Observations on the Phospholipase A_2 of *Crotalus atrox*. Molecular Weight and Other Properties*

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ABSTRACT: Phospholipase A_2 (EC 3.1.1.4.) was isolated from *Crotalus atrox* venom essentially by a procedure previously developed for the same enzyme activity from *Crotalus adamanteus* venom (M. A. Wells and D. J. Hanahan (1969), *Biochemistry 8*, 414). The purified enzyme from the *C. atrox* venom was shown to have a molecular weight of 29,500 as indicated by gel filtration and low-speed sedimentation equilibrium. The specific activity of this preparation was near 1500 equiv of fatty acid released per minute per milligram of protein. On disc gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol, the enzyme was found to have a molecular weight of 14,700. This evidence would support a dimer structure for the native enzyme. The amino acid composition was determined and the results would be consistent with a molecular weight of 29,500. Similar to the phospholipase A_2 isolated from other sources, the enzyme from *C. atrox* venom exhibited a characteristically high content of cystine residues. These results were contrary to those reported by T. W. Wu and D. O. Tinker ((1969), *Biochemistry 8*, 1558), who isolated a phospholipase A_2 from *C. atrox* venom with a molecular weight of 14,500, as judged by gel filtration only, and a specific activity of 188. A discussion of the possible reasons for these discordant results is presented.

Over the past several years this laboratory has maintained a continuing interest in the biochemical characterization of phospholipase A_2 (EC 3.1.1.4.) present in snake venoms. In an earlier study on the phospholipase A_2 present in *C. adamanteus* venom (Saito and Hanahan, 1962), two species of the enzyme were isolated and their molecular weight estimated to be in the range of 30,000–35,000. Later, a more detailed and comprehensive purification of the two species of this enzyme from the same source by Wells and Hanahan (1969) showed each form to have a molecular weight of 29,800. It was interesting and yet puzzling that Wu and Tinker (1969) studying a similar enzyme activity in *C. atrox* venom, reported a molecular weight of 14,500. Further, the occurrence in closely related species of an enzyme with widely differing molecular weights, in fact by a factor of two, was indeed provocative. One obvious explanation, of course, was that the phospholipase A_2 isolated from *Crotalus adamanteus* was a dimer whereas the phospholipase from *C. atrox* was best represented as monomer.

In order to more fully understand these observations, we were prompted to isolate the phospholipase A_2 from the readily available *C. atrox* venom and to study its behavior. Wu and Tinker (1969) had previously reported isolation of this enzyme from this source with a molecular weight of 14,500 and a specific activity of 188. The results of our experiments are contrary to those reported by these investigators and show essentially that the one form of phospholipase A_2 isolated from this venom has a molecular weight of 29,500 and a specific activity of 1500. Only under strongly dissociating conditions was evidence obtained for a lower molecular weight species comparable to that noted by Wu and Tinker. However, it was clear that the phospholipase A_2 of *C. atrox* does exist as a dimer as isolated. Recently, Wells (1971) has presented conclusive evidence on a dimer structure for the phospholipase A_2 obtained from *Crotalus adamanteus* venom.

Experimental Section

Materials

Lyophilized *Crotalus atrox* (Western diamondback rattlesnake) venom was purchased from Miami Serpentarium (Miami, Fla., lot no. CXA 34F). Aluminum oxide (Bio-Rad, Richmond) and silica gel G (Merck, Darmstadt) were employed for column chromatography and thin-layer chromatography, respectively. DEAE-cellulose (DE-52) was a Whatman product; SE-Sephadex (C-50), Sephadex G-75, and Sephadex G-100, beaded forms were obtained from Pharmacia Products, respectively. All solvents were reagent grade and used as received, except for diethyl ether, which was redistilled. Constant-boiling HCl was distilled twice in an all-glass apparatus. Dialysis tubing was boiled in 0.1 M EDTA (pH 7.0) for 30 min, washed with distilled water, and stored in cold water. Sodium dodecyl sulfate (Sigma) was crystallized twice from 80% ethanol and air-dried.

