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†

Linda Fryklund, David Eaker,* and Evert Karlsson

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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(structural types 61-4 and 62-4) and the larger toxins of group II contain 71 or 74 amino acids and five disulfide bridges (structural types 71-5 and 74-5). The toxins of all four structural types are clearly homologous with respect to amino acid sequence (Strydom, 1972; Mebs *et al.*, 1972) and appear to be homologous with regard to disulfide pairing as well (Endo *et al.*, 1971; Yang *et al.*, 1970; Botes, 1971), the fifth or "extra" disulfide bridge in the toxins of group II serving merely to pinch off a short pentapeptide sequence in a stretch of sequence that is partly deleted from the toxins of group I.

The two principal *Enhydrina schistosa* neurotoxins described in the accompanying paper (Karlsson *et al.*, 1972a) clearly fall into size group I, but are unique in two respects among all the curariform neurotoxins described so far: the peptide chain is only 60 amino acids long, and a cysteine residue (free SH group) is present in addition to four disulfide bridges. The *schistosa* toxins 4 and 5 thus represent a new structural type 60-4-1SH. The complete amino acid sequences of both toxins, including the location of the free SH group, have been established as described below.

Materials and Methods

The isolation of the *schistosa* toxins 4 and 5 and the preparation of the reduced and S-carboxymethylated derivatives are described in the accompanying paper (Karlsson *et al.*, 1972a).

Citraconylation of the Reduced and S-Carboxymethylated Neurotoxins. This was done essentially according to Dixon and Perham (1968). To approximately 2 μmol of reduced and S-carboxymethylated toxin in 2 ml of 1 M *N*-ethylmorpholine acetate buffer, pH 8.0, was added 30 μl of citraconic anhydride (British Drug Houses, Ltd., laboratory reagent), corresponding to a 30-fold excess of reagent over toxin amino groups. After 10 min at room temperature under continuous stirring the reaction mixture was rapidly gel-filtered through a 1.4 × 14 cm column of Sephadex G-25 in 0.1 M *N*-ethylmorpholine acetate, pH 8.0, to remove excess reagents. The fractions corresponding to the protein zone were immediately pooled, quick-frozen, and lyophilized.

Tryptic Digestion of the Citraconylated Proteins. The citraconylated reduced and S-carboxymethylated toxins (*ca.* 2 μmol) were treated with trypsin (Worthington, TPCK[1] treated) in 2 ml of 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5, at an enzyme:substrate ratio of 1:50. After incubation for 2 hr at 37°, the digests were applied directly to a 2 × 102 cm column of Sephadex G-50 equilibrated with 0.1 M ammonium acetate buffer, pH 8.9. The pooled fractions corresponding to peaks A and B (Figure 1) were adjusted to pH 3.5 with glacial acetic acid and the "deblocking" was allowed to proceed with continuous stirring for 15 hr at room temperature, after which the solutions were quick-frozen and lyophilized.

Chymotryptic Digestion of the Reduced and S-Carboxymethylated Toxins. Samples (3 μmol) of the reduced and S-carboxymethylated toxins were digested with chymotrypsin (Worthington, three times crystallized) in 2 ml of 0.1 M *N*-ethylmorpholine acetate buffer, pH 7.5. The substrate concentration was about 1% and the enzyme:substrate ratio 1:100. After incubation for 30 min at 37° the digestion was terminated by acidification with acetic acid and the solutions were lyophilized. Group fractionation of the peptide mixtures was then performed by gel filtration through a 1 × 140 cm column of Sephadex G-25 (fine) in 0.2 M acetic acid.

Zone Electrophoretic Separation of Peptides. All digests and fractions thereof were routinely examined by low voltage paper electrophoresis (440 V) at pH 3.2 (0.03 M pyridine–0.1 M formic acid), pH 5.0 (0.05 M pyridine–0.05 M acetic acid), and pH 7.5 (0.05 M *N*-ethylmorpholine–0.03 M acetic acid) either for the assessment of purity or to provide a basis for the selection of the conditions to be used for preparative electrophoretic separation in columns of cellulose powder.

Preparative (0.5–2-μmol scale) zone electrophoretic separations were done in 1 × 86 cm beds of pyridine-extracted ethanolyzed cellulose powder (Porath, 1964) in externally cooled glass columns (Porath, 1956). Details regarding the packing, evaluation, and operation of the columns shall be published shortly elsewhere (Arnberg *et al.*, 1972).[2] Samples were applied routinely in 2 ml of half-concentrated buffer to compensate for residual electrolytes present in the lyophilized samples and to ensure electrophoretic "sharpening" of the starting zones. Following electrophoresis elution was carried out with the column buffer at a flow rate of 10–12 ml/hr and the effluent was collected in 1-ml fractions. For the detection of peptide zones, appropriate aliquots were analyzed by ninhydrin following alkaline hydrolysis at 110° in open polypropylene (Nalgene) tubes.

