Hemorrhagic Toxins from Western Diamondback Rattlesnake (*Crotalus atrox*) Venom: Isolation and Characterization of Five Toxins and the Role of Zinc in Hemorrhagic Toxin e†

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ABSTRACT: Five previously unknown hemorrhagic proteins, designated hemorrhagic toxins a, b, c, d, and e, were isolated from the venom of the western diamondback rattlesnake (*Crotalus atrox*). Molecular weights of hemorrhagic toxins a–e were determined to be 68 000, 24 000, 24 000, 24 000, and 25 700, respectively, by sodium dodecyl sulfate–phosphate gel electrophoresis using various polyacrylamide gel concentrations. Amino acid composition showed a total of 636, 200, 213, 214, and 219 amino acids for hemorrhagic toxins a–e, respectively. All the hemorrhagic toxins were found to lose their hemorrhagic activities with the metal chelators ethylenediaminetetraacetic acid and 1,10-phenanthroline. All the hemorrhagic toxins were found to contain approximately 1 mol of zinc/mol of toxin, and they were all demonstrated to be proteolytic when dimethylcasein and dimethylhemoglobin were used as substrates. When zinc was removed from hemorrhagic toxin e with 1,10-phenanthroline, both the proteolytic and hemorrhagic activities were equally inhibited. When the apohemorrhagic toxin e thus produced was incubated with zinc, the hemorrhagic and proteolytic activities were regenerated to the same extent. CD, UV, and Raman spectroscopy were used to study the structure of native hemorrhagic toxin e as well as the structural changes caused by zinc removal. From CD spectroscopy the native toxin was estimated to consist of 23% α helix, 6% β structure, and 71% random-coil conformation. When over 90% of the zinc was removed, the α-helix content dropped from 23 to 7%.

The manifestations of local tissue damage, such as hemorrhage and myonecrosis, are among the best documented effects of certain types of snakebite. These effects are especially pronounced for the venoms of Crotalidae, which include rattlesnake and copperheads in the United States. Despite the importance of hemorrhage in rattlesnake envenomation, little information has been available on the chemistry and function of the hemorrhagic toxins.

The venom components responsible for hemorrhage have been termed hemorrhagins (Grotto, 1967), hemorrhagic principles (Ohsaka et al., 1960), or hemorrhagic factor (Mandelbaum et al., 1975). Since Crotalidae and Viperidae venoms are strongly hemorrhagic and also contain proteolytic enzymes, it was thought as early as 1930 (Houssay, 1930) that venom proteases were responsible for inducing hemorrhage. However, there is little direct evidence to support this supposition and the absence of proteolytic activity from a hemorrhagic toxin was reported for the first time from the venom of *Trimeresurus flavoviridis* (Ohsaka et al., 1960). Since then there have been conflicting reports on the proteolytic properties of hemorrhagic toxins.

The problem with these conclusions was the use of the casein–trichloroacetic acid precipitation method as the proteolytic enzyme assay. Such an assay can fail to detect a protease with low specificity for the peptide bonds of casein.

It has also been reported that hemorrhagic activity was eliminated by EDTA[1] and other chelating agents (Goucher and Flower, 1964) when they were added to snake venoms and to purified isolated hemorrhagic toxins. No information exists on the nature or functional role of the metal ions.

In this report, the isolation of the five hemorrhagic toxins is described along with some chemical and functional aspects such as the role of zinc in relation to hemorrhagic activity, proteolytic activity, and the protein conformation.

Materials and Methods

Lyophilized crude venom was purchased from Miami Serpentarium Laboratories. Diethylaminoethylcellulose DE 32 and carboxymethylcellulose CM 32 were purchased from Whatman Biochemicals, Ltd. Ultragel AcA 44 and ampholytes over the pH range 3.5–10 were obtained from LKB. 2,4,6-Trinitrobenzenesulfonic acid was purchased from Eastman Kodak Co. Molecular weight protein standards, 1,10-phenanthroline, Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), acrylamide, and collagen were obtained from Sigma Chemical Co. Zinc, magnesium, and calcium standards and Spectropor dialysis tubing were purchased from Fisher Chemical Co. FAGLA (furylacryloylglycyl-L-leucinamide) was obtained from Vega-Fox Biochemicals.

Isolation Procedure. All fractionation steps were performed at 2–4 °C. Two 10-g portions of crude venom were each dissolved in 50 mL of distilled water and extensively dialyzed against 10 mM borate, pH 9.0, containing 0.1 M NaCl and 2 mM $CaCl_2$. The venom was separated by a Whatman DE 32 anion-exchange column (Figure 1). The fractions A-1, A-2, and A-4 were further fractionated (Figures 2–4). Fraction A-1 yielded hemorrhagic toxins a and b, fraction A-2 yielded hemorrhagic toxins c and d, and fraction A-4 yielded hemorrhagic toxin e.

Hemorrhagic Toxins a and b. The combined fraction A-1 from the two runs was lyophilized and dissolved in 75 mL of buffer 0.1 M NaCl, and 2 mM $CaCl_2$ in 5 mM Tris buffer, pH 8.5 (22 °C). This sample was applied in three separate portions to a column of Sephadex G-75 superfine resin (5 × 95 cm)

† From the Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523. *Received December 22, 1977.* This work was supported by National Institutes of Health Grant 5R01 GM15591.

‡ This paper is based on a dissertation submitted by J. B. Bjarnason in partial fulfillment for the degree of Ph.D. at Colorado State University.

[1] Abbreviations used are: CD, circular dichroism; CM, carboxymethyl; EDTA, ethylenediaminetetraacetic acid; FAGLA, furylacryloylglycyl-L-leucinamide; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; K_R, retardation coefficients; p*I*, isoelectric point; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; Tris, tris(hydroxymethyl)aminomethane; UV, ultraviolet.

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equilibrated with the buffer (Figure 2). Fractions B-1 and B-3 containing hemorrhagic activity were lyophilized and equilibrated against their respective starting buffers, as described below.

Fraction B-1 was equilibrated with 10 mM Tris, pH 8.5 (22 °C), containing 0.04 M NaCl and 2 mM $CaCl_2$ by dialysis and loaded on a Whatman DE 32 column (1.5 × 40 cm). The column was developed with a linear gradient from 0.04 to 0.07 M NaCl in 500-mL total volume, followed by a two-step NaCl gradient of 0.1 and 0.2 M NaCl as shown in Figure 2C. Hemorrhagic fraction C-2 was lyophilized and dissolved in 10 mL of distilled water and applied to a column containing ultragel AcA 44 resin (2.5 × 100 cm) equilibrated with 0.1 M NaCl in 5 mM Tris buffer, Ph 8.5. The hemorrhagic fraction (Figure 2D) was further fractionated on the same column to yield 64 mg of hemorrhagic toxin a from 20 g of venom.

Fraction B-3 was equilibrated by dialysis against 0.04 M NaCl and 2 mM $CaCl_2$ in 10 mM Tris, pH 8.5, and applied to a column containing Whatman CM 32 cation-exchange resin (Figure 2F). Hemorrhagic fraction F-3 was further fractionated and yielded 740 mg of hemorrhagic toxin b from 20 g of venom.

Hemorrhagic Toxins c and d. Fraction A-2 was dissolved in distilled water and loaded onto a column of Sephadex G-25 superfine resin (5 × 90 cm) equilibrated with 0.1 M NaCl and 2 mM $CaCl_2$ in 5 mM Tris, pH 8.5. Fraction H-3 was found to be hemorrhagic (Figure 3H) and was lyophilized. It was then equilibrated by dialysis with 10 mM Tris, pH 8.5, containing 2 mM $CaCl_2$ and 0.04 M NaCl and loaded onto a column (1.5 × 40 cm) of Whatman DE 32 anion-exchange resin (Figure 3I). Two hemorrhagic fractions (Figure 3I-1, I-2) were further purified on DE 32 anion-exchange column as shown in Figures 3J and 3K. The yields for hemorrhagic toxin c and d were 34 and 62 mg, respectively, from 20 g of venom.

