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# A Study of the Purification and Properties of the Phospholipase A of *Crotalus adamanteus* Venom*

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Two proteins (I and II) with phospholipase A activity have been isolated from the venom of *Crotalus adamanteus* (Eastern diamond back rattlesnake) by a procedure involving *p*H change, heat treatment, and subsequent chromatography on diethylaminoethyl-cellulose. Proteins I and II were chromatographically separable and had similar sedimentation constants but differed significantly in their electrophoretic mobility and isoelectric points. The purity of the proteins I and II was calculated to be 80 to 85% and the molecular weight estimated to be in the range of 30–35,000. No protease, nucleotidase, phosphodiesterase, or phosphomonoesterase activity was demonstrable in these purified fractions. Although the specific activity of I and II was not similar, there was no observed difference in the nature of the fatty acids liberated by their action on native ovolecithin. No specificity toward the chain length or unsaturation was evident. The presence of ethylenediaminetetraacetic acid was required throughout the purification for the preservation of maximal activity of these enzymes. In evaluating the mode of action of proteins I and II on lecithin in an ether solution, the inclusion of NaCl, $CaCl_2$, and ethylenediaminetetraacetic acid was found to be necessary for optimum enzymatic activity.

It has been well documented that several different species of snake venoms are potent sources of the enzyme phospholipase A (Kates, 1960). This enzyme has been shown to effect the hydrolytic cleavage of the fatty acid ester at the $\beta$ or 2 position of L-$\alpha$-lecithins with the formation of an $\alpha'$-acyl-L-$\alpha$-glycerylphosphorylcholine (lysolecithin) and free fatty acid (Tattrie, 1959; Hanahan *et al.*, 1960; de Haas *et al.*, 1960; de Haas and van Deenen, 1961).

Although there have been several reports on the purification of phospholipase A (Radominski and Deichmann, 1958; Neumann and Habermann, 1955; Boman and Kaletta, 1957; Yang *et al.*, 1959a,b), many of the described assay systems have depended upon quite indirect and inexact techniques for the evaluation of enzyme activity, namely, hemolysis and neurotoxic activity. Recently, however, Kawichi and coworkers (Wakui and Kawichi, 1961; Iwanaga and Kawichi, 1959; Wakui and Kawichi, 1959) have described the purification and properties of phospholipase A in certain Japanese and Formosan snake venoms. In this informative study, in which two separate, active phospholipase A fractions were isolated, the assay system depended on a combined enzymatic and chemical determination of lysolecithin formed in the reaction. This present communication confirms in part the results of Kawichi and collaborators and presents additional information and observations on the purification and properties of the phospholipase A of *Crotalus adamanteus* (Eastern diamond back rattlesnake) venom.

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## EXPERIMENTAL[1]

*Materials.*—Dehydrated *Crotalus adamanteus* (Eastern diamond back rattlesnake) venom was purchased from Ross Allen's Reptile Institute, Silver Spring, Fla. The ovolecithin (hen) was prepared by aluminum oxide (Rhodes and Lea, 1957) and silicic acid (Hanahan *et al.*, 1957)

[1] The following abbreviations are used: DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid (Na salt); AMP, adenosine-5′-phosphate; FAD, flavin adenine dinucleotide.

chromatography.[2] Its composition was as follows: P, 4.17%; N, 1.88%; N:P, molar ratio, 0.995; choline:P, molar ratio, 1.00; fatty acids: P, molar ratio, 2.05; iodine number, 56.8; $[\alpha]_D^5$ + 6.2 (2 dm, 5% in chloroform). Thin layer chromatography of this product, plus the infrared spectrum, showed lecithin as the only component. DEAE-cellulose was obtained from Eastman Organic Chemicals, Distillation Products Industries, Rochester 3, N. Y. Diisopropylphosphofluoridate and *p*-chloromercuriphenylsulfonic acid were generously supplied by Dr. H. Neurath and Dr. F. M. Huennekens, respectively. Ethylene glycol succinate on Chromosorb W was purchased from Applied Sciences Laboratories (State College, Pa.).

Ca(bis[*p*-nitrophenyl]phosphate)$_2$ and *p*-nitrophenyl phosphate were purchased from California Foundation for Biochemical Research. Adenosine-5′-phosphate was a Sigma Chemical Company product and casein (Hammarsten quality) was obtained from Nutritional Biochemical Corporation. Synthetic L-$\alpha$-lecithins were prepared by reacylation of lysolecithins (Hanahan and Brockerhoff, 1960).[3]

*Methods.*—The analytical procedures for phosphorus, nitrogen, choline, total fatty acids, and other components have been described in a previous publication (Dittmer and Hanahan, 1959). The assay of individual fatty acids was accomplished by gas-liquid chromatography of their methyl esters. These esters were prepared either by diazomethane treatment of the fatty acids in a mixture of methanol-ether (1:9, v/v) (Schlenk and Gellerman, 1960) or by transesterification of the intact lecithin with 5% anhydrous methanolic HCl for 2 hours (Stoffel *et al.*, 1959) but without the sublimation step. In the former case, it is necessary to remove completely any trace amount of diazomethane from the reaction mixture, since otherwise additional peaks, due to side-reaction products, will be evident in the gas-liquid chromatogram. After the diazomethane-treated mixture was allowed to stand for 10 minutes at room temperature, the solvents and excess of diazomethane were evaporated on the water bath at 30° for at least 30 minutes under nitrogen. Sufficient *n*-hexane (redistilled from potassium permanganate) was added to bring the concentration to approximately 10 mg per ml. The fatty acid esters in *n*-hexane were applied by the flow interruption technique to a 4-ft. column of ethylene glycol succinate, 19% on Chromosorb W (80 to 100 mesh), contained in a Pye Argon Chromatograph. This unit was equipped with a RaD ionization detector, and the temperature was maintained at 170° with a flow rate of 30 ml per minute. As an index of "efficiency" of the column, stearic and oleic acids could be separated cleanly (separation factor, 1.2) in 10 minutes.

Column chromatography on DEAE-cellulose was accomplished essentially by the procedures of Peterson and Sober (1956) and Keller *et al.* (1958). DEAE-cellulose was washed with 10 volumes of a mixture of 1 M NaOH and 0.5 M NaCl for 30 minutes at 4° with stirring, filtered on a Buchner funnel, and washed with water until the washings were free of alkali. The DEAE-cellulose was suspended in 6 volumes of 0.005 M potassium phosphate buffer, *p*H 7.4, containing $1 \times 10^{-3}$ M EDTA and titrated with 30% phosphoric acid to *p*H 7.4 with stirring. After the mixture had stood for 30 minutes, the *p*H of the supernatant was rechecked and adjusted, if necessary. The slurry was then evacuated at the water pump to remove the entrained air bubbles. The DEAE-cellulose slurry thus prepared was stored at 4° and was stable for at least one month.

The column used for purification of 1.0 g of crude venom measured 1.8 × 40 cm and contained a 200-ml reservoir equipped with a ball joint to which a pressure source (usually nitrogen) could be attached. The column was filled with 0.005 M buffer and the slurry was poured into the column and packed under 10 p.s.i. With this size column and with a gradient of 0.005 M to 0.1 M, 2 liters of each buffer were used.

