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Purification and Biochemical Study of Viriditoxin, Tissue Damaging Toxin, from Prairie Rattlesnake Venom†

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ABSTRACT: Rattlesnake poisoning frequently produces a severe tissue damaging effect including myonecrosis and hemorrhage. In this investigation, viriditoxin was isolated from the venom of the prairie rattlesnake (*Crotalus viridis viridis*), and its chemical properties and biological activities associated with tissue damage were investigated. The toxin has a molecular weight of ~115 000 with an isoelectric point of 4.8. Amino acid composition analyses indicate that the toxin consists of ~1018 residues with high content of acidic amino acid residues. Circular dichroism was used to examine the conformation of viriditoxin. Although there is some indication of a high β-sheet content, it is not possible to identify the exact conformation due to the anomalous CD spectra. Viriditoxin has both myotoxic and hemorrhagic activities. High serum creatine kinase activity of mice injected with the toxin substantiates the destructive action of viriditoxin on muscle. Viriditoxin has an LD_{50} value of ~5.0 μg/g in mice indicating the protein is also moderately lethal in addition to its tissue damaging effect.

Rattlesnake bites in the United States, although rarely fatal, induce considerable damage to the tissues surrounding the site of envenomation. The most common of these local tissue effects are myonecrosis and hemorrhage. Since antivenin treatments may not prevent myonecrosis and hemorrhage unless treatment is administered immediately following envenomation (Minton, 1954; Stahnke et al., 1957), these effects remain a source of serious clinical concern. A number of toxins which induce hermorrhage, myonecrosis, or both have been isolated from pit viper (Crotalidae) and viper (Viperidae) venoms.

Five hemorrhagic toxins, which contain zinc, have been isolated from western diamondback rattlesnake (*Crotalus atrox*) venom (Bjarnason & Tu, 1978). One of these toxins, hemorrhagic toxin *b*, has been found to also induce myonecrosis in addition to its hemorrhagic activity (Ownby et al., 1978). Myotoxin *a*, a nonhemorrhagic muscle degenerating factor, was isolated from prairie rattlesnake (*Crotalus viridis viridis*) venom (Cameron & Tu, 1977). Myotoxin *a* has 42 amino acid residues, and its complete amino acid sequence and the position of the disulfide bonds have been identified (Fox et al., 1979). Despite myotoxin *a*'s small size, it is antigenic and an antiserum capable of neutralizing its myotoxic action has recently been produced (Ownby et al., 1979).

During the course of the isolation of myotoxin *a*, Cameron & Tu (1977) observed that a high molecular weight venom fraction (which eluted in the void volume of Sephadex G-50) induced considerable tissue damage following injection into experimental lab animals. A toxin has been purified from the venom of the prairie rattlesnake (*C. viridis viridis*) in order to study the specific high molecular weight toxin responsible for this tissue damage. This toxin has been designated viriditoxin. This study of viriditoxin is intended to provide for a more complete understanding of venom-induced tissue damage and of the overall toxic action of rattlesnake venoms.

In the present investigation, it has been shown that viriditoxin is a high molecular weight toxin with a molecular weight of approximately ~115 000. Presented in this report are some of the chemical properties of viriditoxin, such as amino acid composition, isoelectric point, secondary structure, and molecular weight. Several biological actions of viriditoxin are also discussed: myotoxicity, hemorrhagic activity, hemolytic activity, proteolytic activity, and the effect upon serum creatine kinase levels.

Materials and Methods

Crude venom was purchased as a lyophilized powder from Miami Serpentarium Laboratories. DEAE[1] Bio-Gel A was purchased from Bio-Rad Laboratories. Sephadex G-150-40, Sephadex G-150-120, insulin, Temed, and creatine kinase diagnostic kit 40-UV were purchased from Sigma Chemical Co. Spectrophor dialysis tubing was purchased from Fisher Chemical Co. Ampholytes over the pH ranges of 3.5-10 and

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[1] Abbreviations used: DEAE, diethylaminoethylagarose; Tris, tris(hydroxymethyl)aminomethane; Temed, *N,N,N',N'*-tetramethylethylenediamine; Bis, *N,N'*-methylenebis(acrylamide); Cl_3CCOOH, trichloroacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; ADP, adenine diphosphate; NADH, reduced nicotinamide adenine dinucleotide; LD_{50}, lethal dose for 50%; CD, circular dichroism; $[\theta]_{mrw}$, mean residue ellipticity; $[\theta]_M$, molar ellipticity.

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4–6.5 were obtained from Pharmacia Chemicals. Hide powder azure was purchased from Calbiochem-Behring Corp. Casein was purchased from J. T. Baker Chemical Co.