Hemorrhagic Toxin e. Fraction A-4 was dissolved in distilled water and divided in half, and each portion was loaded onto a gel-filtration column of Sephadex G-75 superfine resin (5 × 90 cm). Fraction L-2, which was found to be hemorrhagic, was lyophilized and equilibrated against 0.1 M NaCl in 5 mM Tris, pH 8.5 (22 °C), and loaded onto a DE 32 anion-exchange column (1.5 × 40 cm). A total of 320 mg of hemorrhagic toxin e was obtained from 20 g of venom (Figure 4M).

Assay for Hemorrhagic Activity. This assay was performed as reported previously (Kondo et al., 1960). Five Swiss Webster white mice (20 g) were each injected subcutaneously with one dose and sacrificed after 6 h. The minimum hemorrhagic dose was defined as the least quantity of venom or toxin which caused a hemorrhagic reaction of 5 mm in diameter 6 h after subcutaneous injection.

Assay for Proteolytic Activity. Proteolytic activity was assayed using dimethylcasein prepared according to Lin et al. (1969) as substrate and trinitrobenzenesulfonic acid to react with the amino groups. A molar extinction coefficient of 6.46×10^3 M^{-1} cm^{-1} (Goldfarb, 1966) for the trinitrophenyl-α-amino groups was used to estimate the turnover number. Concentrations of the toxins in the reaction mixture were in the range of 5 to 40 μg/mL.

Molecular Weight. The molecular weight was determined by gel filtration and by the $NaDodSO_4$ method. Protein standards used were cytochrome *c* (12 000), ribonuclease (13 700), lysozyme (14 300), myoglobin (17 200), chymotrypsinogen A (25 000), β-lactoglobulin (35 000), ovalbumin (45 000), bovine serum albumin (68 000), and phosphorylase a (100 000).

For the $NaDodSO_4$ method, the toxins and standards were treated with $NaDodSO_4$ and reduced with mercaptoethanol for 5 min at 100 °C and then run on polyacrylamide gels of four different acrylamide concentrations: 5, 7.5, 10, and 12%. Mobilities were determined relative to that of cytochrome *c*, a Ferguson plot was constructed, and K_R (retardation coefficient) values were obtained. A standard curve of K_R vs. log molecular weight was constructed, and the molecular weights of the toxin were determined. Protein standards used in this experiment were myoglobin, chymotrypsinogen A, carbonic anhydrase (29 000), β-lactoglobulin, and bovine serum albumin.

Amino Acid Composition. Amino acid compositions of the carboxamido-methylated hemorrhagic toxins were determined with a JEOL Model JLC-6AH automatic amino acid analyzer. Hydrolysis of samples was done either with constant-boiling HCl or mercaptoethanesulfonic acid (Penke et al., 1974) at 110 °C for 24, 48, and 72 h. Tryptophan was determined spectrophotometrically by the method of Edelhock (1967) and by the method of Penke et al. (1974).

Collagenase and Neutral Protease Assays. Collagenase assay was performed according to the suspension method procedure described by Seifter and Gallop (1962) with the exception that we used bovine achilles tendon collagen instead of ichthyocal (carp swim-bladder collagen). Neutral protease assay using FAGLA as substrate was performed according to the procedure described by Feder (1968). The reaction was monitored at 345 nm with a Beckman DB-G spectrometer.

Carbohydrate Test. Hemorrhagic toxin e was tested for carbohydrates with the electrophoretic glycoprotein stain developed by Keyser (1964).

Metal Analysis. Metal ions were analyzed by atomic absorption spectrophotometry on a Beckman atomic absorption modular system (Model 1301 atomic absorption unit with laminer flow burner, acetylene, and air and DB-G spectrophotometer), and the dialysis buffer was used as a blank. All solutions were prepared with deionized quartz-distilled water.

Zinc Removal and Reincorporation. Hemorrhagic toxin e was dialyzed against 10 mM Hepes, pH 7.2 (22 °C), containing 2 mM 1,10-phenanthroline, 10 mM $CaCl_2$, and 0.1 M NaCl for 24 h to remove zinc. Apohemorrhagic toxin e was also formed by running hemorrhagic toxin e on a Sephadex G-25 column (1.5 × 30 cm) equilibrated with the same buffer, as suggested by Feder and Garrett (1971). The zinc content of apohemorrhagic toxin e was measured. For circular dichroism and ultraviolet spectroscopy, 1,10-phenanthroline was removed by dialysis against 2 mM Tris, pH 7.9 (22 °C), and 10 mM $CaCl_2$ in 0.1 M NaCl for 48 h with six solution changes. For Raman spectroscopy, apohemorrhagic toxin e was dialyzed against distilled water and lyophilized. For hemorrhagic and proteolytic assays, apohemorrhagic toxin e was dialyzed against 10 mM borate buffer, pH 9.0 (22 °C), containing 2 mM $CaCl_2$ and 0.1 M NaCl for 32 h and four solution changes or run on a Sephadex G-25 column (1.5 × 40 cm) equilibrated with the same buffer.

Apohemorrhagic toxin e was incubated with zinc ion to attempt to regenerate native hemorrhagic toxin e as described below. First, apohemorrhagic toxin e was dialyzed against 10 mM Hepes, pH 7.2, containing 10 mM $CaCl_2$ and 0.1 M NaCl for 16 h and two solution changes, then against 10 mM Hepes, pH 7.2, containing 2 mM $CaCl_2$, 1 mM $ZnCl_2$, and 0.2 M NaCl for 48 h, and finally against 10 mM borate, pH 9.0, containing 2 mM $CaCl_2$, 0.03 mM $ZnCl_2$, and 0.1 M NaCl for 16 h. Apohemorrhagic toxin e was also run on a Sephadex G-25 column equilibrated with a 10 mM borate buffer, pH 9.0, containing 2 mM $CaCl_2$, 0.03 mM $ZnCl_2$, and 0.1 M NaCl.

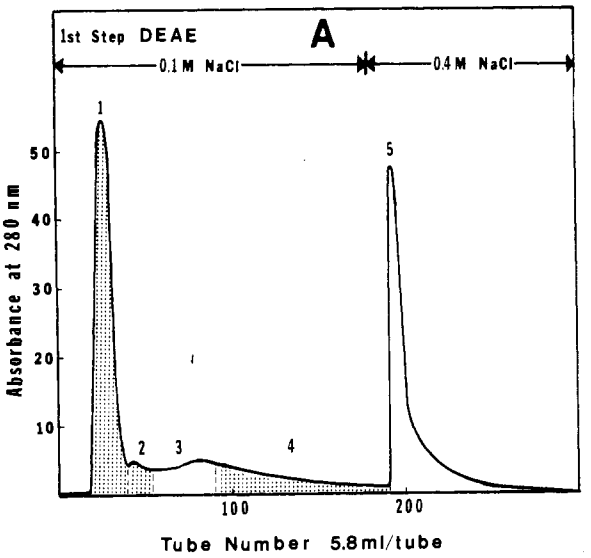

FIGURE 1: DEAE-cellulose chromatography of 10 g of crude *Crotalus atrox* venom on a 2.5 × 35 cm column with a step gradient of 0.1 M and 0.4 M NaCl both in 10 mM borate buffer, pH 9.0, containing 2 mM $CaCl_2$ and operating at a flow rate of 85 mL/h.

