The complete sequence is shown in Figure 8. The alignment has already been shown in the Results section. The sequence Cys-Asn for residues 59–60 is assumed from homology with the lytic factor (Fryklund and Eaker, 1973). For some reason carboxypeptidase A will not digest this molecule; it could be the cysteic acid residue at 59, or perhaps the presence of arginine as opposed to lysine in the lytic factor at residue 58. Neurotoxins with two residues of asparagine at the carboxyl terminal digest readily. The sequence shows the features also present in the *H. haemachates* lytic factor and the *Naja naja atra* cardiotoxin (Narita and Lee, 1971), namely the hydrophobic amino terminal and the cluster of methionines in the center of the molecule. The cysteine residues are also found in homologous positions.

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The Complete Covalent Structure of a Cardiotoxin from the Venom of *Naja nigricollis* (African Black-Necked Spitting Cobra)†

Linda Fryklund* and David Eaker

ABSTRACT: The complete covalent structure of a small, basic protein with cardiotoxic activity is described. This has been isolated from the venom of *Naja nigricollis* by gel filtration on Sephadex G-75 and gradient ion exchange chromatography on Bio-Rex 70. The cardiotoxin, molecular weight 6806 from amino acid composition, consists of 60 amino acids, cross-linked by four disulfide bridges, connecting 3–21, 14–38, 42–53, and 54–59. The protein contains one residue of tryptophan, phenylalanine, and glutamic acid, two residues of arginine and tyrosine, four residues of methionine, and nine residues of lysine. Histidine is absent. The chymotryptic peptides of the oxidized and S-carboxymethylated protein were isolated by gel filtration on Sephadex G-25 and zone electrophoresis on a cellulose column. The sequence was determined by Edman degradation, using the (manual) direct phenylthiohydantoin method and with the use of carboxypeptidase A. Disulfide pairing was determined on thermolysin cleaved peptides from the native protein. The sequence is shown to be homologous to other cardiotoxins and a lytic factor from snake venoms and also shows homology, both in sequence and disulfide pairing to neurotoxins. A partial reduction experiment in the absence of denaturing agent using ^{14}C-labeled iodoacetic acid as S-carboxymethylating agent shows that disulfide bonds 14–38 and 42–53 were reduced fastest followed marginally by 54–59, and then bond 3–21.

This cardiotoxin has the same pharmacological properties as that described in the accompanying paper (Fryklund and Eaker, 1975). The sequence of this cardiotoxin completes the series on small basic proteins and their homology relationships with neurotoxins in Elapid and Hydrophid venoms. The disulfide pairing was also established to see whether homology was apparent even at this structural level (see also Fryklund et al. (1972), Fryklund and Eaker (1973), and accompanying paper). This cardiotoxin has also been characterized immunologically by Boquet et al. (1972) and is denoted Toxin γ by them.

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Experimental Procedure

Isolation of the Cardiotoxin. The *Naja nigricollis* venom

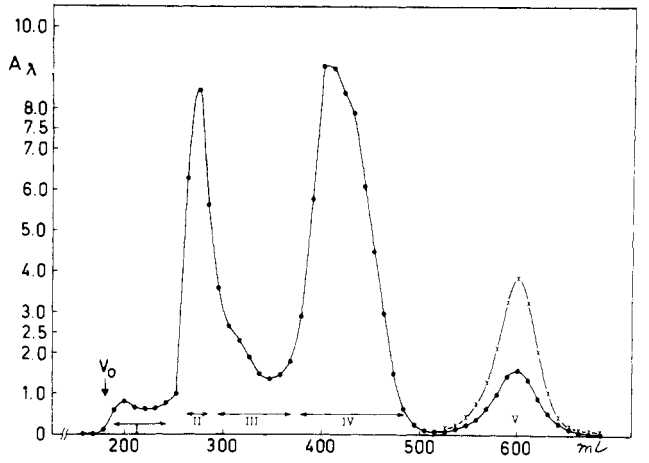


FIGURE 1: Gel filtration of 1.0 g of crude *N. nigricollis* venom on Sephadex G-75, 3.2 × 69 cm, in 0.2 *M* ammonium acetate. Flow rate, 20 ml/hr. (●) $A_{279\ nm}$; (×) $A_{260\ nm}$.

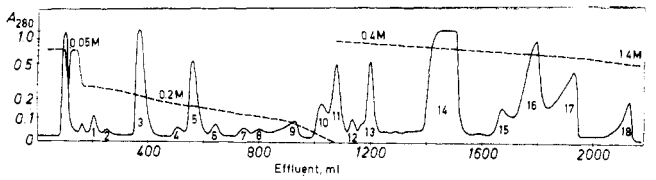


FIGURE 2: Ion-exchange chromatography on Bio-Rex 70, 3.2 × 25 cm, equilibrated in 0.2 *M* ammonium acetate (pH 7.3) of fraction IV, Figure 1; flow rate, 80 ml/hr. The column was eluted with 60 ml of 0.05 *M* ammonium acetate before applying the 10-ml sample. This was followed by 40 ml of 0.05 *M* buffer before applying the concave gradient 0.14–1.4 *M* ammonium acetate with a total volume of 2 l. The figures on the conductivity trace (--) indicate the approximate molarity.

was a gift from Dr. Paul Boquet of the Pasteur Institute, Garches, France, and was collected in Ethiopia, as a single 50-g batch, in 1961; 1.0 g of crude venom (desiccated) was used. For details of the venom separation see the accompanying paper.