Ovalbumin (five-times crystallized) and pepsin (crystallized) were obtained from Pentex (Kankakee). Ribonuclease (bovine pancreas, five-times crystallized), α-chymotrypsinogen (bovine pancreas, crystallized), and cytochrome *c* (bovine heart, crystallized) were Calbiochem (Los Angeles) products.

Phosphatidylcholine was prepared in pure form by the aluminum oxide procedure described in a previous paper (Wells and Hanahan, 1969). On analysis it gave the following values: P, 3.79%; N, 1.76%; N/P, molar ratio, 1.01; fatty acyl ester/P, molar ratio, 2.01; $[\alpha]_{546}^{25}$ +6.55° (in chloroform–methanol, 1:1, v/v). This sample migrated as a single spot on thin-layer chromatography and gave the expected infrared spectrum (Abramson *et al.*, 1965).

Methods

Phospholipase A_2 activity was assayed by a previously described technique (Wells and Hanahan, 1969). During

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TABLE I: Purification of Phospholipase A_2 of *C. atrox* Venom by Wu and Tinker Procedure.

Sample or Treatment	Total Protein		Units of Activity		
	In mg	In % of Original	Total	In % of Original	Sp Act.
1. Crude venom	640.0	100	42,944	100	67
2. Ammonium sulfate treatment					
0–25%	9.4	1.5	179	0.4	10
25–35%	21.6	3.4	1,170	2.7	54
35–50%	282.3	44.1	22,600	52.7	80
50%	221.3	34.6	10,380	24.2	47
3. Sephadex G-25 eluate from 35–50% ammonium sulfate fraction	249.5	39.0	21,470	50.0	86
4. Concentrate from Diaflo membrane treatment of (3) above	186.3	29.2	14,580	34.0	78
5. Sephadex G-100 eluate	75.8	11.8	14,288	33.3	188

titration of the liberated fatty acid, a slow stream of nitrogen gas was bubbled through the solution to maintain a carbon dioxide free atmosphere. On occasion, the assay 2 system of Wu and Tinker (with chloroform–methanol as solvent) was employed for comparison purposes. One unit of enzyme activity was defined as the release of 1.0 μequiv of fatty acid per min. In the purification steps, specific activity is expressed as units per milligram of protein, assuming that $E_{280}^{1\%} = 10.00$. Except for the dialysis and concentration steps which were carried out at 4° all other steps were conducted at room temperature.

Two procedures were used in isolation of this enzyme, one was that described by Wu and Tinker (1969) and the other was that of Wells and Hanahan (1969). In the latter technique the only change was to eliminate the step involving chromatography on Bio-Rex 70 columns since this proved of little value in purification in this instance and also to change the pH of the citrate buffer for chromatography on SE-Sephadex columns from 5.2 to 4.8.

Amino acid analysis were conducted by the accelerated method of Spackman *et al.* (1958) using a Beckman Spinco amino acid analyzer, Model 120C. Subsequent to dialysis of the protein against distilled water for removal of salt, the sample was hydrolyzed as outlined by Moore and Stein (1963). Performic acid oxidation was carried as described by Hirs (1956) and the oxidized protein was hydrolyzed for 24 hr.

Polyacrylamide gel electrophoresis for estimation of protein molecular weight was conducted in the presence of sodium dodecyl sulfate and β-mercaptoethanol, as outlined by Weber and Osborn (1969). Molecular weight determination by gel filtration was accomplished by the technique of Andrews (1964). Sephadex G-100 was hydrated and equilibrated with 0.05 M Tris·HCl (pH 8.0) containing 0.1 M NaCl and 0.001 M EDTA. Elution was conducted with the same buffer. Ultracentrifugation was carried out in a Spinco Model E analytical ultracentrifuge equipped with electronic speed control and a photoelectric scanner. The weight-average molecular weight was determined from a low-speed sedimentation equilibrium run carried out in 0.01 M Tris·HCl–0.1 M NaCl (pH 8.0) at 20.0° and at 14,000 rpm. When successive scans indicated that the system had come to equilibrium, four scans were made. The values of log A_{280} were the average of these four scans.

