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Chromatographic Separation of *Enhydrina schistosa* (Common Sea Snake) Venom and the Characterization of Two Principal Neurotoxins†

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ABSTRACT: The venom of the common sea snake, *Enhydrina schistosa*, was separated into seven protein zones by gel filtration on Sephadex G-75. The most retarded of the protein zones contained all of the toxic material and accounted for 70% of the soluble venom protein. Further separation of the toxic fraction by gradient chromatography on Bio-Rex 70 in ammonium acetate buffers indicated that the venom preparation studied might contain 16 or more different neurotoxins which together account for 60% of the venom protein, or 45% of the weight of the dried crude starting material. Two principal neurotoxins designated *schistosa* 4 and 5 were isolated in yields corresponding to 14 and 25%, respectively, of the soluble venom protein. Each of the two toxins consists of a single peptide chain of 60 amino acids cross-linked by four disulfide bridges and contains a cysteine residue the free SH group of which can be alkylated with iodoacetate without inactivation. Both toxins have methionine at the amino terminus and the carboxyl terminal sequence Asn-Asn. The two toxins differ in amino acid composition only by substitution of a serine for a proline residue.

Sea snakes, *Hydrophiidae*, are widely distributed in the warmer regions of the Indian and Pacific Oceans. Their venoms are extremely poisonous and primarily neurotoxic in action (Cheymol *et al.*, 1967). Remarkedly large swarms consisting of hundreds of thousands of individuals have been observed. The exclusively aquatic habits of these snakes account for the low frequency of casual unpleasant encounters with humans, but the many thousands caught in nets each year constitute a real occupational hazard to fishermen (Werler and Keegan, 1963; Barme, 1963, 1968; Tu and Tu, 1970).

The venom of the common sea snake, *Enhydrina schistosa*, is among the most toxic snake venoms (Boquet, 1964). The mouse LD_{50} of about 100 μg/kg (Carey and Wright, 1960;

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Tu and Toom, 1971) indicates that the dried crude venom is at least half as toxic as any purified curariform neurotoxin so far isolated from elapid or hydrophid venoms, but the high toxicity is apparently accountable to an unusually high (*ca.* 50%) content of postsynaptic neurotoxins of "ordinary" potency.

Recently, *Enhydrina schistosa* venom has been separated by chromatography on carboxymethylcellulose into eight distinct fractions, four of which exhibited neurotoxic activity (Tu and Toom, 1971). Their principal neurotoxin was described as consisting of 62 amino acids, including nine residues of half-cystine, but no evidence was given for the presence of an SH group.

We have fractionated *Enhydrina schistosa* venom by gel filtration on Sephadex G-75 followed by cation exchange chromatography on the polycarboxylic, acrylic type resin Bio-Rex 70, and our results differ from those just cited with regard to both the number of different neurotoxins present and the amino acid composition of the principal ones.

Materials and Methods

Venom. The venom was purchased from the Snake and Venom Research Institute, General Hospital, Penang, Malaysia, through the courtesy of Director T. Devaraj. The snakes were caught off Penang Island in the years 1965–1969. The venom was extracted by pressing the venom glands and dried in desiccators over silica gel. The average yield per snake was 10 mg.

Isolation Procedure. For preparative gel filtration, 1-g samples of venom were dissolved in 9 ml of 0.2 M ammonium acetate, and, after breaking the surface foam by touching with a toothpick moistened with 1-octanol, the volume was adjusted to 10.0 ml. Small amounts of insoluble debris and a fine turbidity were removed by centrifugation for 5 min at 1000*g* followed by 15 min at 20,000*g*. The clear amber supernatant (9.8 ml) was decanted and 50-μl aliquots were taken for total amino acid analysis and toxicity assay. The remaining 9.7 ml was gel filtered on Sephadex G-75 in 0.2 or 0.05 M ammonium acetate. The column effluent was collected in 10-ml fractions, which were analyzed by absorbance measurements at 260 and 280 nm. The material present in each of the eight pools made on the basis of the effluent pattern thus obtained was recovered by lyophilization and tested for neurotoxic activity by intraperitoneal injection into three female albino mice at a dose level of 10 μg, administered in 100 μl of 0.9% NaCl.