## Results

### A. *Assay System and Definition of Activity Unit*

The assay system used was essentially the same as that described previously (Hanahan *et al.*, 1954), except that the dried venom was dissolved in an aqueous solution which contained NaCl, $CaCl_2$, and EDTA in a final concentration of $2.2 \times 10^{-1}$ M, $2 \times 10^{-2}$ M, and $1 \times 10^{-3}$ M, respectively, and adjusted to *p*H 7.4 with 0.1 N KOH. The protein concentration was measured by spectral absorption at 280 m$\mu$, $E_{280}^{1\%} = 23.2$, and by the Kjeldahl nitrogen value. In the usual enzyme reaction, 15 mg of ovolecithin was dissolved in 2 ml of diethyl ether in a 5-ml volumetric flask, and 20 $\mu$l of the above enzyme solution was added.[4] The reaction mixture was shaken for 3 minutes and then set aside for 10 or 15 minutes. At fixed intervals, this mixture was diluted with 95% ethanol to 5 ml and immediately

[2] Ovolecithin stored in 95% ethanol, and also synthetic lecithins isolated via silicic acid chromatography, showed on occasion a resistance to phospholipase A attack. If these samples were passed through an aluminum oxide column (1 mg P per g oxide) in chloroform-methanol (1:1, v/v), the lecithin obtained in this eluate was readily attacked by the enzyme. Whenever silicic acid chromatography was employed in the purification of ovolecithin, initial elution with chloroform was continued until no free fatty acids were detectable in a large sample of the eluate by gas-liquid chromatography.

[3] We are indebted to Dr. Patrick Kemp for his preparation of these compounds.

[4] The concentration of the enzyme was adjusted to make the reaction mixture turbid after approximately 9 minutes' incubation. Usually 3 $\mu$g of enzyme in 20 $\mu$l of the enzyme mixture was sufficient to effect the result.

titrated with 0.02 N methanolic NaOH, with cresol red used as an indicator.

Under the above conditions, it was found that the reaction followed essentially zero-order kinetics (Fig. 1). Therefore, the specific activity of the enzyme is expressed as μmoles of ovolecithin hydrolyzed per minute per mg of enzyme protein. The ovolecithin used in this experiment is assumed to be dipalmitoyllecithin for purposes of calculation of activity. The specific activity of the various batches of crude venom was in the range of 110 to 150.

In the above assay system and particularly with reference to phospholipase A action in general, the ionic environment as well as the *p*H were of considerable importance for optimal activity. The decided effect of NaCl and $CaCl_2$ on the activity of crude venom and purified proteins I and II is evident in Figures 2 and 3, respectively. Long and Penny (1957) noted that calcium ions at certain concentrations increased the rate of degradation of ovolecithin by moccasin venom phospholipase A, but concluded that this influence was more dependent on substrate than on enzyme concentration. In the current investigation a similar effect was not observed, but other factors such as physical state (charge effect) of the substrate, its purity and the nature of the fatty acids, and also the enzyme source, may make the difference. Wakui and Kawichi (1961) reported an increased sensitivity of purified phospholipase A fractions to $Ca^{++}$ and $Hg^{++}$ and argued that most probably this was due to the removal of metal ions in the purification procedure. The optimal *p*H of the assay system, which refers in this instance to the *p*H of the aqueous enzyme solution, was near 7.4 (Fig. 4).

Although no extensive studies were conducted on the influence of inhibitors or protein modification reagents on enzyme activity, the action of certain compounds on the phospholipase A activity of crude venom was investigated. When the crude venom was preincubated with *p*-chloromercuriphenylsulfonic acid ($10^{-4}$ M), L-cysteine ($10^{-4}$ M), or diisopropylphosphofluoridate ($10^{-2}$ to $10^{-4}$ M) for periods ranging from 0.5 to 30 hours and then assayed, no significant change in the specific activity of the venom was observed. While these reagents did not appear to influence the activity of the enzyme preparation, EDTA, under the conditions described below, had a positive effect on the ultimate stabilization of the enzyme.

### B. *Purification Procedure*

In general a combination of *p*H change, heat treatment, and column chromatography was found to be the most profitable route to highly purified phospholipase A. A typical experiment in which crude venom was subjected to these treatments is described below:

1. *p*H CHANGE.—One g of the crude dried venom was mixed with 100 ml of water with the resultant formation of a turbid suspension, *p*H 5.9 to 6.1, $E_{280}/E_{260} = 1.32$. The *p*H was adjusted to 9.0 by addition of approximately 5 ml of 0.1 N KOH. The precipitate was removed by centrifugation at 12,800 × *g* for 10 minutes at 4° and the supernatant (*A*) saved.

2. HEAT TREATMENT AT pH 3.0.—The supernatant (*A*) was adjusted to *p*H 7.0 and dialyzed at 4° against 32 liters of water containing $10^{-3}$ M EDTA for nearly 30 hours and then against $10^{-4}$ M EDTA for an additional 40 hours. At the end of this dialysis the enzyme solution was slightly turbid and had a *p*H of 5.2, $E_{280}/E_{260} = 1.76$. This slightly turbid preparation was adjusted to *p*H 3.0, and the resultant clear solution (*B*) was transferred to a 1-liter Erlenmeyer flask. The solution (*B*) was heated at 90° for 5 minutes in a hot paraffin oil bath (about 140°) with gentle stirring. After rapid cooling in ice-cold water, the *p*H, usually 3.2, was adjusted to 7.4 and the precipitate was removed by centrifugation at 12,800 × *g* for 10 minutes at 4°, $E_{280}/E_{260} = 1.77$; the clear supernatant (*C*) was saved.

3. COLUMN CHROMATOGRAPHY ON DEAE-CELLULOSE.—This entire operation was performed at 4°. The supernatant (*C*) was dialyzed against 32 liters of potassium phosphate buffer, 0.005 M, *p*H 7.4, containing $10^{-3}$ M EDTA. The DEAE-cellulose was equilibrated at 25° with the same buffer and packed into a column under 10 p.s.i., with a final column dimension of 1.8 × 37 cm. The column and the buffer were then placed at 4° and the buffer was allowed to pass through the column overnight. After application of the protein solution (*C*) to the column, a continuous flow of the same buffer was initiated and fractions of 27 ml each were collected. The flow rate was 50 ml per hour. The optical density of each fraction was measured at 280 mμ.

When the breakthrough peak had been reached and the absorption at 280 mμ had returned to a baseline value, gradient elution of the anionic proteins was begun. A linear gradient from 0.005 to 0.1 M potassium phosphate buffer, *p*H 7.4, containing $1 \times 10^{-3}$ M EDTA, was established. Figure 5 presents the elution diagram for this chromatographic separation.

Table I presents a typical protocol, including recoveries and enrichment, and Table IV shows some of the physical properties of the enzymatically active fractions. It was found that two chromatographically separable proteins, both of which were active on ovolecithin, were present in the venom. These two proteins are indicated as I and II in Figure 5.

