Studies on Phospholipase A. I. Isolation and Characterization of Two Enzymes from *Crotalus adamanteus* Venom*

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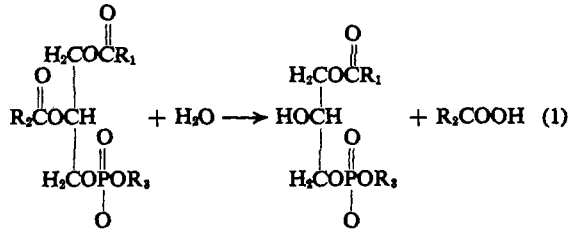
ABSTRACT: Two proteins with phospholipase A activity were purified from *Crotalus adamanteus* venom by a combination of gel filtration; chromatography on a weak cation-exchange resin, DEAE-cellulose, and SE-Sephadex; and crystallization. Both proteins have the same sedimentation coefficient (3.11 S), diffusion coefficient (9.02×10^{-7} cm²/sec), molecular weight (30,000, as determined from sedimentation and diffusion coefficients and high-speed equilibrium ultracentrifugation), extinction coefficient ($E^{1\%}_{280}$ 22.7), frictional coefficient (1.16), partial specific volume (0.718 ml/g), and indistinguishable amino acid analyses. However the two proteins are clearly separated on disc gel electrophoresis. Both proteins have specific activities of 3200 μequiv of fatty acid released/min per mg as assayed in ether–methanol solutions using phosphatidylcholine as substrate. There are some unusual features in the amino acid compositions. Out of a total of 266 residues there are 24 residues of glycine, 16 residues of proline, and 15 residues of cystine. There are no detectable free sulfhydryl groups. Both proteins are extremely stable. One enzymatically active protein is not generated from the other during the isolation procedure.

All attempts to characterize the difference between the two proteins by tryptic fingerprinting have been unsuccessful.

Phospholipase A (EC 3.1.1.4) catalyzes the hydrolysis of the fatty acyl ester at the 2 position of phospholipids (reaction 1).



This activity has been detected in the venoms of snakes, bees, and scorpions, as well as in several mammalian tissues (Kates, 1960). Numerous studies have been reported on the specificity of this enzyme (for a summary, see van Deenen and de Haas, 1964), which is widely used to determine the fatty acid esterified at C-2 of certain phosphoglycerides such as phosphatidylcholine and -ethanolamine. Most of these studies have been done with crude venom with little attention to the use of purified enzymes. A few reports are available on the purification of the enzyme from various venoms (*cf.* Saito and Hanahan, 1962) although there has not been extensive characterization of the products. Saito and Hanahan (1962) reported the partial purification of two proteins with phospholipase A activity from *Crotalus adamanteus* venom, and the enzyme has been reported to exist in two forms in certain Formosan snake venoms (Wakiu and Kawichi, 1959; Wakui and Kawichi, 1961; Iwanaga and Kawichi, 1959). Recently phospholipase A has been purified from porcine pancreas (de Haas *et al.*, 1968a) where it occurs in a zymogen form (de Haas *et al.*, 1968b).

During preparation of purified phospholipase A from *C. adamanteus* for use in studies of lipid–protein interactions, an isolation procedure was developed which gave higher yields and purer products than previously reported. This paper reports the preparation of two highly purified proteins with phospholipase A activity, and a comparison of their properties.

Experimental Section

Materials

Lyophilized *C. adamanteus* venom was obtained from The Miami Serpentarium (Miami, Fla.) or Ross Allen Reptile Institute (Silver Springs, Fla.). Fresh venom was obtained from The Miami Serpentarium. Silicic acid (Mallinckrodt 100 mesh; CC-4 and CC-7 100–200 mesh), aluminum oxide (Merck, and Bio-Rad AG-7), and silica gel G (Brinkman) were used for column chromatography and thin-layer chromatography of lipids. DEAE-cellulose (Whatman DE-52), Bio-Rex 70, 100–200 mesh (Bio-Rad), SE-Sephadex and Sephadex G-25 and G-100 beaded form (Pharmacia) were used for protein chromatography and gel filtration.

Diethyl ether was used from newly opened containers or was distilled from sodium and stored over iron wire.

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Anhydrous methanol was prepared by the method of Lund and Bjerrum (1931). Hexane was distilled from potassium permanganate and the fraction boiling between 67 and 69° was collected. All other solvents were reagent grade and used as received. Urea was crystallized from ethanol, iodoacetic acid (Mann) from petroleum ether (bp 30–60°), and iodoacetamide (Aldrich) from ethyl acetate. Constant-boiling HCl was distilled twice in an all-glass apparatus. Dansyl chloride, dansylamino acids, crystalline trypsin, and Ca[bis(*p*-nitrophenyl)phosphate]$_2$ were purchased from Calbiochem. Casein was a Nutritional Biochemical product. Dialysis tubing was boiled in 0.1 M EDTA (pH 7.0) for 30 min, washed with distilled water, and stored in cold water.

Methods

Purification of Phosphatidylcholine. Hen egg yolks were homogenized with one-half their volume of distilled water, and extracted by the method of Bligh and Dyer (1959). A crude phospholipid fraction was prepared from this extract by acetone precipitation. Phosphatidylcholine was purified from the crude phospholipid fraction by sequential chromatography on aluminum oxide (Merck) and silicic acid (Rhodes and Lea, 1957; Hanahan *et al.*, 1957; Wells and Dittmer, 1966). Occasional preparations were resistant to phospholipase A hydrolysis, but this was remedied by chromatography on neutral alumina as described below.

Alternatively phosphatidylcholine was isolated in a single step as follows. A chloroform solution of the crude phospholipids was placed onto a column of neutral alumina (Bio-Rad AG-7) which had been packed in chloroform. A loading factor of 1 mg of phosphorus/g of alumina was used. The column was eluted with chloroform until no further organic material could be detected in the eluate (Ways, 1963). The column was then eluted with chloroform–methanol (85:15) and the eluate was collected in fractions. (For a 500-g column, 20-ml fractions were taken.) The phosphatidylcholine was located by charring of 0.1-ml aliquots (Ways, 1963) and thin-layer chromatography of appropriate fractions (chloroform–methanol–water, 95:35:6). Those fractions which contained pure phosphatidylcholine were combined and filtered through three layers of Whatman No. 1 filter paper (previously washed with chloroform–methanol, 1:1) to remove fines eluted from the column. Elution of fines occurred only when large amounts of phosphatidylcholine were being eluted, and could not be eliminated by prewashing the alumina. The clear, lightly yellow filtrate was concentrated to dryness *in vacuo*, dissolved in methanol, and treated with charcoal (Darco-G-60) using 100 mg/ml.

Analyses (Dittmer and Wells, 1968) of pure phosphatidylcholine prepared by either method gave as typical results: P, 4.03%; N, 1.81%; N:P molar ratio = 0.99; choline:P molar ratio = 1.01; fatty acyl ester:P molar ratio = 2.03; $[\alpha]_D^{25}$ +6.3° (*c* 5.0, chloroform–methanol, 1:1). In addition the material contained no detectable ash, showed a single spot on thin-layer chromatography, and gave the expected infrared spectrum (Abramson *et al.*, 1965) (in chloroform or carbon tetrachloride). Fatty acid analysis using a Barber Coleman Model 2000 equipped with an ionization detector and 15% ethylene glycol succinate on Anakrom AB (60–70 mesh) at 175° gave results similar to those of Saito and Hanahan (1962).

