.*, 1954; Tattrie, 1959; DeHaas *et al.*, 1960; DeHaas and Van Deenen, 1961). This enzyme possesses several interesting properties, such as heat stability (Saito and Hanahan, 1962) and capacity to act in nonaqueous systems (Hanahan, 1952) and at the surface of lipid particles (Dawson, 1963a) and monolayers (Hughes, 1935; Colacicco and Rapport, 1966; Shah and Schulman, 1967). A review of the literature on this enzyme prior to 1965 has been presented (Condrea and DeVries, 1965).

The structure and mechanism of action of phospholipase A_2 would seem to merit increased study, and to this end several groups have undertaken its isolation from various sources in recent years. The most successful purifications seem to be those of Hanahan and coworkers (Saito and Hanahan, 1962; Wells and Hanahan, 1969), who employed *C. adamanteus* venom as the source, of DeHaas *et al.* (1968) from pancreatic juice, and of Habermann and Reiz (1965) from bee venom.

In the present report, we shall describe a two-step method leading to the isolation of a highly purified phospholipase A_2 preparation from *C. atrox* venom. Some of the properties of this enzyme, a number of which are at variance with previous concepts, are also described. Among other reasons, *C. atrox* venom has been selected for the present study since (a) to the author's knowledge the enzyme had not previously been isolated in high purity from this source, (b) only a few studies involving this venom in the crude state have been reported (Marinetti, 1965; Gottfried and Rapport, 1962; Colacicco and Rapport, 1966), and (c) results obtained with the purified enzyme may be compared with those from studies of the enzyme of *C. adamanteus*, a close taxonomic neighbor, whose venom enzyme is probably the most intensively investigated and best documented, both in the crude and purified states.

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Experimental Section

Materials. Lyophilized *C. atrox* venom was purchased from the Ross Allen Reptile Institute, Silver Springs, Fla., and stored at −20° in the dry state prior to use. Phosphatidylcholine (lecithin) was isolated from fresh egg yolks as described by Tinker and Saunders (1968). Analyses of the preparations gave phosphorus contents of 3.80–3.85% and nitrogen:phosphorus molar ratios of 0.98–1.01. The material appeared homogeneous after thin-layer chromatography on silica gel G, using chloroform–methanol–water (95:35:4, v/v) as eluent. The fatty acid composition, as determined by methanolysis (Morgan *et al.*, 1963) and gas–liquid partition chromatography, was not significantly different from that reported by Kuksis and Marai (1967).

Rat liver phosphatidylcholine was the gift of Dr. A. Kuksis; this was subsequently further purified by passage through an aluminium oxide column.

Highly purified insulin was the gift of Dr. C. C. Yip. This material appeared as one sharp peak after chromatography on Sephadex G-75. Aqueous suspensions of beef liver catalase (twice crystallized) and cytochrome C (twice crystallized) were obtained through the courtesy of Dr. A. Menon. Ribonuclease A (six times crystallized) was a generous gift from Mr. C. Kan.

Sephadex media (bead form) were purchased from Pharmacia Ltd., Uppsala, Sweden, and prepared for use as prescribed by the manufacturer.

Analytical and Chromatographic Methods. Protein was determined both by its absorbance at 280 mμ and by the method of Lowry *et al.*, (1951) using six times-crystallized bovine serum albumin as a working standard. Phosphorus was determined by a modification of the method of Bartlett (1959). Nitrogen was determined by the method of Long (1952) or by a micro-Kjeldahl technique.

Disc electrophoresis was carried out on polyacrylamide gel by a slight modification of the technique of Davis (1964). The buffer solutions described by that author for the reservoirs were diluted fivefold before use, and the bromophenol blue was 0.005% instead of 0.001%. Molecular weight of the purified enzyme was determined using Sephadex G-75 chromatography as described by Whitaker (1963). Column chromatography on Sephadex media was performed at 4°, using columns fitted with flow adapters (purchased from Pharmacia Ltd., Uppsala). Flow rate was maintained using a peristaltic pump.

In the assay of phospholipase A_2 activity, free fatty acids were estimated by titration in methanolic solution with 0.02 N methanolic sodium hydroxide, using cresol red as indicator. The sodium hydroxide solution was prepared and standardized each day against 0.1 N potassium acid phthalate (British Drug House Microanalytical Grade). All solvents used in the phospholipase assays were frequently redistilled, gassed with nitrogen, and stored in bottles fitted with carbon dioxide traps. This was found to be necessary in order to keep the blank values low. Protease activity was measured by the assay technique of Kunitz (1947), and 5′-nucleotidase by the procedure of Bjork and Porath (1959). Phosphodiesterase activity was determined by the method of Cheung (1967).

Methods for hydrolysis of phospholipids, recovery and esterification of released fatty acids, and analysis of fatty acids by gas–liquid partition chromatography are as described by Kuksis *et al.* (1968).

Assay of Phospholipase A_2. Four different assay systems have been used in this work; a description of each follows. Assay 1 (ethereal system) is a slight modification of that used by Hanahan *et al.* (1954). The enzyme preparation is dissolved in 0.005 M sodium phosphate buffer (pH 7.4) or 0.05 M Tris buffer[1] (pH 7.4) containing 0.02 M $CaCl_2$ and 0.001 M EDTA. Crude venom (10–20 μg) or purified enzyme (0.5–2.0 μg) in 20 μl of buffer is added to the substrate solution (28 mg of egg phosphatidylcholine in 2 ml of redistilled diethyl ether). After incubation in a nitrogen atmosphere at room temperature for a specified time the reaction was stopped by the addition of 3 ml of methanol, and the fatty acids were titrated using a 0.5-ml Agla micrometer syringe buret accurate to ±0.0001 ml. A slow stream of nitrogen was bubbled through the solution during the titration, serving to stir the mixture and maintain a carbon dioxide free atmosphere. Under these conditions the blank titers were 0.002–0.003 ml (0.08–0.12 μequiv of base). The assay mixtures containing hydrolyzed substrate required at least 0.1 ml of titrant (2 μequiv of base).

Assay 2 (chloroform system) is similar to assay 1 except that chloroform containing 2% methanol was used instead of ether.

Assay 3 (aqueous Tris system) is similar to the above systems except that the substrate solution is an ultrasonically irradiated phosphatidylcholine sol in 0.05 M Tris buffer, prepared essentially according to Dawson (1963b). The enzyme is added in Tris buffer.

Assay 4 (aqueous phosphate system) is essentially that used by Levene *et al.* (1924) and Chargaff and Cohen (1939). The enzyme and substrate were incubated in the phosphate buffer described under assay 1 (above). The lipid dispersion was not treated with ultrasonic radiation. Appropriate blanks were run in the case of all assay systems, and these showed negligible acidity released in the absence of enzyme. In all of the above systems, 1 unit of enzyme activity is defined as the amount of enzyme which hydrolyzes 1 μmole of egg phosphatidylcholine/min under conditions where zero-order kinetics applies. Specific activity is expressed in enzyme units per milligram of protein.