The gel filtration cut containing the neurotoxic activity was chromatographed on a short, closed-bed column of Bio-Rex 70, −400 mesh (Bio-Rad Laboratories) equilibrated with 0.2 M ammonium acetate at pH 7.30. Prior to applying the sample, 20–40 ml of 0.06 M ammonium acetate was pumped into the column to displace the stronger buffer from the upper part of the resin bed. Elution was done using a 2-l. concave gradient of 0.06 *vs.* 1.4 M ammonium acetate formed by means of a Beckman Model 131 gradient pump fitted with the standard cam no. 324812, and the column effluent was monitored continuously with a Beckman Model 130 Spectrochrom analyzer. Details regarding the preparation and equilibration of the resin, the packing and operation of the columns, and the lyophilization procedure used to recover the separated fractions are given elsewhere (Karlsson *et al.*, 1971).

Amino Acid Analysis. Hydrolysis was done at 110° in thoroughly evacuated tubes using 6 N HCl (Merck Suprapur) containing 10 mg/ml of reagent grade phenol. Total half-cystine was determined as cysteic acid (Moore, 1963). The hydrolysates were analyzed with a Bio-Cal BC-200 analyzer equipped with a Infotronics CRS-110A integrator.

Preparation of the Reduced and S-Carboxymethylated Derivative and the Estimation of Free SH Groups. The principal neurotoxins were completely reduced with mercaptoethanol and alkylated with iodoacetate according to the standard procedure (Crestfield *et al.*, 1963), and the derivatives were recovered by lyophilization after removal of urea and reagents by gel filtration on Sephadex G-75 in 10% acetic acid.

Free SH groups were estimated as CM-cysteine[1] following treatment of the toxins with iodoacetate in 8 M urea in the manner described above, but without prior reduction.

Ultraviolet Spectra and the Estimation of Tryptophan Content. Ultraviolet spectra were run in 0.2 M ammonium acetate (native toxins) or 10% acetic acid (reduced and S-carboxymethylated derivatives) at concentrations of about 0.5 mg/ml. To permit calculation of molar absorptivities, the protein concentration in the solutions used for the spectral measurements was accurately determined by total amino acid analysis of suitable aliquots. Tryptophan content was estimated from the absorption spectra of the reduced and S-carboxymethylated derivatives, using the values 1200 and 5579 for the molar absorptivities of tyrosine and tryptophan, respectively, at 279 nm (Sober, 1970).

Determination of Amino and Carboxyl Terminii. The amino and carboxyl terminal residues of the principal neurotoxins were determined by the direct phenyl isothiocyanate method (Edman, 1970) and by digestion with carboxypeptidase A, respectively, using the reduced and S-carboxymethylated derivatives of the toxins. The carboxypeptidase digestions were done using an enzyme:substrate ratio of 1:50, and the digests were analyzed directly with a Beckman 120B amino acid analyzer using the lithium–citrate buffers (Benson *et al.*, 1967; Eaker, 1970).

Toxicity Assays. These were done by intravenous injection (caudal vein) into female albino mice weighing 20–25 g. The injections were done in 0.1 or 0.2 ml of 0.9% NaCl, and in the determination of LD_{100} values at least three mice were used at each dose level. In the case of the two principal neurotoxins, the toxin concentrations were accurately established by absorbance measurements using the predetermined molar absorptivity values.

Results

Gel Filtration of Crude Venom on Sephadex G-75. Zones I–V, VI, and VII in Figure 1 represent 7.7, 21.5, and 70.8%, respectively, of the total soluble venom protein, which accounts for 75% of the dried crude venom as determined by total amino acid analysis. Essentially the same gel filtration pattern was obtained when 0.05 M ammonium acetate was used as eluent.

At the dose level of 10 μg used in the screening assays, none of the individual protein fractions I–VI or the nonprotein fraction of 690–850 ml caused any visible symptoms in mice, while fraction VII killed all the animals within 30 min.

Ion Exchange Chromatography of the Neurotoxic Fraction VII. The pattern shown in Figure 2 was obtained with half of lyophilized fraction VII from the gram-scale gel filtration separation done in 0.2 M ammonium acetate (Figure 1). An identical chromatogram was obtained with the other half after storage for 6 months at room temperature. All of the numbered peaks 1–16 exhibit neurotoxic activity. Another pair of

[1] Abbreviation used is: CM-cysteine, *S*-carboxymethylcysteine.