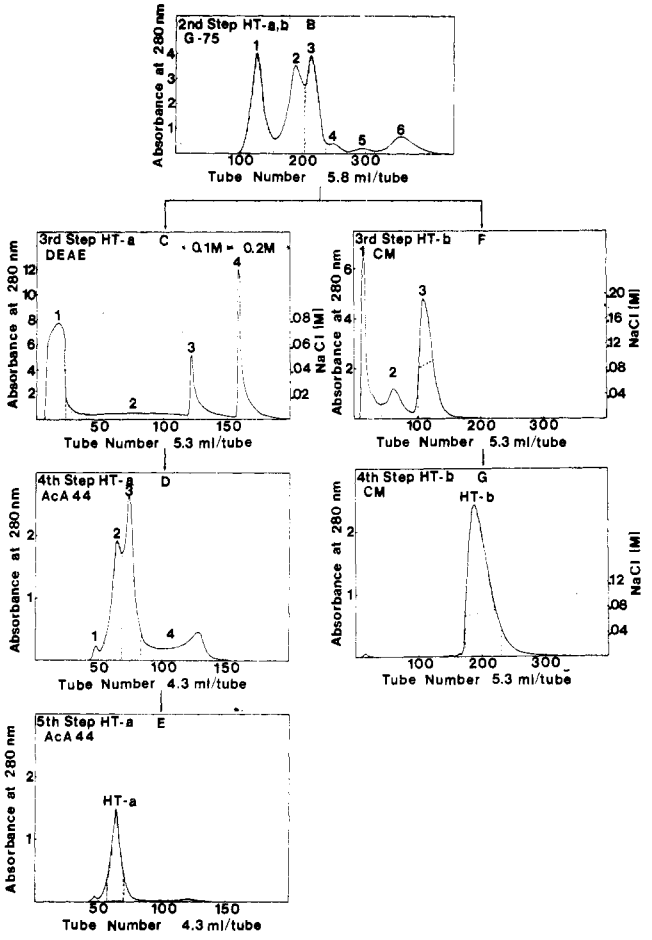

FIGURE 2: Isolation scheme of hemorrhagic toxins a and b from fraction A-1 (Figure 1). The isolation procedure was described under Materials and Methods section.

Regenerated hemorrhagic toxin e was prepared for CD and UV spectroscopy by dialysis against 2 mM Tris, pH 7.9 (22 °C), 10 M $CaCl_2$, and 0.1 M NaCl for 24 h.

CD Spectroscopy. The circular dichroic spectra of native, apo-, and regenerated hemorrhagic toxin e were obtained with

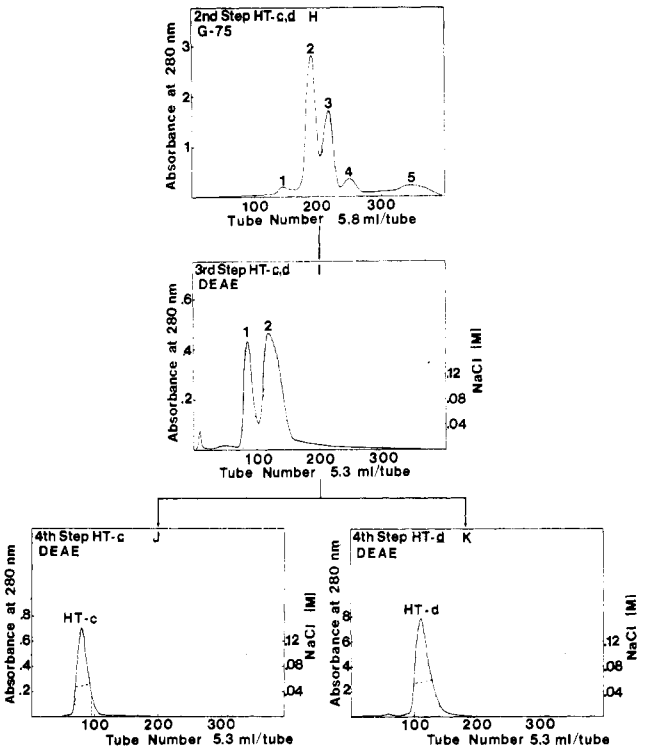

FIGURE 3: Isolation scheme of hemorrhagic toxins c and d from fraction A-2. The isolation procedure was described in the Materials and Methods.

a JASCO Model J-41 C spectropolarimeter at 25 °C. The solvent used was 2 mM Tris, pH 7.9 (22 °C), containing 10 mM $CaCl_2$ and 0.1 M NaCl; 3×10^{-5} M $ZnCl_2$ was also added to regenerated hemorrhagic toxin e.

Raman Spectroscopy. The Raman spectra of native and apohemorrhagic toxin e were obtained with a Spex Ramalog 5 with a Spex M01 double monochromator, Spectro-Physics Model 164 argon-ion laser, and photo-counting electronics. The spectra were obtained with 514.5-nm line excitation and a 5 cm^{-1} spectral slit width.

Results

Isolation. The first fractionation step was chromatography on a column of a DEAE-cellulose (Figure 1) which was loaded with 10 g of venom per run. This yielded three hemorrhagic fractions, fractions 1, 2, and 4, all of which were fractionated further. Fraction 1 yielded hemorrhagic toxin a in four additional steps and hemorrhagic toxin b in three additional isolation steps (Figure 2). Fracture 2 yielded hemorrhagic toxins c and d in three additional column steps (Figure 3) and fraction 3 yielded hemorrhagic toxin e in two additional isolation steps (Figure 4). The yields of hemorrhagic toxins b and e were highest, giving 3.7 and 1.6% of the total material in the venom, respectively, whereas the other toxins yielded less than 1% of the 20 g of venom used in the isolation (Table I). On the other hand, hemorrhagic toxin a had the highest hemorrhagic potency with a minimum hemorrhagic dose of 0.04 μg. Hemorrhagic toxin a made up 16% of the total hemorrhagic activity of the crude venom. Hemorrhagic toxins b and e had activities similar to crude venom (Table I) and together yielded a total of 5.7% of the total hemorrhagic activity of crude venom, but hemorrhagic toxin c and d had somewhat lower activities and together yielded 0.1% of the total activity of the venom. The five hemorrhagic toxins thus yielded 21.8% of the total hemorrhagic activity of the crude *Crotalus atrox* venom.

TABLE I: Yield and Activity of the Hemorrhagic Toxins Isolated from the Venom of *Crotalus atrox*.

	toxin recov (mg)	% protein	min hemorr dose (μg)	% hemorr act	protease[a] act.
crude venom	20 000	100	2	100	+
hemorr toxin					
a	64	0.32	0.04	16	+
b	740	3.70	3	2.5	+
c	34	0.17	8	0.04	+
d	62	0.31	11	0.06	+
e	320	1.60	1	3.2	+

[a] Positive proteolytic activity is indicated by +.

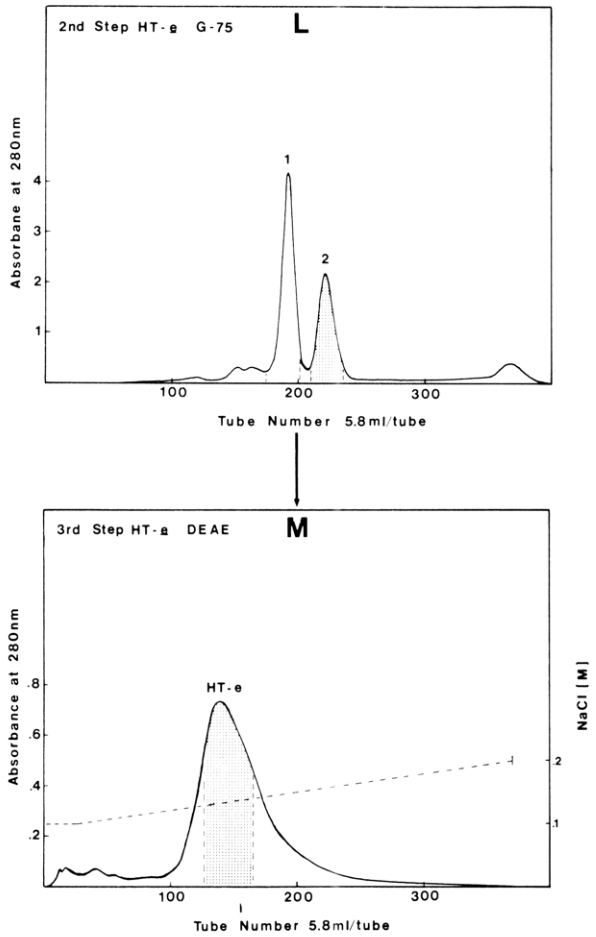

FIGURE 4: Isolation scheme of hemorrhagic toxin e from fraction A-4 (Figure 1).