Ultrasonic treatments were carried out where indicated at 20 kc and cavitating intensity for 30 min using a sonic generator manufactured by Measuring and Scientific Equipment Ltd., London, England. A 0.25-in. titanium probe was employed and the lipid dispersions were maintained at 4° under a nitrogen atmosphere. Sols were centrifuged at 10,000*g* for 30 min, after ultrasonic treatment.

[1] Unless otherwise noted, Tris buffer refers to 0.05 M Tris-hydrochloride (pH 7.4) containing 0.02 M CaCl and 0.001 M EDTA.

TABLE I: Summary of Purification.

Steps	Protein[a]		Specific Activity[a]		Total Activity[a]		No. of Bands after Disc Electrophoresis
	mg	% of Original	Units/mg of Protein	Rel to Venom	Units	% of Original	
Crude dried venom	5,000	100	5.2 (4.9–6.0)	1.0	26,000	100	8 or more
Fraction after salt fractionation	140	2.8 (0.6–5.0)	28.6 (23.4–33.8)	5.5	4,004	15.4 (4.1–19.3)	3–5
Fraction from Sephadex G-75 chromatography	24	0.48 (0.36–0.6)	182.0 (110–252)	35.0	4,368	16.8 (8.1–25.2)	1

[a] Data in brackets are the range of results obtained in ten different preparations using various quantities of crude venom (normalized to give figures equivalent to a preparation using 5 g of venom). The data not enclosed in brackets are means of the ten sets of results.

Enzyme Isolation. The purification procedure involves essentially two steps: ammonium sulfate fractionation and Sephadex G-75 column chromatography.

Lyophilized *C. atrox* venom is dissolved in the Tris buffer described under assay 1 (above) to give a concentration of 20 mg/ml. The insoluble material, containing no phospholipase activity, is removed by centrifugation at 28,000*g* for 30 min. The salt concentration of the solution is then increased from 0 to 70% saturation in 5% steps, correction being made for volume changes at each stage as described by Dixon (1953). (In later preparations, this procedure has been shortened and the following ammonium sulfate cuts used: 0–20, 20–25, 25–35, 35–40, and >40). The mixture is centrifuged at 28,000*g* for 30 min after each step; aliquots of the supernatant are suitably diluted and assayed for protein and enzyme activity. The precipitate is dissolved in 2 ml of Tris buffer and similarly assayed. The temperature is maintained at 4° throughout the fractionation unless otherwise noted.

The precipitates containing enzyme activity are dissolved in Tris buffer (final concentration 1 mg of protein/ml) and freed of ammonium sulfate by passage through a Sephadex G-25 column in Tris buffer. Toluene is added to the enzyme solution (final concentration 10 μl/ml; toluene is necessary at all subsequent stages to preserve activity and inhibit bacterial growth) and the solution chromatographed on a column of Sephadex G-75 (column size 90 cm × 1.5 cm i.d., flow rate 14.5 ml/hr) using Tris buffer (containing toluene) as elunt. Fractions of 3 ml are collected, and the fractions containing the enzyme peak pooled. The latter is rechromatographed on Sephadex G-75 under the same conditions.

The purified enzyme is stored frozen in Tris buffer at −20°, in which its activity remains constant for about 1 week; thereafter, the activity declines gradually and steadily.

Results

Ammonium Sulfate Fractionation. Figure 1 shows the fractionation of *C. atrox* venom by stepwise increase in ammonium sulfate concentration. At 4°, the enzyme activity is localized in a small protein peak precipitating between 25 and 35% saturation, as shown by the specific activity profile. The fractionation is quite temperature sensitive and if the temperature is increased the enzyme peak merges with the protein precipitating above 35% saturation. Figure 1 shows a fractionation performed at 15°, which illustrates this phenomena.

A drastic loss of total enzyme activity occurs after salt fractionation of the crude venom as is evident in Table I.

Sephadex G-75 Chromatography. Figure 2 shows the elution profile of the enzyme fraction from step 1 after chromatography on Sephadex G-75. Three major protein peaks are obtained; as shown in Figure 2, all peaks contain enzyme activity, the first two in minor amounts. The bulk of the enzyme is located in the third peak as shown in Figure 2; this material has a specific activity 34–35 times that of the crude venom, and on rechromatography yields one sharp peak with constant specific activity (Figure 3).

Amounts (1 mg) of material from all three fractions shown in Figure 2 were examined qualitatively for the presence of lipids by thin-layer chromatography on silica gel G, using either chloroform–methanol–water (95:35:4, v/v) or hexane–diethyl ether–glacial acetic acid (90:10:1, v/v) as solvents. No lipid was detectable in any fraction by this technique.

Table I gives a summary of the purification protocol, with yields and specific activities after each step. The range of results obtained in ten different preparations is given in Table I in order to provide an index of reproducibility of these results.

Criteria of Purity. The number of protein constituents in various fractions was estimated after micro zone disc electrophoresis as desctibed above. Figure 4 shows the electrophoretic patterns of crude venom (at least eight components), 25–35% ammonium sulfate fraction (two major components and at least one minor component), and the purified enzyme, after electrophoresis using the pH 8.4 system of Davis (1964). Using buffer systems of various pH values in the range 4.5–8.4, the purified enzyme reveals consistently a single protein band after electrophoresis. In Figure 4, band 3, there appears to

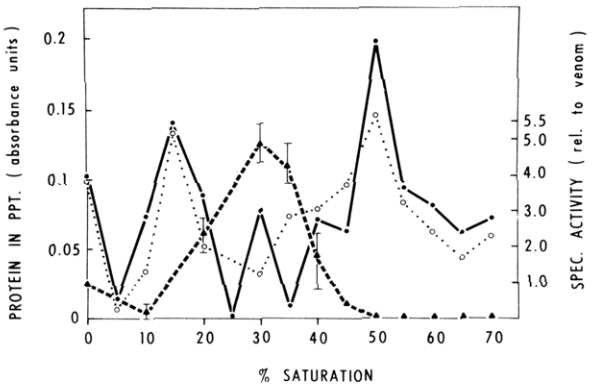

FIGURE 1: Ammonium sulfate fractionations of 5 g of *C. atrox* venom at 4 and 15°. Details as in text. (●——●) Protein profile, 4°. (○.....○) Protein profile, 15°. (▲------▲) Phospholipase A_2 specific activity profile, 4°. Vertical bars indicate absolute range of results from five similar fractionations.