Edman Degradation. Sequential phenyl isothiocyanate degradations were done manually using the direct three-step procedure of Edman (1970) essentially as described by Iwanaga *et al.* (1969). The ethyl acetate soluble PTH derivatives were identified by thin-layer chromatography on commercial silica gel plates (Merck Fertigplatten F_{254}) in the following four solvent systems: solvent E (Edman and Sjöquist, 1956), solvent V (Jeppson and Sjöquist, 1967), and solvents II and III (Brenner *et al.*, 1962). The water-soluble derivatives of histidine, arginine, and cysteic acid were identified by low voltage (440 V) paper electrophoresis for 2–3 hr at pH 6.5 in a sodium phosphate buffer containing 1.34 g of $Na_2HPO_4 \cdot 2H_2O$ (0.015 mol), 2.07 g of $NaH_2PO_4 \cdot H_2O$ (0.03 mol), 1 g of disodium EDTA, and 5 g of soluble starch per liter. The PTH derivatives were detected as white spots on a coffee-colored background by means of the iodine–azide reagent (Edman, 1970).

Labeling of the Free SH Group with [^{14}C]Iodoacetate. [1-^{14}C]Iodoacetic acid with a specific radioactivity of 13.4 Ci/mol was obtained from New England Nuclear Chemicals, GmbH, lot no. 465-243. To 1.4 mg (0.2 μmol) of neurotoxin *schistosa* 4 monomer in 1.43 M Tris-HCl buffer, pH 8.6, was added 2.5 μCi (187 nmol) of [^{14}C]iodoacetate, and solid urea was then dissolved to a concentration of 8 M. After 15 min at room temperature the solution was gel filtered through a 3.2 × 32 cm column of Sephadex G-50 in 0.2 M ammonium acetate. The protein was recovered by lyophilization and was then completely reduced with mercaptoethanol and alkylated with cold iodoacetate in the usual way (Crestfield *et al.*, 1963). The labeled, reduced, and S-carboxymethylated derivative was then recovered by gel filtration and lyophilization. The product was dissolved in water and a 10% aliquot was taken for amino acid analysis. The remainder of the material was subjected to four stages of Edman degradation.

The amino acid analysis was done with an updated Beckman 120 amino acid analyzer equipped with scale expansion (0.1 o.d., full scale) and the radioactivity in the column effluent was monitored continuously with a Nuclear-Chicago 6352 Chroma-Cell system (6353, 8770, 6770). The anthracene flow scintillation cell of 2-ml capacity was connected between the

[1] Abbreviations used are: CM-cysteine, *S*-carboxymethylcysteine; PTH, phenylthiohydantoin; TPCK, tosylphenylalanine chloromethyl ketone.

[2] Arnberg, H., Eaker, D., and Karlsson, E. (1972), manuscript in preparation.

TABLE I: Amino Acid Compositions of Reduced and S-Carboxymethylated Toxins.

Amino Acid	*schistosa* 4	*schistosa* 5
Tryptophan	(1)	(1)
Lysine	5.00	4.96
Histidine	2.00	2.04
Arginine	3.00	3.00
CM-cysteine	8.99	8.60
Aspartic acid	5.72	6.09
Threonine	6.63	7.16
Serine	*4.95*	*5.88*
Glutamic acid	7.74	7.99
Proline	*3.00*	*1.90*
Glycine	4.02	3.94
Alanine	0.99	1.01
Valine	0.96	0.92
Methionine	0.95	0.97
Isoleucine	1.91	1.76
Leucine	0.98	0.95
Tyrosine	1.02	1.04
Phenylalanine	0	0
Total	60	60

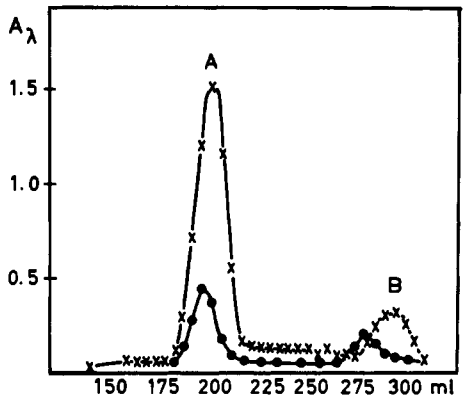

FIGURE 1: Gel filtration on a 2 × 102 cm Sephadex-G-50 column in 0.1 M ammonium acetate of tryptic digest of 1.7 μmol of citraconylated reduced and S-carboxymethylated *schistosa* 5. The intact reduced and S-carboxymethylated derivative elutes at 155 ml under the same conditions. The enzyme eluted at 130 ml (not shown): (●) absorbance at 275 nm; (×) absorbance at 570 nm following alkaline hydrolysis and ninhydrin reaction.

bottom of the column and the ninhydrin mixing block of the analyzer. Counts were listed at 1-min intervals. As determined by static and steady-state experiments with standards of known radioactivity, the counting efficiency for [^{14}C]amino acids in the analyzer buffers at the voltage and gate settings used was about 40%.