Phospholipase A Assay. The assay procedure used was similar to that described by Saito and Hanahan (1962). A freshly prepared solution of phosphatidylcholine, 15 μmoles/ml, in 95% diethyl ether (peroxide free) and 5% methanol (anhydrous) was prepared for each series of assays. No attempt was made to use a solution for more than 1 day. Even after storage for only a few days the solution turned yellow and became resistant to hydrolysis.

The substrate solution (2 ml) was placed in a 5-ml volumetric flask and 25 μl of enzyme preparation added. After vigorous shaking for 30 sec, the reaction was allowed to proceed for 10 min at 20–25°. The reaction was stopped by the addition of 95% ethanol to a total volume of 5 ml. The fatty acids were immediately titrated with 0.02 N NaOH in 90% ethanol (dilution of stock aqueous 0.2 N NaOH with 99% ethanol) using 1 drop of 0.1% cresol red as an indicator and an Ultraburet (Scientific Industries) to dispense the base. Ethanolic solutions of base were prepared as needed, and were standardized during each assay with potassium acid phthalate (National Bureau of Standards). Zero-time reaction mixtures required 0.005–0.010 ml of base and the reproducibility of titration was 0.005 ml. In order to obtain data with an error of about 5%, the amount of enzyme was adjusted such that 0.100–0.150 ml of base was required above the blank. This figure corresponded to 5–10% hydrolysis. In numerous experiments zero-order kinetics were observed up to 25% hydrolysis or for 30 min.

One unit of enzyme activity is defined as the release of 1.0 μequiv of fatty acid in 1 min. For purposes of purification specific activity was expressed as units per milligram of protein, assuming $E_{280}^{1\%}$ 10.00.

Phosphate buffer at high concentration was inhibitory. In order to avoid this problem and other possible effects of the buffers used in chromatographic procedures, all enzyme solutions were diluted at least 25-fold with a solution containing 0.22 M NaCl, 0.02 M $CaCl_2$, and 0.001 M EDTA (pH 7.5) (Saito and Hanahan, 1962). Under these conditions no inhibitory effects were observed.

The exact amount of water in the ether–methanol reaction mixture was quite critical. Therefore 25 μl of enzyme solution was always used with 2.0 ml of substrate solution. Solutions with enzymatic activity too great to be measured were reassayed after appropriate dilution.

Purification Procedure. All steps except dialysis and concentration were carried out at room temperature. Dry venom (5 g) was suspended in 50 ml of a mixture of 0.1 M NaCl, 0.05 M Tris-HCl, and 0.001 M EDTA (pH 8.0). The suspension was mixed for 5 min, then centrifuged at 5000 rpm for 15 min. The clear, yellow, supernatant solution (I) contained all the phospholipase A activity and was subjected to gel filtration.

Gel Filtration. Sephadex G-100 was swollen in a mixture of 0.1 M NaCl, 0.05 M Tris-HCl, and 0.001 M EDTA

(pH 8.0) as recommended by the manufacturer. After degassing, the gel was packed into a glass column (Pharmacia) under a pressure head of 20 cm. A column (2.5 × 90 cm) equipped for upward flow and containing a small T in the inlet line was used. After equilibration overnight under a pressure head of 20 cm, the flow rate was adjusted to 25 ml/hr with a pressure head of 10–15 cm (Marriotte flask). Solution I was applied to the column, and washed into the column with 10 ml of 5% sucrose in buffer solution. Elution was initiated with the same solution used to pack the column, and the eluate was collected in 5-ml fractions. The peak phospholipase A activity was found at an elution volume, V_e/V_0, of 1.75. All the recovered activity was found in six to seven tubes on either side of the peak tube. The fractions containing phospholipase A activity were pooled and dialyzed at 4° against 0.15 M sodium phosphate (pH 6.8). The sample was then concentrated to approximately 10 ml at 4° using an Amicon Ultrafiltration Cell, Model 50, containing a UM-1 Diaflo Ultrafiltration Membrane (Scientific Systems Division, Amicon Corp., Lexington, Mass.). This solution (II) was chromatographed on Bio-Rex 70.

Bio-Rex 70 Chromatography. Bio-Rex 70 (100–200 mesh) was washed with 0.15 M sodium phosphate (pH 6.8) and transferred to a 2.5 × 45 cm column. Solution II was applied and the column eluted with the same phosphate buffer at a flow rate of 15 ml/hr. The eluate was collected in 5-ml fractions. Two well-separated protein peaks were eluted from the column. All the phospholipase A activity recovered was found in the first peak. Those fractions containing phospholipase A activity were pooled, dialyzed against distilled water, and then several changes of 0.05 M Tris-HCl (pH 8.0). The solution was concentrated to 20 ml as described above. This latter solution (III) was chromatographed on DEAE-cellulose.

DEAE-cellulose Chromatography. Whatman microcrystalline DEAE-cellulose (DE-52) was washed as recommended by the manufacturer. After equilibration in a solution of 0.2 M NaCl and 0.05 M Tris-HCl (pH 8.0), the DE-52 was packed into a 2.5 × 45 cm column. The column was then washed with 0.05 M Tris-HCl (pH 8.0). Solution III was applied to the column; the column was eluted with 200 ml of a 0.05 M Tris-HCl (pH 8.0) and then with a linear gradient formed from 1 l. of 0.05 M Tris-HCl (pH 8.0) and 1 l. of a solution of 0.2 M NaCl in 0.05 M Tris-HCl (pH 8.0). A flow rate of 80 ml/hr was maintained with a metering pump and the entire eluate was collected in 5-ml fractions.

It is at this point that two enzymatically active components are separated. The first active fraction, α, is eluted at 0.08 M NaCl, and the second, β, at 0.12 M NaCl. In each case those fractions which contained phospholipase A of approximately constant specific activity were pooled and dialyzed against 0.01 M sodium citrate (pH 5.2). Both enzyme solutions were concentrated to 10 ml, and these solutions (IV-α and IV-β) were chromatographed on SE-Sephadex.

SE-Sephadex Chromatography. SE-Sephadex (C-50) was washed several times with 0.01 M sodium citrate (pH 5.2) to remove fines and then packed into a 2.5 × 45 cm column. Solution IV-α or IV-β was applied to the column, the column was eluted with citrate buffer at a flow rate of 30 ml/hr, and the eluate was collected in 4-ml fractions. In both cases, those fractions of constant specific activity were pooled and concentrated to a protein concentration of 5 mg/ml (A_{280}).

Crystallization. The concentrated solution from the SE-Sephadex column was chilled in an ice bath and $(NH_4)_2SO_4$ was added slowly during 1 hr until the onset of turbidity (about 2.2 M). After standing overnight the crystals were collected by centrifugation. Phospholipase A_α was recrystallized by dialyzing a solution in 0.01 M sodium citrate (pH 5.2) against 2.0 M $(NH_4)_2SO_4$ in 0.01 M sodium citrate (pH 5.2), adjusting the protein concentration to 2 mg/ml, and further dialysis at 4°. The crystals grew in about 2–3 weeks. The same procedure was used for phospholipase A_β except that 1.8 M $(NH_4)_2SO_4$ was used, and dialysis was carried out at room temperature.

