Isolation and Characterization of the Toxic Component of Enhydrina schistosa (Common Sea Snake) Venom*

(August 25, 1970)

ANTHONY T. TÜ‡ AND PAUL M. TOOM§

From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80521

SUMMARY

The toxic component from the venom of the common sea snake (Enhydrina schistosa) was isolated by passage of the venom through a carboxymethyl cellulose column. The preparation, which was shown to be homogeneous by zonal electrophoresis, ultracentrifugation, isoelectric focusing, and gel filtration, had a molecular weight of 0.044 mg per g of body weight. The molecular weight of the toxic protein, as determined by sedimentation velocity and diffusion experiments, was found to be 7200. The minimum molecular weight calculated from amino acid analysis was 7000. The isoelectric point, determined by isoelectric focusing, was 9.2. Although hyaluronidase, alkaline phosphatase, phosphodiesterase, phospholipase A, acetylcholinesterase, DNase, leucine amino peptidase, and clotting activity could all be detected in unfractionated venom; no enzymatic activity could be found in the purified toxin. The compound N-bromosuccinimide completely destroyed the toxicity of the preparation, which, under the conditions employed, strongly suggests that tryptophan is necessary for toxicity.

It is generally believed that the over-all action of snake venoms is caused by the combined effect of a number of different proteins (both enzymatic and nonenzymatic) found in the venoms (1–3). Recent studies have shown that venom from snakes of the family Hydrophiidae (Sea Snake) are much simpler in composition than the venoms of the venomous land snakes (Families: Crotalidae, Viperidae, and Elapidae (4)). However, to date, very few investigations have been undertaken on sea snake venoms. Many of these snakes spend their entire lives in the ocean, which makes capturing enough specimens to do a detailed study of their venom most difficult. In addition, the amount of venom which can be extracted from a venomous sea snake is only 3 to 4 mg as compared to up to 1 g from land snakes.

In 1961, Carey and Wright (5) studied the venom of Enhydrina schistosa and concluded that the initial effect of envenomation by this snake is at the neuromuscular junction. Chan and Geh (6) found that this same venom produced muscle weakness and respiratory paralysis, while Cheymol et al. (7) attributed toxicity of this venom to an almost irreversible blocking of specific receptors of the postsynaptic membrane.

The only chemical investigations reported to date on toxins from sea snakes have come from the sea snake subfamily Laticaudinae. Tamiya and Arai (8) isolated two toxins from the venom of Laticauda semifasciata. Each toxin contained 62 amino acids and had a molecular weight of about 6900. Recently, Sato et al. (9) isolated the toxins from the venom Laticauda laticaudata and Laticauda colubrina. Although the amino acid composition of these two toxins varied slightly from that of the toxins of L. semifasciata, the molecular weight of each was about 7000.

This paper will describe a procedure for the isolation of the toxin from the Common Sea Snake (E. schistosa) commonly found in the Gulf of Thailand, Strait of Malacca, Vietnam Coastal waters, and the Bay of Bengal. This toxin will be shown to be homogeneous by a number of criteria, and report on some of its chemical and physical properties.

EXPERIMENTAL PROCEDURE

Materials—Venom from the sea snake E. schistosa was collected by the authors in the Strait of Malacca during the summer of 1967. The venom was milked, dried under desiccation, and stored at 4°C, until used in this investigation. Cellex-CM (Cm-cellulose) was purchased from Bio-Rad Laboratories, Richmond, California, and prepared according to the manufacturer's suggestions. Sephadex G-10 and G-75 were obtained from Pharmacia. The Ampholnle carrier ampholytes and electrofocusing column used in the electrofocusing experiments were purchased from LKB Instruments (Stockholm, Sweden).

Electroin was purchased from Mann and purified as previously described (10). Other substrates used in the enzymatic assays were purchased from Sigma, Calbiochem, Fisher, and Mann.

Isolation Procedure—Previously washed and charged CM-cellulose was equilibrated with the initial buffer (0.01 M sodium phosphate, pH 7.5) to be used in the separation. The flow rate was adjusted to 15 ml per hour and the column was loaded with 0.500 g of E. schistosa venom which had been dissolved in 1.0 ml of 0.01 M sodium phosphate, pH 7.5. Fractions were eluted by means of increasing salt concentrations added in a stepwise manner. The column effluent was continuously monitored with

* This investigation was supported by National Institutes of Health Grant 5 RO1 GM 15891, Office of Naval Research Contract N00014-67-A-0299-0005, NR 305-780.
‡ Recipient of Career Development Award K 4 GM 41780.
§ Present address, Department of Chemistry, University of Southern Mississippi, Hattiesburg, Mississippi 39401.
an ISCO model U-A2 ultraviolet analyzer up to absorbance one, and fractions of 3.0 ml were collected. For the fractions with absorbance greater than one, the absorbance of each tube was measured with a Beckman DB-G spectrophotometer. The fraction which exhibited the highest toxicity toward mice was desalted by passage through a column of Sephadex G-10 and lyophilized. This fraction was then rechromatographed on a second Cm-cellulose column which had been previously equilibrated with 0.01 M glycine buffer, pH 9.5. Fractions were again eluted by increasing salt concentrations, the column effluent continuously monitored with an ISCO model UA-2 ultraviolet analyzer, and fractions of 3.0 ml were once again collected. The purified toxin from this Cm-cellulose column was lyophilized, desalted by passage through Sephadex G-10, and stored at 4°C until used for characterization studies.