### C. *Comments on Purification Procedure*

1. THE EFFECT OF DIALYSIS.—As shown in Table I, the main loss in total activity occurred during dialysis (step 3), wherein approximately 50% of the substances measured by the absorption at 280 mμ were lost; nonetheless, the specific

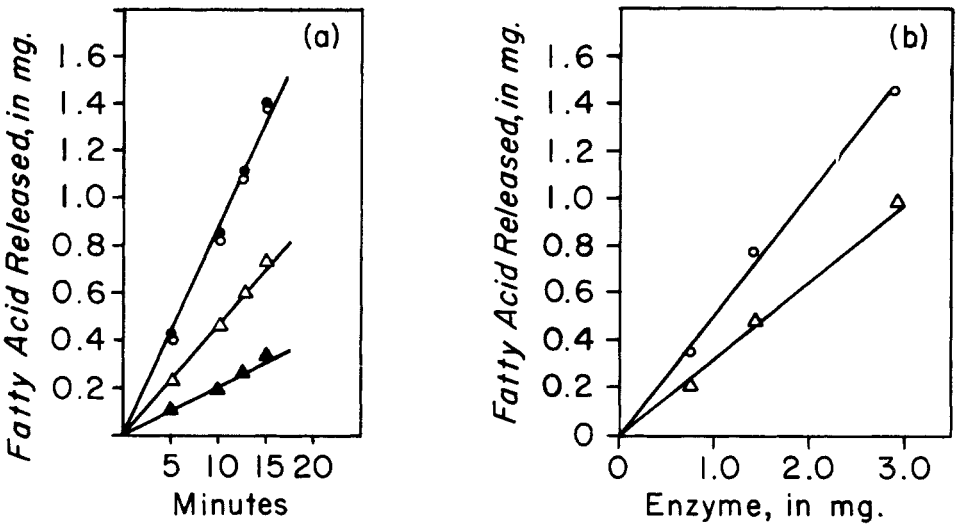


FIG. 1.—The rate of reaction of crude venom on ovolecithin, as influenced by (*a*) substrate concentration. 2.9 μg of venom incubated with 30 mg (●–●), 15 mg (O–O), 7.5 mg (△–△), and 3.8 mg (×–×) substrate, respectively; (*b*) Enzyme concentration. 15 mg substrate incubated with indicated amounts of enzyme for 10 minutes (△–△) and 15 minutes (O–O). Other details of reaction system given in text.

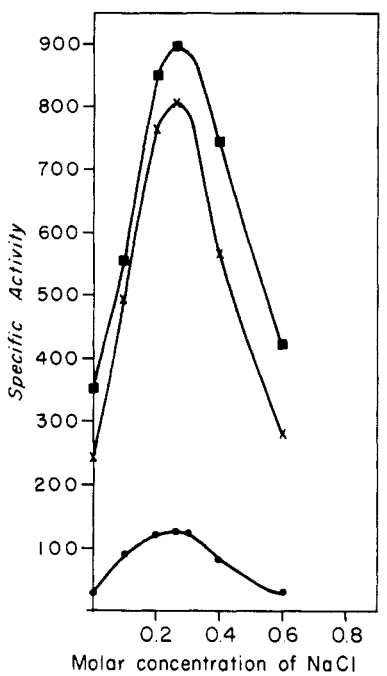


FIG. 2.—Influence of NaCl on phospholipase A activity. To a mixture of $CaCl_2$ ($2 \times 10^{-2}$ M), EDTA ($1 \times 10^{-3}$ M), and several different molar concentrations of NaCl, crude (●–●) and purified phospholipase A (I, ×–×; II, ■–■) fractions were added to make the final concentration of enzymes approximately 0.150, 0.030, 0.030 mg per ml (by optical density at 280 mμ), respectively, and the *p*H adjusted to 7.4. Twenty μl of this enzyme solution was added to a 2-ml ether solution containing 15 mg ovolecithin. After 10 minutes' incubation at 25°, the reaction mixture was diluted with 95% ethanol to 5 ml and directly titrated with 0.02 N methanolic NaOH, with 0.1% cresol red as an indicator.

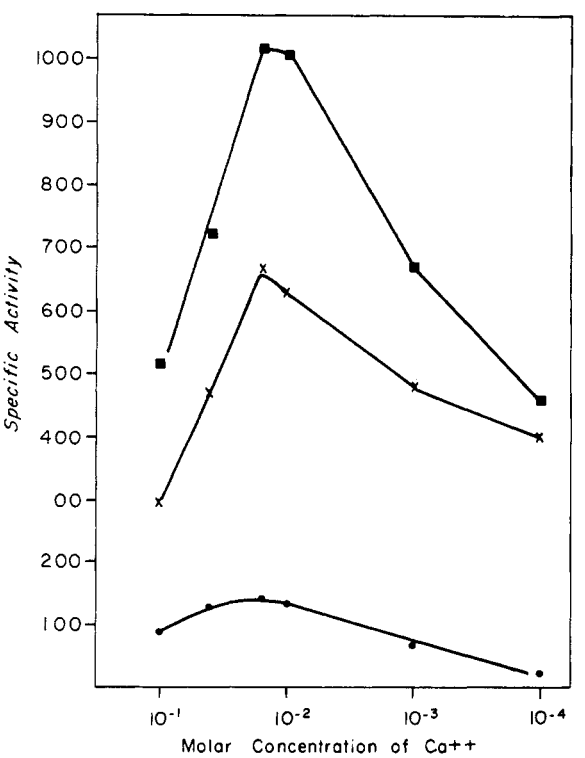


FIG. 3.—Influence of $Ca^{++}$ on phospholipase A activity. To a mixture of NaCl ($2.2 \times 10^{-1}$ M), EDTA ($1 \times 10^{-3}$ M), and several molar concentrations of $CaCl_2$, crude (●–●) and purified phospholipase A (I, ×–×; II, ■–■) fractions were added. Twenty μl of these enzyme solutions, adjusted to *p*H 7.4, was tested for activity on ovolecithin. Conditions were similar to those indicated in Figure 2.

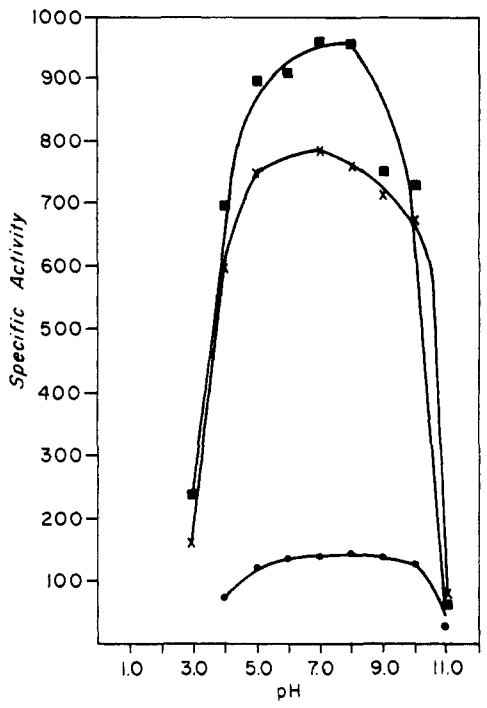


FIG. 4.—*p*H optimum. The solution of crude (●–●) and purified phospholipase A (I, ×–×; II, ■–■) fractions in a mixture of $CaCl_2$ ($2 \times 10^{-2}$ M), NaCl ($2.2 \times 10^{-1}$ M) and EDTA ($1 \times 10^{-3}$ M) was adjusted to desired *p*H values, with other conditions the same as in Figure 2.

activity (as compared with the original venom) did not increase twofold after dialysis. Similar observations are presented in Table II, in which specific activity and recovery of the protein and total activity were measured on 10 mg of the dried crude venom after three separate dialysis periods.