Ultracentrifugation was carried out in a Spinco Model E analytical ultracentrifuge focused at the two-thirds plane (Svenson, 1954, 1956; Yphantis, 1964). The protein solution was dialyzed against 0.1 M NaCl in 0.001 M Tris-HCl (pH 8.0). The final dialysis solution was used for reference cells in double-sector cells and for gravimetric dilutions. All runs were made at 20.0°. Plates were read on a Nikon microcomparator. Sedimentation and diffusion coefficients were corrected to standard conditions as described by Svedberg and Pedersen (1940).

High-speed equilibrium centrifugation runs (Yphantis, 1964) were performed with a modified overspeeding and underspeeding technique (Teller, 1965, and personal communication, 1967). A double-sector cell equipped with sapphire windows was filled with 10 μl of fluorocarbon (Spinco) and 0.12 ml of protein solution. Both proteins were analyzed using an initial protein concentration of 0.96 mg/ml as calculated from the measured $E^{1\%}_{280}$. The centrifuge was run at 35,600 rpm for 3 hr (complete depletion of the meniscus), at 18,200 rpm for 20 min, then at 25,980 rpm for 16 hr, when photographs, taken every hour for 4 hr, indicated that equilibrium had been obtained. Interference photographs were read up to the point where the fringes could no longer be resolved. The data were analyzed with an IBM 7090–7094 IBSYS system using the program of Teller (1965, and personal communication, 1967). Sedimentation velocity was carried out at 59,780 rpm using double-sector cells equipped with sapphire windows. Concentration dependence of the sedimentation coefficient was evaluated at the initial protein concentration.

Diffusion coefficients were determined in the analytical ultracentrifuge using a synthetic boundary cell which layered at 3000 rpm. Boundary spreading was followed on schlieren photographs taken every 4 min for 3 hr. The diffusion coefficient was determined from plots of the square of the inflection point *vs.* time (Svedberg and Pedersen, 1940). Concentration dependence was evaluated at initial protein concentration. The partial specific volume was calculated from the amino acid analysis using the data of McMeekin *et al.* (1949).

$E^{1\%}_{280}$ was determined using the ultracentrifuge as a differential refractometer. The synthetic boundary cell was the same as used for diffusion coefficient measure-

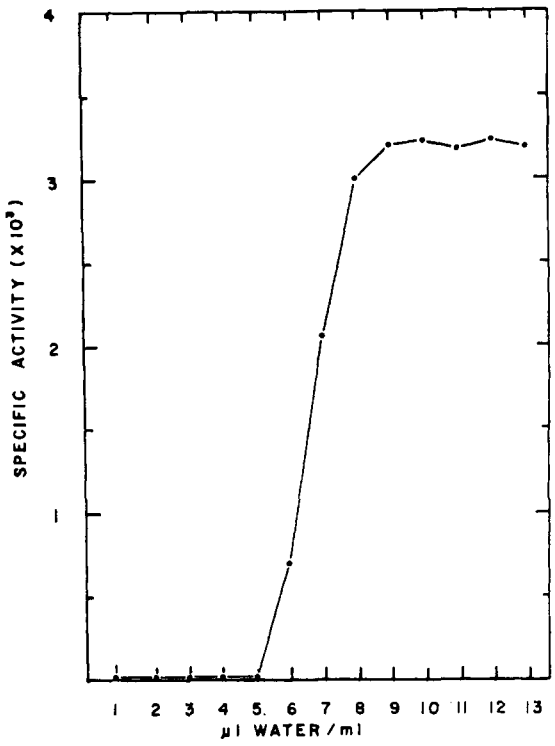

FIGURE 1: Effect of water concentration on phospholipase A activity. Phosphatidylcholine (50 μmoles) was dissolved in 5 ml of ether–methanol (95:5). Each assay contained 1 μl of 1.25 M $CaCl_2$, 1 μl of purified phospholipase A_α (0.25 μg/μl), and sufficient water to give the indicated concentration of water. Aliquots (2 ml) were titrated after a 10- and 20-min reaction. The specific activity was calculated using $E^{1\%}_{280}$ 22.7.

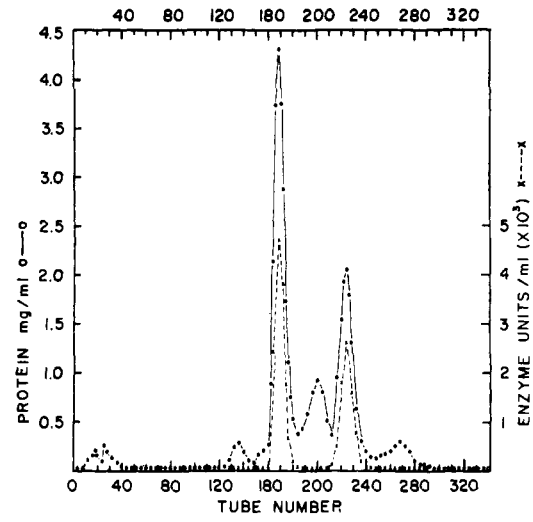

FIGURE 2: Separation of two phospholipases A on DEAE-cellulose. Whatman DE-52 was packed in a 2.5 × 45 cm column in 0.05 M Tris-HCl (pH 8.0). The eluate from a Bio-Rex 70 column containing 833 mg of protein and 613,000 units of phospholipase A activity was applied to the column. The column was eluted with 200 ml of 0.05 M Tris-HCl (pH 8.0) and then with a linear gradient formed from 1 l. of 0.05 M Tris-HCl (pH 8.0) and 1 l. of 0.2 M NaCl in 0.05 M Tris-HCl (pH 8.0). A flow rate of 80 ml/hr was maintained with a metering pump and 5-ml fractions were collected.

ments. After boundary formation, interference photographs were taken as soon as sufficient diffusion had occurred to allow clear resolution of the fringes. The protein concentration was calculated using refractive index gradient of 1.86×10^{-3} (Armstrong *et al.*, 1947). The protein concentration of all solutions used for sedimentation and diffusion studies was determined in this manner. The A_{280} was measured after gravimetric dilution.

Amino acid analyses were carried out by the accelerated method of Spackman *et al.* (1958) on a Spinco amino acid analyzer. The protein solution was dialyzed against distilled water for removal of salt. Protein (1 mg) was hydrolyzed according to the method of Moore and Stein (1963). Performic acid oxidation was carried out according to Hirs (1956). The oxidized protein was hydrolyzed for 24 hr. All analyses were carried out in duplicate. Serine, threonine, and ammonia were calculated after extrapolation to zero time. Hydrophobic amino acids were calculated from 72- and 100-hr hydrolysates. Tyrosine and tryptophan were determined by the method of Goodwin and Morton (1946). The latter tyrosine value agreed within 2% of that found from amino acid analysis.

Disc gel electrophoresis was carried out as described by Ornstein (1964) using 7% acrylamide and the buffer system of Davis (1964). Protein in the gel was stained with amido black.