The absolute radioactivity of effluent peaks was estimated by means of an empirically established relationship between net counts and rated absolute activity. The calibration curve was made with a number (Thr, Ser, Gly, Ala, and Phe) of [^{14}C]amino acids having a rated specific activity of 10.0 mCi/mmol (kit NEC-483, New England Nuclear), using a constant level (0.25 μmol) of cold carrier. Plots of net observed counts per peak *vs.* applied disintegrations per minute (dpm) were linear over the range from 450 to 45,000 counts, and the slope was 1.25 counts/dpm. The background counting rate was 25–30 cpm.

Labeling of the SH Group with 4-Vinylpyridine. To 0.5 μmol of neurotoxin in 4 ml of 0.2 M *N*-ethylmorpholine–acetic acid buffer, pH 8.6, containing urea at a concentration of 8 M was added 100 μl (1 mmol) of 4-vinylpyridine (Fluka, stabilized monomer, practical grade). The medium was saturated with nitrogen prior to dissolving the toxin and the reaction was carried out under nitrogen in a closed vessel at room temperature with continuous magnetic stirring. After 3 hr the reaction mixture was gel filtered through a 2 × 102 cm column of Sephadex G-50 in 0.2 M ammonium acetate. The protein eluted in three peaks at 144, 183, and 218 ml, which accounted for 16, 74, and 10% of the total protein, respectively. The main component was recovered by lyophilization.

The content of *S*-β-(4-pyridylethyl)-L-cysteine was estimated both by spectrophotometric measurements and amino acid analysis. For reference use, authentic *S*-β-(4-pyridylethyl)-L-cysteine was synthesized according to Cavins and Friedman (1970). Elementary analysis of the crystalline product (mp 210°) gave C, H, N, and S values of 52.91, 6.01, 12.24, and 14.09%, in excellent agreement with the respective theoretical values 53.08, 6.24, 12.38, and 14.17 .The molar absorptivity at 254 nm in water at pH 6.5 was 4.38×10^3. The substance was completely homogeneous on the amino acid analyzer, eluting ahead of, and well separated from, arginine on a 0.9 × 8 cm column of Bio-Rad A-5 resin operated at 56° with 0.25 M sodium citrate buffer, pH 5.20 (prepared simply by dilution and pH adjustment of the standard 0.35 M buffer of pH 5.28). Oxidation of the *S*-β-(4-pyridylethyl)-L-cysteine with preformed performic acid at 0° resulted in a single product, presumably the sulfone, that eluted between lysine and histidine in the short-column system described above. Excess performic acid was of course removed by repeated lyophilization, rather than by addition of HBr. We did not succeed in preparing the sulfoxide(s).

The phenylthiohydantoin of *S*-β-(4-pyridylethyl)-L-cysteine was prepared essentially by the general procedure (Edman, 1970), except that trifluoroacetic acid was substituted for HCl. The derivative migrated like PTH-threonine in solvent II (see above), remained at the origin in the acidic systems III and V, and ran as a "smear" up the lower third of the plate in solvent E.

Amino Acid Analysis. Routine amino acid analyses were done with a Bio-Cal BC-200 equipped with an automatic sample injector (Chromotronix) and an electronic integrator (Infotronics CRS-110A) using the 3- or 15-mm cuvets as required, following hydrolysis at 110° in thoroughly evacuated sealed tubes in 2 ml of 6 N HCl (Merck Suprapur) containing 10 mg/ml of reagent grade phenol.

Results

The sequences of the toxins *schistosa* 4 and 5 were done completely in parallel using the reduced and S-carboxymethylated derivatives, and in all of the peptide sequences written below -Cys- therefore represents a residue of CM-cysteine unless stated otherwise. The amino acid data for the particular reduced and S-carboxymethylated preparations used in the sequence analyses are given in Table I.

Amino Terminal Sequence. The intact reduced and S-carboxymethylated derivatives of *schistosa* 4 and 5 were subjected to 21 cycles of sequential degradation. Interpretable results

TABLE II: Tryptic Peptides from Citraconylated Reduced and S-Carboxymethylated Toxins 4 and 5.

	Fragment[a]									
	Toxins 4 and 5						Toxin 4		Toxin 5	
Amino Acid	1–31		32–34		35–37		38–60		38–60	
Tryptophan	1.0	1								
Lysine	3.2	3					2.04	2	1.9	2
Histidine	1.0	1					1.01	1	0.7	1
Arginine	1.0	1	1.0	1	1.0	1				
CM-cysteine	3.8	4					4.30	5	4.7	5
Aspartic acid	3.1	3					3.24	3	3.0	3
Threonine	5.2	5	0.95	1			1.15	1	1.2	1
Serine	5.0	5					0.24	*0*	1.2	*1*
Glutamic acid	3.9	4			1.04	1	3.29	3	3.3	3
Proline	0.6	1					2.04	*2*	1.0	*1*
Glycine			1.11	1			3.01	3	2.9	3
Alanine	0.9	1								
Valine							0.95	1	1.1	1
Methionine	0.9	1								
Isoleucine					0.96	1	0.99	1	0.8	1
Leucine							1.09	1	1.2	1
Tyrosine	0.9	1								
Phenylalanine										
Total		31		3		3		23		23
Yield (%)		85		100		100		100		100