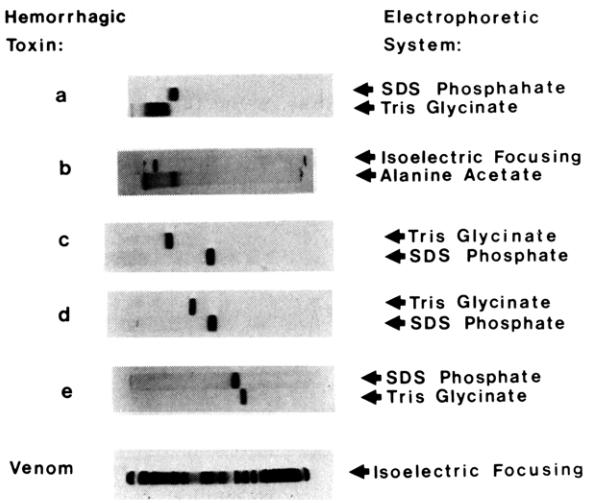

FIGURE 5: Electrophoresis of 50–100 μg of the hemorrhagic toxins and 300 μg of crude venom on polyacrylamide gels.

Homogeneity. As shown in Figure 5, the hemorrhagic toxins were electrophoretically homogeneous in the respective types of gel electrophoresis. From isoelectric focusing experiments, hemorrhagic toxin b was found to be basic, all the other hemorrhagic toxins were acidic. The p*I* of hemorrhagic toxin e was 5.6 as can be seen in Figure 6.

Amino Acid Composition. The amino acid composition of reduced and carboxyamidomethylated hemorrhagic toxins a–e are shown in Table II. The values in Table II are nearest integers of the corrected values from three hydrolysis times: 24, 48, and 72 h. The values for serine and threonine were obtained by extrapolating to zero hydrolysis time; other amino acid values were averages of the three hydrolyses. The most striking aspect of the amino acid compositions is the unusually high amount of carboxymethylcysteine of hemorrhagic toxin a and the very similar amino acid compositions of hemorrhagic toxins c and d. A difference of only one residue is observed for five amino acids; all the others are the same. Hemorrhagic toxin e was investigated in more detail than the others. The reduced and alkylated toxin was found to contain eight carboxymethylcysteine residues, whereas the nonreduced and alkylated toxin contained eight half-cystines and no carboxymethylcysteine residues. Also, from the Raman spectra of hemorrhagic holotoxin e in water, it was possible to observe a disulfide peak appearing at 509 cm^{-1}, but no peak was observed in the sulfhydryl region of 2500 to 2600 cm^{-1}. Hemorrhagic toxin e did not stain with the periodic acid–Schiff base stain used to detect carbohydrates on proteins. It is therefore concluded that hemorrhagic toxin e is not a glycoprotein.

Molecular Weights. The molecular weight of hemorrhagic toxin a was quite high, 68 000 g/mol, while the molecular weights of the other hemorrhagic toxins were lower and quite similar (see Table III). Minimum molecular weights calculated on the basis of amino acid composition and zinc content are also reported in Table III and are in good agreement with those determined with the $NaDodSO_4$–phosphate method. Another important observation from the $NaDodSO_4$–phosphate gel electrophoresis of the reduced hemorrhagic toxins is that they are all composed of one polypeptide chain.

Metal Composition. The crude venom of *Crotalus atrox* was tested for a wide variety of metal ions and found to contain Na, K, Mg, Ca, and Zn. Three different preparations of hemorrhagic toxin e were assayed for calcium, magnesium, and zinc. The three preparations were found to contain 0.42, 0.58, and 0.25 mol of calcium/mol of toxin e, having an aver-

TABLE II: Amino Acid Compositions of Hemorrhagic Toxins a–e.[a]

amino acid	hemorrhagic toxin a	b	c	d	e
Lys	27	10	7	7	9
His[b]	18	8	8	8	9
Arg	33	12	10	11	8
Asx	85	26	33	33	30
Thr	36	7	11	10	10
Ser	35	14	16	16	14
Glx[b]	55	16	24	25	26
Pro	27	6	6	6	8
Gly[b]	43	13	9	9	14
Ala[b]	36	8	10	9	7
CM-Cys	66	4	2	2	8
Val	35	11	11	11	12
Met	11	6	5	5	8
Ile	36	12	17	18	21
Leu	49	19	25	25	14
Tyr[b]	21	7	7	7	11
Phe[b]	17	7	7	7	6
Trp	6	4	5	5	4
total	635	200	213	214	209

[a] Amino acid composition is based on the nearest integer to the corrected value for three hydrolysis times: 24, 48, and 72 h. The corrected value was an average of the values at the three hydrolysis times except for serine and threonine, whose values were obtained by extrapolation to zero time and tryptophan which was obtained spectrophotometrically by the method of Edelhoch for all the toxins except toxin e, in which case tryptophan was obtained with hydrolysis with mercaptoethanesulfonic acid. [b] Recoveries for histidine, glutamic acid, glycine, alanine, tyrosine, and phenylalanine were averaged to obtain normalized integration values for each hydrolysis. [c] The amino acid analysis values for 24, 48, and 72 h are available upon request.

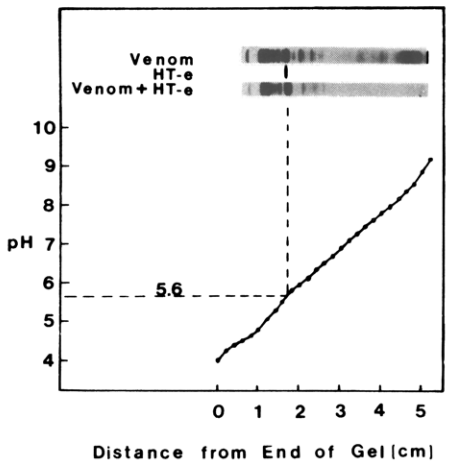

FIGURE 6: Isoelectric focusing of crude *Crotalus atrox* venom, hemorrhagic toxin e, and a mixture of venom and hemorrhagic toxin e on polyacrylamide gels with ampholine carrier ampholytes in the range pH 3.5–10. Anode is to the left. The gels are compared to the pH gradient of a standard gel from the same electrophoretic run.