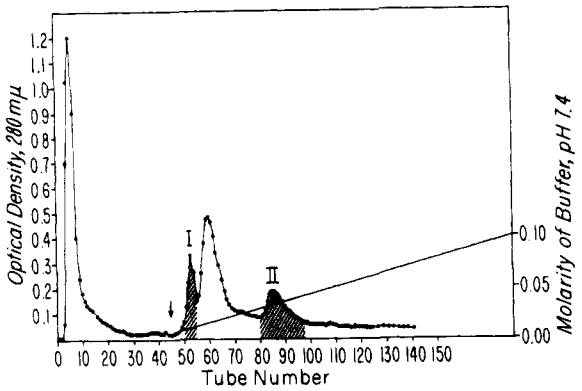


FIG. 5.—Chromatographic resolution of the anionic proteins of the partially purified enzyme (*p*H change and heat treatment) on DEAE-cellulose. The gradient was initiated at the point indicated by the arrow. The shaded areas I and II represent the fractions in which there was phospholipase A activity. The conditions of the experiment are given in the text. Volume in each tube was 27 ml.

One could possibly explain the loss of activity units after dialysis in the presence of EDTA by assuming that an activator or an enzymatically active, low-molecular-weight peptide(s) was removed; this appeared more likely than extensive proteolysis. If the latter situation had prevailed, the specific activity of the phospholipase A would have decreased with an increased dialysis period. Since the specific activity of the enzyme remained constant throughout a 3-day dialysis period, whereas the total units of activity decreased by 50%, proteolytic degradation evidently did not occur.

2. THE INFLUENCE OF HEAT TREATMENT.—Although the loss of activity during heat treatment is very small, treatment at 90° for 5 minutes at *p*H

TABLE I

PROTOCOL FOR PURIFICATION OF PHOSPHOLIPASE A

One g of the dried venom was subjected to treatment noted below and described in more detail in the text and in Figure 5. Rechromatography data are presented in Table III.

| | Protein[a] | | | Total Activity | |
|---|---|---|---|---|---|
| | Mg | % of Original | Specific Activity | Units | % of Original |
| 1. Crude, dried venom | 2000[b] | 100 (1.3)[c] | 112 (1.0)[d] | 224,000 | 100 |
| 2. Fraction soluble at *p*H 9.0 | 1728 | 86 (1.3) | 152 (1.4) | 262,656 | 117 |
| 3. Dialysis against EDTA ($10^{-3}$ M) | 859 | 43 (1.8) | 144 (1.3) | 123,696 | 55 |
| 4. Heat treatment at *p*H 3.0 | 470 | 24 (1.8) | 225 (2.0) | 105,750 | 47 |
| 5. Column chromatography | | | | | |
| First active peak (I) (Fractions 51–55) | 28 | 1.4 (1.5) | 460 (4.2) | 12,880 | 6 |
| Second active peak (II) (Fractions 80–97) | 69 | 3.4 (1.5) | 500 (4.5) | 34,500 | 15 |

[a] Measured by optical density at 280 mμ. [b] Owing to the obvious presence of colored material in the crude venom, the amount of "protein" calculated from the $E_{280}$ absorption value was higher than that actually weighed. [c] The numbers in parentheses represent the $E_{280}/E_{260}$ ratios of the particular fraction. [d] The numbers in parentheses represent the increase in specific activity obtained at each step.

TABLE II
EFFECT OF DIALYSIS ON ACTIVITY OF CRUDE VENOM

10 mg crude venom dialyzed against $1 \times 10^{-4}$ M EDTA for the intervals indicated and then assayed for phospholipase A activity.

| | Protein (mg) | Specific Activity | Total Activity | |
|---|---|---|---|---|
| | | | Units | % of Original |
| Before dialysis | 10.0[a] | | | |
| | 20.5[b] (1.3)[c] | 120 | 2460 | 100 |
| After dialysis | | | | |
| One day | 12.8[b] (1.8) | 108 | 1382 | 56 |
| Two days | 12.5[b] (1.7) | 114 | 1425 | 58 |
| Three days | 12.7[b] (1.7) | 107 | 1359 | 55 |

[a] Measured by dry weight. [b] Measured by optical absorption at 280 mμ. [c] The numbers in parentheses represent the $E_{280}/E_{260}$ ratios of the particular fraction.

3.0 is nevertheless drastic, and it is possible that one of the two active proteins may be an artifact, *e.g.*, one deriving from the other. In order to ascertain whether these two active proteins are present in the crude venom, an attempt was made to purify the crude venom without heat treatment. Fifty-four mg of the crude venom, specific activity 120, was dissolved in 5 ml of 0.7% EDTA and dialyzed overnight at 4° against 6 liters of water containing $1 \times 10^{-3}$ M EDTA, and then against 6 liters of potassium phosphate buffer, 0.005 M, *p*H 7.4, plus $1 \times 10^{-3}$ M EDTA for 24 hours. A small amount of precipitate formed and was spun down at 12,800 $\times g$ at 4° for 10 minutes. The supernatant was applied to a DEAE-cellulose column (0.9 × 20 cm), and fractions of 2.1 ml were collected at a flow rate of 4 ml per hour. A linear gradient from 0.005 to 0.1 M potassium phosphate buffer, *p*H 7.4, containing $1 \times 10^{-3}$ M and $5 \times 10^{-3}$ M EDTA, respectively, was established as above.

The elution diagram, which was similar to that shown in Figure 5, showed the presence of two major phospholipase A active protein peaks. The first peak, I, had a specific activity of 43 and represented 2% of the total activity units, and

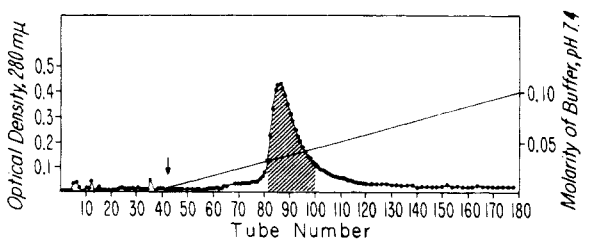


FIG. 6.—Column chromatography of the reheated second active protein (II, Fig. 5) on DEAE-cellulose. The gradient was initiated at the point indicated by the arrow, and the shaded area represents the fractions in which there was phospholipase A activity. The conditions of the experiment are given in the text. Volume in each tube was 3.5 ml.

the second peak, II, had a specific activity of 170 and contained 6.5% of the total activity units. A small amount of phospholipase A activity was present in the fractions between the two major peaks, but this represented only 0.4% of the total activity units applied to the column. On the basis of this evidence, it was concluded that the two active proteins I and II are present in the crude venom, with the major enzymatic activity being present in the second peak (II). In other words, the two active peaks are not artifacts resulting from the heat treatment. On the other hand, the recovery of total activity in the procedure without heat treatment is only 9%, *i.e.*, the activity of each peak is not stable or constant. Another possibility would be that the enzymatically active protein is irreversibly adsorbed on the ion exchanger. The recovery of the activity from heat-treated samples is consistent and is near 21%. Consequently, heat treatment can afford optimum "protection" of the activity of the enzyme. One possible explanation of the general success of this approach may reside in the heat lability or metal requirements, or both, of certain of the proteases of the crude venom. Deutsch and Diniz (1955) noted that the proteolytic activity of various snake venoms toward hemoglobin was inhibited by EDTA. Hence, since proteases were present in the crude venom used here, a comparable situation may exist.