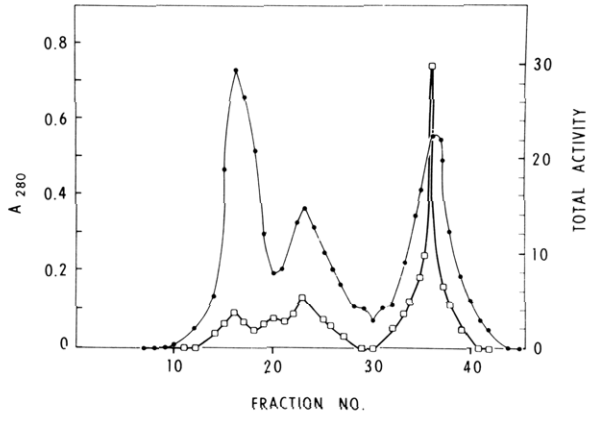

FIGURE 2: Chromatography of 25–30% ammonium sulfate fraction from *C. atrox* venom on Sephadex G-75. Column size 90 cm × 1.5 cm i.d., flow rate 14.5 ml/hr, temperature 4°, fraction size 3 ml. Load applied was 120 mg of protein. (●——●) Protein concentration, absorbance units. (□——□) Total phospholipase A_2 activity, in enzyme units per fraction.

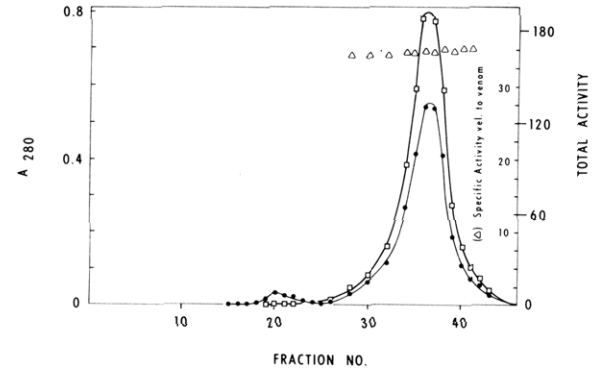

FIGURE 3: Rechromatography of major enzyme peak from Figure 2 on Sephadex G-75. Conditions as in Figure 2. (●——●) Protein concentration, absorbance units. (□——□) Total phospholipase A_2 activity in enzyme units per fraction. (△) Specific activity of protein relative to crude venom.

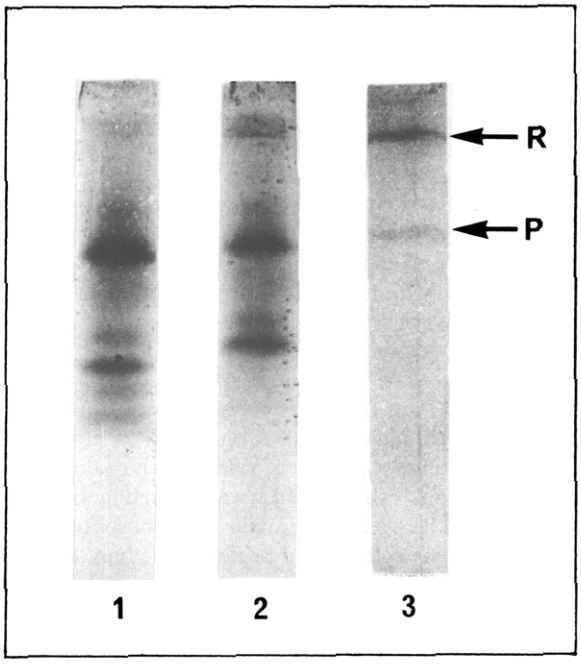

FIGURE 4: Polyacrylamide gel disc electrophoretograms of various protein fractions; stained with Amido Black. (1) *C. atrox* venom, (2) 25–35% ammonium sulfate fraction from *C. atrox* venom, and (3) purified phospholipase A_2 fraction after Sephadex chromatography. (R) Bromophenol blue reference marker. P indicates the position of the single protein zone containing phospholipase A_2 activity in band 3 (see text). The pH 8.4 system of Davis (1964), modified as described in the text, was employed. Conditions: 80–100 V/cm at 22° for 105 min, current 5 mA/tube. Point of sample application was at the bottom of the band in each case.

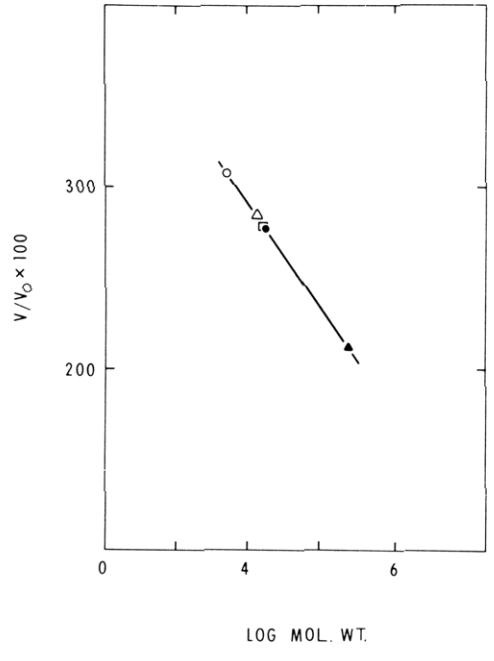

FIGURE 5: Molecular weight determination using Sephadex G-75 chromatography. V/V_0, retention volume relative to void volume. (○) Insulin, (△) cytochrome *C*, (□) ribonuclease A, (●) phospholipase A_2 from *C. atrox* venom, and (▲) beef liver catalase.

be a small amount of material moving ahead of the bromophenol blue reference, R. This was not always present; it may represent a minor contaminant in the preparation or it may be an artifact. At any rate, no protein or phospholipase A_2 activity could be detected in this region (see below).

Amounts (100 μg) of purified enzyme were subjected simultaneously to electrophoresis on 12 identical polyacrylamide columns (pH 8.4 system); of these three were stained to locate the protein bands; the remaining gels were sliced into 3-mm sections, and corresponding sections from the unstained gels pooled and homogenized in Tris buffer. After this extraction, the gel material was removed by centrifugation and the eluate passed through a small Sephadex G-25 column, using Tris buffer containing calcium chloride and EDTA. Material extracted from each section of the gels was then assayed for protein and phospholipase activity. Enzymic activity and protein were only found in areas of the gels corresponding to the zone-labeled P in Figure 4, band 3, the recovery of applied enzyme units being 50% or more.