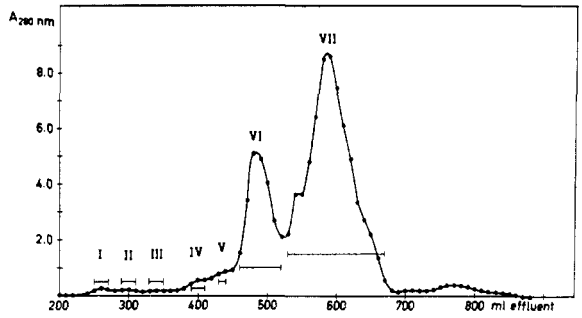

FIGURE 1: Gram-scale separation of clarified *Enhydrina schistosa* venom on a 3.2 × 92 cm column of Sephadex G-75 in 0.2 M ammonium acetate: V_0 = 250 ml, V_t = 740 ml. Cytochrome *c* (mol wt 12,000) elutes at 475 ml, coincident with the maximum of peak VI.

gradient runs was done without intermediate lyophilization. To accomplish this, half (80 ml) of the pooled fraction VII from a gram-scale G-75 separation done in 0.05 M ammonium acetate was diluted to 120 ml with distilled water and applied directly to the ion exchange column, preceded and followed by 40 ml of 0.03 M ammonium acetate. Gradient elution was then performed as described for the run shown in Figure 2. The material corresponding to the unretarded, inactive peaks between 150 and 250 ml in Figure 2 eluted as very broad flat zones in the 0.033 M application buffer, but components 1 and 2 eluted as discrete peaks about 140 and 180 ml following the breakthrough of the first (0.06 M) gradient buffer and the pattern for the remainder of the run was identical with that shown in Figure 2 for the lyophilized material. The same result was obtained with the other half of fraction VII after storage for 4 months at 4° in 0.05 M ammonium acetate.

The two principal neurotoxic components corresponding to peaks 4 and 5 have been selected for further study, and shall be designated *schistosa* 4 and *schistosa* 5.

Rechromatography of Toxins schistosa 4 and 5. The overlap of peaks 4 and 5 in Figure 2 is exaggerated by the logarithmic ordinate scale. The absorbance at the minimum is only 8% of that at the lower (peak 4) of the two maxima, and when the single 10-ml fraction corresponding to the minimum was excluded from the pools, no cross-contamination of *schistosa* 4 and 5 was detected by rechromatography in 0.1 M ammonium acetate at the pH (7.3) used in the gradient separation. Both of the principal neurotoxins are also chromatographically homogeneous at pH 6.50, as evidenced by the analytical elution patterns shown in Figures 3a (*schistosa* 4) and 3b (*schistosa* 5). At this pH the two toxins do not differ significantly in elution behavior and cannot be separated chromatographically in the systems used, but each again elutes in a single peak that accounts for 95% of the absorbance units applied to the column.

Amino Acid Composition of the Principal Neurotoxins. The data given in Table I are based on four analyses of *schistosa*

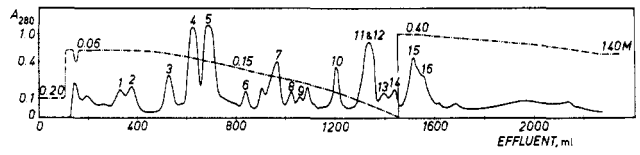

FIGURE 2: Separation on a 3.2 × 28 cm column of Bio-Rex 70 of 326 mg of lyophilized fraction VII from G-75 filtration (Figure 1). Elution at 80 ml/hr with a concave gradient of ammonium acetate. The numbers affixed to the conductivity trace (— ·) indicate the approximate ammonium acetate concentration in the effluent.

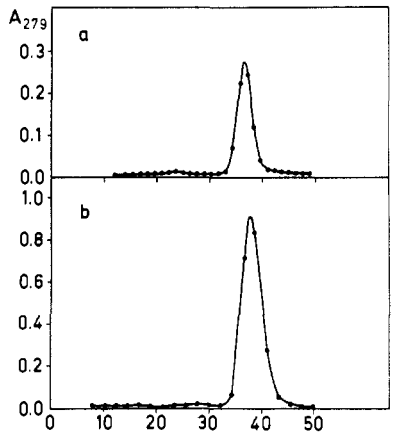

FIGURE 3: Elution chromatography of *schistosa* toxins on a 1 × 34 cm column of Bio-Rex 70 in 0.25 M ammonium acetate at pH 6.50: a and b, direct rechromatography of aliquots from peaks 4 (*ca.* 1 mg) and 5 (*ca.* 3 mg), respectively, of gradient run.