A partial specific volume of 0.708 was calculated from the amino acid composition (see Table III), as outlined by McMeekin *et al.* (1949).

Results

A. Chromatographic Purification. Inasmuch as our results, using an entirely different isolation technique, were in disagreement with those of Wu and Tinker, a comparison of these two purification approaches was undertaken and are described below.

1. Wu and Tinker Procedure. A sample of venom, weighting 501.7 mg, was dissolved in 10 ml of a mixture containing 0.05 M Tris·HCl–0.02 M $CaCl_2$–0.001 M EDTA (pH 7.4) and stirred gently for 5 min. The mixture was centrifuged at 3500*g* (5000 rpm) for 30 min and the supernatant removed and saved. The residue was washed once with 10 ml of the above buffer mixture, centrifuged, and the supernatant mixed with the first supernatant. This latter solution was the starting sample and was submitted to the ammonium sulfate fractionation as described by Wu and Tinker. However, in this experiment the majority of the activity was precipitated by 35–50% saturation whereas Wu and Tinker found their maximum yield at 25–35% saturation. The enzyme containing fraction was then desalted on Sephadex G-25 concentrated on a Diaflo membrane, and chromatographed on Sephadex G-100, using the same buffer as above for elution. The results of these procedures are summarized in Table I. Chromatography on Sephadex G-75 yielded an enzyme preparation with similar specific activity and behavior to that found on Sephadex G-100.

2. Wells and Hanahan Procedure. The venom was dissolved in 0.1 M NaCl–0.05 M Tris·HCl–0.001 M EDTA (pH 8.0) centrifuged as described in section 1 above and the soluble portion was treated as follows.

A. SEPHADEX G-100. Chromatography of the venom sample on Sephadex G-100 resulted in the elution of three major peaks. Only the second peak contained phospholipase A_2 activity (Figure 1). The elution volume, V_e/V_0, for the protein peak was 1.76 as compared to a value of 1.86 for the enzymatic activity. The pigmented (yellow) material in the venom was well separated from the phospholipase activity peak.

B. DEAE-CELLULOSE. The phospholipase A_2 containing

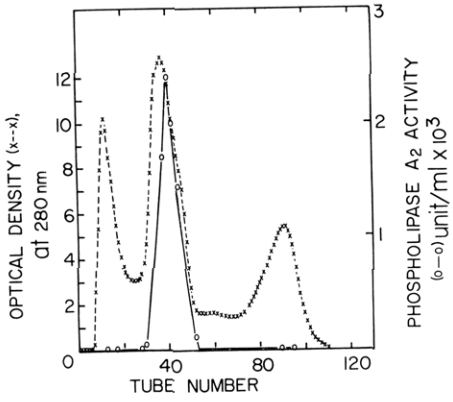

FIGURE 1: Elution profile of venom of *C. atrox* on Sephadex G-100 chromatography. Dried venom (0.5 mg) was suspended in 4 ml of buffer containing 0.05 M Tris·HCl–0.001 M EDTA–0.1 M NaCl (pH 8.0). This mixture was stirred slowly for 5 min and centrifuged at 3500*g* (5000 rpm) for 20 min. The clear supernatant was applied to a Sephadex G-100 column measuring 2.5 × 33 cm. Flow rate, 6.0 ml/hr; (×) protein, (○) phospholipase A_2 activity. The volume of each collection tube was 1.3 ml.