[a] Identification refers to position in sequence. See Figure 7.

were obtained in the first 19 stages, indicating the identical sequences

```
                5                   10
Met-Thr-Cys-Cys-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Lys-Thr-Thr-
                                                    15
                                                Thr-Asn-Cys-Ala-Glu-
```

Tryptic Peptides from the Citraconylated Reduced and S-Carboxymethylated Derivatives. The gel filtration pattern obtained with the tryptic digest of citraconylated *schistosa* 5 is shown in Figure 1. Fractions A and B (into which was included the small ultraviolet (uv) absorbing peak at 275 ml) were examined by paper electrophoresis following deblocking and lyophilization. Fraction A showed only two spots, one neutral and the other moderately anodic at pH 5.0. At pH 7.5 all the material was anodic, and a diffuse zone was observed between the two main spots.

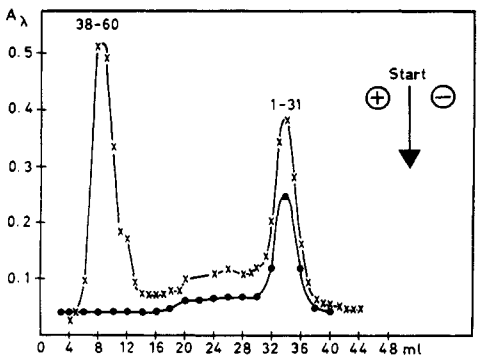

FIGURE 2: Electrophoretic separation on a 1 × 86 cm cellulose powder column of deblocked fraction A (Figure 1) from tryptic digest of citraconylated reduced and S-carboxymethylated *schistosa* 5. Conditions: 24-hr electrophoresis at 1000 V in 0.05 M *N*-ethylmorpholine–acetate acid buffer, pH 7.5; current strength 8 mA. The arrow indicates the position of the starting zone: (●) absorbance at 279 nm; (×) absorbance at 570 nm following alkaline hydrolysis and ninhydrin reaction.

The preparative column electrophoretic separation of fraction A is illustrated in Figure 2. On the basis of the amino acid composition listed in Table II and the amino terminal sequence determined with the intact reduced and S-carboxymethylated toxin, the least anodic of the two peaks observed in Figure 2 is unambiguously assigned to the N-terminal end of the molecule, evidently representing residues 1–31. The presence of arginine at the carboxyl terminus of the peptide was inferred from tryptic specificity and confirmed by digestion with carboxypeptidase B.

The most anodic of the two peaks in Figure 2 contains 23 amino acids but is without arginine, and is on this basis assigned to the C terminus of the molecule, evidently representing residues 38–60.

The smaller peptides of fraction B (Figure 1) were isolated by paper electrophoresis at pH 5.0. Only three ninhydrin-positive zones were observed in the guide strips cut from either side of the paper. Strips corresponding to the three zones were cut from the central unstained portion of the paper and the material eluted with 10% acetic acid was subjected to amino acid analysis. The zone exhibiting neutral behavior was thus identified as a tripeptide with the composition (Ile,Glu)-Arg. The first cathodic zone correpsonded to another tripeptide (Gly,Thr)-Arg. The lemon yellow color observed with this peptide upon staining the papers with the poly-

TABLE III: Chymotryptic Peptides from Reduced and S-Carboxymethylated Toxins.

Amino Acid	Fragment[a] 1–23		24–27		28–33		51–60	
Tryptophan			0.95	1				
Lysine	0.96	1	2.0	2				
Histidine					0.91	1	0.96	1
Arginine					0.93	1		
CM-Cysteine	4.09	4					2.74	3
Aspartic acid	2.27	2			1.00	1	3.04	3
Threonine	3.96	4	0.99	1	1.08	1	1.03	1
Serine	3.97	4			1.08	1		
Glutamic acid	4.23	4					1.99	2
Proline	1.16	1						
Glycine					1.03	3		
Alanine	1.00	1						
Valine								
Methionine	0.65	1						
Isoleucine								
Leucine								
Tyrosine	1.00	1						
Phenylalanine								
Total		23		4		6		10

[a] Identification refers to position in sequence. See Figure 7.

chromatic ninhydrin–collidine "color dip" (Bennett, 1967) is indicative of amino terminal glycine.

The second cathodic peptide, which migrated about 25% faster than the tripeptide (Gly,Thr)-Arg, was not obtained in an amount sufficient to permit unambiguous identification. However, this peptide probably corresponds to the material responsible for the uv absorption (tryptophan spectrum) observed in zone B of Figure 1, and can be tentatively identified later on when the complete sequence is proved.

The two tripeptides (Gly,Thr)-Arg and (Ile,Glu)-Arg together with the large fragments 1–31 and 38–60 account for the 60 residues present in the toxin molecule, and are thus assigned to the central region 32–37.