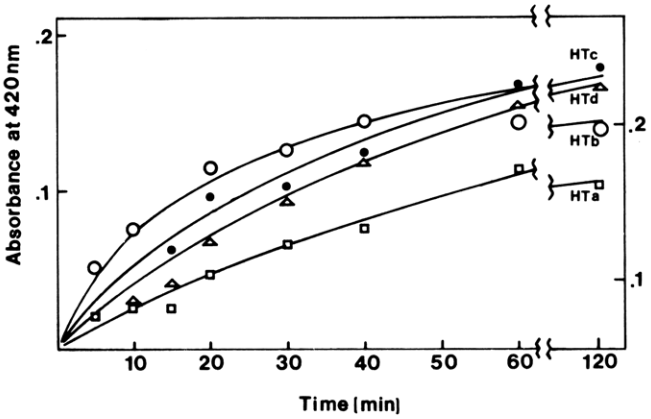

FIGURE 7: Proteolytic progression curves of hemorrhagic toxins a (□), b (○), c (●), and d (△) on a dimethylcasein substrate. Concentrations of the hemorrhagic toxins in the reaction mixture were HT-a, 4.9 μg/mL; HT-b, 7.0 μg/mL; HT-c, 6.0 μg/mL; HT-d, 7.2 μ/mL.

age of 0.42 mol of calcium/mol of toxin e. Magnesium content per mole of toxin e was 0.07, 0.06, and 0.03 mol or an average of 0.05 mol of magnesium/mol of toxin e. Zinc content per mol of toxin e was determined as 0.98, 1.06, and 1.06 mol, averaging 1.03 mol of zinc/mol of hemorrhagic toxin e. Zinc content was also measured for other hemorrhagic toxins (Table IV) and the average was close to 1 mol of zinc/mol of protein for all hemorrhagic toxins.

Proteolytic Activity. All hemorrhagic toxins were tested for proteolytic activity with dimethylcasein as substrate. The most striking aspect of the proteolytic progression curves (Figure 7) is the lack of direct relationship between proteolytic activity on dimethylcasein and hemorrhagic activity. Hemorrhagic toxin a, the most potent hemorrhagic toxin, has the least proteolytic activity on dimethylcasein. Another interesting observation is the close similarity of the progression curves of hemorrhagic toxins c and d, which also have a very similar amino acid composition, molecular weight, and zinc content.

When the initial velocity of dimethylcasein proteolysis was plotted against the concentration of hemorrhagic toxin e, a linear relationship was obtained. Dimethylhemoglobin was also used for hemorrhagic toxin e, and positive results were shown. Collagenase activity was not detected using native collagen as a substrate. Furylacryloylglycyl-L-leucinamide, a substrate for neutral proteases, was also used for assay with negative results.

TABLE III: Molecular Weights of Hemorrhagic Toxins a–e.

method of mol wt determinat.	hemorrhagic toxin a	b	c	d	e
min mol wt[a] from AA comp	6 400	6 000	12 300	12 300	6 400
min mol wt from Zn content	68 700	29 300	27 900	27 900	25 000
mol wt by $NaDodSO_4$	68 000	24 000	24 000	24 000	25 700
mol wt by gel[b] filtrat	83 000	19 000	20 500	21 000	22 000

[a] Minimum molecular weight was estimated assuming methionine to be one residue in toxin a, carboxymethylcysteine to be one residue in toxins b–d and tryptophan to be one residue in toxin e. [b] Molecular weight of hemorrhagic toxin by a gel filtration was determined using an ultragel AcA 44 column, while the molecular weights of the other hemorrhagic toxins were determined using a Sephadex G-75 superfine column.

TABLE IV: Zinc Content of Hemorrhagic Toxins a-e in mol of Zinc/mol of Toxin.

toxin prep	hemorrhagic toxin a	b	c	d	e
1st					0.98
2nd	0.77	0.72			1.06
3rd	1.22	0.92	0.86	0.86	1.06
av	0.99	0.82	0.86	0.86	1.03
based on[a] mol wt of	68 000	24 000	24 000	24 000	25 700

[a] Molecular weights based on the $NaDodSO_4$-phosphate polyacrylamide gel electrophoresis method using four different gel concentrations were used as a basis for the zinc-content calculations.

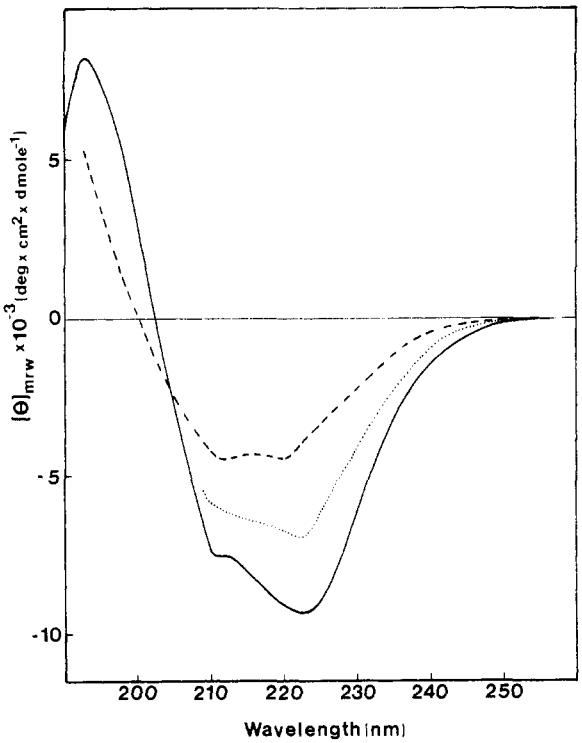

FIGURE 8: Circular dichroic spectra of native hemorrhagic toxin e (—), hemorrhagic apotoxin e (---), and zinc-regenerated hemorrhagic apotoxin e (···) in the peptide region. Mean residue ellipticities (θ_{mrw}) based on mean residue weight of 115.

Role of Zinc. Approximately 90% of the zinc was removed using the gel-filtration method with 12% of the original proteolytic activity and approximately 10% of the original hemorrhagic activity still associated with the apotoxin. When the dialysis method was used to remove zinc, less than 10% of original zinc content was found associated with the apotoxin, according to atomic absorption measurements. Less than 5% of the original proteolytic activity remained with the apotoxin and approximately 5% of the original hemorrhagic toxicity was still associated with the apotoxin. When regeneration of hemorrhagic toxin e was attempted by the gel-filtration method, 25% of the proteolytic and hemorrhagic activities was recovered. When the apotoxin was incubated with zinc ion using the dialysis method, 20% of the original proteolytic activity was regenerated and 25% of the hemorrhagic activity was found associated with the regenerated toxin. A minimum hemorrhagic dose of 4 μg was obtained as compared to 1 μg for the original hemorrhagic toxin e. The apo- and regenerated

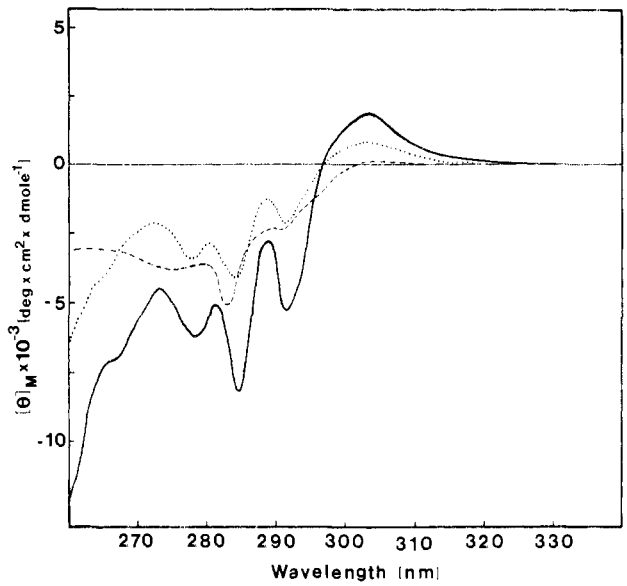

FIGURE 9: Circular dichroic spectra of native hemorrhagic toxin e (—), hemorrhagic apotoxin e (---), and zinc-regenerated hemorrhagic toxin e (···) in the aromatic region. Molar ellipticities (θ_M) are based on a molecular weight of 25 000.

hemorrhagic toxins e obtained by the dialysis method were used in the following spectroscopic studies.