TABLE II: Purification of Phospholipase A_2 from *C. atrox* Venom.[a]

Step[b]	Total[b] (mg)	Protein Recov (%)	Total Act. Units	Total Act. % Recov	Sp Act.
5000 rpm	(a) 1656	100	107,640	100	65
supernatant	(b) 3092	100	217,980	100	70
Sephadex	(a) 393	23.7	72,759	67.6	185
G-100	(b) 905	29.3	146,477	67.2	162
DE-52	(a) 55.7	3.4	33,030	30.1	593
	(b) 71.0	2.3	57,865	26.5	815
SE-Sephadex	(a) 13.6	0.8	21,096	19.6	1551
	(b) 29.1	0.9	43,500	20.0	1495

[a] Procedure essentially that of Wells and Hanahan (1969). Further details are given in the text. [b] Results from two experiments (a and b) on one batch of venom.

fraction from the Sephadex G-100 step above was chromatographed on a DEAE-cellulose column and a total of eight protein peaks were obtained. Only the seventh peak, which was eluted with 0.14 M NaCl, showed phospholipase A_2 activity. When this latter eluate was rechromatographed on DEAE-cellulose under exactly the same conditions, the enzymatic activity was eluted at the identical point in the gradient as it was on the original chromatogram. On polyacrylamide gel electrophoresis, this fraction revealed one major and a minor protein band.

C. SE-Sephadex. Chromatography of the above DEAE-cellulose phospholipase A_2 containing eluate on SE-Sephadex showed two peaks, one a major component and the other only a trace. The major component showed constant specific activity across the entire protein peak and on disc gel electrophoresis revealed only a single band (Figure 2).

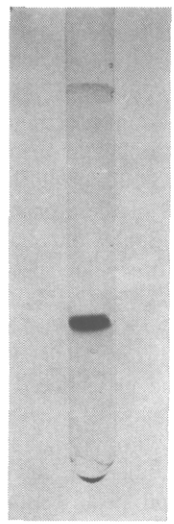

FIGURE 2: Behavior of purified phospholipase A_2 isolated from *C. atrox* venom on polyacrylamide gel electrophoresis. The general procedure was accomplsihed as described by Ornstein (1964), using the Tris–glycine buffer system of Davis (1964). Migration was from top to bottom. Current, 4 mA/gel. Protein was stained with Amido Black. Approximately 100 μg of enzyme was applied to the gel.

The data from two separate experiments with a single batch of venom are summarized in Table II.

B. Characteristics of Purified Enzyme. Except where specifically noted, the data in this section were obtained on samples prepared as described in Table II.

1. Molecular Weight. This parameter was determined by three different techniques: (a) Sephadex gel filtration, (b) low-speed sedimentation equilibrium, and (c) polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol.

The results obtained by gel filtration on Sephadex G-100 are given in Figure 3. The elution volume for the standards and for the phospholipase A_2 from *C. atrox* was plotted against the logarithm of the molecular weight. The V_e/V_0 value for the phospholipase A_2 of *C. atrox* venom was 1.86, which corresponded to a molecular weight of 29,500.

The weight-average molecular weight, as determined by ultracentrifugation was 30,100 ± 150 (standard error of log A_{280} *vs.* r^2 plot).

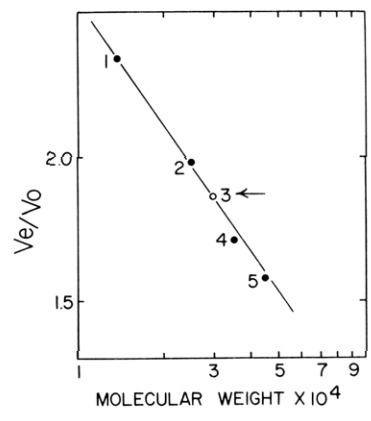

FIGURE 3: Molecular weight values of phospholipase A_2 (*C. atrox*) and standard proteins as indicated by elution pattern on Sephadex G-100. The ratio of elution volume to void volume, V_e/V_0, is plotted against logarithm of molecular weight. The specific proteins and their reported molecular weight are: 1, ribonuclease 13,700; 2, chymotrypsinogen, 25,000; 4, pepsin, 35,000; 5, ovalbumin, 43,000. The molecular weight of the phospholipase A_2 (3, in graph) was calculated to be 29,500.

TABLE III: Amino Acid Composition of Phospholipase A_2 Residues/34 Residues of Aspartic Acid.