Since it is well established that besides phospholipase A_2, snake venoms contain a variety of other enzymes, the crude venom and purified enzyme fraction were tested for three of the most prominent of these activities, *viz.*, protease, phosphodiesterase, and 5′-nucleotidase activity. The results indicated that the crude venom contains these activities, while the purified phospholipase A_2 fraction had no other detectable enzyme at the levels tested. Examination of the ultraviolet and visible spectrum of the preparations revealed the absence of any flavin components.

Molecular Weight Determination. A column of Sephadex G-75 was prepared and calibrated according to the manufacturer's specifications and the method of Whitaker (1963); Blue Dextran 2000 (purchased from Pharmacia Ltd., Uppsala) was used as a standard for determination of the void volume, v_0. Four different proteins (insulin, mol wt 5500; cytochrome *c*, mol wt 12,500; ribonuclease A, mol wt 13,700; and beef liver catalase, mol wt 24,000) serving as standards were applied to this column and the characteristic elution volumes, v, determined. A plot of the logarithm of molecular weight against v/v_0 was linear (Figure 5); the purified phospholipase A_2 from *C. atrox* venom had a value of v/v_0 of 2.76 $\pm$ 0.03, indicating a molecular weight of 14,500 $\pm$ 500 (average of five determinations).

Assay System and Kinetics. In the initial stages of this work, the assay system of Hanahan *et al.* (1954) was employed. This method exhibited several disadvantages in our hands; a lag period of several minutes sometimes occurred before hydrolysis of substrate commenced, necessitating numerous assays in order to ensure accuracy of activity measurements. Moreover, formation of a precipitate (lysolecithin) during the course of the incubations was accompanied by a decrease in hydrolysis rate. The latter phenomenon could be eliminated by the addition of 2% ethanol to the ethereal substrate mixture.

The chloroform assay system (assay 2) was introduced to obviate the above disadvantages, and in fact was employed for all the activity determinations reported in Table I. Two other systems were employed in certain cases. Assay 3 employed an ultrasonically treated lipid sol in Tris buffer, as substrate, while assay 4 employed a dispersion of phosphatidylcholine in phosphate buffer. Progress curves for hydrolysis of 7 μmoles of phosphatidylcholine by identical amounts of purified enzyme in the four systems are shown in Figure 6. The order of activities in the various systems was system 3 > system 2 > system 1 $\gg$ system 4. However, increased variability was noted in the extent of hydrolysis in system 3 at later times. The addition of ether to system 3 had no effect upon the hydrolysis rate. Progress curves were determined for periods of up to

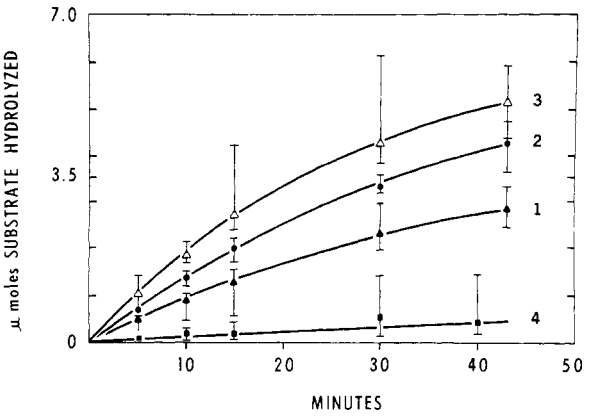

FIGURE 6: Progress curves for hydrolysis of 7 μmoles of egg phosphatidylcholine in the presence of 0.5 μg of purified phospholipase A_2. Assay systems: (1) ethereal, (2) chloroform, (3) aqueous Tris buffer, ultrasonicated, and (4) aqueous phosphate buffer, not ultrasonicated. Details of all assay systems are in text. Vertical bars indicate absolute range of results from ten identical experiments; points are means. Temperature, 22°.

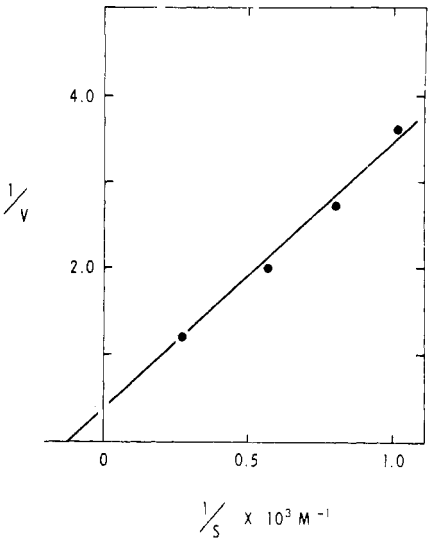

FIGURE 7: Dependence of initial velocity of substrate hydrolysis on substrate concentration in assay system 2 (chloroform). Data plotted according to Lineweaver and Burk (1934). Temperature was 22°. The apparent Michaelis constant, K_m, was 8.5 mM.

TABLE II: Fatty Acid Composition of Products of Phospholipase A_2 Hydrolysis of Rat Liver Lecithin.[a]

Fatty Acid[b]	Original Lecithin	α′-Acids[c]	β-Acids[d]	Reconstituted Lecithin[e]
14:0	0.5	0.8	1.0	0.9
15:0	0.2	0.4	0.1	0.3
16:0	17.5	30.5	4.1	17.3
16:1	1.4	1.2	1.5	1.4
18:0	18.8	47.8	2.5	25.2
18:1	13.3	13.9	12.5	13.2
18:2	15.6	2.2	27.3	14.8
20:1	0.1	0.2	0.2	0.2
20:2	0.5	2.9	0.8	1.9
20:3	2.4		4.4	2.2
20:4	24.2		39.9	19.9
20:5	Tr		0.1	Tr
22:0	0.7		1.4	0.7
22:3	0.1		0.5	0.3
22:4	0.7		0.4	0.2
22:5	0.4		0.7	0.4
22:6	3.5		5.0	2.5

[a] Results given in mole per cent; Tr signifies less than 0.1 mole %. See text for details of experiment. [b] Fatty acid notation: first number, number of carbon atoms; second number, number of double bonds. [c] Fatty acids of lysolecithin released by phospholipase A_2. [d] Free fatty acids released by phospholipase A_2. [e] Arithmetic average of third and fourth columns.

10 days in order to ascertain the maximum extent of hydrolysis obtainable. (Theory indicates a maximum of 1 mole of fatty acid released/mole of phosphatide). It was found that 100% hydrolysis to lysolecithin plus fatty acid could be achieved within 2 hr using assay systems 1–3. However in assay system 4, only 50% hydrolysis could be achieved even after prolonged incubation.

The effect of substrate concentration on initial velocity of the enzymic reaction in system 2 is shown in Figure 7. The data are plotted according to Lineweaver and Burk (1934). The reaction apparently follows Michaelis–Menten kinetics, with a K_m value of 8.5 mM. Similar results were obtained using the ethereal system (assay 1), the K_m value in this case being 8.3 mM. In routine assays using system 2, a substrate concentration of 18 mM (28 mg of phosphatidylcholine in 2 ml of chloroform) and an incubation of 10 or 15 min were employed. Under these conditions the release of fatty acids was linearly proportional to the amount of enzyme employed, in the range 0.5–2 μg of purified enzyme.