4 (one of the native toxin and three of the reduced and S-carboxymethylated derivative) and seven analyses of *schistosa* 5 (three of the native toxin and four of the reduced and S-carboxymethylated derivative). The CM-cysteine values of 8.7 and 8.6 obtained with the reduced and S-carboxymethylated derivatives of toxins 4 and 5, respectively, are taken to indicate that each toxin contains a total of nine residues of half-cystine plus cysteine. The implied CM-cysteine recovery of about 96% is consistent with recoveries observed in this laboratory for several other neurotoxins and related proteins of established sequence.

Analyses of samples of toxins *schistosa* 4 and 5 following treatment with iodoacetate in 8 M urea without prior reduction yielded 0.88 residue of CM-cysteine in each case, indicating that both toxins contain one SH group.

The proline values given in Table I were obtained with the reduced and S-carboxymethylated derivatives, where interference by cysteine, which coelutes with proline in the analyzer system used (Moore and Stein, 1963), is ruled out. The apparent proline values observed in the analyses of the native toxins were one–two residues higher. The inclusion of phenol in the hydrolysis medium ensures quantitative recoveries of tyrosine, but somehow considerably enhances the conversion of cystine to cysteine during the hydrolysis.

The molar absorptivity at 279 nm of the reduced and S-carboxymethylated derivatives of both toxins was 6800 M^{-1} cm^{-1}, which is exactly the sum of the molar absorptivities of tryptophan (5579) and tyrosine (1220) at the same wavelength. Each toxin therefore contains one residue of tryptophan in addition to the single residue of tyrosine observed in the acid hydrolysates.

The two principal neurotoxins *schistosa* 4 and 5 thus contain 60 amino acid residues each, and differ in amino acid composition only by substitution of a proline for a serine residue.

Amino and Carboxyl Terminal Residues. Since the complete amino acid sequences of toxins 4 and 5 are given in the companion paper (Fryklund *et al.*, 1972) we shall only mention here that both toxins have methionine at the amino terminus, and that 1.8 residues of asparagine were observed in 4-hr carboxypeptidase A digests of the reduced and S-carboxymethylated derivatives, indicating the carboxyl terminal sequence: -Asn-Asn-OH.

Toxicity. The LD_{100} of the monomeric forms of the toxins *schistosa* 4 and 5 is 1.6–1.7 μg per 20-g mouse, or 80–85 μg/kg.

TABLE I: Amino Acid Compositions of *Enhydrina schistosa* Toxins 4 and 5.

Amino Acid	Toxin 4		Toxin 5	
Tryptophan		1		1
Lysine	4.94 ± 0.18	5	4.96 ± 0.09	5
Histidine	2.01 ± 0.03	2	2.00 ± 0.03	2
Amide NH_3	8.69 ± 0.70[a]	9	9.12 ± 0.51[a]	9
Arginine	3.06 ± 0.05	3	3.01 ± 0.04	3
Aspartic acid	5.97 ± 0.09	6	6.05 ± 0.06	6
Threonine	6.95 ± 0.19	7	7.05 ± 0.13	7
Serine	4.90 ± 0.08	5	6.00 ± 0.11	6
Glutamic acid	7.89 ± 0.07	8	8.05 ± 0.10	8
Proline	2.91 ± 0.09[b]	3	1.93 ± 0.03[b]	2
Glycine	4.00 ± 0.08	4	3.94 ± 0.04	4
Alanine	1.02 ± 0.02	1	1.00 ± 0.01	1
Half-cystine				
As CM-cysteine	8.66 ± 0.30[b]	9[d]	8.57 ± 0.07[c]	9[d]
As cysteic acid	9.04		9.04	
Valine	0.98 ± 0.02	1	0.92 ± 0.04	1
Methionine	0.94 ± 0.03	1	0.98 ± 0.08	1
Isoleucine	1.97 ± 0.02	2	1.84 ± 0.07	2
Leucine	1.00 ± 0.05	1	0.97 ± 0.03	1
Tyrosine	1.05 ± 0.03	1	0.99 ± 0.03	1
Phenylalanine	0.00	0	0.00	0
Total residues		60		60
Formula wt		6689		6679
Molar absorptivity, 279 nm		8400		8400
Free SH (as CM-cysteine)		0.88		0.88
LD_{100} (μg/kg mouse)		85		85
% total soluble venom protein		14.4		24.6

[a] Two determinations. [b] Three determinations (reduced and S-carboxymethylated derivative). [c] Four determinations (reduced and S-carboxymethylated derivative). [d] Eight half-cystine residues and one cysteine residue.