Disulfide reduction and modification of the protein were done in an attempt to increase sensitivity to tryptic hydrolysis. Disulfide reduction was carried out according to Anfinsen and Haber (1961). Reaction of the free sulfhydryl groups with either iodoacetic acid, iodoacetamide, or acrylonitrile was accomplished as described by Anfinsen and Haber (1961) for iodoacetic acid. In all three reactions the protein precipitated from the reaction mixture and was isolated by centrifugation. It was washed several times with water to remove excess reagents. Completely reduced protein was isolated using Sephadex gel filtration (Anfinsen and Haber, 1961) in 0.1 M acetic acid. Free sulfhydryl groups were determined by a slight modification of the method of Ellman (1959). One sample of protein was diluted into the buffer to measure the protein blank, but for assay the protein was diluted into buffer which contained Ellman's reagent. The rate of reoxidation was so fast that considerably lower values were obtained if the protein stood for only a few minutes at alkaline pH in the absence of the reagent.

N-Terminal amino acid analysis was carried out using dansyl chloride as described by Gray and Hartley (1963). Approximately 1 mg of native or modified protein was dissolved in 1 ml of 0.1 M $NaHCO_3$. To this solution was added 1 ml of dansyl chloride solution (20 mg/ml) in acetone. The clear solution was allowed to stand for 4 hr at room temperature. In the case of the native protein the dansylated product was isolated after gel filtration on Sephadex G-25 in 0.2 M NH_4OH. The dansylated products from modified proteins were retained irreversibly on Sephadex. They were isolated by precipitation from the reaction mixture by addition of acetone and washing with 0.1 N HCl to remove excess reagents. The dansylated proteins were hydrolyzed in redistilled HCl in sealed, evacuated tubes at 110° for 24 hr. The dansyl

TABLE I: Purification of Phospholipase A from *C. adamanteus* Venom.[a]

Step	Vol (ml)	Total Protein (mg)	% Protein	Total Units	% Act.	Sp Act.[b]	Purifcn
Crude suspension	50	7250.0	100	716,000	100	100	
	50	7500.0	100	760,000	100	102	
	50	7425.0	100	760,000	100	102	
5000 rpm supernatant (I)	50	7250.0	100	716,000	100	100	
	50	7475.0	99.7	760,000	100	102	
	50	7400.0	99.6	756,000	99.5	102	
Sephadex G-100 (II)	140	2730.0	37.6	640,000	89.5	236	2.4
	160	3240.0	43.2	716,000	94.5	222	2.2
	160	3160.0	42.5	716,000	94.5	228	2.3
Bio Rex 70 (III)	50	787.5	10.9	560,000	78.2	710	7.1
	55	869.0	11.6	624,000	82.8	718	7.0
	56	833.0	11.2	613,000	81.0	738	7.2
DE-52α	97	260	3.6	255,000	35.6	999	10
	93	253	3.4	288,000	38.0	1060	10.6
	100	252	3.4	247,000	32.5	998	10
DE-52β	96	157	2.2	194,000	27.2	1190	11.9
	100	175	2.3	194,000	25.6	1110	11.1
	100	155	2.10	176,000	23.2	1140	11.4
Total α DE-52 combined		756	3.45	790,000	35.5	1030	10.3
Total α DE-52 combined		487	2.20	564,000	25.3	1160	11.6
SE-Sephadex α	242	532.4	2.40	733,260	32.8	1375	13.75
SE-Sephadex β	110	287	1.3	394,000	17.6	1370	13.7
First α crystals	35	412	1.85	565,000	25.3	1415	14.5
First β crystals	35	210	0.95	299,250	13.4	1425	14.25
α, second crystallization						1410	14.1
β-second crystallization						1400	14.0

[a] Results from three experiments on one batch of venom. Details are given in the text. [b] Calculated assuming $E_{280}^{1\%}$ 10.0.

amino acids were separated by thin-layer chromatography on silica gel G using the solvent systems of Deyl and Rosmus (1965).

Tryptic hydrolyses were carried out on 6 mg of either native or modified protein. The protein in 1.0 ml of water was heat denatured, then adjusted to pH 8.0 in a Radiometer pH-Stat. After addition of 0.1 mg of trypsin, the reaction mixture was maintained at pH 8.0 until the uptake of base stopped. The reaction mixture was adjusted to pH 7.0 and centrifuged to remove any precipitate that formed. The clear supernatant solution was lyophilized and dissolved in water; the peptides were separated by thin-layer chromatography and electrophoresis (Savant apparatus) as described by Ritschard (1964). The solvent system which gave the best results was chloroform–methanol–NH_4OH (40:40:20). The peptides were visualized with ninhydrin.

Protease activity was assayed by the procedure of Kunitz (1947), and phosphodiesterase activity by the procedure of Boman and Kaletta (1957).

Results

Assay Procedure. The procedure used was similar to that described by Saito and Hanahan (1962) except that methanol was included in the reaction mixture. The presence of methanol at levels up to 5% was not inhibitory. In the presence of 5% methanol the lysolecithin formed remained in solution until approximately 25% hydrolysis had taken place, whereas in pure ether, the lysolecithin precipitated after approximately 5% hydrolysis. The results were more reproducible in the presence of methanol since the lysolecithin remained in solution.

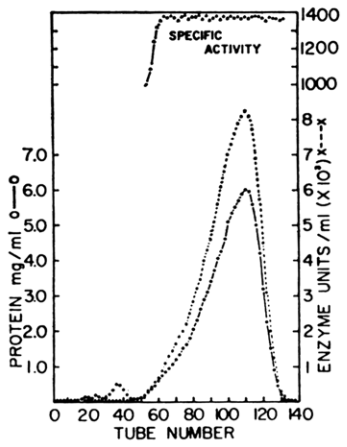

FIGURE 3: Purification of phospholipase A_α on SE-sephadex. A 2.5 × 45 cm column of SE-sephadex (C-50) in 0.01 M sodium citrate was used. The combined phospholipase A_α fractions from three DE-52 columns containing 765 mg of protein and 790,000 units of phospholipase A_α were chromatographed. The column was run at a flow rate of 30 ml/hr and 4-ml fractions were collected. Specific activity was calculated assuming $E^{1\%}_{280}$ 10.0.

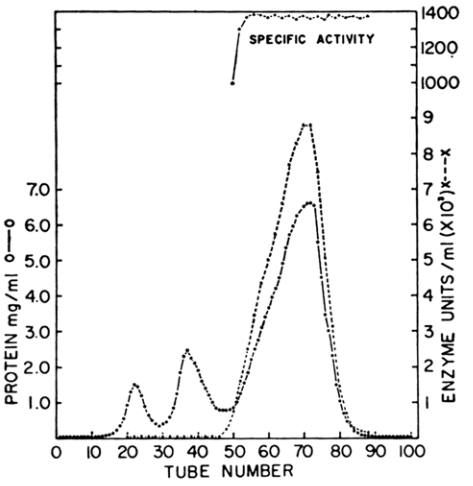

FIGURE 4: Purification of phospholipase A_β on SE-sephadex. A 2.5 × 45 cm column of SE-sephadex (C-50) in 0.01 M sodium citrate was used. The combined phospholipase A_β fractions from three DE-52 columns containing 487 mg of protein and 564,000 units of phospholipase A_β activity were chromatographed. The column was run at a flow rate of 30 ml/hr and 4-ml fractions were collected. Specific activity was calculated assuming $E^{1\%}_{280}$ 10.0.

When lysolecithin precipitates, as in pure ether, the reaction proceeds in the precipitate but at an altered state.