3. OTHER OBSERVATIONS.—It was important to prove further whether the heat treatment possibly caused a cleavage of one molecule of phospholipase A into two active fragments. The second active protein (Fig. 5, peak II) was rechromatographed (as described below) and was approximately 85% homogeneous electrophoretically; 37 mg of this material was dissolved in 3 ml of water containing $1 \times 10^{-4}$ M EDTA, adjusted to *p*H 3.0, and subjected to heat-treatment at 90° for 5 minutes. A completely clear solution resulted, and after adjustment to *p*H 7.4 this solution was dialyzed and chromatographed on a DEAE-column (0.9 × 20 cm) in the same manner as described previously.

The elution diagram, illustrated in Figure 6, shows the presence of only a single peak, *i.e.*, the major enzymatically active protein (Fig. 5, peak II). The recoveries of protein and activity on the reheated sample were 68% and 93%, respectively; the specific activity was 790. Since the recovery of protein was low, the column was further eluted with 1 M phosphate buffer, *p*H 7.4, but less than 1% of the added protein was recovered.

The influence of EDTA on the activity of the purified enzyme was also quite definite. This effect was illustrated with a partially purified enzyme preparation which was obtained by *p*H change, dialysis against EDTA, and heat treatment in the presence of EDTA. This sample was applied to a DEAE-column without any added EDTA and eluted with the same buffer as above. The recovery of the total activity was

nearly 13%, or approximately half of the amount recovered in the presence of EDTA. Hence, a protective action of EDTA on the stability of the enzyme was strongly indicated.

### *D. Large-Scale Preparation of Proteins I and II*

A total of 15 g of the dried venom was purified in three separate batches of 5 g each exactly as described above, except that the column chromatography was undertaken at the same loading ratio on a larger column (3.0 × 70 cm). The separation obtained in this experiment was similar to that described in Figure 5. Although the two active proteins thus obtained were approximately 70% homogeneous by electrophoretic examination, the purity could be increased further to nearly 85% by rechromatography under the same conditions. These results are presented in Table III and Figure 7. Subsequently, proteins I and II were dialyzed against two changes of 6 liters of water containing $10^{-3}$ M EDTA for 2 days for removal of phosphate buffer. Thereafter, each protein solution was lyophilized and the resultant white powder, containing a small amount of EDTA, was stored at −25°. The activity remained constant for several months.

### *E. Properties of Proteins I and II*

The two enzymatically active proteins obtained by the procedure described in the preceding section (*D*) were subjected to the following physical measurements, the results of which are summarized in Table IV.[5]

[5] An examination of the second active protein (II) revealed the presence of lipid. Although little or no lipid was extracted from an acidified solution of protein II, a detectable amount of lipid was released as fatty acid upon hydrolysis of this fraction at reflux in 6 N HCl for 8 hours. Subsequent analyses of this fatty acid fraction by gas-liquid chromatography indicated approximately 4 moles of fatty acid per mole of enzyme (assuming a molecular weight of 30,000). Interestingly, nearly 85 mole % of this fatty acid fraction was composed of a $C_{18}$ compound. Lack of sufficient amounts of protein II prevented a more detailed study.

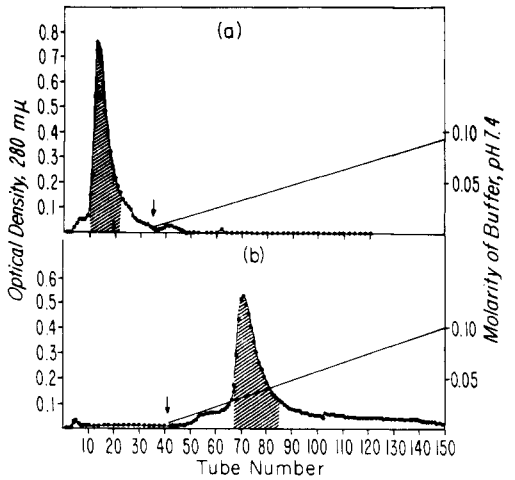


FIG. 7.—Rechromatography of the first (*a*) and second (*b*) active proteins (I and II, respectively, Fig. 5) on DEAE-cellulose. The gradient was initiated at the point indicated by the arrow and the shaded areas represent fractions in which there was phospholipase A activity. The details are given in the text.

1. ELECTROPHORETIC ANALYSIS.—Electrophoresis was performed in monovalent buffers (sodium acetate and potassium phosphate) of ionic strength 0.1 over a *p*H range of 4.4 to 7.4 in a Spinco model H electrophoresis apparatus. Figure 8 shows typical electrophoretic patterns of the crude venom and proteins I and II. The *p*H-mobility curve of the two active proteins is shown in Figure 9.

2. SEDIMENTATION ANALYSIS.—Solutions of proteins I and II were analyzed in the Spinco model E ultracentrifuge at 20°, in the standard cell. The buffer was potassium phosphate, ionic strength 0.1, *p*H 7.4. Sedimentation constants were obtained over the concentration range of 0.1 to 1.0% protein. Extrapolation of these values to infinite dilution gave values $s_{20,w}$ = 3.08 for protein I and 3.03 for protein II. On the basis of the sedimentation equilibrium data, the molecular weights of protein I and protein II were estimated to be in the range of 30–35,000.

3. SPECTRAL ABSORPTION CHARACTERISTICS.—

TABLE III

RECHROMATOGRAPHY OF TWO ACTIVE PROTEINS

The two enzymatically active proteins (I and II, Figure 5) were rechromatographed on DEAE cellulose columns as described in the text and Figure 8.

| Sample | Conditions | Protein[a] Mg | Protein[a] % of Original | Specific Activity | Total Activity Units | Total Activity % of Original |
|---|---|---|---|---|---|---|
| First active protein (I) | Before rechromatography | 314 | 100 | 465 (1.0)[c] | 146,010 | 100 |
| | After rechromatography | 190[b] | 60 | 575 (1.2) | 109,250 | 75 |
| Second active protein (II) | Before rechromatography | 336 | 100 | 500 (1.0) | 168,000 | 100 |
| | After rechromatography | 187[b] | 56 | 850 (1.7) | 158,950 | 95 |

[a] Measured by optical density at 280 mμ. [b] Indicates the areas shaded in Figure 8. [c] The numbers in parentheses indicate the increase in specific activity during rechromatography.