CD, UV, and Raman Spectroscopy. The circular dichroism (CD) spectra of hemorrhagic toxin e and apo- and regenerated hemorrhagic toxins e are shown in Figures 8 and 9. In Figure 8 the CD spectra of the peptide region is expressed in terms of mean residue weight elipticity, and in Figure 9 the CD spectra of the aromatic region is expressed in terms of molar elipticity. The CD spectra for the native hemorrhagic toxin e (solid line in Figure 8) in the peptide region has a minimum at 222 ($[\theta]_{mrw} = -9300$) and at 211 nm ($[\theta_{mrw} = -7500$) and a maximum at 193 nm ($[\theta]_{mrw} = 8200$). This spectrum is highly suggestive of substantial amounts of α-helical formation, which were estimated at 23% by the method suggested by Chen et al. (1972) using the equation $\theta_{222} = -30\,300 f_H - 2340$. Assuming f_H to be 0.23, f_β and f_R were estimated using the equations

$$\theta_{mrw} = f_H X_H + f_\beta X_\beta + f_R X_R$$

$$f_H + f_\beta + F_R = 1$$

and X values calculated by Chen et al. (1972). The f_H, f_β, f_R refer to the fraction of α helix, β-sheeted structure, and random-coil conformation. Mean residue weight elipticities at 193, 222, 228, and 240 nm were used in the calculations. Estimates of 6% β structure and 71% random coil were thus obtained for native hemorrhagic toxin e. The CD spectra of hemorrhagic apotoxin e (dashed line in Figure 8) had minima at 221 and 212 nm, both with $\theta_{mrw} = 4500$ indicating a 7% α-helix content. It was not possible to have enough wavelengths to determine the maximum for the apotoxin. The regenerated toxin e (dotted line in Figure 8) had a CD spectrum about midway between those for the native toxin and the apotoxin; it has a minimum at 222 nm ($\theta_{mrw} = -6900$) indicative of about 15% α-helix content.

Similarly, the CD spectra in the aromatic region suggests structural changes with removal of zinc (Figure 9). The CD spectrum of native hemorrhagic toxin e has a positive peak at about 303 nm which is probably due to disulfides. Three negative peaks appear at 291.5 ($[\theta]_M = -5250$), 284.5 ($[\theta]_M = -8125$), and 278.5 nm ($[\theta]_M = -6250$). Tryptophan is prob-

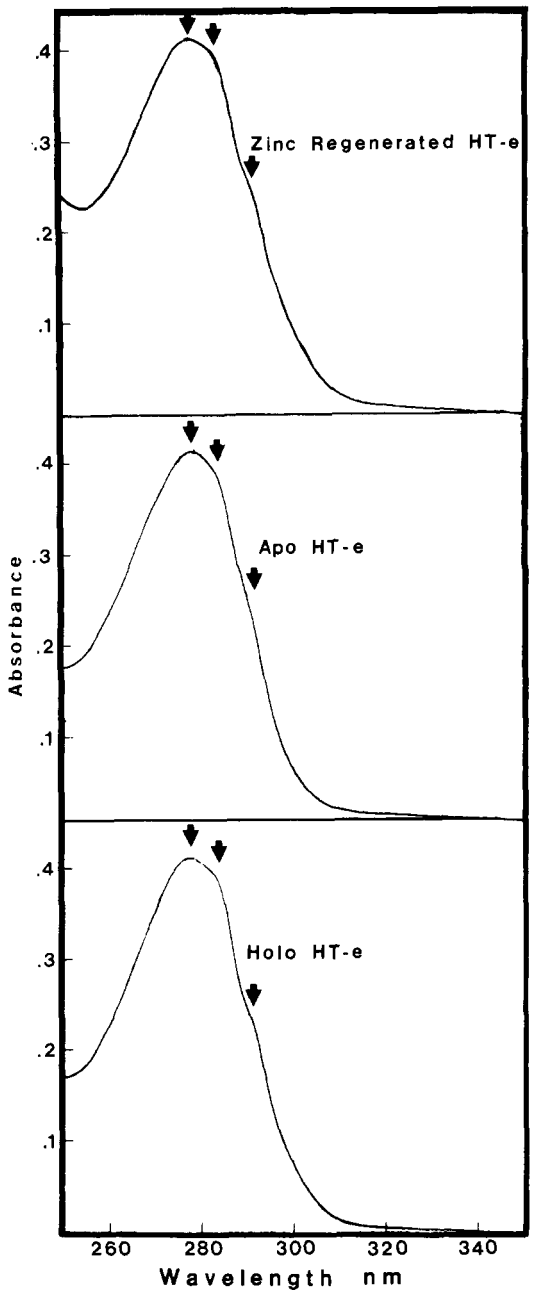

FIGURE 10: Ultraviolet spectra of native hemorrhagic toxin e (bottom), hemorrhagic apotoxin e (middle), and zinc-regenerated hemorrhagic toxin e (top) in the aromatic region. Concentration was 0.37 mg/mL in all cases. Note the shoulders at 291 and 284 nm which are greatly diminished upon zinc removal and regenerated when the apotoxin was incubated with zinc.

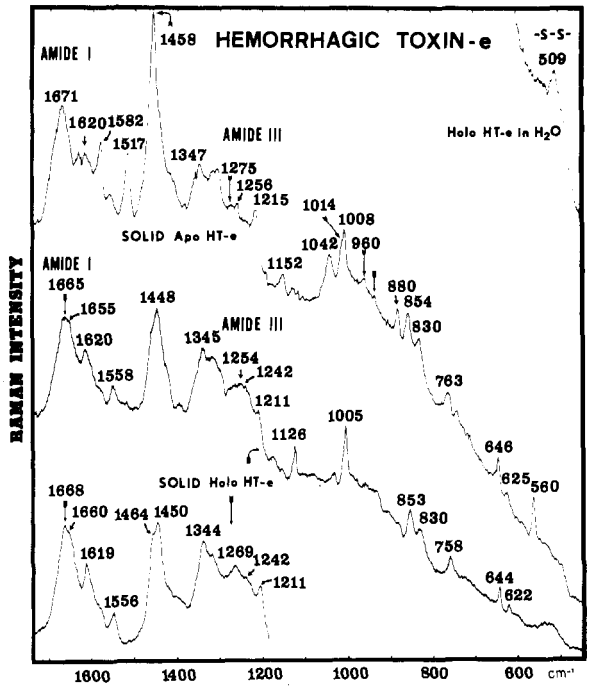

FIGURE 11: Laser Raman spectra of native hemorrhagic toxin e (two bottom curves) and hemorrhagic apotoxin e (top curve) in the solid state, and the disulfide region of aqueous native hemorrhagic toxin e (upper right-hand corner).

ably responsible for the 291.5- and 284.5-nm peaks, while the 278.5-nm peak is likely due to tyrosine or tryptophan or both. Tyrosine could also contribute to the 284.5-nm peak. A shoulder can be seen at 267 nm which is probably due to phenylalanine (Strickland, 1974).

The UV spectrum of native hemorrhagic toxin e shown in Figure 10 has peaks and shoulders roughly corresponding to the negative peaks of the CD spectrum, i.e., shoulders of 291 and 284 nm and it peaks at 278 nm. The shoulders at 291 and 284 nm become much less pronounced when zinc is removed, as seen in the UV spectrum of hemorrhagic apotoxin e (Figure 10). This change in UV absorbance is accompanied by a similar change in the CD spectrum (dashed line, Figure 9), where there is a great decrease in the fine structure of the spectrum and an apparent blue shift, making it markedly different from the spectra of the native toxin. The positive peak at 303 nm disappears, and a negative shoulder is found at 290.5 nm, along with a fairly strong negative peak at 283 nm and a broad negative peak at 275.5 nm. The fine structure observed for the native toxin reappears at identical wavelengths but at lower intensities in the CD spectrum of regenerated hemorrhagic toxin e (Figure 9) as do the shoulders in the UV spectrum of zinc-regnerated hemorrhagic toxin e (Figure 10).