The amount of water added to the ether–methanol solution had a pronounced effect on the phospholipase A activity at all phases of its purification. Figure 1 shows results of the effect of water addition on the activity of purified phospholipase A_α in ether–methanol solution. Identical results were obtained with phospholipase A_β and with crude samples of venom. Similar results were obtained in pure ether, except the curve was displaced toward lower water concentration. The amount of water required for maximal activity was 100 times the amount of water consumed in the reaction. Preliminary studies show a complex relationship between water, substrate, and Ca ion concentration.

Changes in these parameters from those reported in this paper can give quite different specific activities for the purified phospholipases A. A more detailed study of the effect of water is underway.

Purification Procedure. Table I contains data from three experiments with a single batch of venom. The fractions were combined after the DEAE-cellulose step.

Gel Filtration. Gel filtration was chosen for the first step since it removes the need for dialysis and eliminates larger proteins which are eluted between the two phospholipases A during DEAE-cellulose chromatography. In our hands satisfactory results were obtained only with glass columns and careful attention to the recommendations for running the columns provided by Pharmacia. Lucite columns gave rise to hyperbolic band shapes which resulted in poor resolution. The use of Sephadex G-150 or G-200 did not result in higher purification, whereas G-75 did not result in a material of lower specific activity.

Concentration and Dialysis. Saito and Hanahan (1962) reported considerable loss of enzymatic activity during dialysis. No such losses were ever encountered during this study. Dialysis tubing which was boiled in EDTA was used, whereas Saito and Hanahan (1962) apparently did not follow this procedure. Although no specific study of this problem was made, it may be that the difference lies in the treatment of the dialysis tubing. The use of a membrane concentrator is of considerable advantage in concentrating protein solutions. Recoveries of 95–100% were routinely obtained in this procedure.

Bio-Rex 70 Chromatography. Bio-Rex 70, a weak cation-exchange resin, was used by Neumann and Haber-

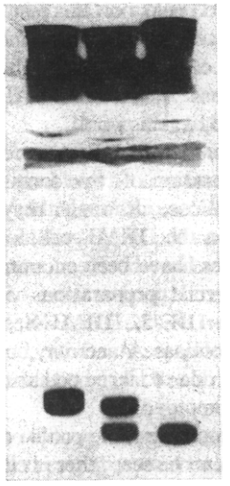

FIGURE 5: Disc gel electrophoresis of purified phospholipases A. Disc gel electrophoresis was carried out by the methods of Ornstein (1964) and Davis (1964). Migration was from top to bottom with a current flow of 5 mA/gel. The proteins were stained with amido black. For this photograph the gels were cut at the point where the bromothymol blue band was located. The gel on the left contains phospholipase A_α (500 μg), the gel in the middle a mixture of phospholipase A_α (250 μg) and phospholipase A_β (250 μg), and the gel on the right phospholipase A_β (500 μg).

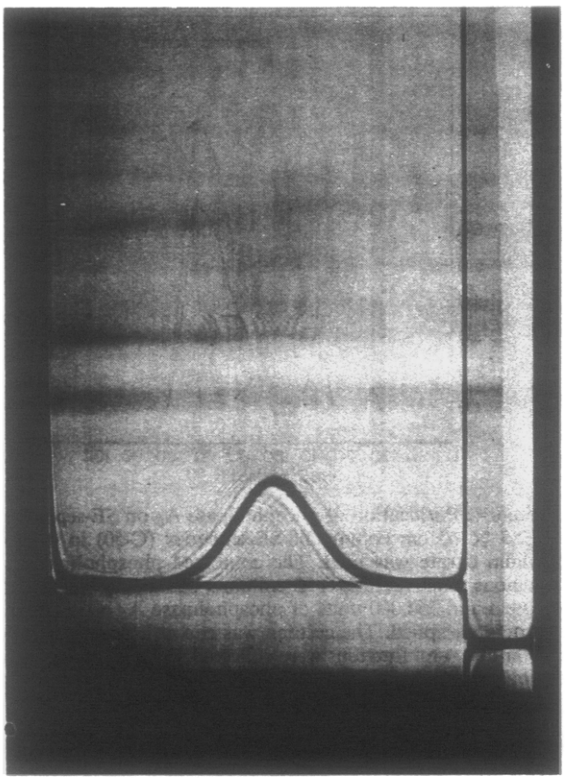

FIGURE 6: Sedimentation velocity pattern of phospholipase A_α. Sedimentation velocity was carried out at 59,780 rpm using a double-sector cell with sapphire windows, the protein concentration was 11.4 mg/ml. Sedimentation is from right to left. The photograph was taken after 144 min with a bar angle of 65°.

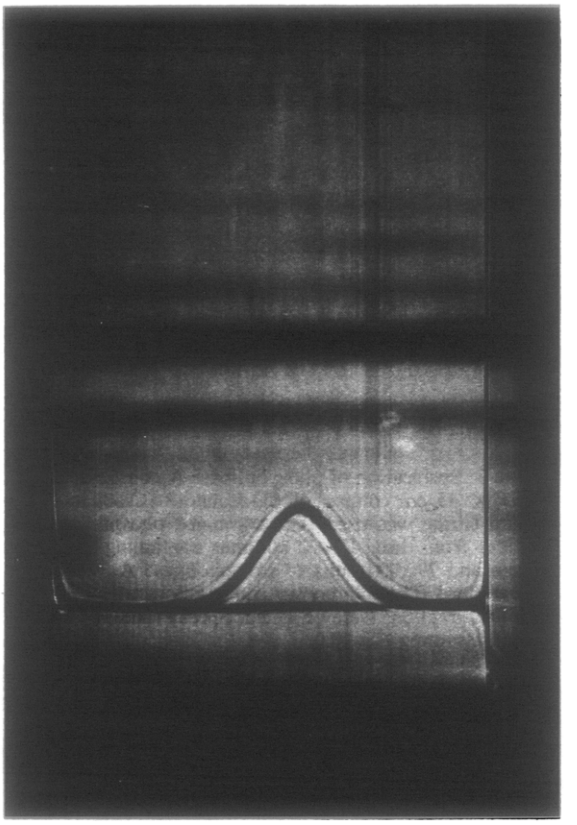

FIGURE 7: Sedimentation velocity pattern of phospholipase A_β. Sedimentation velocity was carried out at 59,780 rpm using a double-sector cell with sapphire windows, the protein concentration was 10.6 mg/ml. Sedimentation is from right to left. The photograph was taken after 144 min with a bar angle of 65°.

mann (1955) for the separation of toxins in snake venom, and proved useful here. The proteins are resolved into two well-separated peaks on this resin. The first, rapidly eluted peak contains all the phospholipase A activity. The second peak contains proteins which would be eluted with phospholipase A_β from DEAE-cellulose if they were not removed at this point.

DE-52 Chromatography. Saito and Hanahan (1962) observed the separation of two forms of phospholipase A on DEAE-cellulose, although they experienced considerable losses on the DEAE-cellulose used. Similarly, considerable losses have been encountered in this study with all commercial preparations of DEAE-cellulose except Whatman DE-52. DEAE-Sephadex gave good yields of phospholipase A activity, but was more difficult to work with due to large bed shrinkage in the ionic strength range employed.

A typical chromatographic profile on DE-52 is shown in Figure 2. As can be seen, there is clean separation of two protein peaks with phospholipase A activity. When the protein from each peak was rechromatographed under the same conditions, only a single peak was observed, which was eluted at the same point in the gradient as it was on the first column.