Rechromatography of the Cardiotoxin. The homogeneity of the cardiotoxin was demonstrated by analytical elution chromatography on a 1 × 30 cm column of Bio-Rex 70 equilibrated with 0.60 *M* ammonium acetate at pH 7.3, and preparative rechromatography was also done on a dipolar adsorbant, β-alanyl-Sephadex, of the type described by Porath and Fryklund (1970). Only one symmetrical peak was observed in each case.

For determination of the molar extinction coefficient, toxicity assays, oxidation and enzymic digestion of the oxidized derivative, Edman degradation, and amino acid analysis of the peptides see the accompanying paper (Fryklund and Eaker, 1975).

Reduction and S-carboxymethylation of the protein was performed according to the procedure described by Crestfield et al. (1963). The protein was recovered by gel filtration on Sephadex G-75 in 10% acetic acid.

Thermolytic Digest of the Native Cardiotoxin. Native cardiotoxin (2.0 μmol) was dissolved in 2.0 ml of 0.1 *M N*-ethylmorpholine acetate buffer (pH 7.0); 2.3 mg of thermolysin (a gift from V. Mutt and obtained from Chugai Boyeki Co. Ltd., Importers and Exporters, Sanwa Building, Kawaramachi Higashiku, Osaka, Japan) was added in 100 μl of the same medium. Digestion was allowed to proceed for 16 hr at 40°. The solution was then acidified, centrifuged, and gel filtered on a Sephadex G-50 column, 1 × 92 cm, 0.02 *N* HCl.

Table I: Amino Acid Composition of the Cardiotoxin.[a]

Amino Acid	Native[b]		Reduced and S-Carboxymethylated		Oxidized	
Tryptophan[c]	1.00	1	1.00	1		(1)
Lysine	9.00	9	8.80	9	9.07	9
Arginine	1.93	2	2.18	2	1.91	2
Cysteic acid					7.50	8
Cm-cysteine			8.20	8		
Aspartic acid	6.14	6	5.99	6	6.10	6
Methionine sulfone					3.85	4
Threonine	3.04	3	2.99	3	3.02	3
Serine	2.00	2	1.98	2	1.95	2
Glutamic acid	1.17	1	1.12	1	1.22	1
Proline	6.77	6	6.00	6	5.80	6
Glycine	1.99	2	1.95	2	2.01	2
Alanine	2.01	2	1.95	2	2.01	2
Half-cystine[d]		8				
Valine	2.82	3	2.80	3	3.10	3
Methionine	3.74	4	2.65[e]	4		
Isoleucine	2.80	3	2.70	3	2.80	3
Leucine	4.94	5	4.78	5	4.95	5
Tyrosine	1.96	2	1.93	2	2.00	2
Phenylalanine	0.96	1	0.93	1	1.00	1
Total		60		60		60

[a] Mol wt 6806; $A_{279nm}{}^{1cm}(0.1\%) = 1.33$; $\epsilon_{279nm} = 9.02 \times 10^3$. [b] Average of 24- and 72-hr hydrolysis. [c] Spectrophotometric determination. [d] As cysteic acid [e] Low value with the reduced and S-carboxymethylated protein.

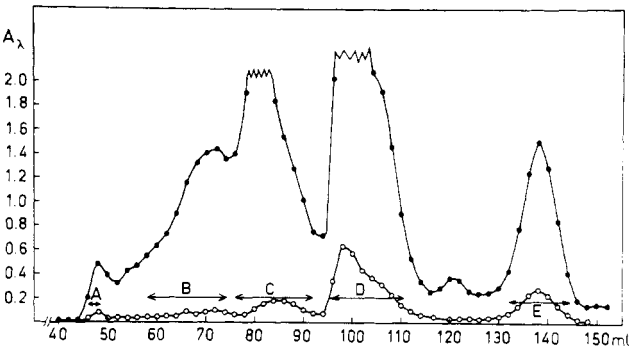


FIGURE 3: Gel filtration on Sephadex G-25, 1 × 145 cm, in 0.02 *N* HCl, of the chymotryptic digest of the reduced and S-carboxymethylated protein. (●) $A_{225\ nm}$; (○) $A_{280\ nm}$.

Analysis and Separation of the Thermolytic Peptides. High voltage paper electrophoresis of the thermolytic peptides was done in a Gilson electrophorator, Model D, using Whatman No. 1 or Whatman 3MM paper for analytical or preparative scale, respectively, in pyridine acetate buffer (pH 3.50) (0.043 *M* pyridine–0.58 *M* acetic acid) at 3000 V for 40 min. Disulfide peptides were located by the diagonal method of Hartley (1970). The oxidation was performed by hanging the paper strips in a closed glass jar containing preformed performic acid vapor for a minimum of 2 hr. Simple mixtures were purified on the zone electrophoresis column, while complex mixtures were separated on paper. The paper was then lightly sprayed with ninhydrin and the strips were eluted by descending chromatography in 10% acetic acid.