The fragment 38–60 was subjected to 12 cycles of sequential degradation, thereby establishing the sequence

40 45
Gly-Cys-Gly-Cys-Pro-Gln-Val-Lys-Ser-Gly-Ile-Lys-

60
(Cys_3,Asx,Thr,Glx_2,Leu,His)-Asn-Asn

The carboxyl terminal sequence -Asn-Asn is inferred from the results of carboxypeptidase experiments done with the intact reduced and S-carboxymethylated derivative of the toxin (Karlsson *et al.*, 1972a).

Identical results were obtained with the citraconylated reduced and S-carboxymethylated *schistosa* 4, except that the fragment 38–60 contained two (rather than one) residues of proline and serine was absent (Table II). The latter fragment showed proline instead of serine in position 46, but was otherwise identical with the corresponding *schistosa* 5 peptide described above.

Chymotryptic Fragments of the Reduced and S-Carboxymethylated Toxins. The gel filtration pattern obtained with a 30-min chymotryptic digest of the reduced and S-carboxymethylated *schistosa* 5 is shown in Figure 3. The most retarded zone D showing the high uv absorption at 279 nm consisted of the pure tetrapeptide Lys-Lys-Thr-Trp (Table III). PTH-lysine was obtained in one cycle of Edman degradation. A second cycle was not attempted since the residual peptide was extracted along with the end group in the first cycle and was consequently exposed to the conversion conditions. Digestion of the intact tetrapeptide with carboxypeptidase A indicated the carboxyl terminal sequence -Thr-Trp, thereby placing the second lysine at position 2 in the peptide.

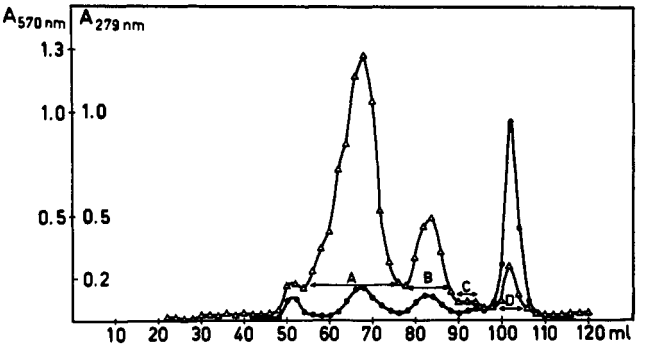

FIGURE 3: Gel filtration on a 1 × 140 cm column of Sephadex G-25 in 0.2 M acetic acid of chymotryptic digest of 2 μmol of reduced and S-carboxymethylated *schistosa* 5: (○) absorbance at 279 nm; (△) absorbance at 570 nm following alkaline hydrolysis and ninhydrin reaction.

Fractions A and B (Figure 3) were complex mixtures. The column electrophoretic separation of fraction B is illustrated in Figure 4. Two pure peptides were obtained. The large anodic peak 6 consists of the decapeptide, Glu-Cys-Cys-His-Thr-Asn-Glu-Cys-Asn-Asn (Table III). The sequence was established by nine consecutive stages of Edman degradation. This peptide is unambiguously assignable to the carboxyl terminus of the toxin molecule, thereby representing residues 51–60. Since the sequence 38–49 was established by degradation of the fragment 38–60 isolated from the tryptic digest of the citraconylated reduced and S-carboxymethylated derivative, the sole leucine residues present in the fragment can be assigned to position 50, thereby establishing the sequence

40 45 50
Gly-Cys-Gly-Cys-Pro-Gln-Val-Lys-Ser-Gly-Ile-Lys-Leu-Glu-Cys-

55 60
Cys-His-Thr-Asn-Glu-Cys-Asn-Asn

The leucine residue also accounts for the chymotryptic cleavage.

Peak 2 eluting at 25 ml in Figure 4 corresponds to the hexapeptide, Ser-Asp-His-Arg-Gly-Thr (Table III), as established by five cycles of Edman degradation. Since the fragment 1–31 isolated from the tryptic digest of the citraconylated reduced and S-carboxymethylated derivative showed carboxyl terminal arginine, and since the remaining two arginine residues present in the molecule were accounted for in the two tripeptides (Gly,Thr)-Arg and (Ile,Glu)-Arg, the above hexapeptide can only be assigned to positions 28–33 in the molecule, thereby establishing the sequence 28–37 as

30 37
Ser-Asp-His-Arg-Gly-Thr-Arg-(Ile,Glu)-Arg

The column electrophoretic separation of fraction A (Figure 3) is illustrated in Figure 5. The large anodic peak 5 consisted