TABLE IV
COMPARISON OF PROPERTIES OF THE TWO PHOSPHOLIPASE A FRACTIONS

| Properties | First Active Protein | Second Active Protein |
|---|---|---|
| $u \cdot cm^2 \cdot volt^{-1} \cdot sec^{-1}$ ($pH$ 7.4) | $-3.94 \times 10^{-5}$ | $-4.88 \times 10^{-5}$ |
| Isoelectric point, univalent buffer, $\Gamma/2 = 0.1$ | 4.55 | 4.40 |
| $s_{20,w}$ | 3.08 $s$ | 3.03 $s$ |
| $E^{1\%}_{280}$, based on the Kjeldahl N | 23.2 | 21.3 |
| Specific activity on native ovolecithin | 575 | 850 |

These two active proteins exhibited a typical protein spectral pattern, with a maximal extinction at 280 m$\mu$, a minimal extinction at 252 m$\mu$, and a ratio of extinction, $E_{280}/E_{260}$, of 1.96 for each protein. $E^{1\%}_{280} = 23.2$ for the first and 21.3 for the second active protein, as calculated from the Kjeldahl nitrogen values.

### *F. Action of Crude Venom and Purified Phospholipase A on Native Ovolecithin*

The enzymatic action of the crude venom and of the purified phospholipase A fractions on ovolecithin was followed in an ethereal solution; the amount of fatty acids liberated from ovolecithin at specified time intervals was measured, and composition of the liberated fatty acids at these times intervals was determined (Table V). In all cases the reaction was 95% complete at 120 minutes. Experimentally, 15 mg of ovolecithin dissolved in 2 ml of diethyl ether was incubated with 3.0, 0.8, and 0.7 $\mu$g, respectively, of the crude venom and of the chromatographically purified proteins I and II (Fig. 5) phospholipase A. At fixed intervals, the reaction mixture was diluted with 95% ethanol to 5 ml and divided into two parts; (*a*) the liberated fatty acids in 2 ml of the reaction mixture were directly titrated with 0.02 N methanolic NaOH, and (*b*) the remainder was dried *in vacuo*, dissolved in 5 ml chloroform, and placed in a 1-g silicic acid column (12 × 8 mm). The column was washed with 50 ml of chloroform and the eluate, which was free of phosphorus, was evaporated, methylated with diazomethane, and analyzed by gas-liquid chromatography.

The only products of these enzymatic reactions were lysolecithin and free fatty acid. These compounds were isolated by column chromatography (Hanahan *et al.*, 1960) and identified by chemical analysis, by their behavior on thin layer chromatography, and by the nature of their infrared spectra.

The data obtained from the reaction of proteins I and II on native ovolecithin were plotted by the double reciprocal method (Neilands and Stumpf, 1958), and these results are shown in Figure 10. In both instances the curves were identical and yielded a $K_M$ value of $3.9 \times 10^{-2}$ M. Similar results were obtained in experiments with the synthetic lecithins, $\alpha'$-palmitoyl-$\beta$-oleoyl-L-$\alpha$-glycerylphosphoryl choline and $\alpha'$-oleoyl-$\beta$-palmitoyl-L-$\alpha$-glycerylphosphoryl choline.

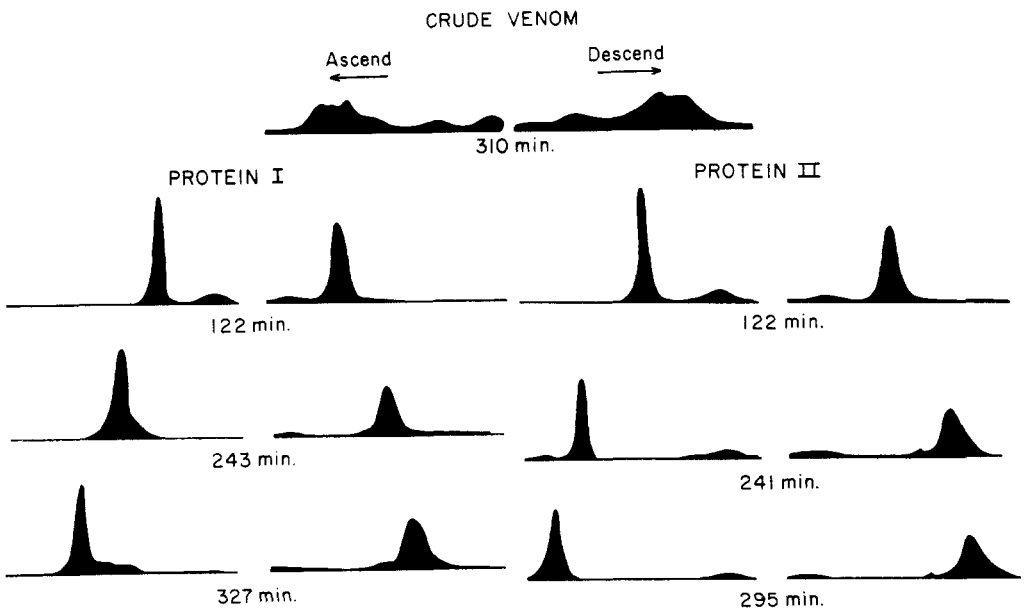


FIG. 8.—Electrophoretic patterns of crude venom and of the active proteins, (I) and (II), in potassium phosphate buffer, $pH$ 7.4, ionic strength 0.1, at several time intervals. Field strength, 5.80 v per cm for crude venom and 5.87 v per cm for (I) and (II).

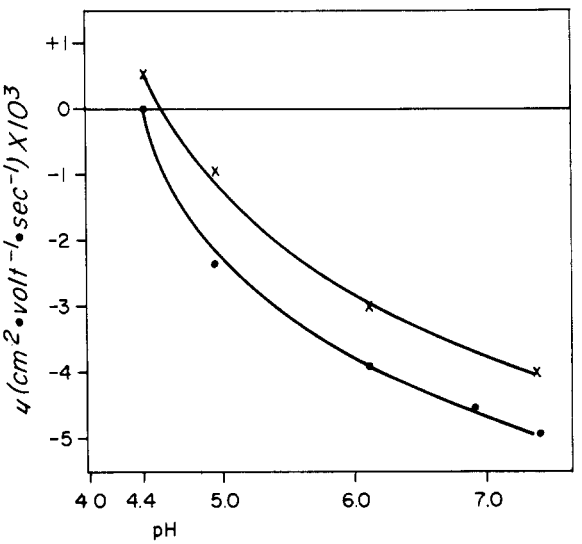


FIG. 9.—*p*H-mobility curves of phospholipase A active protein I (×) and II (●). Monovalent buffers of ionic strength 0.1 were used in both series.

### G. *Other Enzymatic Activities in Crude and Purified Phospholipase A Fraction*

It is well established that, in addition to phospholipase A, snake venom contains many other enzymes, *e.g.*, proteases, phosphatases, nucleotidases (AMPase), and L-amino acid oxidase.[6] In the present study, the purified phos-

[6] The L-amino acid oxidase activity of proteins I and II was not determined. Although no FAD was detectable spectrophotometrically, it is interesting to note that FAD was found only in the highly colored fraction intermediate between the two phospholipase A active peaks. On the basis of the observations of Wellner and Meister (1960) on the presence of 2 moles of FAD per mole of crystalline L-amino acid oxidase of *Crotalus adamanteus*, we have assumed that no L-amino acid oxidase was present in proteins I and II.