Amino Acid	24 hr	48 hr	72 hr	100 hr	Calcd Amino Acid Composition
Asp	34.0	34.0	34.0	34.0	34
Thr[b]	13.8	13.2	11.2	9.2	16
Ser[b]	13.9	13.2	8.8	27.8	16
Glu	27.6	28.0	28.3	7.6	28
Pro	18.4	18.0	18.0	18.0	18
Gly	30.0	29.0	29.0	30.0	30
Ala	17.8	18.4	19.3	18.5	18
Half-Cys[c]					28[c]
Val[d]	7.0	7.2	7.8	8.0	8[d]
Met[e]	1.6		1.0	1.0	4[e]
Ile	11.6	13.8	13.0	13.6	14
Leu	11.2	12.4	13.8	13.6	14
Tyr	14.3	13.0	11.9	14.2	14
Phe	7.7	8.6	8.2	7.6	8
Lys	14.0	15.0	13.5	14.0	14
His	4.2	4.3	4.2	4.1	4
Arg	9.8	8.7	9.4	9.3	10
Trp[f]					2[f]

[a] The analyses were run on a hydrolysate of 0.1 mg of protein. The data are calculated assuming 34 aspartic acids/molecule. [b] Calculated by extrapolation to zero time. [c] Half-cystine residues were determined after 24-hr hydrolysis of performic acid oxidized protein. [d] Calculated from 100-hr hydrolysis. [e] Data from performic acid oxidation showed four residues of methionine present. [f] Numbers of tryptophan residues were calculated spectrophotometrically. Tyrosine and tryptophan ratio was determined by the method of Goodwin and Morton (1946). The calculation from two experiments showed a tyrosine to tryptophan molar ratio, 6.4:1.

A plot of mobility of the phospholipase A_2 and standard proteins on gel electrophoresis (in sodium dodecyl sulfate and β-mercaptoethanol) against the logarithm of molecular weight is presented in Figure 4. On the basis of these data, the phospholipase A_2 of *C. atrox* has a minimal molecular weight of 14,700, and together with data presented above strongly support a dimer structure for the phospholipase A_2.

2. Amino Acid Composition. The data on the purified enzyme are presented in Table III. The average yield of amino acids from hydrolysis of 1 mg of protein was 8.8 μmoles. This would calculate to be 258 residues for a molecular weight of 29,400. Again, as with the enzyme isolated from *C. adamanteus* venom, the data in Table III show the presence of a large number of half-cystine residues.

3. Enzymatic Attack on Phosphatidylcholine. The specificity of attack of the purified enzyme on phosphatidylcholine isolated from hen's eggs was studied and the results confirmed that this enzyme attacked preferentially the C-2 ester group of this particular substrate. These results are in agreement with those reported by Wu and Tinker (1969).

In a typical experiment, 13.3 μmoles of phosphatidylcholine (based on P content) was dissolved in 2 ml of diethyl ether–methanol (95:5, v/v) and to this solution was added

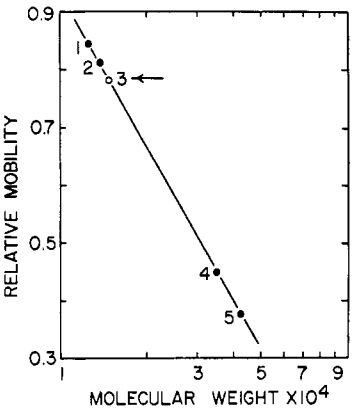

FIGURE 4: Molecular weight values of phospholipase A_2 (*C. atrox*) and standard proteins as obtained from migration pattern on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol. The specific proteins and their reported molecular weight values are: 1, cytochrome *c*, 12,400; 2, ribonuclease, 13,700; 4, pepsin, 35,000; 5, ovalbumin, 43,000. In this instance, the molecular weight of the phospholipase A_2 (3, in graph) was calculated to be 14,700.