**Enzyme Assays**—Tests for enzymatic activities against the substrates casein, hemoglobin, N-benzoyl-L-arginine ethyl ester, p-toluenesulfonyl-L-arginine methyl ester, acetyl-L-tyrosine ethyl ester, and N-benzoyl-L-tyrosoe ethyl ester, were carried out as described previously (11). Acid and alkaline phosphatase activities were determined spectrophotometrically on the substrates p-nitrophenylphosphate and O-carboxyphenylphosphate (12). Acetylcholine esterase activity was assayed by following the hydrolysis of indophenyl acetate as described by Kramer and Gamson (13). The hydrolysis of RNA was followed by the method of Babbina (14) while DNA hydrolysis followed the procedure described by Maeno and Mitsuhashi (15). Phosphodiesterase activity was measured (spectrophotometrically) by following the hydrolysis of the substrate calcium bis-(p-nitrophenyl)phosphate (14). 

**Hyaluronidase activity** was followed by measuring the decrease in turbidity of a hyaluronic acid-protein complex (16). Phospholipase A was measured titrimetrically as previously described (10).

**Analytical Ultracentrifugation**—A Spinco model E ultracentrifuge with an AN-D rotor, an Epon double sector, and an Epon double sector capillary synthetic boundary cell was used to measure the sedimentation and diffusion coefficients. Diffusion measurements were made at 9,945 rpm with 0.10 M glycine, pH 9.2, as solvent. Photographs were taken of the Schlieren patterns at 4-min intervals for a total of 60 min. Sedimentation velocity measurements were made at 59,780 rpm in the same buffer, where photographs were also taken at 4-min intervals for 60 min. Physical measurements were made with a Nikon profile projector, model 6c microcomparator.

**Isoelectric Focusing and Polyacetate Electrophoresis**—Isoelectric focusing was performed in a 115-ml column as described previously (11). Zone electrophoresis experiments were carried out on Gelman polyacetate strips. Buffers used were: 0.028 M Na-barbital, pH 8.8; 0.01 M Na-acetate, pH 5.0, and 0.01 M Na-phosphate, pH 7.5 and 6.2. The strips were subjected to electrophoresis for 2 hours at 200 volts (1.5 ma per strip). After 2 hours, the strips were stained in 0.25% Amido black dissolved in methanol acetic acid water (4:4:1 v/v/v). The strips were then washed clear of stain in anhydrous methanol and cleared in 10% acetic acid.

**Amino Acid Analysis**—Amino acid analyses were performed according to the procedure of Moore and Stein (17). Hydrolysis was carried out at 110°C for 18, 36, and 54 hours in constantly boiling HCl. Amino acid analyses were done on a Beckman amino acid analyzer model 120 B. For analysis of cysteine, a sample of toxin was first treated with performic acid as described by Schram, Moore, and Digwood (18).

**Toxicity Tests**—Toxicity was determined by injecting 0.25 ml of toxin dissolved in 0.85% NaCl solution into the tail vein of 20-gram Swiss white mice. Ten to fifteen mice were injected at each of 15 dosage levels for each toxicity determined. The number of mice which died within 24 hours was recorded, and the median lethal dose 50% (LD₅₀) determined by the method of Litchfield and Wilcoxon (19).
TABLE I

| Fraction       | Total protein | LD50<sup>a</sup> |
|----------------|---------------|------------------|
| Venom          | 500           | 0.098 (0.093-0.103) |
| First fractionation<sup>b</sup> |               |                  |
| I              | 47            | Nontoxic         |
| II             | 7             | Nontoxic         |
| III            | 32            | 0.230 (0.242-0.219) |
| IV             | 155           | 0.073 (0.068-0.078) |
| V              | 27            | 0.181 (0.174-0.188) |
| VI             | 108           | 0.102 (0.097-0.107) |
| VII            | 40            | Nontoxic         |
| Recovery       |               | 450              |
| Second fractionation<sup>c</sup> |               |                  |
| V-A            | 8             | Nontoxic         |
| V-B            | 22            | 0.098 (0.093-0.103) |
| V-C            | 124           | 0.044 (0.042-0.046) |
| Recovery       |               | 154              |

<sup>a</sup> LD50, lethal dose 50%; numbers in parentheses represent 95% confidence limits.