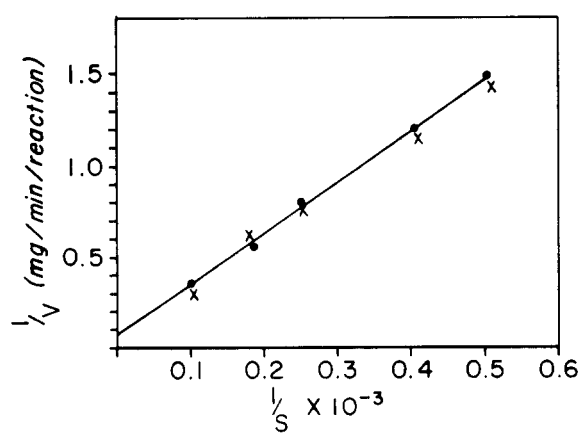


FIG. 10.—The reciprocal plot of fatty acid released by action of proteins I (●–●) and II (×–×) *against* substrate (lecithin) concentration. The details of these experiments are described in the text. Reaction time, 15 minutes.

TABLE V

FATTY ACIDS RELEASED AT VARIOUS TIME INTERVALS BY ACTION OF CRUDE VENOM (CV), ACTIVE PEAK I (FIG. 5), AND ACTIVE PEAK II (FIG. 5) ON OVOLECITHIN[a]

| Fatty Acid[b] | Chemical Hydrolysis | Enzymatic Hydrolysis | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 10 min. | | | 20 min. | | | 40 min. | | | 60 min. | | | 90 min. | | | 120 min. | | |
| | | CV | I | II | CV | I | II | CV | I | II | CV | I | II | CV | I | II | CV | I | II |
| 16:0 | 40.7 | 1.7 | 1.8 | 2.3 | 1.7 | 1.8 | 2.0 | 1.3 | 1.4 | 1.3 | 1.3 | 1.4 | 1.7 | 1.3 | 1.3 | 1.5 | 1.3 | 1.1 | 1.3 |
| 16:1 | 1.2 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.9 | 0.7 | 0.6 | 0.6 | 0.7 | 0.9 | 0.8 | 0.5 | 0.6 | 0.6 | 0.6 | 0.8 | 0.5 |
| 18:0 | 11.7 | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | tr. | — |
| 18:1 | 33.0 | 66.8 | 67.1 | 66.9 | 66.3 | 64.2 | 64.0 | 63.9 | 65.2 | 64.0 | 64.3 | 64.0 | 65.8 | 66.6 | 63.5 | 61.8 | 65.5 | 68.8 | 62.8 |
| 18:2 | 11.9 | 26.5 | 26.2 | 26.3 | 26.5 | 23.0 | 23.0 | 26.5 | 28.1 | 29.1 | 29.1 | 28.9 | 27.7 | 27.4 | 31.2 | 32.1 | 28.7 | 25.2 | 30.9 |
| 20:4 | 1.5 | 4.4 | 4.2 | 3.8 | 4.9 | 5.4 | 5.0 | 4.6 | 4.7 | 5.0 | 4.6 | 4.8 | 4.2 | 4.2 | 3.3 | 3.9 | 4.0 | 4.0 | 4.5 |

[a] Results expressed as mole %. [b] This notation system for the fatty acids indicates chain lengths and number of double bonds. 16:0, a saturated acid such as palmitic acid; 18:1, a monenoic acid such as oleic acid, etc. Trace amounts (less than 0.2 mole %) of the following acids (tentative) were detected in all fractions. 17:0, 18:3, 22:6, and 24:0.

pholipase A fractions I and II as well as the crude venom were assayed for protease, phosphodiesterase, and AMPase activity.

1. PROTEASE ACTIVITY.—This activity was measured by the assay technique of Kunitz (1947). In a typical experiment, 1 ml of a 2% casein solution was incubated at 25° with 3 ml 0.05 M phosphate buffer, *p*H 7.4, which contained 2.10, 2.25, and 2.13 mg (measured by optical density at 280 mμ), respectively, of crude and purified phospholipase A fractions I and II. At fixed intervals a 1-ml aliquot was mixed with 2 ml 10% trichloroacetic acid and centrifuged, and the optical density of the supernatant was measured at 280 mμ. The results showed that proteases were not present in the purified phospholipase A fractions I and II, but, as expected, were present in the crude venom. In the latter instance, 0.34 μmole tyrosine was released in 8 hours by 1.0 mg protein.

2. PHOSPHODIESTERASE ASSAY. —Essentially the technique of Boman and Kaletta (1957) was used, wherein the quantity of *p*-nitrophenol liberated from Ca(bis[*p*-nitrophenyl]phosphate)$_2$ by enzymatic attack was measured by its optical absorption at 420 mμ. As an example, 0.3 ml of the enzyme solution was incubated at 37° with 2 ml 0.05 M Tris buffer, *p*H 8.9, which contained 0.01 N Ca(bis(*p*-nitrophenyl)phosphate)$_2$ and 0.01 M $MgSO_4$. Each of the enzyme solutions contained 0.215, 0.230, and 0.220 mg per ml, respectively, of the crude venom and the purified phospholipase A fractions I and II in 0.5% NaCl. At fixed intervals, a 0.2-ml aliquot was diluted with 2 ml 0.1 N NaOH and the optical absorption was immediately measured at 420 mμ. No phosphodiesterase activity was detectable in the purified phospholipase A fractions I and II, but, as expected, the crude venom had a significant diesterase activity. As an example, 8.1 μmoles *p*-nitrophenol were released by 1.0 mg crude venom protein in 6.5 hours.

In addition, the phosphomonoesterase activity in the crude venom, as measured with *p*-nitrophenylphosphate as substrate, was very low in comparison to the phosphodiesterase activity.

3. 5′-NUCLEOTIDASE.—The procedure of Björk and Porath (1959) was used for assay purposes. Experimentally, 10 ml of substrate solution which contained 0.01 M adenosine-5′-phosphate, 0.01 M $MgSO_4$, and 0.05 M Tris buffer, *p*H 8.9, was incubated at 37° with 1 ml of enzyme in 0.5% NaCl. The concentrations of the crude venom and of phospholipase A fractions I and II were 0.308, 0.315, and 0.320 mg per ml, respectively.

At fixed intervals, a 1-ml aliquot was removed and assayed for inorganic phosphate. AMPase activity was not detectable in the purified phospholipase A fractions I and II but was present in the crude venom. For example, 83.3 μmoles inorganic phosphate were released by 1.0 mg crude venom protein in 60 minutes and 121 μmoles in 90 minutes; thereafter, the rate of release became constant.