SE-Sephadex. Examinations of the two fractions obtained from DE-52 by disc gel electrophoresis revealed the presence of one major and two minor bands. Figures 3 and 4 show the chromatographic profiles of the two preparations on SE-Sephadex. It will be noted that the bulk of the enzymatic activity is eluted from the column with constant specific activity. In addition disc gel electrophoresis showed only a single band.

Crystallization. Several experiments indicated that crystallization occurred most readily at about pH 5.0–5.5. Therefore the eluate from the SE-Sephadex was readily crystallized, after concentration by addition of $(NH_4)_2SO_4$. Recrystallization using the technique of slow dialysis did not increase the specific activity of the preparations.

Properties of the Purified Enzymes. Each enzyme showed a single band on disc gel electrophoresis. A mixture of the two is clearly resolved into two bands (Figure 5). Figure 6 shows the sedimentation velocity pattern for phospholipase A_α at a protein concentration of 11.4 mg/ml, and Figure 7 shows the pattern for phospholipase A_β at a protein concentration of 10.6 mg/ml. Examination of several plates failed to reveal any detectable asymmetry to the patterns. Figure 8 shows the concentration dependence of the sedimentation and diffusion coefficients. There is no significant difference in the values for the two proteins. The molecular weight calculated from s and D at infinite dilution is 29,800 for both proteins. Figure 9 shows plots of M_n and M_w calculated at various points in the centrifuge cell from

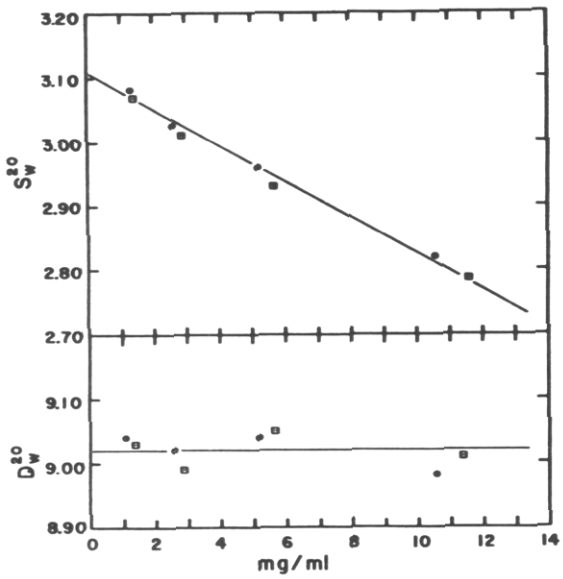

FIGURE 8: Concentration dependence of s_w^{20} and D_w^{20} of purified phospholipases A. Top: Concentration dependence of s_w^{20}. Bottom: Concentration dependence of D_w^{20}; ⊡ are for phospholipase A_α, ⊙ for phospholipase A_β.

a single high-speed equilibrium run. Although the molecular weights calculated are slightly different, it cannot be stated that there is a significant difference in molecular weight on the basis of a single run. Table II summarizes the physical properties of the two phospholipases A. The only property in which they differ significantly is that of electrophoretic mobility.

The amino acid composition of the two proteins is summarized in Table III. The average yield of amino acids from the hydrolysis of 1 mg of protein (correcting for losses of serine and threonine, low values of hydrophobic amino acids at early stages of hydrolysis, and adding the tryptophan value) was 8.70 ± 0.2 μmoles.

TABLE II: Physical Properties.*

	Phospholipase A_α	Phospholipase A_β
M_n	29,900	31,100
M_w	29,500	31,200
M_z	29,900	31,900
$M_{z,D}$	29,800	29,800
M_{aa}	29,864	29,864
s_w^{20} (S)	3.11	3.11
D_w^{20} (cm²/-sec)	9.02×10^{-7}	9.02×10^{-7}
$\bar{V}_{aa}$ (ml/g)	0.718	0.718
f/f_0	1.16	1.16
$E_{280}^{1\%}$	22.7	22.7

* Physical properties of the two phospholipases A isolated from *C. adamanteus* venom. Details are given in the text.

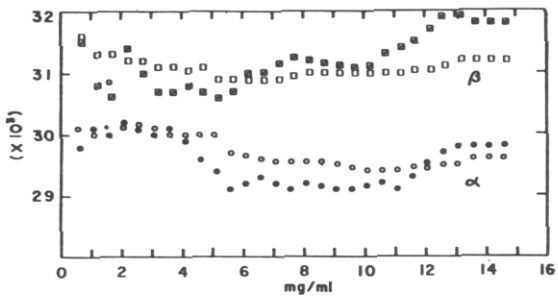

FIGURE 9: Concentration dependence of M_n and M_w of purified phospholipases A. M_n and M_w calculated at various points in an ultracentrifuge cell after attaining equilibrium. High-speed equilibrium was used as described in the text. (○) M_n for phospholipase A_α, (◑) M_w for phospholipase A_α; (□) M_n for phospholipase A_β, (◪) M_w for phospholipase A_β.

This would indicate 261 residues for a molecular weight of 30,000. The data for alanine were closest to an integral factor, so it was assumed that 15 alanines were present. On the latter basis each protein has 266 residues and a molecular weight of 29,864. As can be seen in Table III there is no fundamental difference between the amino acid composition of the two proteins.

Analysis of free sulfhydryl groups by the method of Ellman indicated less than 0.1 residue/mole of protein. After reduction, which eliminated all enzymatic activity, 28–29 residues of free sulfhydryl groups per mole of protein were found, in good agreement with the cysteic acid content found after performic acid oxidation.

Less than 1% carbohydrate (assayed as described by Winzler, 1955) was found in both proteins. No fatty acid could be detected by thin-layer chromatography of a petroleum ether extract of an acid hydrolysis of 6 mg of either protein. Saito and Hanahan (1962) detected fatty acid in their preparations. Further work on this point is underway.

N-Terminal amino acid analyses on either native or modified proteins were negative. Under no conditions

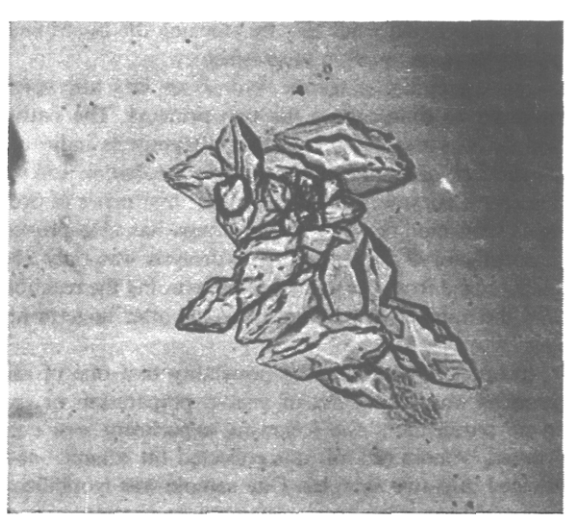

FIGURE 10: Crystals of phospholipase A_α. Crystals were grown by dialysis in 2.2 M $(NH_4)_2SO_4$ as described in the text. × 425.