<sup>b</sup> Venom, 500 mg, was centrifuged and added to the Cm-cellulose column.

<sup>c</sup> Fraction V, 180 mg, from the first fractionation was added to the Cm-cellulose column.

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**RESULTS**

**Purification of Toxin**—A typical fractionation started with 500 mg of lyophilized *E. schistosa* venom. After fractionation with a NaCl salt gradient on a Cm-cellulose column at pH 7.5, the fraction displaying highest toxicity was further purified by passage through a second Cm-cellulose column at pH 9.7. A typical fractionation is illustrated in Figs. 1 and 2. The fractionation was independently repeated five times with the same patterns and yields. A summary of the purification is presented in Table I, where it can be seen that *E. schistosa* consists of five toxic components, the most toxic of which was about 2 times more toxic than the starting material. The 124 mg of this toxin which was obtained represents about 25% of the original material.

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**Criteria of Purity**—Following electrophoresis at pH 8.8 on polyacrylamide gels, 12 components could be detected in unfractionated venom, three components after passage through the first Cm-cellulose column, and only a single component after passage through the second Cm-cellulose column. To further establish homogeneity of the toxin, additional electrophoresis experiments were carried out at pH values of 7.5, 6.0, and 5.0. In each experiment, only a single protein band could be detected.

Isoelectric focusing of unfractionated venom revealed seven components. As can be seen from Fig. 3, when the purified toxin was subjected to isoelectric focusing under the same conditions, only a single component could be found.

Fig. 4 presents the results obtained when the purified toxin was passed through a column of Sephadex G-50. Rechromatography of the peak tube gave a similar molecular sieve pattern with band broadening of a comparable degree. Thus, it can be assumed that the preparation is homogeneous in respect to molecular weight.

Ultracentrifugation of the toxin was performed at four different concentrations, from two separate toxin preparations. In each of the eight experiments, only a single component could be detected.

**Physical Parameters**—At four different concentrations of toxin, 1, 0.72, 0.52, and 0.38%, sedimentation coefficients of 1.31 S, 1.34 S, 1.37 S, and 1.38 S were obtained, respectively. Extrapolation to zero protein concentration gave an \( s_{20, w} \) value of 1.4 S. At the same concentrations of protein, diffusion coefficients (\( D \)) of 11.2 \( \times 10^{-7} \) cm<sup>2</sup> per sec, 12.2 \( \times 10^{-7} \) cm<sup>2</sup> per sec, 13.1 \( \times 10^{-7} \) cm<sup>2</sup> per sec, and 13.8 \( \times 10^{-7} \) cm<sup>2</sup> per sec were obtained. Extrapolation to zero protein concentrations gave a \( D_{20, w} \) value of 15.5 \( \times 10^{-7} \) cm<sup>2</sup> per sec.

By a combination of sedimentation and diffusion coefficients, a molecular weight was determined by means of the Svedberg equation, \( M = RT \bar{s} / (1 - \bar{s} \rho)D \). With values of 0.70 for the partial specific volume (calculated from the amino acid composition by the method described by Schachman (20)), \( \bar{s}_{20, w} \) of 1.4 S and \( D_{20, w} \) of 15.5 \( \times 10^{-7} \) cm<sup>2</sup> per sec, a molecular weight of 7300 was calculated.

Table II presents the amino acid composition of the toxin.
Each value represents the average of three independent determinations. The most probable value represents the average for each amino acid except serine and threonine which are extrapolated to zero hydrolysis time. Cysteine was determined as cysteic acid after oxidation with performic acid (18). Trypsinogen was estimated by the spectrophotometric method of Edelhoch (21), and by titration with N-bromosuccinimide (22). Each method suggested 1 mole of trypsinogen per mole of enzyme. The minimum molecular weight of the toxin as calculated from the amino acid analysis was 6981.

The isoelectric point of the toxin is at or near pH 9.2. This value is taken from isoelectric focusing experiments where the toxin forms a band at pH 9.17 (Fig. 3).

Nonenzymatic Nature of Toxin—The enzymatic activities of both purified toxin and unfractionated common sea snake venom were tested on 16 substrates. Enzymatic activity toward each of these substrates has been shown to be present in venoms of at least some venomous land snakes. Although clotting activity, hyaluronidase, alkaline phosphatase, phosphodiesterase, deoxyribonuclease, acetylcholinesterase, and leucine aminopeptidase, activities could be detected in unfractionated venom, none of these activities could be detected in the purified toxin.