25 μl of the enzyme solution containing 7.6 μg of protein in 0.22 M NaCl–0.02 M $CaCl_2$ plus 0.001 M EDTA (pH 7.4). At the end of a 5-hr period, the only products detectable by thin-layer chromatography on silica gel G in a solvent system of chloroform–methanol–water (95:35:6, v/v) or petroleum ether (bp 30–60°)–diethyl ether–acetic acid (80:20:1, v/v) were lysolecithin and free fatty acid. The starting substrate gave the following fatty acid composition (in mole per cent): *saturates:* 16:0, 42.0; 18:0, 10.3; *unsaturates:* 16:1, 1.7; 16:2, 0.3; 18:1, 32.4, 18:2, 11.0; 18:3, 0.4; 20:4, 1.9. The fatty acids liberated by phospholipase A_2 action showing the following composition (mole per cent): *saturates:* 16:0, 6.6; 18:0, 2.0; *unsaturates:* 16:1, 1,3; 16:2, 0.2; 18:1, 60.0; 18:2, 24.3; 18:3, 1.4; 20:4, 4.2.

4. Factors Influencing Dimer Stability. The above data support a dimer structure for the phospholipase A_2 of *Crotalus atrox* venom and a molecular weight of 29,500. In an attempt to resolve differences in these results and those of Wu and Tinker, who apparently were studying a monomer form, certain of the techniques and procedures of the latter investigators were studied in some detail as possible factors influencing monomer formation. The following sections describe the experimental approach and results.

a. EFFECT OF AMMONIUM SULFATE. In each of these studies, the sample was 5 ml of the eluate from the Sephadex G-100 column (Table I) and contained 1.42 mg of protein/ml in 0.05 M Tris·HCl containing 0.001 M EDTA and 0.02 M $CaCl_2$ (pH 8.0). It was treated as follows. (1) The sample was adjusted to pH 4.0 with 1 N HCl, solid ammonium sulfate added to 50% saturation at 4°, and the pH readjusted to 4.0. After 2 hr the resulting precipitate was dissolved in 0.05 M Tris·HCl, 0.1 M NaCl, and 0.001 M EDTA (pH 8.0) and passed through a Sephadex G-100 column. The V_e/V_0 was 1.86, which was identical with that obtained for an untreated sample. The molecular weight was calculated to be 29,500. (2) The sample was adjusted to pH 3.0 with 1 N HCl made to 50% saturation with ammonium sulfate at 4°. The pH was readjusted to 3.0 after standing at 4° for 2 hr, the mixture was centrifuged at 9750*g* (9000 rpm), the precipitate dissolved in buffer (see section 1 above), and passed through a Sephadex G-75 column. The V_e/V_0 was 1.39 for this sample.

Standard samples of ovalbumin (mol wt 43,000) and cytochrome *c* (mol wt 12,400) gave V_e/V_0 values of 1.25 and 1.73, respectively, on the same column. The molecular weight of the phospholipase A_2 was calculated to be 29,000. (3) The sample was adjusted to pH 3.6 with 1 N HCl and incubated at 90° for 5 min. The solution was cooled rapidly and solid ammonium sulfate added to 50% saturation at 4° and treated the same as in 2 above. The V_e/V_0 on Sephadex G-75 was 1.42 and the molecular weight was calculated to be 27,800.

On the basis of these experimental results there appears to be no direct effect of ammonium sulfate on the molecular weight (or size) of this protein.

b. Effect of lyophilization. A sample of purified phospholipase A_2 (similar to that described in Table II), containing 0.702 mg in 1 ml of 0.01 M citrate buffer (pH 4.8), was lyophilized and the residue dissolved in 1 ml of 0.05 M Tris·HCl, containing 0.02 M $CaCl_2$ and 0.001 M EDTA (pH 7.4). The enzymatic activity was measured immediately by the procedure described above. At the same time another sample containing exactly the same components, but not lyophilized, was assayed for its enzymatic activity. The specific activity of these two samples was exactly the same, *i.e.*, 1209 units/mg.