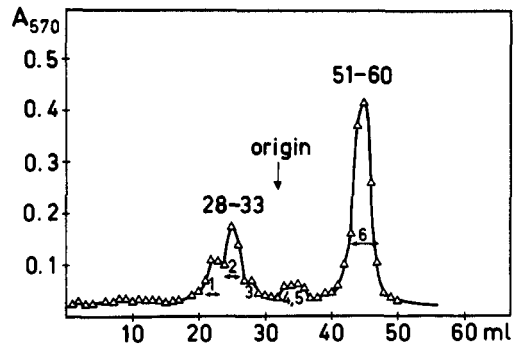

FIGURE 4: Column zone electrophoretic separation of chymotryptic peptide fraction B (Figure 3). Electrophoresis for 7 hr, at 1000 V in 0.05 M pyridine–acetic acid buffer, pH 5.0; current strength 7.5 mA. The arrow indicates the position of the starting zone; cathode is at the right; ordinate, ninhydrin color following alkaline hydrolysis.

of a pure peptide which was readily identified as the fragment 1–23 on the basis of the amino acid composition data (Table III). This fragment was treated with trypsin and the digest was gel filtered on Sephadex G-25. The resulting pattern is shown in Figure 6. The amino acid composition of peak I (Table IV) corresponded to the known sequence 1–12. Peak II was the undecapeptide, Thr-Thr-Thr-Asn-Cys-Ala-Glu-Ser-Ser-Cys-Tyr (Table IV). After ten cycles of consecutive degradation performed with 0.4 μmol of peptide, free tyrosine was obtained in a yield of 10% as determined by direct analysis (*e.g.*, without hydrolysis) of the residue on the 50-cm column of the analyzer.

Peaks 1–3 in Figure 5 were mixtures of overlapping peptides from the region 28–60 in the molecule. The absence of fragments from the amino terminal end was evident from the total absence of methionine, alanine, and tyrosine, which occur only at positions 1, 18, and 23 in the molecule, respectively. Peak 1 was mainly the fragment 28–50 (expected charge 2+). Peak 2 was a 1:2 mixture of the fragments 28–45 and 34–50 (expected charge 1+ and 1+). Peak 3 appeared to consist mainly of the fragment 34–45 (expected charge 0). Peak 4 was mainly the fragment 34–60 (expected charge 2−) but was apparently contaminated with some of the fragment 1–27 (expected charge 2−) as well.

Eight cycles of sequential degradation were performed on the mixture of the fragments 28–45 and 34–50. Two PTH amino acids were obtained in each of the cycles 1–3 and 6–8.

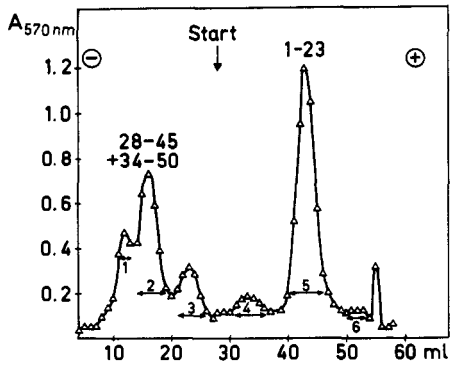

FIGURE 5: Column electrophoretic separation of chymotryptic peptide fraction A (Figure 3). Electrophoresis for 15 hr at 1000 V in 0.05 M pyridine–acetic acid buffer, pH 5.0; current strength 7.5 mA; arrow indicates the position of the starting zone; ordinate, ninhydrin color following alkaline hydrolysis.

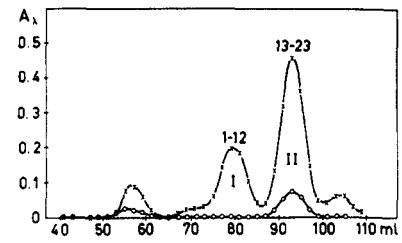

FIGURE 6: Gel filtration on a 1 × 140 cm Sephadex G-25 column in 0.2 M acetic acid of tryptic digest of chymotryptic fragment 1–23 (peak 5 in Figure 5) from reduced and S-carboxymethylated *schistosa* 5: (×) absorbance at 230 nm; (○) absorbance at 275 nm.

Stage 4 showed only PTH-Arg and stage 5 showed only PTH-Gly. Since the hexapeptide sequence 28–33 was already known, the results were easily interpreted to indicate the sequences

$$\overset{30}{\text{Ser-Asp-His-Arg-Gly-Thr-Arg-Ile-(36-45)}}$$

and

$$\overset{35}{\text{Arg-Ile-Glu-Arg-Gly-Cys-Gly-Cys-(42-50)}}$$

The sequence Arg-Gly occurs at positions 3 and 4 in both peptides.

Identical results were obtained with the chymotryptic digest of reduced and S-carboxymethylated *schistosa* 4 with regard to the isolation and tryptic subsplitting of fragment 1–23 (yielding fragments 1–12 and 13–23), and the isolation and sequencing of fragments 13–23, 24–37, 28–33, and 51–60). The separation of the larger fragments from the region 28–60 was

TABLE IV: Tryptic Fragments of Chymotryptic Peptide 1–23.