Partial Reduction and Subsequent Alkylation. Native cardiotoxin (1.0 μmol) was dissolved in 500 μl of Tris-HCl buffer, 1.4 *M*, pH 8.5, which had previously been saturated with nitrogen; 2.0 μmol of dithiothreitol in 100 μl of the same buffer was added at 0 time, at room temperature,

Table II: Chymotryptic Peptides from the Reduced and S-Carboxymethylated Protein.

	Fragment[a]									
	RCm-[b] Chy E				RCm-[c] Chy C-1		RCm-[c] Chy C-4		RCm-[c] Chy C-6	
Amino Acid	5–11		21–22		32–36		45–48		37–44	
Tryptophan[d]	1.00	1								
Lysine					1.06	1			1.00	1
Arginine					1.00	1				
Cm-cysteine			0.95	1					2.00	2
Aspartic acid									1.00	1
Serine							2.01	2		
Glutamic acid	1.04	1								
Proline	1.96	2			1.00	1			1.03	1
Glycine									1.00	1
Valine					1.94	2			0.99	1
Isoleucine	1.03	1							0.96	1
Leucine	1.03	1					2.00	2		
Tyrosine			1.00	1						
Phenylalanine	0.98	1								
Total		7		2		5		4		8

[a] See text and Figure 9 for identification. [b] See Figure 3. [c] See Figures 3 and 4. [d] Spectrophotometric determination.

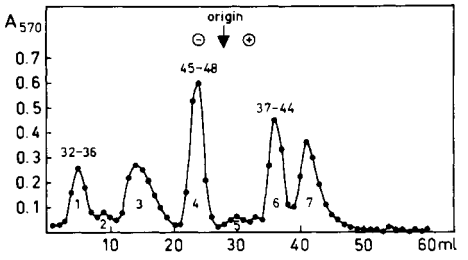


FIGURE 4: Zone electrophoresis on a cellulose column, 1 × 86 cm, of fraction RCm-Chy C, Figure 3, in 0.05 *M* pyridine acetate buffer (pH 5.0) for 10 hr at 1000 V and 8 mA. The arrow indicates the position of the starting zone. Fractions analyzed by ninhydrin after alkaline hydrolysis.

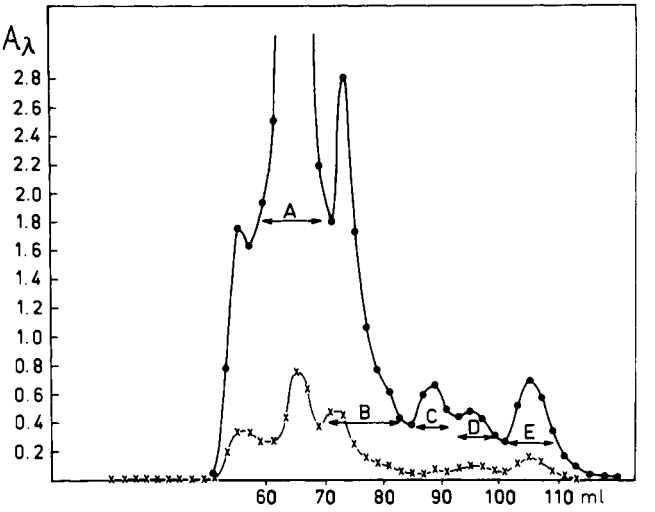


FIGURE 5: Gel filtration on Sephadex G-25 using the same column and conditions as for Figure 3, of the chymotryptic digest of the oxidized protein. (●) $A_{230\ nm}$; (×) $A_{275\ nm}$.

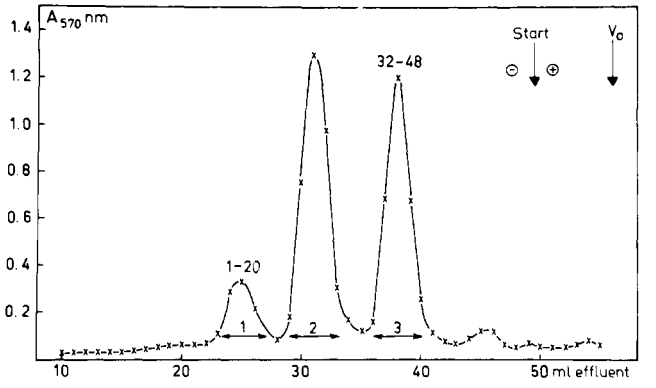


FIGURE 6: Zone electrophoresis of fraction Chy A, Figure 5, on the same column and in the same medium as described for Figure 4, for 20 hr at 1000 V and 8 mA. The arrow indicates the position of the starting zone; V_0 is the void volume of the column. Fractions were analyzed by ninhydrin after alkaline hydrolysis.

under nitrogen. After 5 min a 4:1 mixture of [^{12}C]- and [^{14}C]iodoacetic acid (total 5 μmol) was added, in 1 *N* NaOH. The iodoacetic-*1*-^{14}*C* acid was obtained from New England Nuclear Corp., and had a specific activity of 13.4 Ci/mol. After a further 5-min reaction time the solution was gel filtered on a Sephadex G-75 column in 0.2 *M* ammonium acetate. The fractions were pooled and then totally reduced and alkylated as already described. Four times the amount of cold (unlabeled) reduced and S-carboxymethylated protein was added as carrier before trypsin digestion.