TABLE III: Amino Acid Composition of Phospholipases A.[a]

Amino Acid	Residues/15 Residues of Alanine								Calcd Amino Acid Composition	
	24 hr		28 hr		72 hr		100 hr			
Asp	30.4	30.5	30.3	29.9	30.1	30.2	30.0	30.0	30.0	30.0
Thr	12.3	12.2	11.7	11.5	11.1	11.0	10.5	10.6	13.0	13.0[b]
Ser	12.2	12.1	11.2	11.1	10.4	10.3	9.5	9.7	13.0	13.0[b]
Glu	24.3	23.6	23.9	24.2	24.0	24.3	24.2	23.9	24.0	24.0
Pro	16.1	15.7	15.9	16.3	16.2	16.1	15.8	15.0	16.0	16.0
Gly	23.8	23.6	24.2	24.0	24.3	24.1	24.1	24.0	24.0	24.0
Ala	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Half-Cys	28.6	28.8	29.2	29.5	28.4	29.0	28.3	28.0	30.0	30.0[c]
Val	9.7	9.5	10.5	10.6	10.9	11.0	10.8	11.1	11.0	11.0[d]
Met	2.0	2.1	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0[e]
Ile	8.6	8.9	10.0	10.2	10.8	11.0	11.0	10.9	11.0	11.0[d]
Leu	9.6	9.3	10.3	10.1	10.9	10.8	11.2	11.1	11.0	11.0[d]
Tyr	16.2	16.0	15.9	16.2	16.3	16.0	16.1	16.0	16.0	16.0
Phe	9.0	9.3	9.7	9.5	9.9	10.0	10.1	10.1	10.0	10.0[d]
Lys	16.1	16.0	15.8	16.2	16.2	16.0	16.2	16.1	16.0	16.0
His	4.8	4.9	4.9	5.1	4.8	4.9	4.8	5.0	5.0	5.0
Arg	12.1	12.0	12.3	11.8	11.9	12.1	11.8	12.1	12.0	12.0
NH_3	16.9	16.8	17.8	18.0	18.7	18.8	19.7	20.0	16.0	16.0[b]
Trp	7.1	7.0	7.0	7.1	7.1	7.0	7.1	7.0	7.0	7.0[f]

[a] The analyses were run on hydrolysates of 1 mg of protein. The data are calculated assuming 15 alanines per mole. The data for phospholipase A_α are in the left column, that for phospholipase A_β in the right of each period of hydrolysis. [b] Calculated by extrapolation to zero time. [c] Determined after 24-hr hydrolysis of performic acid oxidized protein. Duplicates gave 29.8, 30.1, and 30.0, 30.3 residues per 15 residues of alanine for α and β, respectively. [d] Calculated from 72- and 100-hr hydrolyses. [e] The same results were obtained after performic acid oxidation. [f] Calculated from measured extinction coefficient which showed a Tyr to Trp molar ratio of 2.28.

could any dansylated amino acids, other than those with reactive groups not involved in peptide bonds, be detected. On the basis of this result it is tentatively concluded that neither phospholipase A has a free N-terminal amino acid. Thus far hydrazinolysis of the proteins to ascertain what may be blocking the N-terminal amino acid has proven unsatisfactory.

Fingerprinting of tryptic hydrolyses was attempted in order to differentiate the two proteins. The native protein was essentially resistant to trypsin as indicated by the lack of uptake of base during incubation and retention of enzymatic activity. Tryptic hydrolysis of proteins with modified sulfhydryl groups has also proven unsatisfactory. The extent of hydrolysis was only 25–30% judged from the uptake of base during the reaction and the number of peptides observed after fingerprinting.

In order to eliminate the possibility that one of the proteins was an artifact of venom preparation or enzyme purification, the following experiment was performed. Venom (20 ml) was collected (at Miami)[1] and divided into two samples. One sample was lyophilized as usual, and the other was frozen. Both samples were shipped in Dry Ice and kept at −25° until used. Aliquots of each containing 750 mg (A_{280}) of protein and 75,000 units of activity were chromatographed directly on a 2.5 × 45 cm column of DE-52 as described in the purification procedure. In each case two active fractions were eluted at the same points in the gradient as described above in the purification procedure. In the case of the lyophilized sample 39,000 units of activity (52%) were recovered in the phospholipase A_α fraction, and 28,500 units (38%) in the phospholipase A_β fraction. The ratio of units in the first eluted fraction to those in the second eluted fraction was 1.37 with a recovery of 90%. In the case of the native venom 40,000 units (53.3%) were recovered in the first fraction, and 29,000 units (38.6%) in the second fraction. The ratio was 1.39 with a recovery of 91.9%. In the three experiments described in Table I the ratio of units in the phospholipase A_α to A_β fractions was 1.34, 1.46, and 1.40. On the basis of these data it seems unlikely that one enzyme is derived from the other during collection of the venom and isolation of the proteins. A more definitive answer to this question must await elucidation of the differences between the two proteins.

Both phospholipase A preparations were free from phosphodiesterase and protease activity. The absence

[1] These samples of venom were kindly provided by the Miami Serpentarium.

of absorbance at 340 mμ was taken to indicate the absence of L-amino acid oxidase.

The specific activity of the purified phospholipases A was 3150–3250 units/mg. Under the conditions of assay used there is no difference between the two enzymes. The turnover number of both enzymes is 1600 moles of substrate hydrolyzed/mole of enzyme per sec. This is probably a low estimate, since preliminary attempts to determine V_m indicate that V_m is higher than 3200 μmoles min. As discussed above, the kinetics of the reaction in ether–methanol are complex and are under investigation. Thus far no studies have been carried out with synthetic substrates or with phospholipids other than phosphatidylcholine.

Both enzyme preparations are extremely stable. Solutions containing 0.05 to 5 mg/ml of protein in 0.01 M $CaCl_2$ at pH 7.0 have been kept at 4° for 18 months without loss in activity. Similar solutions stored at room temperature for several weeks have not lost activity nor developed bacterial contamination.

Discussion

The specific activity of 3200 μmoles/min per mg (based on measured $E^{1\%}_{280}$) of the purified proteins is 4- to 5-fold higher than reported by Saito and Hanahan, although the over-all purification of 14-fold is only about 2- to 2.5-fold greater than observed by these authors. However, when the initial protein concentration is calculated on the basis of the weight of starting venom, instead of an arbitrary $E^{1\%}_{280}$, these differences are resolved. On the basis of the weight of starting venom the specific activity of the crude venom is 152, while that of the purified enzymes is 3200. This gives a purification of 21-fold. Similar calculations from the data of Saito and Hanahan give their purification as approximately four-fold. Thus our preparations are 4- to 5-fold purer based both on a comparison of the specific activity of the final product and the over-all purification.

On the basis of the criteria thus far applied the two enzymatically active proteins appear to be nearly homogeneous. There is an indication of some heavy material in the high-speed equilibrium runs, but it represents probably less than 5% of the total protein. The molecular weight of 30,000 is consistent with the sedimentation velocity and diffusion measurements, high-speed equilibrium measurements, and amino acid analysis.

There are several interesting properties of the two phospholipases A that deserve some comment at this point. The high content of disulfide bonds, glycine, and proline probably account for the high stability of the proteins. In this regard it is of interest to note that the enzyme from porcine pancreas (de Haas *et al.*, 1968a) which has a molecular weight of 14,000 has seven disulfide bonds and a high proportion of glycine and proline. The content of hydrophobic amino acids in the phospholipases A is lower than generally found in proteins, and although there is a high proportion of charged amino acids, the net charge is small. This is consistent with the low ionic strength required to elute the proteins from DEAE-cellulose. Preliminary data on the solubility of the proteins as a function of pH would indicate that the isoelectric point is near 4. This is in agreement with the values determined by Saito and Hanahan (1962).