The rate of hydrolysis of substrate in the aqueous systems was independent of substrate concentration in the range of 10–50 mg/ml.

Proof of Site of Attack. Rat liver phosphatidylcholine (15 mg) was dissolved in 2 ml of diethyl ether containing 2% ethanol and incubated for 2 hr at room temperature with 2 μg of the purified phospholipase in 20 μl of Tris buffer containing calcium chloride and EDTA. The reaction was stopped by the addition of methanol and the products were examined by thin-layer chromatography. This revealed the complete absence of phosphatidylcholine and the presence of lysophosphatidylcholine and fatty acids. Titrations of identical hydrolysis mixtures run in parallel revealed the reaction to be 99.5% complete.

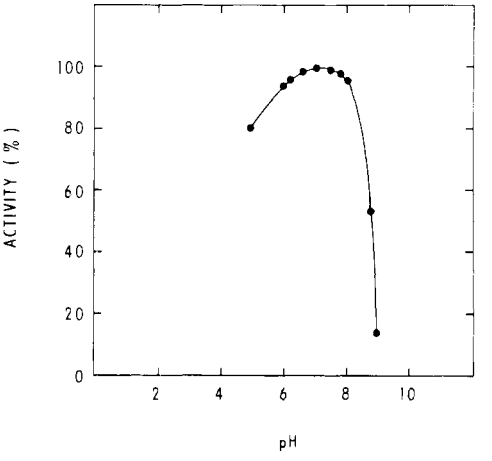

FIGURE 8: Effect of pH on initial velocity of substrate hydrolysis in assay system 3. Temperature, 22°. Activity is per cent of maximum velocity.

Recovery of the reaction products was accomplished by thin-layer chromatographic separation and the fatty acid compositions of the lysophosphatide, released fatty acid, and also of the original phosphatidylcholine were determined by gas–liquid partition chromatography as described by Kuksis *et al.* (1968). The results of these analyses, shown in Table II, indicated that virtually all the polyenoic fatty acids but very little of the palmitic and stearic acids of the original phosphatidylcholine were liberated by enzymic hydrolysis.

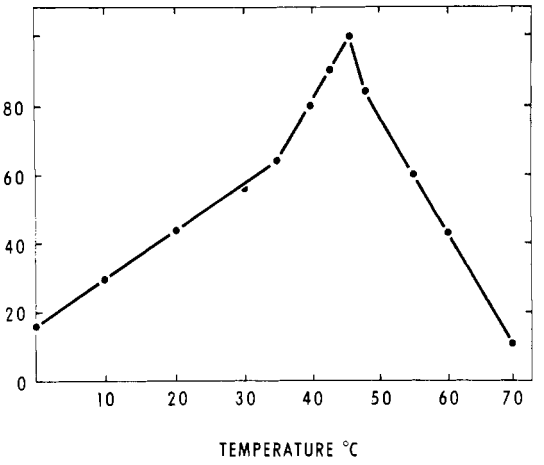

FIGURE 9: Effect of temperature on initial velocity of substrate hydrolysis in assay system 3. pH, 7.4. Activity is per cent of maximum velocity.

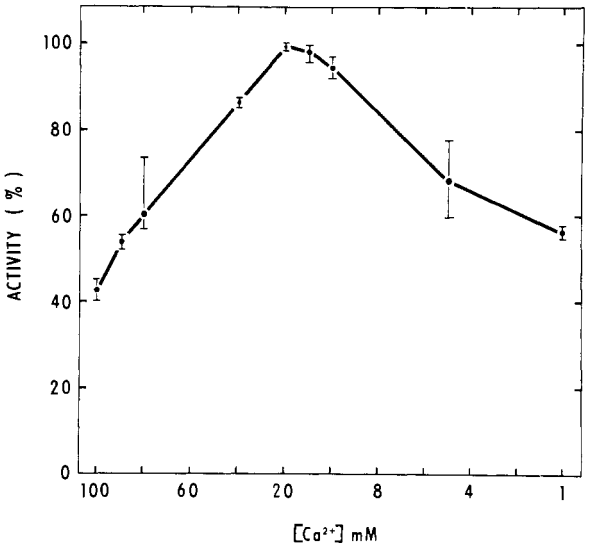

FIGURE 10: Effect of calcium ion concentration on initial velocity of substrate hydrolysis in assay system 3. pH 7.4; temperature, 22°. Activity is per cent of maximum velocity. Note reversal of abscissa scale.

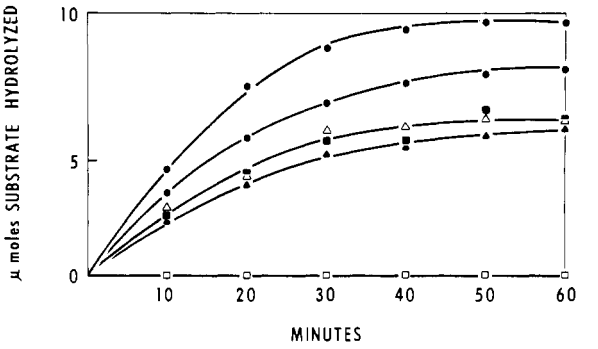

FIGURE 11: Effects of various divalent metal ion salts on progress curves of hydrolysis of 28 mg of egg phosphatidylcholine sonicated in 2 ml of Tris buffer. No EDTA was present and all metal ions were present alone at 0.02 M. (●——●, upper) $CaCl_2$, (●——●, lower) $NiCl_2$, (△——△) $CoCl_2$, (■——■) $MgCl_2$, (▲—▲) $CdCl_2$, and (□) $ZnSO_4$, $HgCl_2$, $CuSO_4$, $BaCl_2$, and no ions; 10 μg of phospholipase A_2 used in each case.