Trypsin digestion, separation, and analysis of the ^{14}*C-labeled peptides* was performed as described by Fryklund et al. (1972) using the same Nuclear Chicago Flow scintillation system to monitor radioactivity.

Pepsin Digestion of the ^{14}*C-Labeled Tryptic Peptide.* The peptide was dissolved in 2.0 ml of 0.01 *N* HCl; pepsin (Worthington) was added to give molar ratio 1:100 of enzyme/substrate. After incubation at room temperature overnight, the digest was separated by low voltage paper electrophoresis, 400 V for 2 hr in 0.05 *M* pyridine acetate buffer (pH 5.0). Radioactive peptides, located by a guide strip, were eluted and analyzed after hydrolysis.

Results

Isolation of the Protein. The gel filtration pattern obtained with the crude venom is shown in Figure 1. Five peaks were obtained; peak IV contained the neurotoxic and cardiotoxic activity.

This fraction was then subjected to ion-exchange chromatography on Bio-Rex 70, giving the pattern shown in Figure 2. Peak 3 is toxin α (Eaker and Porath 1967); peak five has nearly the same composition as α, but contains an additional residue of isoleucine and methionine, and is short one residue each of leucine and glutamic acid. Fifteen cycles of Edman degradation on the reduced and S-carboxymethylated derivative of this molecule indicated the sequence Met-Ile-Cys-His-Asn-Gln-Gln-Ser-Ser-Gln-Pro-Pro-Thr-Thr-Lys, which differs from the amino terminal sequence of toxin α only by residues 1 and 2 which are Leu and Glu in the latter, thereby accounting for the only differences in amino acid composition observed between the two toxins. Peak 14 is the cardiotoxin described below. The amino acid composition is given in Table I. The molar extinction coefficient was 9020 at 279 nm, which is consistent with a content of one tryptophan, two tyrosines, and four disulfide bridges (molar absorptivities 5579, 1200, and 150 for tryptophan, tyrosine, and cystine, respectively. The LD_{100} for a 20-g mouse is 15 μg, intravenous injection, and 40–50 μg by intraperitoneal injection (P. Boquet, personal communication). Death was a result of ventricular fibrilla-

Table III: Amino Acid Composition of the Chymotryptic Peptides and One Tryptic Peptide Obtained from the Oxidized Protein.

	Fragment[a]																	
											Chy C-1[e]							
Amino Acid	Chy A-1[c] 1–20		Chy D[b] 21–26		Chy A-2[c] 27–48		Chy A-3[c] 32–48		Chy B-5[d] 49–60		23–26 a		27–31 b		Trp A 28–35	
Lysine	4.05	4	1.00	1	2.43	2	1.97	2	2.00	2	1.03	1			1.00	1
Arginine					1.94	2	1.01	1					0.96	1		
Cysteic acid	2.10	2	1.08	1	1.71	2	2.09	2	2.91	3						
Aspartic acid	2.2	2			1.22	1	1.31	1	3.16	3						
Methionine sulfone			2.17	2	0.92	1			1.08	1	2.00	2	1.01	1	1.01	1
Threonine	1.1	1	0.99	1					1.11	1	0.97	1				
Serine					1.91	2	2.00	2								
Glutamic acid	0.95	1														
Proline	3.05	3			3.06	3	2.08	2					0.92	1	2.01	2
Glycine	1.20	2			1.29	1	0.95	1								
Alanine					1.96	2							2.03	2	1.94	2
Valine					2.97	3	3.09	3							2.06	2
Isoleucine	1.00	1			1.02	1	0.92	1	0.89	1						
Leucine	2.95	3			1.95	2	1.82	2								
Tyrosine			1.00	1					1.00	1						
Phenylalanine	1.00	1														
Total		20		6		22		17		12		4		5		8

[a] See Figure 9 and text for identification. [b] See Figure 5. [c] See Figures 5 and 6. [d] See Figures 5 and 7. [e] See Figures 5 and 8.

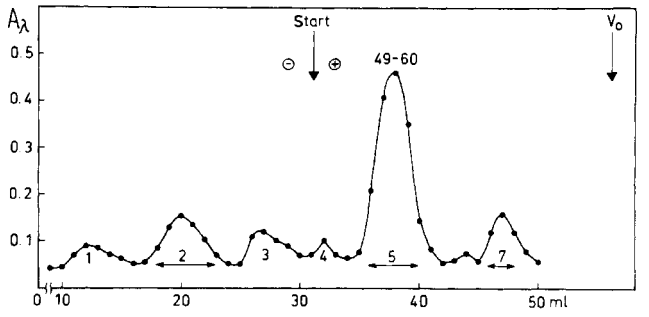


FIGURE 7: Zone electrophoresis of fraction Chy B, Figure 5, using the same column and conditions as for Figure 4, for 10 hr at 1000 V and 8 mA. Arrows and method of analysis of fractions as for Figure 6.