The corresponding values for the crude venom used are 3 and 150 μg, respectively. Although these neurotoxins have not been tested with myoneural preparations, the nature and time course of the paralytic effects observed in the mouse assay indicate that they have the same kind of "curariform" activity as the *siamensis* 3 toxin (Karlsson *et al.*, 1971; Lester, 1970).

Reactivity and Importance of the Free SH Group. In the absence of 8 M urea, the sulfhydryl group of toxins 4 and 5 did not react with iodoacetate, iodoacetamide, or 4-vinylpyridine, indicating that the SH group is "buried" in the native toxins or is perhaps masked by a firmly bound metal ion. No metal analyses have been carried out.

In the presence of 8 M urea the reaction of the SH group with any of the above reagents is complete within 2–3 hr and is accompanied by severe (iodoacetate, iodoacetamide) or complete (4-vinylpyridine) losses of activity. In the latter case the loss of activity might well be a direct consequence of the S-pyridylethylation, but in the other cases the activity losses are definitely due to alterations in addition to the alkylation of the SH group. In one experiment, 25 mg of *schistosa* 5 was treated with iodoacetate overnight in 8 M urea. The crude product isolated by gel filtration on Sephadex G-25 in 0.2 M ammonium acetate showed 0.9 residue of CM-cysteine/mol and had a residual activity of about 20%. The pooled fractions containing the crude product were diluted to an ammonium acetate concentration of 0.05 M and the material was directly adsorbed to a column of Bio-Rex 70 equilibrated with 0.2 M ammonium acetate at pH 6.50. Elution was then performed with a concave gradient of 0.06 *vs.* 1.4 M ammonium acetate. The protein eluted in a complex series of overlapping peaks between 0.12 and 0.22 M ammonium acetate. No native toxin was observed at 0.25 M eluent. The tallest and sharpest peak, which eluted at 0.17 M ammonium acetate approximately in the middle of the series, showed 0.88 residue of CM-cysteine/mol and killed three out of three mice at a dose level of 2 μg. Although treatment of the toxin with iodoacetate in 8 M urea gives rise to a complex mixture of products, mostly inactive, that probably differ as a result of aggregation, denaturation, N-carboxymethylation, S-alkylation of the amino terminal methionine, or carbamylation (no precautions were taken to exclude cyanate from the 8 M urea), the isolation in about 10% yield of a fully active mono-CM-cysteine derivative conclusively shows that the SH group is not required for activity.

Gel Filtration Behavior and Toxicity of Dried Preparations. Aliquots taken directly from the pooled fractions corresponding to peaks 4 and 5 in the gradient separation (Figure 2) are completely homogeneous with regard to gel filtration behavior on Sephadex G-50. However, lyophilized preparations of *schistosa* 4 and 5 invariably contain material that elutes ahead of the native toxin. An extreme example is the pattern shown in Figure 4 for a preparation of *schistosa* 5 that had inadvertently thawed during the final stages of the lyophilization. The zones A + B, C, and D account for 41, 15, and 43% of the applied protein, respectively, are identical with regard to amino acid composition, and became indistinguishable upon reduction and S-carboxymethylation. Zone

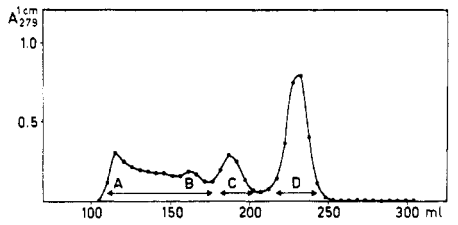

FIGURE 4: Gel filtration on a 2 × 102 cm column of Sephadex G-50 in 0.2 M ammonium acetate of a badly lyophilized (see text) preparation (25 mg) of toxin *schistosa* 5.