## DISCUSSION

In the present study, the phospholipase A activity in the venom of *Crotalus adamanteus* was isolated in a reasonable state of purity through a procedure utilizing *p*H change, heat treatment, and chromatographic separation on a DEAE-cellulose column. A novel aspect of this study was the finding of two chromatographically separable proteins with phospholipase A activity (proteins I and II, Fig. 5). Proteins I and II possessed similar sedimentation constants but had significantly different electrophoretic mobilities and isoelectric points. These observations suggested that these two enzymatically active proteins might be artifacts of the isolation technique. This situation could have resulted from the heat treatment and subsequent chromatographic process, or from the conversion of protein I to II either by a dimerization process or by physical or enzymatic means. On an experimental basis, however, it was observed that these two proteins could be isolated (though in lower yields) from the crude venom by a procedure not involving heat treatment. Further, rechromatography of proteins I and II separately on DEAE-cellulose columns yielded no additional protein peaks. Finally, the similarity in sedimentation constants of I and II tends to obviate a consideration of dimer formation. Consequently, it was reasonable to assume that these two phospholipase A active proteins do exist in this venom. As added support of this conclusion Kawichi and collaborators (Iwanaga and Kawichi, 1959; Wakui and Kawichi, 1959, 1961) demonstrated that two phospholipase A active proteins could be obtained from Mamushi and Habu snake venoms by starch zone electrophoresis and by carboxymethyl and DEAE-cellulose chromatography. While these two venoms apparently contained two species of phospholipase A, these authors noted that cobra venom contained only one protein with phospholipase A activity.

Although no adequate explanation of the finding of two distinct phospholipase A proteins in venom can be offered now, similar observations have been reported on other enzyme systems. Two proteolytic enzymes with identical enzymatic behavior have been isolated from stem bromelain (Murachi and Neurath, 1960). As further examples of this phenomenon, two forms of ribonuclease, A and B, with the same activity have been observed in pancreas (Tanford and Houenstein, 1956), and several phosphatases with similar enzymatic behavior have been detected in peas (Pierpoint, 1957). Boman and Kaletta (1957) have reported the separation of three phosphodiesterases with comparable activity from *Crotalus adamanteus* venom. Hence, the isolation of two phospholipase A active proteins is certainly not unique, as is well substantiated by the observations of Markert and Møller (1959) on the existence of "isozymes."

The purity of proteins I and II must be con-

sidered with some reservation. The phospholipase A activity of I and II was comparable with respect to mode and specificity of attack but differed with respect to specific activity. This difference could be attributed to the adventitious inclusion of proteins with no phospholipase A activity. In this regard, proteins I and II contained no proteases, nucleotidases (AMPase), or phosphodiesterases (or phosphomonoesterases), and presumably no L-amino acid oxidase. Thus, on this basis alone, the purification achieved in this fractionation appears of a high order but again does not rule out the presence of non-enzymatically active protein. This latter point gained support through observation of the physical behavior of these two protein fractions. Although I and II could be separated by free boundary electrophoresis, it was evident from their patterns that they were not entirely homogeneous proteins, and with some reservation could be considered near 85% in purity.

The results of this present investigation show that the two phospholipase A active proteins from *Crotalus adamanteus* venom have an identical mode of action on ovolecithin. It was observed that the fatty acids liberated by the attack of crude venom and proteins I and II on lecithin were predominantly unsaturated, mainly oleic and linoleic acids. Nevertheless these latter acids were always accompanied by 1 to 2% saturated acids, mainly palmitic acid, even after a short incubation period. As shown in Table V, there was no apparent difference in the composition of the fatty acids liberated by the crude venom and by the two purified phospholipase A proteins. This distribution of fatty acids was the same at the theoretical limit of the reaction, namely, after liberation of one mole of fatty acid per mole of substrate as in the initial phases of the reaction. Only limited success was achieved with use of a completely saturated substrate (owing to difficulties in proper solubilization), but there appeared to be no difference in the reactivity of the proteins I and II to this type of substrate. Finally, the Michaelis constant, $K_M$, for these two enzymes, $3.9 \times 10^{-2}$ M, was in the range expected for hydrolytic enzymes. These observations support the conclusion that proteins I and II have similar phospholipase A activity and differ primarily in their physical and chemical structures or both. Recently, however, Bennett and Tattrie (1961) have reopened the question of phospholipase A specificity. These authors claim from their experimental evidence that although phospholipase A directs its attack primarily at one carboxylic ester position of "semisynthetic" lecithins, it also can attack the other position to a limited extent. Nonetheless, on the basis of enzyme incubation periods of nearly 40 hours and the acidic nature of their reaction mixture, the significance of these observations must be questioned. Inasmuch as no control reactions (without added enzyme) were reported, it would appear that nonspecific degradation could conceivably have occurred in this long reaction period. Further, the reason for a long lag period (up to 21 hours) during which no enzymatic degradation occurred was not readily apparent. In our current study, the conditions of the experiments were such that lecithin could be completely degraded to lysolecithin and free fatty acids in 2 to 3 hours.

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# Phospholipid Patterns of the Developing Chick Embryo*

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The phospholipids of unincubated egg yolks and of chick embryos of eggs incubated 4, 6, 12, 14, and 18 days have been separated by silicic acid chromatography. The major components have been identified as lipids containing ethanolamine, choline, sphingosine, and inositol in the egg and the embryos. Serine-containing lipids were found in the embryo but not in the egg yolk. The phospholipid pattern in the developing embryo was similar throughout the period studied, although changes in the relative amounts of the individual phosphatides were apparent as development progressed. Serine lipids appeared to increase, and lecithin decreased. The most prominent change occurred in the sphingomyelins, which at least doubled in relative amount between 4 and 18 days of incubation. It has been suggested that the change in the sphingomyelins is a reflection of nerve myelination in the chick embryo. The lipid phosphorus content was found to remain relatively constant throughout the period studied when determined from DNA content. When determined on the basis of fat-free Kjeldahl nitrogen or fat-free dry weight, lipid phosphorus declined steadily.

Although there have been reports in the literature on the changes of lipid phosphorus in the egg yolk and the chick embryo during incubation, little information on the separation of the phospholipids has been reported. Egg yolk phospholipids have been extensively studied by Rhodes and Lea (1957). Studies of ether-soluble lipid phosphorus in the developing chick embryo and the residual yolk have been carried out by Plimmer *et al.* (1909), Masia and Fukutomi (1923), Kugler (1936), and Tsuji *et al.* (1955). In the above studies of the embryo phospholipids, separation of the individual phospholipids was not attempted, and the results, reported in terms of cephalin, lecithin, and sphingomyelin, were based primarily on analysis of nitrogen bases.

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The purpose of the present paper is to present the results of studies on the quantitative separation and identification of the major phospholipid components of unincubated egg yolk and chick embryos at 4, 6, 12, 14, and 18 days of incubation. It is hoped that this information can be used to correlate changes in phospholipid patterns with morphologic changes during embryogenesis.

## Materials and Methods

Fertilized eggs from White Leghorn chickens were obtained from Hanson's Hatchery, Corvallis, Ore. The eggs were incubated in electric incubators as specified by the manufacturer.

The lipid isolation technique used was the same for all samples. The wet tissue was homogenized for 2 minutes in a Waring Blendor with a solution of chloroform–methanol (2:1 v/v). The total volume of solvent used was ten times the wet weight of the tissue. One half of the solvent was used in the first extraction, with an extraction time of fifteen minutes. After centrifugation the precipitate was extracted with the second portion of the solvent for 30 minutes. After centrifugation of this mixture the first and second extracts were combined and washed with solutions of KCl as described by Folch *et al.* (1957). In the first wash, a volume of 0.88% KCl equal to 0.2 of the volume of the lipid extract was thoroughly mixed