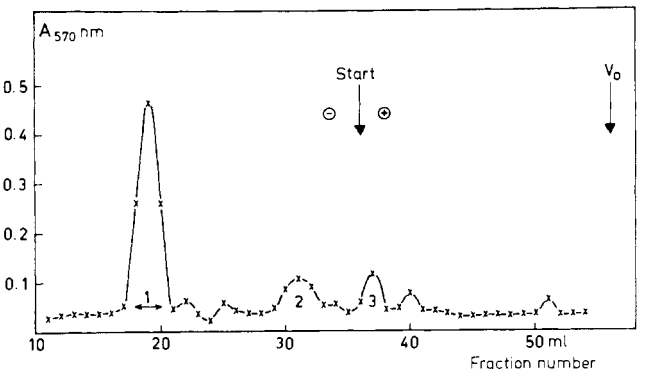


FIGURE 8: Zone electrophoresis of fraction Chy C, Figure 5, using the same conditions as for Figure 7.

tion. This cardiotoxin is identical with toxin γ described by Izard et al. (1969a) and does not have direct lytic activity (Izard et al. 1969b).

Determination of the Sequence. Edman degradation on the reduced and S-carboxymethylated protein gave unambiguous data for the first 20 stages, as follows:

1 5 10
Leu-Lys-Cys-Asn-Gln-Leu-Ile-Pro-Pro-Phe-

15 20
Trp-Lys-Thr-Cys-Pro-Lys-Gly-Lys-Asn-Leu

PTH-tryptophan[1] was obtained in good yield in stage 11.

Carboxypeptidase A digestion of the reduced and S-carboxymethylated derivative yielded 1 mol of asparagine/mol of protein. Residue 60 is therefore Asn.

Results of the Chymotryptic Digest with the Reduced and S-Carboxymethylated Protein. Gel filtration of the chymotryptic digest on Sephadex G-25 gave the pattern shown in Figure 3. Fraction RCM-Chy E was a mixture of two peptides, one was identified as residues 5–11, from the degradation through residue 20, the other had the composition (Cys, Tyr); the sequence Cys-Tyr is inferred from chymotrypsin specificity. The amino acid compositions of the chymotryptic peptides are shown in Table II.

Zone electrophoresis of fraction RCM-Chy C, Figure 3, gave five main peaks as shown in Figure 4. The most cathodic, RCm-Chy C-1 (peak 1) was a pentapeptide. Amino acid composition and four stages of degradation gave the sequence Val-Pro-Val-Lys-Arg-OH. RCm-Chy C-4 (peak 4, Figure 4) was a mixture of two peptides, containing mostly (Ser_2, Leu_2). Two stages of degradation on this peptide gave the sequence Ser-Ser-Leu-Leu-OH. Seven stages of degradation on the anodic octapeptide RCm-Chy C-6 gave the sequence as follows: Gly-Cys-Ile-Asp-Val-Cys-Pro-Lys-OH.

Results of the Chymotryptic Digest with the Oxidized Protein. The gel-filtration pattern obtained on Sephadex G-25 is shown in Figure 5. Fraction Chy A was further fractionated by zone electrophoresis, as shown in Figure 6. Chy A-1 (peak 1, Figure 6) corresponds to the amino terminal sequence, residues 1–20; Chy A-2 (peak 2, Figure 6) was a mixture of two peptides, which were separated by

[1] Abbreviations used are: Cm-cysteine, *S*-carboxymethylcysteine; PTH-amino acid, phenylthiohydantoyl amino acid; PTC, phenylthiocarbamyl.