D corresponds to the fully active monomeric form of the toxin, while none of the earlier eluting fractions A (110–150 ml), B, or C caused any symptoms in mice at a dose level (10 μg) corresponding to 6 LD_{100} of the native toxin. In the absence of any direct proof, we can only suggest that zones C, B, and A in Figure 2 correspond to dimers, trimers, and higher polymers of the toxin, respectively.

Discussion

The characterization data presented above indicate that each of the two principal neurotoxins *schistosa* 4 and 5 consists of a single peptide chain of 60 amino acids cross-linked by four disulfide bridges. The sequence analyses described in the companion paper (Fryklund *et al.*, 1972) confirm these conclusions and further show that the toxins differ in primary structure only by a serine–proline substitution at position 46, in complete accord with the amino acid composition data recorded in Table I.

In addition to the four disulfide bridges, each toxin contains a "masked" SH group which becomes accessible to sulfhydryl reagents in 8 M urea. However, the isolation of a fully active mono-S-carboxymethylated derivative from the complex mixture of products obtained upon treatment of the *schistosa* 5 toxin with iodoacetate in 8 M urea indicates that the structural alteration involved in the "unmasking" is either reversible or does not involve the active site.

All of the curariform elapid and hydrophid toxins that have been fully characterized heretofore contain either 61 or 62 amino acids and 4 disulfide bridges (structural types 61-4 and 62-4) or 71 and 74 amino acids and 5 disulfide bridges (structural types 71-5 and 74-5) and are devoid of free SH groups. The *schistosa* toxins 4 and 5 thus represent a new structural type 60-4-1SH. We do not yet have any concise information regarding the serological relationships between these toxins and representatives of the four structural types recognized earlier.

Dried preparations of toxins *schistosa* 4 and 5 obtained by lyophilization from 0.2 M ammonium acetate routinely show 5–6% of altered, less active or perhaps inactive forms that elute earlier than the native toxins on Sephadex G-50, but the production of these earlier eluting froms, which probably consist of dimers, trimers, and higher polymers of the toxins, is greatly enhanced if thawing occurs during the drying operation. We doubt that the apparent polymerization involves the formation of intermolecular disulfide bridges because: (1) all of the earlier eluting forms show 0.5 residue or more of CM-cysteine upon alkylation in 8 M urea, (2) the mono-S-carboxymethylated derivative exhibits similar behavior following lyophilization or exposure to 8 M urea, and (3) we have observed similar effects with several other neurotoxins that contain all their eight or ten half-cystine residues in disulfide form. For example, the *Naja nigricollis* toxin α (Karlsson *et al.*, 1966) gives a gel filtration pattern very similar to that shown in Figure 4 after lyophilization from acidic media of low ionic strength (*e.g.*, 0.2 M acetic acid).

The two principal toxins *schistosa* 4 and 5 together account for 39% of the soluble venom protein, or 29% of the weight of the dried crude venom. The gel filtration fraction VII in which they elute in the initial group separation step (Figure 1) contains 71% of the total soluble protein, or 53% of the total venom material, and since neurotoxic activity is present in all of the numbered areas of the gradient chromatogram of fraction VII shown in Figure 2 we conclude that neurotoxins might account for more than 60% of the soluble venom protein, or more than 45% of the dried crude venom. The high toxicity of *Enhydrina schistosa* venom thus seems simply accountable to an exceptionally high content of curariform neurotoxins of ordinary (mouse LD_{100} *ca.* 100 μg/kg) potency.

The gradient pattern shown in Figure 2 provides chromatographic evidence for the presence in gel filtration fraction VII of at least 25 components, and the assay evidence indicates that the venom contains 14 or more different neurotoxins in addition to *schistosa* 4 and 5. The multiplicity of curariform neurotoxins in elapid and hydrophid venoms has become thoroughly established within the last few years. For example, from *Naja naja siamensis* (Thailand cobra) venom we have isolated three distinctly different minor neurotoxic components, two of the 62-4 structural type (toxins *siamensis* 3C and 7C) and one of the 61-4 type (toxin *siamensis* 5) in addition to the overwhelmingly dominant principal toxin (*siamensis* 3) of the 71-5 type (Karlsson *et al.*, 1971). Furthermore, all four toxins were observed in the venom of a single snake.