The Raman spectra of native hemorrhagic toxin e and hemorrhagic apotoxin e are shown in Figure 11. In the upper right-hand corner of the figure, the disulfide region of aqueous hemorrhagic toxin e is shown. The disulfide peak appears at 509 cm^{-1}, indicative of a gauche-gauche-gauche configuration of the CCSSCC networks. The upper spectrum in Figure 11 is of hemorrhagic apotoxin e in the solid form and the lower two spectra are of native hemorrhagic toxin e in the solid form. Five spectra were obtained of native hemorrhagic toxin e in the solid form and the two that differed most are shown in Figure 11, to demonstrate the variance in the Raman spectra. The most significant difference between the Raman spectra of the holo- and apotoxins is observed in the amide I and III regions. The holotoxin has an amide I doublet, one peak in the region of 1655–1660 cm^{-1} indicative of α-helix conformation and the other peak in the region of 1665–1668 cm^{-1} suggestive of random-coil conformation. This latter peak could also be due to β structure and/or β-turn structures. The β-reverse turn conformation shows the amide I band at 1663–1666 cm^{-1} and the amide III band at 1260–1266 cm^{-1} (Tu et al., 1978).

The "random coil" we observed here does not imply that the peptide chain is free to assume any orientation, but it merely refers to different structure from normal α-helical or β-sheet structures. In the Raman spectrum of hemorrhagic apotoxin e, the amide I peak appears at 1671 cm^{-1} but no peak or shoulder due to α-helix conformation is obvious. The amide III band at 1269 cm^{-1} for hemorrhagic holotoxin e may be a reflection of the presence of β-reverse turn conformation.

TABLE V: Comparative Amino Acid Compositions of Biologically Active Proteins Isolated from the Venoms of Rattlesnakes.[a]

genus: species: subspecies:	*Crotalus atrox*				*Crotalus adamantus*		*Crotalus durrissus terrificus*		*Crotalus scutulatus*	*Crotalus viridis viridis*
	hemorrhagic toxin[b]				phospholipase A_α-α	crotalase	crotoxin basic protein	crotamine	mojave toxin	myotoxin a
	a	b	c	e						
biol act.	hemorrhage				hemolysis	coagulation	neurotoxin[c]	paralytic	cardiotoxin	myotoxin
Lys	27	10	7	9	7	11	9	9	4	10
His	18	8	8	9	2	9	2	2	1	2
Arg	33	12	10	8	5	12	8	2	3	1
Asx	85	26	33	30	16	31	9	2	7	2
Thr	36	7	11	10	7	14	6	0	3	0
Ser	35	14	16	14	7	16	6	3	3	3
Glx	55	16	24	26	13	23	8	2	6	2
Pro	27	6	6	8	9	22	5	3	2	3
Gly	43	13	9	14	13	20	10	5	6	5
Ala	36	8	10	7	8	11	6	0	3	0
½-Cys	66	4	2	8	14	14	10	6	8	4
Val	35	11	11	12	5	17	2	0	1	0
Met	11	6	5	8	1	2	1	1	1	1
Ile	36	12	17	21	5	18	4	1	3	2
Leu	49	19	25	14	5	21	6	1	3	1
Tyr	21	7	7	11	8	7	9	1	5	1
Phe	17	7	7	6	5	13	6	2	3	1
Trp	6	4	5	4	3	6	2	2		1
total	636	200	213	219	133	267	110	42		39
ref	present investigation				Tsao et al. (1975)	Markland & Damus (1971)	Hendon & Frankel-Conrat (1972)	Laure (1975)	Bieber et al. (1975)	Cameron & Tu (1977)

[a] Representative proteins are included in the table. Many more chemically similar proteins are known but cannot be included due to space limitations. [b] The amino acid composition of hemorrhagic toxin d is not presented here because of its similarity to hemorrhagic toxin c. [c] Crotoxin basic protein, a basic phospholipase A_2, becomes neurotoxic in combination with crotoxin acidic protein.

Disappearance of this peak for hemorrhagic apotoxin e may suggest that the β-turn conformation disappears together with the α conformation by removing zinc ion. It seems that the findings from Raman spectra are in fairly good agreement with the results obtained from CD spectral investigation.

Several other differences of interest between the Raman spectra of holo- and apotoxin e are apparent. A sharp 560-cm^{-1} peak is visible in the apotoxin spectrum but not in the holotoxin spectrum. The apotoxin spectrum has a sharp peak at 880 cm^{-1} probably assignable to tryptophan, where there is none detectable in the holotoxin spectrum. Two sharp peaks also appear at 1517 and 1582 cm^{-1} in the spectrum of the apotoxin, but are altogether lacking in the holotoxin spectrum. This change probably reflects the change of environment for phenylalanine and tryptophan, as does the appearance of a shoulder at 1368 cm^{-1} (Figure 11). The I_{850}/I_{830} ratio for the holotoxin is 1.25 and for the apotoxin is 1.16, indicative of exposed tyrosines in both cases (Yu and Jo, 1973).

Discussion

Toxins from the venoms of cobras and sea snakes have been extensively studied by many investigators (Tu, 1973, 1977). In contrast, the toxic components of rattlesnake venoms, especially from rattlesnakes indigenous to North America, have been studied very little. A lethal toxin has been isolated from the venoms of Mojave rattlesnake (*Crotalus scutulatus*), indigenous to Arizona (Bieber et al., 1975), and a myotoxin was recently isolated from the venom of the prairie rattlesnake (*Crotalus viridis viridis*) (Cameron and Tu, 1977) (see Table V). Hemorrhage is one of the serious toxic effects of rattlesnake bite (Ownby et al., 1974). The isolation of hemorrhagic toxins from venoms of Asian snakes has been reported (Oshima et al., 1968; Takahashi and Ohsaka, 1970; Grotto et al., 1967), but no isolations of hemorrhagic toxins from rattlesnake venom have been reported prior to the current investigation. The complexity of the crude rattlesnake venom and the relatively low amounts of each toxin in the venom have been detrimental to this effect. Our isolation represents the first reported purification of hemorrhagic proteins from rattlesnake venom, although several hemorrhagic toxins have been isolated from venoms other than rattlesnakes (Table VI). It was reported that the basement membranes can be hydrolyzed by a hemorrhagic toxin (Ohsaka et al., 1973). This suggests that hemorrhagic toxin possesses enzymatic activity. In two recent reports (Mandelbaum et al., 1975; Nikai et al., 1977) hemorrhagic toxins isolated from venoms of South American and Japanese snakes were found to contain proteolytic activity. Proteolytic activity has been a central issue in the study of hemorrhagic toxins since it was proposed by Houssay (1930). Our investigation definitely corroborates that hemorrhagic action is due to proteolytic action.