Isolation Procedure. All fractionation steps were performed at 2–4 °C. Approximately 500 mg of lyophilized prairie rattlensnake crude venom was dissolved in 6 mL of 0.05 M Tris, pH 9.0, containing 0.1 M KCl. This was then applied to a column of Sephadex G-150-120 (2.5 × 90 cm) which had been equilibrated with Tris buffer. The column was developed at a flow rate of 10 mL/h, and eluate fractions of 2.3 mL/tube were collected. The absorbance at 280 nm of each tube was measured with a Beckman Model DG-B spectrometer.

The appropriate tubes of fraction A-2 (see Results) from two successive gel filtration steps were combined and prepared for further fractionation by dialysis against 1 L of 4 mM monoethanolamine buffer, pH 9.0, containing 2 mM KCl. The sample was then applied to a DEAE Bio-Gel A ion-exchange column (1.5 × 40 cm) equilibrated with the monoethanolamine buffer. Fraction tubes up to number 55 were eluted with monoethanolamine buffer. A linear salt gradient of 2–300 mM KCl in a total of 100 mL of monoethanolamine buffer was initiated at tube 55. The flow rate was 30 mL/h with fractions of 2.3 mL/tube collected. The absorbance of each tube was monitored at 280 nm.

The combined B-4 fractions from two runs on the DEAE column were then concentrated on a Pasteur pipet column of DEAE Bio-Gel A. The B-4 fraction was dialyzed against 4 mM monoethanolamine buffer, pH 9.0, containing 2 mM KCl and applied to the pipet DEAE column. The sample was then eluted with 0.05 M Tris buffer, pH 9.0, containing 0.1 M KCl. The volume of the B-4 fractions was reduced to 3–4 mL.

This concentrated sample was then applied to a Sephadex G-150-40 gel filtration column (2.5 × 90 cm) which had been equilibrated with the Tris buffer. Elution was carried out at a flow rate of 5 mL/h, and fractions of 2 mL/tube were collected. The absorbance at 280 nm of each tube was determined, and the appropriate tubes of fraction C-1 were pooled and stored at 2 °C.

Homogeneity. Disc gel electrophoresis was performed by using a Tris–glycinate disc gel system (Weber & Osborn, 1975). Solution and gel compositions were as follows: stacking gel, 2.25% acrylamide monomer, 0.62% methylenebis(acrylamide), and 0.1% ammonium persulfate in 30 mM Tris containing 1 mL/L Temed, at pH 6.8; resolving gel, 7.5% acrylamide monomer, 0.27% methylenebis(acrylamide), and 0.1% ammonium persulfate in 50 mM Tris containing 1 mL/L Temed, and pH 8.8; reservoir buffer, 50 mM Tris and 385 mM glycine at pH 8.3.

Gels were electrophoresed at 2 °C, with normal polarity, at 0.5 mA/tube for 30 min, followed by 1 mA/tube for 1 h and then 2 mA/tube until the bromophenol blue dye marker migrated to the bottom of the gel. Gels were stained for 3 h in 0.25% Coomassie blue in a solution of 50% methanol and 10% acetic acid, destained with a solution of 5% acetic acid and 16% isopropyl alcohol, and stored in 7.5% acetic acid at 4 °C.

Estimate of Isoelectric Point. The p*I* of viriditoxin was estimated by isoelectric focusing gel electrophoresis over the pH ranges 3.5–10 and 4–6.5 in 6-cm gels. Gel and solution compositions were as follows: focusing gel, 5% ampholytes, 8.1% acrylamide monomer, 0.2% Bis, and 0.5% ammonium persulfate containing 1 mL/L Temed; upper reservoir buffer, 2 mL of concentrated H_2SO_4/L; lower reservoir buffer, 8 mL of monoethanolamine/L. Gels were focused, with reverse polarity, at 65 V for 3 h and 100 V for 4 h, following which they were soaked in Cl_3CCOOH for 48 h. The gels were then stained, destained, and stored as described above. For determination of the pH gradient within the gels, a companion gel which was not loaded with sample was focused along with the sample gels. This gel was cut into 2-mm slices, and the pH of each slice was measured in 1 mL of distilled water immediately following electrophoresis.

Carbohydrate Test. Viriditoxin was tested for carbohydrates with the electrophoretic glycoprotein stain developed by Segrest & Jackson (1972).

Estimates of Molecular Weight. The molecular weight of viriditoxin was estimated by $NaDodSO_4$ gel electrophoresis by a method similar to that described for Tris–glycinate electrophoresis with the following exceptions. Prior to electrophoresis the toxin was reduced and alkylated. Stacking gels of 10, 12, and 14% acrylamide monomer were used. Stacking and resolving gels, and the reservoir buffer, were made to contain 0.1% $NaDodSO_4$.

Following electrophoresis the gels were soaked for 2 h in 20% Cl_3CCOOH and then stained and destained as previously described. The protein mobility relative to the dye front was determined for each of the three gel concentrations. From these data a plot of the mobility vs. acrylamide gel concentration allowed for an estimate of molecular weight as described by Hendrick & Smith (1968).