Thus, these results, unlike those of Wu and Tinker (1969) show no lag period in development of enzymatic activity subsequent to lyophilization of an active enzyme preparation.

c. Stability of enzyme. A solution of highly purified phospholipase A_2 (Table I), containing 1.84 mg of protein/ml in 0.01 M citrate buffer (pH 5.0) showed no loss in activity on storage for 3 months at 4°. It was also observed that toluene (10 μl/ml), which was used by Wu and Tinker (1969) to inhibit bacterial growth, did not show any detectable effect on either the molecular weight as determined by sedimentation equilibrium or enzymatic activity of the purified phospholipase A_2.

Discussion

The results of the present investigation show that the phospholipase A_2 isolated from *Crotalus atrox* venom by a procedure previously described for preparation of the same enzyme from *C. adamanteus* venom has a molecular weight of 29,500 and a specific activity 1500. These observations differ sharply from those reported by Wu and Tinker (1969) who noted a molecular weight of 14,500 and a specific activity of 182. It is in order at this point to examine some possible reasons for these discordant results and to attempt to resolve some of these differences.

Some comment should be made on certain facts of the experimental procedures and results of these two laboratories. First, the specific activity of our starting material was some 14-fold greater than reported by Wu and Tinker. These results were the same whether their assay system or the assay developed in this laboratory was used.

Although we have obtained consistently reproducible results with numerous batches of substrate, it is possible that variation in substrate composition could account for the differences in the observed activities. It is evident that the nature of the starting material in our two laboratories was quite different.[1] In our hands, chromatography of the *C. atrox* venom on Sephadex G-100 revealed only one phospholipase A_2 activity peak, whereas Wu and Tinker using G-75 in their study found one major and two minor (but definitive) peaks. It was conceivable that their initial treatment of the venom with ammonium sulfate casued some alteration in the physical or chemical structure of the enzyme, but our experimental results with the crude venom, or partially purified enzyme, showed no effect of ammonium sulfate on molecular weight. The overall yield of enzymatic activity was comparable in the two procedures, *i.e.*, 16.8% (Wu and Tinker, 1996), 19.8% (this study), though the steps at which activity loss was encountered were quite different. For example, Wu and Tinker reported that their major loss occurred in the initial salt fractionation step, whereas chromatography on DEAE-cellulose resulted in the greatest loss in our experimental protocol. Further, Wu and Tinker reported that lyophilization of their purified enzyme resulted in almost complete loss of enzymatic activity. However, they did observe that solution of the lyophilized preparation in buffer allowed regain of enzymatic activity *but* only after a lag period of several hours. Our results with purified phospholipase A_2 from *C. atrox* venom showed no such lag period after lyophilization. Previous experience with the purified enzyme from *C. adamanteus* venom also showed no effect of lyophilization on enzymatic activity.

Perhaps the major point of criticism of the study of Wu and Tinker would center on the determination of the molecular weight of the phospholipase A_2. These investigators used only a single technique, namely that of gel filtration, for calculating their reported molecular weight of 14,500. Some question, must be raised, however, on their gel filtration data. Even though a molecular weight of 24,000 for beef liver catalase was given in the text, it is obvious from their log M value (5.4) that the former was a typographical error.[2] A more fundamental question concerned the V_e/V_0 value of approximately 2, reported by Wu and Tinker, for catalase on Sephadex G-75. This protein, which has a molecular weight of 240,000 should be excluded from G-75 (presumed limit, 70,000) and hence a linear plot between insulin and catalase, as reported by these investigators, would appear to be meaningless. These observations do not explain the molecular weight which they obtained, but do suggest that their data should be interpreted with caution. In our studies, three different procedures, namely, gel filtration, analytical ultracentrifugation, and amino acid analysis were employed to arrive at a molecular weight of 29,500. However, it is important to emphasize that the mobility of this enzyme on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and β-mercaptoethanol corresponded to a molecular weight of 14,700. This result supports the proposal that the phospholipase A_2, as isolated from *C. atrox* venom, exists as a dimer. A similar observation has been noted for the same activity obtained from *C. adamanteus* by Wells (1971). Nonetheless, these latter data do not explain the results of Wu and Tinker since a repeat of their procedure in this laboratory did not give rise to any detectable monomer form.