The proteolytic progression curves (Figure 7) are different for the different hemorrhagic toxins, suggesting different proteolytic specificities and furthermore that the toxin with the greatest hemorrhagic activity, hemorrhagic toxin a, seems to have the lowest activity for the peptide bond of dimethylcasein. Thus, there does not seem to be a direct relationship between specific hemorrhagic activity of the different toxins and their proteolytic activity on dimethylcasein. These observations are suggestive of a highly selective specificity of the

TABLE VI: Comparative Properties of Hemorrhagic Toxins Isolated to Date.

family:	Crotalidae				Crotalidae	Crotalidae	Crotalidae	Crotalidae			Viperidae
genus:	*Crotalus*				*Agkistrodon*	*Agkistrodon*	*Bothrops*	*Trimeresurus*			*Vipera*
species:	*atrox*				*acutus*	*halys blomhoffii*	*jararaca*	*flavoviridis*			*palestinae*
subspecies:											
	hemorrhagic toxin[d]						hemorr	hemorrhagic principle			hemor-
	a	b	c	e	$A_{Cl\text{-proteinase}}$	proteinase b	factor HF_2	HR-1	HR-2a	HR-2e	rhagin
mol wt	68 000	24 000	24 000	25 700	24 500	95 000	50 000	100 000	nd[c]	nd	44 000
Zn content	0.99	0.82	0.86	1.03	nd	nd	nd	nd	nd	nd	nd
inhib[a] by EDTA	+	+	+	+	+	+	+	+	+	+	+
proteolytic[b]	+	+	+	+	+	+	+	−	−	−	−
isoelect pt	acidic	basic	acidic	5.6	4.7	4.18	nd	4.3	nd	nd	acidic
ref	present investigation				Nikai et al. (1977)	Oshima et al. (1972)	Mandel-baum et al. (1975)	Omori-Satoh & Osaka (1970)	Takehshi & Osaka (1970)	Grotto et al. (1967)	

[a] Inhibition of hemorrhagic activity by EDTA is indicated by +. [b] Presence of proteolytic activity is indicated by +; nondetection of proteolytic activity is indicated by −. [c] Not determined. [d] All data presented here for hemorrhagic toxin c is identical to hemorrhagic toxin d.

toxins, especially hemorrhagic toxin a, the most potent.

Comparison of the amino acid compositions of other proteins isolated from rattlesnake (Table V) demonstrates that the hemorrhagic toxins are indeed quite dissimilar from the known proteins isolated from *Crotalus* venoms. All hemorrhagic toxins isolated to this date have been shown to have their hemorrhagic activity inhibited by EDTA and other metal chelators (Table VI). It is therefore of interest that all the hemorrhagic toxins isolated from the venom of the western diamondback rattlesnake were found to contain approximately 1 mol of zinc/mol of toxin (Table IV), especially when the toxins were also proteolytic, since zinc is a common prosthetic group for proteolytic enzymes (Chlebowski and Coleman, 1976) and collagenases (Seifter and Harper, 1970).

On the basis of the data presented in the previous section, it appears that hemorrhagic toxin e is a slightly acidic protein (pI = 5.6) containing no carbohydrates. It is of interest that hemorrhagic fractions isolated from the venom of *Agkistrodon rhodostoma* (Malayan pitviper) are also slightly acidic, having the pI values of 5.6 and 6.1, respectively (Toom et al., 1969). The amino acid composition of hemorrhagic toxin e is reflective of its acidity containing 26% acidic residues and their amidated counterparts. Hemorrhagic toxin e has eight half-cystines which form four disulfide bridges in the native toxin. When the amino acid composition of hemorrhagic toxin e is compared to a snake venom protease, leucostoma peptidase, some similarities can be seen (Wagner et al., 1968). Other similarities between leucostoma peptidase A and the hemorrhagic toxins can be found. Leucostoma peptidase A is a zinc metalloprotease with a molecular weight of 22 500 g/mol containing 1 mol of zinc and 2 mol of calcium per mol of enzyme. Its proteolytic activity was inhibited by exposure of the enzyme to 1,10-phenanthroline, but the activity was restored by the addition of zinc ions (Spiekerman et al., 1973). Unfortunately, leucostoma peptidase A has not been tested for hemorrhagic activity. When zinc was removed from hemorrhagic toxin e with 1,10-phenanthroline, the proteolytic and hemorrhagic activities were equally inhibited and in direct proportion to the zinc removal. When the hemorrhagic apotoxin e thus produced was incubated with zinc, the hemorrhagic and proteolytic activities were regenerated to the same extent. This strongly suggests that there is a direct relationship between the hemorrhagic and proteolytic activities of hemorrhagic toxin e. It also partly explains the inhibition of the hemorrhagic activity of the toxins by chelating agents.

CD, UV, and Raman spectroscopy were used to study the structure of native hemorrhagic toxin e as well as structural changes caused by zinc removal. With CD spectroscopy the native toxin was estimated to consist of 23% α-helix, 6% β-structure, and 71% random-coil conformation. When over 90% of the zinc was removed, the α-helix content dropped from 23 to 7%. This change in CD was paralleled by a disappearance of the α-helix peak in the amide I region of the Raman spectrum of 1655–1660 cm^{-1}. When the apotoxin was incubated with zinc, an increase in α-helix content was observed from the peptide region of the CD spectrum (Figure 8). Similarly, changes in the environments of the aromatic residues with removal of zinc from the toxin were reflected in the CD, UV, and Raman spectra, although these changes are not readily interpretable. Parallel changes can be seen in the aromatic regions of the CD and UV spectra, which appear indicative of changes in the tryptophan environments (Strickland, 1974). These changes are supported by appearances of peaks in the Raman spectrum of the apotoxin attributable to tryptophan which were not visible in the Raman spectra of the holotoxin.

We have thus demonstrated in the present investigation that there is a direct and equal relationship between hemorrhagic and proteolytic activity of hemorrhagic toxin e, as well as between the zinc content of the toxin and the activities. There also appears to be a very significant conformational change in hemorrhagic toxin e with the removal of zinc from the toxin.

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Covalent Structure of Collagen: Amino Acid Sequence of Five Consecutive CNBr Peptides from Type III Collagen of Human Liver†

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ABSTRACT: Type III collagen was solubilized from human liver by limited pepsin digestion and purified by differential salt precipitation and carboxymethylcellulose chromatography. Digestion with cyanogen bromide yielded the nine distinct peptides previously described and an additional tripeptide not recognized in earlier studies. Five of these peptides, α1(III)-CB1, 2, 4, 8, and 10, were further purified by molecular sieve and/or ion exchange chromatography. They contained 12, 40, 149, 125 and 3 amino acid residues, respectively. The amino acid sequence of these peptides was determined by automated Edman degradation of tryptic (before and after maleylation), chymotryptic, thermolytic or hydroxylamine-derived peptide fragments as well as the intact peptides. The alignment of these five peptides within the collagen chain is deduced to be 1-8-10-2-4 by homology with known α1(I) sequences. The known CNBr peptide alignment of the NH_2-terminal portion of type III collagen so far would, therefore, be α1(III)-CB3-7-6-1-8-10-2-4 and correspond to the homologous region of α1(I)-CB0-1-2-4-5-8-3 or residues 11–567 of the α1(III) collagen chain.

Collagen represents nearly one-third of the total body protein of most vertebrates. At least four genetic types are known. The most abundant and extensively studied is type I collagen consisting of two α1(I) chains and one α2 chain as a triple-stranded helix of over 1000 amino acids per chain (Traub & Piez, 1971; Gallop et al., 1972). A composite amino acid sequence of the α1(I) chain can now be constructed from CNBr peptides of calf, chick, and rat skin collagen (Hulmes et al., 1973; Gallop & Paz, 1975; Piez, 1976; Fietzek & Kuhn, 1976) and the sequence of α2 is also nearly complete (Fietzek & Rexrodt, 1975; Dixit et al., 1977a,b).

The genetically distinct type III collagen has recently been identified and found to be nearly as widespread in tissue distribution as type I collagen. Both collagen types I and III occur simultaneously in most connective tissues with the exception of bone which contains type I only, and cartilage, which contains only type II collagen (Miller & Lunde, 1973; Chung & Miller, 1974; Epstein, 1974). Type III collagen, unlike type I, contains intramolecular cysteine-derived cross-linkages and, therefore, can be separated chromatographically as a 280 000 molecular weight species containing three identical α1(III)

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