Amino Acid	Fragment[a] 1–12		13–23	
Tryptophan				
Lysine	0.88	1		
Histidine				
Arginine				
CM-Cysteine	1.87	2	1.71	2
Aspartic acid	1.22	1	1.24	1
Threonine	1.01	1	2.95	3
Serine	2.05	2	2.95	3
Glutamic acid	2.93	3	1.21	1
Proline	0.99	1		
Glycine	0.10	0		
Alanine			1.00	1
Valine				
Methionine	0.50	1		
Isoleucine				
Leucine				
Tyrosine			0.91	1
Phenylalanine				
Total		12		11

[a] Identification refers to position in sequence (Figure 7).

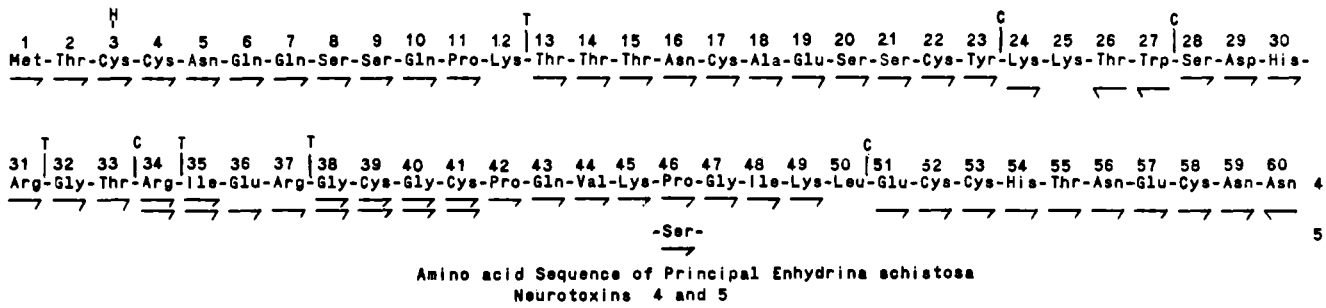

FIGURE 7: Alignment of *schistosa* toxins 4 and 5 indicating sequences established by Edman degradation (→) and carboxypeptidase digestion (←) of intact reduced and S-carboxymethylated derivatives and fragments derived therefrom by tryptic (T) and chymotryptic (C) cleavage. Quantitative tryptic cleavage of the bonds 31–32, 34–35, and 37–38 was observed with the citraconylated reduced and S-carboxymethylated derivatives. The tryptic cleavage of the bond 12–13 was done with the chymotryptic fragment 1–23. The principal sites of chymotryptic cleavage observed with 30-min digests of the reduced and S-carboxymethylated toxins were the bonds 23–24, 27–28, and 50–51 (80–90% each) and the bond 33–34 (*ca.* 50%). In the case of toxin 5 the Lys–Ser bond 45–46 was 25% split as well.

somewhat cleaner than that obtained with *schistosa* 5 owing to the fact that no cleavage occurred at the

45 46
Lys-Pro

bond, but no sequencing was done on these fragments.

Localization of the SH Group. The [^{14}C]carboxymethyl-labeled reduced and S-carboxymethylated derivative of *schistosa* 4 had a specific radioactivity of 0.1 Ci/mol, and in the amino acid run monitored with the flow scintillation counter radioactivity was observed only in the position of the CM-cysteine peak. In the Edman degradation done with 0.1 μmol of this material, PTH-Met and PTH-Thr were obtained in stages 1 and 2. PTH-CM-Cys was obtained in stages 3 and 4. The CM-Cys liberated in stages 3 and 4 accounted for one-third and one-sixth of the total radioactivity, respectively, the residual protein accounting for the remainder. This result was interpreted to indicate that the cysteine residue labeled with [^{14}C]iodoacetate in 8 M urea was located at position 3 in the molecule, the radioactivity observed in stage 4 being attributed to "carry-over." The high radioactivity of the residual protein after four cycles of degradation was not taken to indicate the presence of labeled CM-cysteine elsewhere in the molecule, but was instead attributed to incomplete removal of residue 3, since the yield of end group was scarcely half that expected from the yield of PTH-Thr in stage 2. A similar result was obtained with the *schistosa* 5 toxin.

The main product obtained upon reaction of the *schistosa* 4 toxin with 4-vinylpyridine in 8 M urea eluted from Sephadex G-50 exactly in the position of one of the altered forms, presumably dimer, observed in dried preparations of the native toxins (Karlsson *et al.*, 1972a). The material produced no symptoms in mice at a dose level of 28 μg. The preparation showed one residue of *S*-β-(4-pyridylethyl)cysteine per 60 amino acid residues upon total amino acid analysis, and the presence of one pyridylethyl group per toxin molecule was also indicated by spectral data. The monopyridylethylated toxin (0.37 μmol) was subjected to five cycles of sequential degradation without prior modification of the remaining eight half-cystine residues (*i.e.*, the four disulfide bridges were left intact. Individual PTH-amino acids were obtained in cycles 1–3 and 5, indicating the sequence Met-Thr-PECys···Asn-, where PECys represents the pyridylethylcysteine residue. The end group fraction obtained in stage 4 was completely blank, indicating that the half-cystine residue remained attached to its partner further along in the molecule. The overall yield of asparagine in stage 5 was 27%, as compared to a yield of 89% for methionine in the first cycle, based on the amount of protein taken for degradation.