On the basis of the observations reported in this paper, we conclude that the phospholipase A_2 of *C. atrox* has a

[1] It should be noted that the sources of the venom used in the two studies were different, with our samples obtained from the Miami Serpentarium and the Wu and Tinker starting material from Ross Allen Reptile Institute.

[2] Private communication, D. O. Tinker.

molecular weight of 29,500 and does exist as a dimer. It is not possible at this time to explain the differences between these data and those of Wu and Tinker.[2]

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Reduction of the Selenotrisulfide Derivative of Glutathione to a Persulfide Analog by Glutathione Reductase*

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ABSTRACT: The compounds formed by the reaction of selenious acid with glutathione were studied with regard to the effects of pH and glutathione:selenious acid ratio. At ratios of 4:1 or less, and a pH below 2 and above 4, the first stable product is the selenotrisulfide derivative of glutathione (GSSeSG) plus an equimolar quantity of GSSG:

$$4GSH + H_2SeO_3 \longrightarrow GSSeSG + GSSG + 3H_2O$$

GSSeSG free of GSSG was prepared by separating the two compounds on a Dowex 50 column equilibrated with 0.01 M $NiCl_2$ in 0.1 M sodium acetate (pH 4.7) followed by adsorption of the GSSeSG fraction to Dowex 50 Na^+ at pH 3 and elution with ammonium acetate at pH 5.5. In order to determine the type of selenium compound formed under conditions more nearly simulating physiological conditions of pH and reactant concentrations, ^{75}Se-labeled selenite (1×10^{-6} M) was treated with 4×10^{-3} M GSH at pH 7, 25°, followed by 50 mM iodoacetate. The major selenium compound thus formed was the *Se*-carboxymethyl derivative of glutathione selenopersulfide (GSSeH); this persulfide is believed to be formed by reduction of the initial selenotrisulfide product with excess GSH. Glutathione and elemental selenium were rapidly liberated from GSSeSG at pH 7 by 0.1 μg or less of highly purified glutathione reductase from yeast. The reduction of GSSeSG was not catalyzed by lipoyl dehydrogenase, nor was glutathione reductase active when DPNH or selenodicysteine (CySSeSCy) was substituted for TPNH or selenodiglutathione. The velocity of GSSeSG reduction was similar to that for GSSG reduction. Evidence was obtained that the initial reaction products were GSH and the selenopersulfide (GSSeH):

$$GSSeSG + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} GSH + GSSeH + TPN^+$$

The selenopersulfide rapidly decomposed to GSH and elemental selenium but could be trapped in the presence of 50 mM iodoacetate as the carboxymethylated derivative, which was identified by thin-layer electrophoresis, thin-layer chromatography, and gel filtration. It is believed that this work provides the first evidence for the selenopersulfide class of compounds. These reactions may have important applications to selenium metabolism and the mechanism of action of selenium as an essential nutrient.

The fate of selenious acid, or selenite, in systems containing thiols is relevant to many problems regarding the nutritional and toxicological aspects of this substance. Compounds of the type RSSeSR (2-selena-1,3-disulfides or selenotrisulfides) have been shown in previous studies to be formed by the reaction of selenious acid with a variety of thiols (Ganther, 1968; Ganther and Corcoran, 1969) in accord with the equation first proposed by Painter (1941)

$$4RSH + H_2SeO_3 \longrightarrow RSSeSR + RSSR + 3H_2O \quad (1)$$

The most abundant thiol in biological systems is glutathione. A specific requirement for glutathione in selenite metabolism has been described (Ganther, 1966). The reaction of glutathione with selenious acid was indicated previously to be more complex than for other thiols (Ganther, 1968) and has now been studied in more detail. The results show that at least three selenium derivatives of glutathione can be formed, depending on pH and the glutathione:selenious acid ratio. One

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