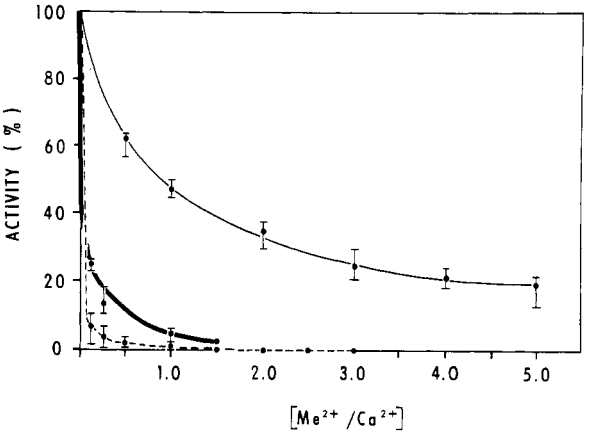

FIGURE 12: Effects of various metal ion salts on initial velocity of hydrolysis of 28 mg of egg phosphatidylcholine sonicated in 2 ml of Tris buffer containing 10^{-2} M $CaCl_2$ and no EDTA. Ordinate: ratio of added metal ion to calcium ion. (●——●) $BaCl_2$, (●▬▬●) $CuSo_4$, and (●------●) $ZnSO_4$; 10 μg of phospholipase A_2 used in each case. Vertical bars represent absolute range of results from ten identical experiments.

Temperature, pH, and Ionic Effects. The following experiments were performed using assay system 3 (aqueous), since the enzyme has been shown to be fully active in this system, and ionic concentrations could be more easily interpreted than in the nonaqueous systems.

The effects of pH and temperature upon the initial velocity of the enzyme-catalyzed reaction are shown in Figures 8 and 9; the enzyme exhibits a broad pH optimum between 7 and 8 and a sharp temperature optimum at 46°. The effects of calcium chloride concentration on the initial velocity are shown in Figure 10; an optimal calcium ion concentration of 0.02 M was observed. In the absence of any added divalent cations, the enzyme exhibited less than 5% of its optimal activity.

The effects of other divalent ions on the activity are shown in Figures 11 and 12. Figure 11 shows progress curves for the enzyme-catalyzed reaction in the presence of various divalent cations, all at 0.02 M. Two types of ionic effects were noted: activation (Ca^{2+}, Ni^{2+}, Co^{2+}, Mg^{2+}, and Cd^{2+}) and inhibition (Hg^{2+}, Zn^{2+}, Cu^{2+}, and Ba^{2+}). The inhibitory nature of the last three cations is more clearly shown in Figure 12, in which the reaction was carried out in the presence of a constant, suboptimal amount of calcium ion and varying amounts of zinc, barium, or copper ion.

Figure 13 shows the results of a time-course experiment designed to elucidate the role of EDTA. Purified enzyme was assayed in the presence of $CaCl_2$ (0.01 M) and $ZnSO_4$ (0.001 M) in 0.05 M Tris buffer (pH 7.4) containing no EDTA. Parallel control reactions were run in the presence of 10^{-2} M $CaCl_2$ only. After 10 min EDTA sufficient to give a final concentration of 10^{-3} M was added to the reaction mixtures containing $ZnSO_4$. As shown in Figure 13, no activity was noted

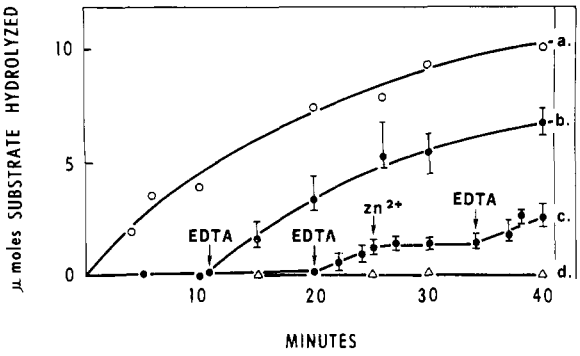

FIGURE 13: Effect of EDTA on progress curves of hydrolysis of egg phosphatidylcholine when an inhibitory metal ion (zinc) is present. (a) Control: assay 3, but with 10^{-2} M $CaCl_2$ and 10^{-3} M EDTA throughout incubation period. (b) Initially assay 3 with 10^{-2} M $CaCl_2$ and 10^{-3} M $ZnCl_2$; addition after 10 min: EDTA to 10^{-3}. (c) Initially as b, above. Additions: after 20 min, EDTA to 10^{-3} M; after 25 min, $ZnCl_2$ to 2×10^{-3} M; after 35 min, EDTA to 2×10^{-3} M. (d) Assay 3, but with 10^{-2} M $CaCl_2$ and 10^{-3} M $ZnCl_2$ in EDTA-free medium throughout incubation period.

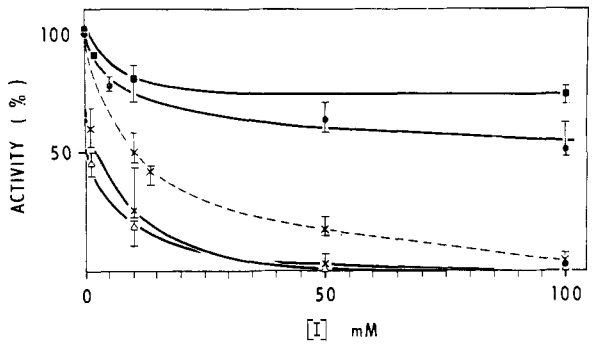

FIGURE 14: Effects of inhibitors on phospholipase A_2 activity. Amounts (1 mg) of the purified enzyme were incubated at 22° in the presence of various inhibitors (at the concentrations shown) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.02 M $CaCl_2$ and 10^{-3} M EDTA. After 30 min, aliquots of the solution containing 50 μg of enzyme were assayed in system 2. Activity, initial velocity as per cent of control. Vertical bars represent absolute range of results in ten identical experiments; points are means. Inhibitors: (■——■) mercaptoethanol, (●——●) *p*-mercuribenzoate, (x------x) *O*-methylisourea, (x——x) DFP, and (△——△) sodium iodoacetate.

in the presence of zinc ions prior to the addition of EDTA, after which almost maximal activity was restored. The activity can be shown to rise and fall with alternate additions of EDTA and additional $ZnSO_4$ as long as Ca^{2+} is in excess of both in concentration. An additional experiment (results not shown) showed that in the absence of calcium ion, EDTA has no activating effect whatsoever on the enzyme.

Effects of Inhibitors. Various reagents known to interact with side-chain functional groups in other types of proteins and enzymes were incubated with the purified phospholipase A_2 for 30 min, at a pH of 7.4 in the absence of substrate (but with calcium chloride and EDTA present). The enzyme was then assayed in assay system 2 in the presence of the inhibitor. The results are shown in Figure 14. Sodium iodoacetate, *O*-methylisourea, and diisopropyl fluorophosphate in moderate to high concentrations all produced complete and irreversible inhibition of the enzyme under these conditions. Mercaptoethanol and *p*-mercuribenzoate produced little inhibition.

Heat Stability, Denaturation, and Renaturation. The purified enzyme was completely stable to heating for 30 min at 80° at a pH of 3.0 At pH 7.4 heat treatment rapidly destroyed the activity. Other effective denaturing agents included ethanol and methanol. Dialysis of the pure enzyme against isotonic buffer, using dialysis tubing which retained the enzyme protein, resulted in a total loss of activity.