Both the original venom and the purified toxin are devoid of the following enzymatic activities: ribonuclease, acid phosphatase, amino acid esterase (with N-benzoyl-L-arginine ethyl ester, N-benzoyl-L-tyrosine ethyl ester, p-toluenesulfonyl-n-arginine methyl ester, and acetyl-L-tyrosine ethyl ester as substrates), and proteases (with casein and hemoglobin as substrates).

### Table II

| Amino acid | Time of hydrolysis | Most probable value | Average integral number to leucine, alanine, and valine | Nearest integral number |
|------------|-------------------|---------------------|-------------------------------------------------------|------------------------|
|            | 18 hrs | 36 hrs | 54 hrs | amoles | amoles | |
| Aspartic acid | 620 | 640 | 680 | 680 | 6.1 | 0 |
| Threonine | 825 | 760 | 900 | 880 | 8.4 | 8 |
| Serine | 390 | 555 | 520 | 540 | 8.2 | 6 |
| Glutamic acid | 880 | 800 | 800 | 870 | 8.3 | 8 |
| Proline | 290 | 270 | 290 | 280 | 2.7 | 3 |
| Glycine | 560 | 560 | 550 | 560 | 5.3 | 5 |
| Alanine | 100 | 100 | 100 | 100 | 1.0 | 1 |
| Valine | 90 | 110 | 100 | 100 | 1.0 | 1 |
| Methionine | 0 | 0 | 0 | 0 | 0.0 | 0 |
| Isoleucine | 190 | 210 | 210 | 200 | 1.9 | 2 |
| Leucine | 110 | 110 | 105 | 110 | 1.0 | 1 |
| Tyrosine | 100 | 100 | 100 | 100 | 1.0 | 1 |
| Phenylalanine | 0 | 0 | 0 | 0 | 0.0 | 0 |
| Lysine | 520 | 550 | 540 | 540 | 5.2 | 5 |
| Histidine | 220 | 230 | 230 | 230 | 2.2 | 2 |
| Arginine | 320 | 320 | 320 | 320 | 3.1 | 3 |
| Cysteine<sup>a</sup> | 900 | 900 | 8.6 | 9 |
| Tryptophan<sup>a</sup> | 0.9 | 1 |

Total residues | 62 |
Minimum molecular weight | 6981 |

<sup>a</sup> Extrapolated to zero time of hydrolysis.
<sup>b</sup> Determined as cysteic acid.
<sup>c</sup> Determined spectrophotometrically and by titration with N-bromosuccinimide.

Importance of Tryptophan in Toxicity—It has been shown that titration of a protein at pH 4.0 in acetate buffer with N-bromosuccinimide results in almost complete destruction of trypsin with minimal destruction of other amino acids. Experiments carried out with N-bromosuccinimide showed that toxicity was destroyed at a proportional rate to the decrease in absorbance of the protein at 280 nm as N-bromosuccinimide was added.

**DISCUSSION**

A two-step purification of the toxic component of the Common Sea Snake (E. schistosa) has been achieved. The preparation was shown to be homogeneous by a number of criteria. The toxin appeared as a single, sharp, symmetrical peak when subjected to isoelectric focusing, and to Sephadex gel filtration. Only one band could be detected with zonal electrophoresis on polyacrylamide gels at various pH values. The preparation also appeared to settle as a single component when the preparation was centrifuged at 59,780 rpm.

The amino acid analysis of the toxin showed that the toxin consists of 62 amino acids with a molecular weight of about 7000. It would thus appear that this toxin is very similar to the toxins isolated from L. seminifasciata captured in Japan by Tamiya and Arni (8) who found 61 amino acids in their toxins with molecular weights of 6900; and to the toxins isolated from L. laticaudata and L. colubrina by Sato et al. (9), who also found 62 amino acids in their toxins with a molecular weight of 7000. The toxins isolated from L. seminifasciata captured in the Philippines are also found to be similar to that of E. schistosa venom (23). The amino acid composition of this preparation is also similar to the above preparations; the major differences being in a higher threonine content, less proline, and the absence of phenylalanine. This preparation also contained 1 mole of alanine, while the above toxins were devoid of this amino acid. It should also be pointed out that each of the above studies reported more than one toxic component which agrees with the findings in this investigation.

The inhibition of the toxin by N-bromosuccinimide under the conditions employed, strongly suggests that tryptophan is necessary for the toxic action of this venom. This finding is in agreement with Carey and Wright (24), who found that the toxic factor of this snake could be separated from phospholipase A by dialysis, and of Chan and Geh (6) who found that there was no acetylcholine esterase activity in the toxin of this venom.

The inhibition of the toxin by N-bromosuccinimide under the conditions employed, strongly suggests that tryptophan is necessary for the toxic action of this venom. Future studies, which have as their goal the mechanism by which tryptophan is involved, should help us to understand the mode of action of Common Sea Snake venom.

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*J. Biol. Chem.* 1971, 246:1012-1016.

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