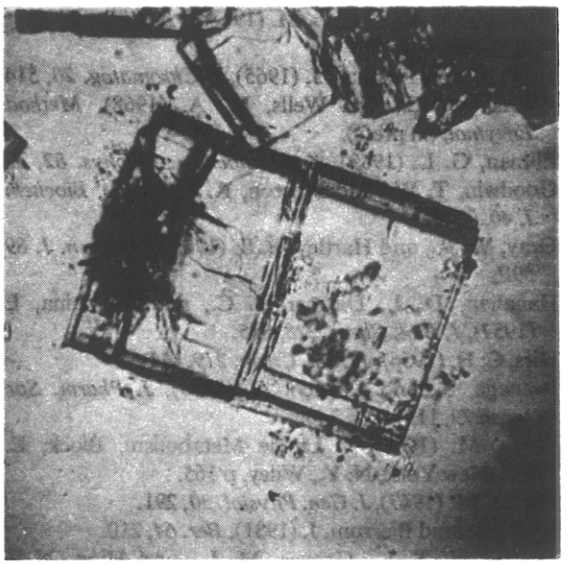

FIGURE 11: Crystals of phospholipase A_β. Crystals were grown by dialysis in 2.0 M $(NH_4)_2SO_4$ as described in the text. × 425.

The difficulty encountered thus far in attempting to obtain tryptic fingerprints may be related to the fact that the disulfide bridges need to be cleaved before significant hydrolysis can take place. The effect of the introduction of 30 residues into the protein during modification of the sulfhydryl group on the availability of trypsin-sensitive sites is unknown. Several methods of tryptic hydrolysis are now under investigation. Similar consideration may apply to our inability to find a free N terminal or the lack of definitive results in attempting to locate an *N*-acyl group.

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References

Abramson, M. B., Norton, W. T., and Katzman, R. (1965), *J. Biol. Chem. 240*, 2389.

Anfinsen, C. B., and Haber, E. (1961), *J. Biol. Chem. 219*, 611.

Armstrong, S. J., Jr., Budka, M. J. R., Morrison, K. C., and Hasson, M. (1947), *J. Am. Chem. Soc. 69*, 1747.

Bligh, E. G., and Dyer, W. J. (1959), *Can. J. Biochem. Physiol. 37*, 911.

Boman, H. G., and Kaletta, U. (1957), *Biochim. Biophys. Acta 24*, 619.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

de Haas, G. H., Postema, N. M., Nieuwehuizen, W., and van Deenen, L. L. M. (1968a), *Biochim. Biophys. Acta 159*, 103.

de Haas, G. H., Postema, N. M., Nieuwhuizen, W.,

and van Deenen, L. L. M. (1968b), *Biochim. Biophys. Acta 159*, 118.
Deyl, Z., and Rosmus, J. (1965), *J. Chromatog. 20*, 514.
Dittmer, J. C., and Wells, M. A. (1968), *Methods Enzymol.* (in press).
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Goodwin, T. W., and Morton, R. A. (1946), *Biochem. J. 40*, 628.
Gray, W. R., and Hartley, B. S. (1963), *Biochem. J. 89*, 59P.
Hanahan, D. J., Dittmer, J. C., and Warshina, E. (1957), *J. Biol. Chem. 228*, 685.
Hirs, C. H. (1956), *J. Biol. Chem. 219*, 611.
Iwanaga, S., and Kawichi, S. (1959), *J. Pharm. Soc. Japan 79*, 1177.
Kates, M. (1960), *in* Lipide Metabolism, Block, E., Ed., New York, N. Y., Wiley, p 165.
Kunitz, M. (1947), *J. Gen. Physiol. 30*, 291.
Lund, H., and Bjerrum, J. (1931), *Ber. 64*, 210.
McMeekin, T. L., Groves, M. L., and Hipp, N. J. (1949), *J. Am. Chem. Soc. 71*, 3298.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Neumann, W. P., and Habermann, E. (1955), *Biochem. Z. 327*, 170.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Rhodes, D. N., and Lea, C. H. (1957), *Biochem. J. 65*, 526.
Ritschard, W. J. (1964), *J. Chromatog. 16*, 327.
Saito, K., and Hanahan, D. J. (1962), *Biochemistry 1*, 521.
Spackman, D. H., Moore, S., and Stein, W. H. (1958), *Anal. Chem. 30*, 1190.
Svedberg, T., and Pedersen, K. O. (1940), The Ultracentrifuge, London, Oxford University.
Svenson, H. (1954), *Opt. Acta 1*, 25.
Svenson, H. (1956), *Opt. Acta 3*, 164.
Teller, D. (1965), Ph.D. Thesis, University of California, Berkeley, Calif.
van Deenen, L. L. M., and de Haas, G. H. (1964), *Advan. Lipid Res. 2*, 167.
Wakui, K., and Kawichi, S. (1959), *J. Pharm. Soc. Japan 79*, 1177.
Wakui, K., and Kawichi, S. (1961), *J. Pharm. Soc. Japan 81*, 1394.
Ways, P. (1963), *J. Lipid Res. 4*, 101.
Wells, M. A., and Dittmer, J. C. (1966), *Biochemistry 5*, 3405.
Winzler, R. J. (1955), *Methods Biochem. Anal. 2*, 279.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Thermal Denaturation of Ribosomes*

Moshe Tal

ABSTRACT: In dilute buffer of Tris-acetate (0.001 M, pH 7.2) where the nuclease-free ribosomes from *Escherichia coli* MRE 600 can be heated without aggregation, reversible ~23% hyperchromicity can be observed with ultraviolet spectrophotometry or by optical rotation (α_D line). The T_m is 62°. Mg^{2+} elevates the T_m whereas EDTA, urea, salt, and various organic reagents lower it. Analysis of ribosomal ash by emission spectrography revealed zinc and nickel in significant amounts and magnesium, calcium, and iron in smaller amounts. The sedimentation constants of natural ribosomes in Tris-acetate (0.001 M) are 24 S + 42 S. However, after heating to 65° and cooling their S values dropped to 17 S + 24 S ("heated particles"). Viscometric analysis showed that the conformation of heated particles is between that of natural ribosomes and an open ribosomal structure which exists at 65°. Reversible interconversion between the "open-molecule" and "heated-particle" conformations was found in repeated cycle of heating and cooling, as was measured by spectrophotometric, viscometric and optical rotation methods. Examination of the composition of "heated particles" showed that the ribosomal ribonucleic acid remained intact and that the particles retain virtually all their proteins. On the basis of these findings a model of thermal denaturation of ribosomes is proposed.

The function of ribosomes demands a high degree of internal order. This well-defined structure enables them to bind mRNA and aminoacyl-tRNA effectively and to form peptide bonds, by means of interaction with cytoplasmic factors. In order to clarify the dependence of the biological activities of the ribosomes on their structure, a system is needed which lends itself to the study of the secondary structure of ribosomes.

By means of several physical methods, ribosomes were found to have a high degree of internal order (Hall and Doty, 1959; Blake and Peacocke, 1965; McPhie and Gratzer, 1966; Bush and Scheraga, 1967).

Thermal denaturation studies can contribute to our understanding of the ribosomal structure. However,

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