It was discovered at an early stage that lyophilization of the purified enzyme destroyed virtually all the catalytic activity. However, on dissolution of the lyophilized protein in buffer solution, almost total renaturation of the enzymic activity was ultimately achieved. Figure 15 shows the time course of a typical renaturation. A lag period of several hours was observed during which no regain of activity occurred; after the lag period, catalytic activity appeared suddenly and increased over a period of 10–12 hr to about

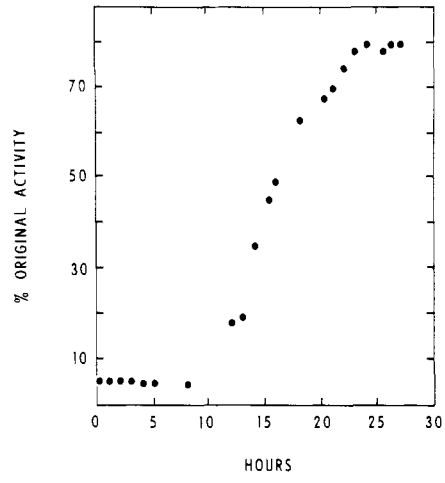

FIGURE 15: Regeneration of activity of lyophilized phospholipase A_2 on dissolution in 0.05 M Tris-HCl containing $CaCl_2$ and EDTA. Freshly lyophilized enzyme (10 mg) was dissolved in 10 ml of buffer and 10-μl aliquots were assayed in system 2 at various times. Ordinate is specific activity expressed as per cent of the preparation previous to lyophilization. Points are means of ten closely agreeing assays in each case.

80% of the activity of the enzyme before lyophilization. This observation was extremely repeatable, except that the lag period varied greatly in length.

Discussion

On the basis of several criteria it would appear that a significant purification of phospholipase A_2 from *C. atrox* venom has been achieved by the two-step method described. The preparation appears as a single, sharp protein band after disc electrophoresis at various pH values. Chromatography on Sephadex G-75 yields a

symmetrical peak with constant enzyme specific activity throughout, indicating uniformity with respect to size. Other enzyme activities present in the crude venom were not detectable in the pure fraction. In addition, ion-exchange chromatography on DEAE-cellulose (data not presented) has led to no resolution of the pure enzyme into more than one component; however, a drastic loss of total activity was observed after this treatment. Clearly, further physical and chemical data are required to assess the homogeneity of the preparation, and these experiments are currently under way. From the increase in specific activity over that of the crude venom, it would appear that the present preparation is the most highly purified phospholipase A_2 obtained from this source to date.

Proof that the enzyme isolated is in fact phospholipase A_2 comes from an examination of the products and stoichiometry of the reaction. Under optimal conditions, a maximum of 1 mole of fatty acid is released from each mole of phosphatidylcholine.

Studies using the well-documented phospholipase A_2 activity of *C. adamanteus* venom have shown that rat liver phosphatidylcholine contains predominantly saturated fatty acids (16:0 and 18:0) esterified at the 1 position, but unsaturated acids (in order of decreasing mole per cent: 20:4, 18:2, 18:1, 22:6, 20:5, and 16:1) at the 2 position (Menzel and Alcott, 1964; Hanahan *et al.*, 1964). This has been confirmed by Kuksis *et al.* (1968). It has indeed been shown (Table II) that the free fatty acids released by the *C. atrox* enzyme are largely polyunsaturated and in the same order of decreasing mole per cent as above, while the fatty acids remaining esterified in lysophosphatidylcholine are largely saturated. Arithmetic averaging of the fatty acid composition of the hydrolyzed and nonhydrolyzed fatty acids yields satisfactory agreement with the fatty acid composition of the original substrate; discrepancies between the original and reconstituted fatty acid compositions in Table II are of the order reported by Lands and Hart (1966). Most of the discrepancy arises from incomplete recovery of polyunsaturated components; if corrections for this were made, much closer agreement between the original and reconstituted values could be obtained.

Two features of the purification should be reemphasized. The first is the drastic loss of total activity following ammonium sulfate fractionations. This may be due to the loss of one or more proteins formerly associated with, and conferring stability upon, the phospholipase A_2. There have indeed been several reports (Ghosh and De, 1937; De, 1945; Yang, 1963) that snake venom phospholipase A_2 is closely associated with basic proteins, the removal of which by ion-exchange chromatography (Bjork, 1961) or salt precipitation (Pang, 1965) led to significant loss of total activity. Alternately, an activator or enzymically active low molecular weight substance may have been removed. The second feature of interest is the existence of minor amounts of phospholipase activity in fractions of higher molecular weight than the major enzyme fraction. These may represent complexes of the enzyme with higher molecular weight proteins; the possibility that the enzyme forms a reversibly associating monomer–polymer system seems unlikely since neither reassociation of the monomer nor dissociation of the higher molecular weight species occurs on rechromatography on Sephadex G-75. The possibility that the higher molecular weight species are lipoproteins seems to be excluded by the lack of detectable quantities of lipid in these fractions. There is no evidence for the existence of isozymes of phospholipase A_2 of closely similar molecular weight in *C. atrox* venom, as has been found to be the case in the venom of *C. adamanteus* (Saito and Hanahan, 1962).

The apparent molecular weight of the *C. atrox* enzyme has been found to be 14,500 ± 500 by Sephadex G-75 chromatography. In contrast the molecular weights of the two isozymes in *C. adamanteus* venom, as measured both by Sephadex chromatography and ultracentrifugal techniques, are close to 30,000 (Saito and Hanahan, 1962; Wells and Hanahan, 1969), or about twice the size of the *C. atrox* enzyme. The existence of this discrepancy in two closely related species raises interesting questions. It may be that the *C. atrox* enzyme represents a monomer of phospholipase A_2, while the *C. adamanteus* enzyme is a dimer. This could arise if gene duplication has occurred, producing an altered monomer which can form dimers either with "normal" monomer or with itself, giving rise to the two isozymes found. Such speculations are without experimental foundation at present, but suggest fruitful areas of exploration. It is interesting that the molecular weight of the *C. atrox* enzyme is in the same range as that of enzymes from other sources, *viz.*, the pancreatic enzyme, mol wt 13,800 ± 500 (DeHaas *et al.*, 1968), and the bee venom enzyme, mol wt 19,700 (Habermann and Reiz, 1965).