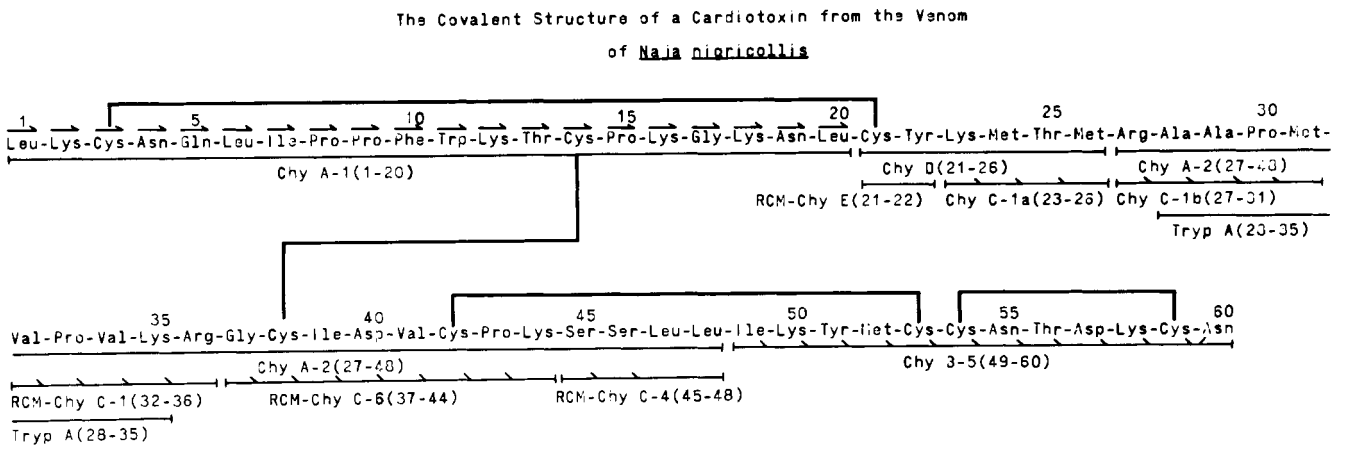


FIGURE 9: The amino acid sequence and the disulfide pairing of the cardiotoxin. Edman degradation indicated by →, carboxypeptidase A, by ⇀. Stages 1–20 on the whole reduced and S-carboxymethylated protein; 23–26 on chymotryptic peptide 23–26 Chy C-1a; 27–30 on chymotryptic peptide 27–31 Chy C-1b; 32–35 on chymotryptic peptide 32–36 RCm-Chy C-1; 37–43 on chymotryptic peptide 37–44 RCm-Chy C-6; 45–46 on chymotryptic peptide 45–48 RCm-Chy C-4; 49–60 on chymotryptic peptide 49–60 Chy B-5.

zone electrophoresis at pH 3.2 in 0.03 *M* pyridine formate. One of them (Chy A-2) had the amino acid composition given in the third column of Table III. The other peptide also contained 22 amino acids and corresponded in amino acid composition to residues 1–20 plus a residue of tyrosine and a third residue of cysteic acid, thereby placing Cys-Tyr (RCm-Chy E) at residues 21–22. Chy A-3 (peak 3, Figure 6) corresponds in composition to Chy A-2 less Chy C-1b (Table III).

Chy B (Figure 5) was further separated by zone electrophoresis, as shown in Figure 7. The major peak 5, Chy B-5, was a dodecapeptide. Eleven stages of degradation and analysis of the residual peptide (free asparagine) gave the sequence as follows: Ile-Lys-Tyr-Met-Cys-Cys-Asn-Thr-Asp-Lys-Cys-Asn-OH.

Chy D (Figure 5) was a hexapeptide with the composition Lys, Cys, Tyr, Met_2, Thr.

Chy C (Figure 5) was further separated by zone electrophoresis, as shown in Figure 8. Chy C-1 (peak 1, Figure 8) was a mixture of two peptides in a 2:1 ratio, Lys, Met_2, Thr and Arg, Ala_2, Pro, Met. Four stages of degradation indicated the sequences Lys-Met-Thr-Met-OH, Chy C-1a, and Arg-Ala-Ala-Pro-Met-OH, Chy C-1b, indicating chymotryptic cleavage at methionine sulfone.

Alignment of the Peptides. The hexapeptide Chy D (Table III) with the amino acid composition Cys, Tyr, Lys, Met, Thr, Met, Arg overlaps Chy C-1a with the N-terminal fragment 1–22 mentioned above, establishing the consecutive sequence 1–26. Since carboxypeptidase A liberates one residue of asparagine from the intact reduced and S-carboxymethylated derivative of the toxin, the peptide Chy B-5 is unambiguously assignable to the carboxyl terminus of the molecule thereby representing residues 49–60. The alignment of the peptides RCm-Chy C-1, RCm-Chy C-6, and RCm-Chy C-4 within Chy A-3, and the assignment of the latter to positions 32–48 is done on the basis of homology with the *Haemachatus haemachates* lytic factor and the *Naja naja* cardiotoxin F8. Since the amino acid composition of Chy A-2 is the sum of Chy A-3 and Chy C-1b, the latter is then assigned to positions 27–31, establishing the consecutive alignment 27–60. The overlap of Chy C-1b with RCm-Chy C-1 is confirmed by the isolation of the tryptic peptide Trp A, corresponding to residues 28–35. The connection between 1–26 and 27–60 is then inferred from the

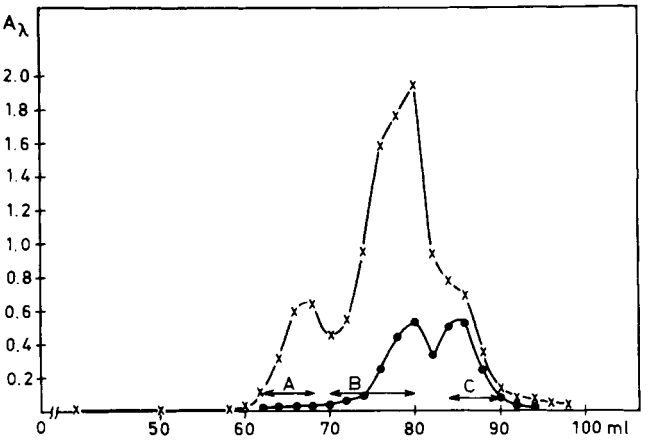


FIGURE 10: Gel filtration of the thermolysin digest of the native cardiotoxin on Sephadex G-50, 1 × 92 cm, in 0.02 *N* HCl. (×) $A_{230\ nm}$; (●) $A_{279\ nm}$.

total composition. The alignment of the molecule thus obtained is shown in Figure 9.