The venom of spectacled *Naja naja naja* cobras from eastern India showed a similar multiplicity of structural types, but in the pooled venom preparation first studied the principal structural type (71-5) was approximately equally represented by two toxins (*naja* 3 and 4) that differ only by a discrete Ser–Ile substitution at position 32 in the peptide chain. Samples of venom from two individual cobras were examined, and, by chance, one snake showed only the *naja* 3 and the other only the *naja* 4 form of the principal toxin (Karlsson and Eaker, 1972). Probably similar allotypic situations occur among the minor neurotoxic components as well.

The 2 g of *Enhydrina schistosa* venom used in the present investigation was supplied from a larger stock containing venom from several hundred individual snakes, and might well display the full spectrum of allotypic variation within the species. The toxins *schistosa* 4 and 5 would thus represent the predominant allotypic pair, and further variation in these and in each of perhaps three or four additional distinct toxin forms produced by every individual could easily account for the complexity of the chromatographic pattern. Since our chromatographic system is readily applicable on the 10-mg scale and any component present at a level of 100 μg or more could be adequately characterized with regard to amino acid composition and LD_{100} dose, the latter hypothesis could be tested directly by examination of venom samples from several individual snakes, but we have not yet succeeded in obtaining such material.

The separation of the *schistosa* toxins 4 and 5 again illustrates the exquisite selectivity of the ammonium acetate–Bio-Rex 70 (or IRC-50) system toward small basic proteins of this type, as we demonstrated earlier in the separation of the *naja* toxins 3 and 4 (Karlsson *et al.*, 1971) and the six isomeric monoacetyl derivatives of toxin *siamensis* 3 (Karlsson *et al.*, 1972). In each of these cases there is no difference in formal charge between or among the molecules separated. The ammonium acetate seems to be important for the high selectivity since a much simpler pattern showing only seven smooth

peaks was obtained when fraction VII was chromatographed in an analogous gradient of sodium phosphate–NaCl buffers on a Bio-Rex 70 column equilibrated with 0.2 M Na^+–phosphate at pH 7.30, and *schistosa* 4 and 5 did not separate. Neither have we been able to separate the toxins *naja* 3 and 4 in sodium phosphate buffers, while they separate readily in ammonium acetate at either pH 6.5 or 7.3.

The very high selectivity of our chromatographic separation system might account for some of the differences between our findings and the recent report of Tu and Toom (1971), who obtained eight peaks, four of which were highly neurotoxic, upon fractionation of crude *Enhydrina schistosa* venom (rather than a defined gel filtration fraction thereof) on CM-cellulose using stepwise elution with sodium phosphate–NaCl buffers. The principal neurotoxin obtained by Tu and Toom (1971) accounted for about 30% of the total venom protein and was described as containing 62 amino acids, including nine residues of half-cystine plus cysteine, but no direct evidence was given for the presence of a free SH group. As compared, for example, with toxin *schistosa* 5 (Table I), their toxin contains an additional residue each of threonine, proline, and glycine and is devoid of methionine. Although an isoelectric point of 9.2 was obtained by isoelectric focusing, their toxin was retarded on CM-cellulose at pH 9.7.

Our venom does not appear to contain any significant amount of a neurotoxic component that lacks methionine, but which is otherwise so similar in amino acid composition to the *schistosa* toxins 4 and 5. The *Enhydrina schistosa* venom used by Tu and Toom (1971) was collected by the authors themselves in the same geographic area as ours (Straits of Malacca) and probably represents a comparable number of individual snakes, so it is difficult to believe that the apparent differences between our principal neurotoxins and theirs can be accountable to selective sampling of segregated subspecies. An amino terminal analysis of their toxin would certainly clarify the issue.

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Amino Acid Sequences of the Two Principal Neurotoxins of *Enhydrina schistosa* Venom†

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ABSTRACT: The complete amino acid sequences of two curariform neurotoxins isolated from the venom of the common sea snake, *Enhydrina schistosa* have been elucidated. The two toxins, designated *schistosa* 4 and *schistosa* 5, differ in sequence only by a proline–serine substitution at position 46 in the 60-residue peptide chain. The arrangement of the four disulfide bridges has not been determined, but the free SH group present in the toxins has been localized at position 3, immediately adjacent to a half-cystine residue

1 2 3 4 5
Met-Thr-CysH-Cys-Asn-

All of the curariform venom neurotoxins for which complete sequence data are available fall into one or the other of two distinct size groups: the smaller toxins of group I contain 61 or 62 amino acids and four disulfide bridges

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