Primary Structure of schistosa Toxins 4 and 5. The two toxins differ in sequence only by a Ser–Pro replacement at position 46, as indicated in Figure 7.

Discussion

The *schistosa* toxins 4 and 5 are obviously homologous to all of the curariform elapid and hydrophid neurotoxins for which sequence data are available, but show two novel features. The first is the presence of a free SH group, which we have located at position 3 in the molecule. The second novel feature is the length of the peptide sequence between half-cystine residues 17 and 22. All of the curariform toxins described so far contain either five (*N. nigricollis* α, Eaker and Porath, 1967; *N. haje* α, Botes and Strydom, 1969; *N. nivea* δ, Botes *et al.*, 1971; *H. hemachates* II and IV, Strydom and Botes, 1971; *N. nivea* α and β, Botes, 1971; *siamensis* 3, Karlsson *et al.*, 1972b; *N. naja* A, Nakai *et al.*, 1971) or six (cobrotoxin, Yang *et al.*, 1969; erabutoxins a and b, Sato and Tamiya, 1971; α-bungarotoxin, Mebs *et al.*, 1972) amino acids in the corresponding region. The *schistosa* toxins 4 and 5 show only four residues between the two half-cystines, which accounts for the shortening of the total peptide chain to 60 amino acids. The *schistosa* toxins 4 and 5 and cobrotoxin are so far the only toxins in which the first of the above-mentioned half-cystines (residue 17 in *schistosa* 4 and 5) is not followed by proline.

The SH group in the *schistosa* toxins reacts with the sulfhydryl reagents tested (iodoacetate, iodoacetamide, and 4-vinylpyridine) only under strongly denaturing conditions (*e.g.*, 8 M urea) and the cysteine residue in question appears to be immediately adjacent to a disulfide bridge, both of which situations invoke the specter of disulfide interchange. If, as suggested by the results obtained with cobrotoxin (Yang *et al.*, 1970), the erabutoxins (Endo *et al.*, 1971), and *N. nivea* α (Botes, 1971), the curariform toxins are homologous with regard to disulfide pairing as well as amino acid sequence, then the SH group in the *schistosa* toxins could only be located at position 3 or 4. The results obtained upon degradation of the [^{14}C]iodoacetate labeled toxins following conversion to the reduced and S-carboxymethylated derivatives indicated that a *minimum* of one-third of the labeled CM-cysteine was located at position 3, but permitted no clear decision regarding the amount of label incorporated at position 4 or at any other of

the remaining seven sites at which [^{14}C]CM-cysteine might be formed because of the low yield obtained in the degradation. We generally observe an approximately 50% drop in yield upon degrading through Cys-Cys sequences wherein Cys is present as CM-cysteine or cysteic acid.

However, in the case of the pyridylethylated derivative, the pyridylethylcysteine appeared to be located exclusively at position 3, but this need not apply for the *native* toxins, since we cannot exclude the possibility that disulfide interchange precedes the derivatization. The assignment o the cysteine residue to position 3 in the *native* toxin must therefore be regarded as tentative.

Methodological Aspects of the Sequence Determination. The remarkable cleanness of the results obtained with the tryptic digests of the citraconylated reduced and S-carboxymentylated derivatives deserves comment. The yields of the fragments 32–34, 35–37, and 38–60 were quantitative. The yield of the fragment 1–31 was about 85%, apparently owing in part to about 10% "parasitic" cleavage of the

23 24
Tyr-Lys

linkage, as evidenced by the presence in the digest (zone B in Figure 1) of a rather basic peptide containing tryptophan which we tentatively identify as residues 24–31. The long shoulder of uv absorbing material on the anodic side of the peak corresponding to the intact deblocked peptide 1–31 in Figure 2 probably contains the fragment 1–23, incompletely deblocked forms of the intact peptide 1–31, and perhaps some small amount of the fragment 1–27 that would result from "parasitic" cleavage of the

27 28
Trp-Ser

linkage. The protection of the lysine residues appears to have been complete, and the deblocking nearly so. Presumably the use of purified β-trypsin would eliminate the problem of parasitic cleavage at Tyr and Trp (Keil-Dlouha *et al.*, 1971a, 1971b).

The limited chymotryptic digestion was performed mainly to obtain a fragment that would establish the alignment of the two central tripeptides isolated from the citraconylated derivatives. The required hexapeptide corresponding to residues 28–33 was isolated in 35% yield. In the case of *schistosa* 5, an atypical chymotryptic split occurred at

45 46
Lys-Ser

whereas the

45 46
Lys-Pro

linkage in *schistosa* 4 was completely resistant. Chymotryptic cleavage at the corresponding lysine residue was observed with the toxins *N. haje* α (Botes and Strydom, 1969) and *N. nivea* δ (Botes *et al.*, 1971) wherein the linkage is Lys-Lys. Apparently some structural feature of these toxins favors a tryptic-like chymotryptic split at this site when the following residue is not proline.

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