Pairing of the Disulfides. The gel-filtration pattern obtained with the thermolysin digest on Sephadex G-50 is shown in Figure 10. Three peaks were observed, the first without absorption at 275 nm. Analytical electrophoresis using the diagonal technique showed that fraction Thermo A (Figure 10) was one disulfide peptide, with a little impurity, readily purified by column electrophoresis at pH 5.0 which gave one major fraction (Thermo A1). Amino acid analysis of the peptide with and without oxidation showed that two disulfide bridges must be present in the peptide, which had the composition (Table IV) Lys_2, Cys_4, Asx_3, Met, Thr, Pro, Val. On the basis of the amino acid composition shown in Table IV, the purified peptide was identified as residues 41–44 + 52–60. Rather than resort to complicated enzyme mixtures to split between residues 53 and 54, the pairing was worked out by Edman degradation, since we already know that peptides containing disulfide bonds can be degraded past the half-cystine residues (Fryklund et al., 1973). The end groups obtained at stage 1 were Val, Met, and Asn (this meant that a thermolytic split had also occurred between residues 54 and 55). At stage 2 the spectrum indicated a yield of at least two end groups, one of which was threonine (since the absorption maximum at 320

Table IV: Amino Acid Composition of Thermolytic Peptides from Native Cardiotoxin.[a] Peptides Separated by Zone Electrophoresis.

Amino Acid	ox Thermo A 1[b] 41–44 + 52–60		Thermo A 1[c] Residual 54 + 57–60		ox Thermo A 1[b] Residual 54 + 57–60		H_2O Phase from 2nd Stage 43–44	
Lysine	1.80	2	1.00	1	1.16	1	1.00	1
Cysteic acid	3.80	4			1.65	2		
Aspartic acid	3.00	3	2.40	2	2.00	2		
Methionine sulfone	1.05	1						
Threonine	1.08	1						
Proline	1.03	1					1.23	1
Half-cystine			1.0	2				
Valine	1.00	1						
Total		13		5		5		2

[a] See Figure 9 and 10. [b] Analytical oxidation. [c] Without phenol.

Table V: Amino Acid Composition of Thermolytic Peptides from Native Cardiotoxin. Peptides Separated by High Voltage Electrophoresis Using the Diagonal Method.

Amino Acid	B 1[a] a 20–21		b 1–3	
Lysine			0.80	1
Cysteic acid	1.00	1	1.00	1
Aspartic acid				
Threonine				
Proline				
Glycine				
Leucine	0.68	1	1.00	1
Total		2		3

[a] Figures 9 and 10.

Table VI: Specific Activities (dpm/μmol of Cm-cysteine) for the ^{14}C-Labeled Tryptic Peptides.

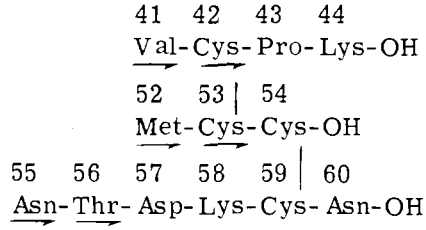
Peptide	Cysteine No.	Specific Activity[a]
3–12	3	51,000
13–16	14	62,000
19–23	21	50,000
37–39[b]	38	60,000
40–44[b]	42	60,000
51–60	53, 54, 59	57,000

[a] Average value of two determinations. [b] Obtained by peptic cleavage.

nm was present, which corresponds to dehydro-PTH-threonine) but only threonine could be identified on thin-layer chromatography. We therefore supposed that this other derivative must be bis-PTH-cystine. This was synthetized by the usual method (Edman, 1970) but did not give any distinct spot on thin layer, though the spectrum was normal.

If we had in fact split off cystine, then analysis of the residual peptide should also show the presence of cystine. This was the case as shown in Table IV. The water phase from the end-group fraction obtained in stage 2 also contained the dipeptide Pro-(PTC)Lys. The dipeptide Pro-(PTC)Lys is soluble in the 1-chlorobutane which was used for extracting the thiazolinone end group following the cleavage reaction. After conversion of the thiazolinones to thiohydantoins and extraction with ethyl acetate, the Pro-(PTC)Lys is left in the aqueous phase and the end groups in the ethyl acetate phase.

Since cystine was present in the residual peptide after two stages of degradation the disulfide pairing must be as shown, since any other derivative of cystine will not yield cystine on acid hydrolysis. The presence of Pro-(PTC)Lys means that Cys-42 has been removed. Residues 41, 42, 52, 53, 55, and 56 have therefore been removed by degradation. Residues 43 and 44 are lost as a dipeptide.