The activation of the *C. atrox* enzyme by numerous divalent cations is a novel and remarkable observation. It has long been felt and even unequivocally stated that calcium is the only ion which activates the snake venom phospholipases (Condrea and DeVries, 1965). To the authors' knowledge, only two reports mention activation of the enzymes by other ions, *viz.* Mn^{2+} in the case of the Habu venom enzyme (Maeno *et al.*, 1962) and Mg^{2+} in the case of the *Agkistrodon piscivorus piscivorus* enzyme (Marinetti, 1965). There has also been some controversy (caused by the use of impure enzymes or substrates containing endogenous calcium) as to whether the enzyme is active in the absence of calcium ion; we can unequivocally state that the *C. atrox* venom exhibits an obligatory requirement for divalent metal ions in its catalytic activity.

Concerning the role of calcium in the enzyme's action, it has been suggested (Roholt and Schlamowitz, 1961; Dawson, 1963a) that a Ca^{2+}–enzyme specific complex is formed prior to the adsorption of the enzyme to the substrate micelle surface. The Ca^{2+} would then serve as a specific bridge between the enzyme and substrate. A corollary of this view is that Ca^{2+} cannot be replaced by any other divalent cation. The present data rule out this hypothesis, and it seems more likely that divalent ions modify the active site of the enzyme to "fit" the substrate, without serving as a

specific bridge. Direct effects of Ca^{2+} on the surface properties of the substrate seem unlikely, since a recent study shows there is insignificant binding of Ca^{2+} to phosphatidylcholine monolayers below pH 10 (Joos and Carr, 1967), while above this pH, venom phospholipase A_2's exhibit little or no catalytic activity. The role of divalent ions in the action of this enzyme is currently under study in this laboratory.

Roholt and Schlamowitz (1961) have advanced the view that the activating effect of EDTA seen with crude venom enzymes is due to removal of traces of inhibitory heavy metals. The data of Figure 13 certainly favor this hypothesis, since in the presence of excess calcium ion, the catalytic action can be halted and restarted at will by the addition of metal ion inhibitor (Zn^{2+}) and EDTA. In the absence of calcium and zinc ions, EDTA has no activating effect on the purified enzyme.

The present report contains the first evidence of inhibition of purified phospholipase A_2, by covalent inhibitors, *viz.*, DFP, iodoacetate, and *O*-methylisourea. The first two of these have no effect on the activity of the *C. adamanteus* venom enzyme (Saito and Hanahan, 1962) or the pancreatic enzyme (DeHaas *et al.*, 1968); perhaps the sites of attack of inhibitors are somehow masked in these proteins. It seems unlikely that IAA reacts with a sulfydryl group, since *p*-mercuribenzoate has little inhibitory effect on the enzyme. The possibility of covalent enzyme–inhibitor adduct formation is now being studied with the availability of larger amounts of purified enzyme.

The denaturation of the *C. atrox* enzyme by lyophilization and its reversal on dissolution are striking observations. In the authors' view, this phenomenon explains some of the spectacular lag periods which have been reported in the literature (Long and Penny, 1957; Bennett and Tattrie, 1961). The kinetics of renaturation are difficult to explain; certainly some cooperative refolding is implied by the renaturation progress curve, which can be interpreted as an exaggerated sigmoidal curve. Physical factors affecting denaturation and renaturation, and optical studies of the native, denatured, and renatured enzyme will be reported subsequently. Understanding of the renaturation process may aid in explaining the remarkable heat stability of this enzyme.

The isolation and preliminary characterization of phospholipase A_2 reported in this paper have cast new light on previous concepts regarding this enzyme, and opened numerous avenues of future investigation, a few of which are being explored in this laboratory. It is to be hoped that as further data become available, an understanding of the structure and mechanism of action of this important class of lipolytic enzymes may be reached.

Acknowledgments

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The Solid-Phase Synthesis of Polyglutamates of Folic Acid*

Carlos L. Krumdieck† and Charles M. Baugh

ABSTRACT: Details of an unambiguous chemical synthesis by which pteroyl-(γ-L-glutamyl)$_n$-L-glutamic acid of any desired chain length (up to $n = 6$) may be prepared are reported. The procedures employed are modifications of the Merrifield solid-phase peptide synthetic system. Glutamic acid was attached to the resin by esterification of *t*-butyloxycarbonyl-L-glutamic acid α-benzyl ester (I) to the benzyl chloride groups on the resin. Deprotection was carried out by treatment for 30 min with 20% v/v trifluoroacetic acid in methylene chloride at room temperature. Peptide formation was accomplished by reaction of the mixed anhydride of I and isobutyl formate with the amino-deprotected aminoacyl-resin. After cycles of deprotection and coupling to the desired chain length, the peptide was terminated by coupling *N*-2,*N*-10-bis(trifluoroacetyl)-pteroic acid (II). The mixed anhydride of II and isobutyl formate was used for the peptide formation. The synthesis of II is described. Cleavage of the product from the resin was carried out by gassing the resin-bound product suspended in trifluoroacetic acid with HBr. Deprotection of the cleaved product was carried out in 0.5 N NaOH under N_2, after removal of the trifluoroacetic acid. Details of the chromatographic behavior and biological activity of the products are reported.

Since the discovery and characterization of folic acid (Snell and Peterson, 1940; Mitchell *et al.*, 1941; Hutchings *et al.*, 1948; Stokstad *et al.*, 1948), more than 20 years ago, a great deal of experimental evidence has accumulated indicating that most natural sources contain a large fraction of their total folate activity in the form of polyglutamyl derivatives (see Figure 1). Polyglutamates of folic acid have been demonstrated in algae (Ericson *et al.*, 1953; Banhidi and Ericson, 1953), lichens (Sjøstrom and Ericson, 1953), yeast (Pfiffner *et al.*, 1945–1947), a number of species of bacteria (Hutchings *et al.*, 1948; Hakala and Welch, 1955; Vora and Tamband, 1966; Wright, 1955; Wood and Wise, 1965; Sirotnak *et al.*, 1963), as well as in animal tissues such as blood (Noronha and Aboobaker, 1963) and liver (Noronha and Silverman, 1962; Wright and Welch, 1943). This widespread distribution led Rabinowitz to state that the *in vivo* functioning form of this coenzyme must be a polyglutamyl derivative of pteroylglutamic acid, and that the latter should be considered as a convenient synthetic analog of the natural cofactor (Rabinowitz *et al.*, 1960). The study of the biological functions of the glutamyl side chain has been, however, limited to a few but very intriguing reports, all showing that the polyglutamates function as either better coenzymes than pteroylglutamic acid (Wright, 1955, 1956; Large and Quayle, 1963) or are indeed absolute requirements for enzyme activity (Guest and Jones, 1960). The main obstacle to further investigations on the biological functions of the polyglutamates of folic acid has been their unavailability. In fact, all the studies conducted until now have made use of the triglutamate, pteroyl-di-γ-glutamylglutamic acid, synthesized by the Lederle group in 1948 (Boothe *et al.*, 1948) and/or of polyglutamate preparations of uncertain structure and

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