Fraction Thermo B (Figure 10) on diagonal paper electrophoresis was seen to be a very complicated mixture, containing at least two disulfide peptides and was fractionated preparatively this way. The eluted pairs of peptides were then analyzed with the results shown in Table V. Fraction Thermo B-1 gave two peptides Leu, Lys, Cys and Leu, Cys. These are unambiguously identified as residues 1–3 and 20–21, respectively, so that Cys-3 is paired with Cys-21. Since the pair 3–21 has been placed the final disulfide pair 14–38 is placed by elimination. The complete disulfide pairing must therefore be 3–21, 14–38, 42–53, 54–59. Figure 9 shows the covalent structure. The results of the partial reduction are shown in Table VI. Cys-3 and Cys-21 have the lowest specific activity and Cys-14, -38, and -42 have the highest (this must also include residue 53), leaving Cys-54 and -59 with an intermediate value. The pair 3–21 is considered proven since a 16% difference in specific activity of the Cm-cysteine is far greater than the experimental error in determining the [^{14}C]Cm-cysteine content with amino acid analysis. The maximum difference in dpm between duplicate different determinations was less than 1000 dpm.

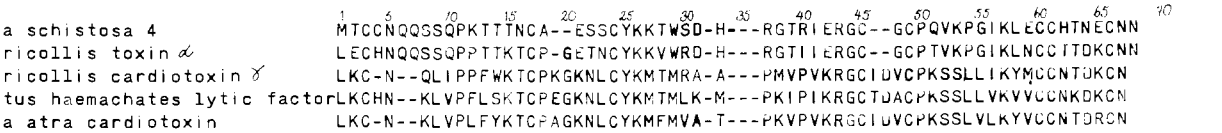


FIGURE 11: Sequence comparison between cardiotoxins, neurotoxins, and a lytic factor. 1-letter code as in Dayhoff (1969). Sequence references in order, Fryklund et al. (1972), Eaker and Porath (1967), this paper, Fryklund and Eaker (1973), Narita and Lee (1970), Fryklund and Eaker (1975), H. Arnberg, D. Eaker, and E. Karlsson (unpublished). *Enhydrina schistosa* 4, *Naja nigricollis* toxin α, and *Naja naja siamensis* 3 are neurotoxins.

Discussion

The cardiotoxin described here is identical with the toxin γ characterized by Boquet et al. (1972). Broadley (1968) has renamed the *Naja nigricollis* in Ethiopia *Naja mossambica pallida*. Our own findings support this conclusion, since several different commercial samples of *Naja nigricollis* venom (Miami Serpentarium, Miami, Fla.) did not contain any cardiotoxin identical with toxin γ. Boquet et al. have also shown that toxin γ is not responsible for the direct lytic activity of *Naja nigricollis* venom (Izard et al., 1969b). Identity between cardiotoxins and direct lytic factors has been proposed, but our own observations indicate that the direct lytic factor from *H. haemachates* (Fryklund and Eaker, 1973) is much less toxic than the cardiotoxins from *N. naja* (Fryklund and Eaker, 1975) and *N. nigricollis*.

Partial reduction and alkylation in the absence of denaturing agents was aimed at locating the four disulfide pairs by means of comparing the specific activities of the labeled half-cystines. The labeled peptides could also be obtained in good yield by trypsin digestion. Since the approach requires that the alkylation reflects the degree of reduction, we employed a very short reduction time and allowed the alkylation to proceed for a few hours by gel filtration of the reaction mixture at neutral pH. However, even 5 min of exposure to 0.5 equiv of reducing agent resulted in labeling of all the half-cystine residues, and only bridge 3–21 could be deduced with certainty. This result suggests that the reduction is highly cooperative.

The complete disulfide pairing was elucidated by conventional methods using thermolysin. It is seen to be identical with that proposed by Takechi and Hayashi (1972) for *N. naja* cytoxin II. It is also homologous to the neurotoxins as shown for cobratoxin (Yang et al., 1970), *Naja nivea* α (Botes, 1971), and erabutoxin (Endo et al., 1971).

This would suggest that the folding of the peptide chains of neurotoxins, cardiotoxins, and lytic factors is at least grossly similar despite differences in pharmacological activity and amino acid sequence. Figure 11 shows an alignment of three typical cardiotoxins, three neurotoxins, and a lytic factor.

The homology between these three types of snake venom components has been discussed at length by Strydom (1973) and used to deduce structure–function relationships (Rydén et al., 1973; Karlsson, 1973). It is sufficient here to indicate the typical cardiotoxin appearance with a high content of hydrophobic and basic amino acids. The amino terminal contains far more of these amino acids than the corresponding neurotoxin section, whereas the carboxyl portions of the molecules are more alike. The typical neurotoxin core of Cys-Tyr-X-Lys-X-Trp is replaced by Cys-Tyr-Lys-Met-X-Met (Figure 11, residues 24–29) in the cardiotoxins, which also lack the sequence Arg-Gly at 37–38 common to all the curari-mimetic neurotoxins.

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