Amino Acid Composition. Amino acid compositions of the carboxamidomethylated toxin were determined with a JEOL Model JLC-6AH automatic amino acid analyzer. Hydrolysis of samples was done with constant boiling HCl at 110 °C for 24, 48, and 72 h (Spackman et al., 1958). Tryptophan was determined spectrophotometrically on a Cary Model 118 recording spectrometer by the method of Edelhoch (1967).

CD Spectroscopy. The circular dichroic spectra of viriditoxin were taken on a Jasco Model J-41C spectropolarimeter at 25 °C. The solvent used was 10 mM phosphate buffer at pH 7. A protein concentration of 0.7 mg/mL was used in 1.0- and 0.05-cm cells to obtain the CD spectra over the range of 200–345 nm. The protein concentration was determined on the basis of dry weight.

Assay for Proteolytic Activity. The method of Rinderknecht et al. (1968) was employed to determine the proteolytic activity of viriditoxin against hide powder azure. Hide powder azure is an insoluble protein complex labeled covalently with Remazobrilliant Blue, which is solubilized by proteolytic cleavage, thus allowing the reaction to be monitored spectrophotometrically at 595 nm.

Proteolytic activity was also assayed by using dimethylcasein as a substrate, prepared according to Lin et al. (1969). Amino groups were determined with 2,4,6-trinitrobenzenesulfonic acid by measuring the absorbance at 420 nm of sulfite complexes of the trinitrophenylated amino groups (Fields, 1971).

A third technique, intended to determine the proteolytic activity of viriditoxin against B chain insulin substrate, was also used. After an incubation period, the insulin–toxin mixture was developed on thin-layer chromatography strips. Following chromatography the strips were reacted with ninhydrin to locate protein positions. The presence of ninhydrin-positive sites on the strips, which were not found on strips developed with insulin and heat-denatured toxin, was taken to indicate proteolysis.

Serum Creatine Kinase Assay. The effects of crude venom, myotoxin *a*, and viriditoxin on serum creatine kinase levels were determined by a method based on the procedure of Tanzer & Gilvarg (1959). This assay technique utilizes the ADP produced in creatine phosphorylation to couple this re-

action to pyruvic acid production from phosphoenolpyruvate by pyruvate kinase. The pyruvic acid is then converted to lactic acid by lactic dehydrogenase which results in lowered NADH levels in the assay mixture, thus allowing for creatine kinase activity to be monitored spectrophotometrically at 340 nm. All necessary substrates and enzymes are included in the reaction mixture with the exception of creatine kinase.

Swiss Webster white mice (20 g) were injected intramuscularly with 10 μg of each toxic substance in a total volume of 0.1 mL of 0.9% saline solution. Controls using uninjected mice and saline-injected mice were also assayed. The effects of myotoxin *a*, prairie rattlesnake crude venom, and viriditoxin were examined by using six mice for each injection type. Blood was extracted from the oribital plexus of the mice 3 h following the injections and centrifuged, and ~100 μL of the serum was used for each assay. The quantity of creatine kinase which will phosphorylate 1 nmol of creatine/min was defined to be 1 unit of activity.

Assay for Myotoxicity. Fractions were tested for myotoxicity by the method of Ownby et al. (1976). Swiss Webster white mice (20 g) were injected intramuscularly into the medial aspect of the thigh with 0.1 mL of 0.9% NaCl solution containing 50 μg of protein from the fraction to be assayed. Control injections of 0.1 mL of 0.9% NaCl were also made concurrently with each assay. The mice were then sacrificed by cervical dislocation 72 h following injection. An approximately cubical tissue sample of 4–5 mm on a side was then removed from the injected thigh. Tissue samples were immediately fixed in Bouin's fixative for 24 h at room temperature. The tissue was then washed for 4 h in running water, dehydrated in an autotechnicon, and embedded in paraffin. The embedded tissue was subsequently sectioned on a microtome and stained for observation under the light microscope. Tissue sections were stained alternately with Van Gieson stain and with hematoxylin and eosin.

Assay for Hemorrhagic Activity. Swiss Webster white mice (20 g) were injected subcutaneously in the center of the back and sacrificed by cervical dislocation after 6 h. The quantity of toxin which induced a hemorrhagic reaction of 5 mm in diameter on the interior surface of the hide was defined to be the minimum hemorrhagic dose.

Assay for Hemolytic Activity. Direct hemolytic activity was assayed with washed rabbit erythrocyte suspensions in 0.9% NaCl adjusted so that 100% hemolysis gave an A_{540} reading of ~1.0 (Jeng et al., 1978). The reaction mixture contained 10–400 μg of toxin in 1 mL of 0.9% NaCl and was incubated at 37 °C for 30 min, followed by an incubation for 20 min at 4 °C. The reaction mixture was then centrifuged, and the supernatant was read for released hemoglobin at 540 nm. Indirect hemolysis was assayed by the same method with the addition of phosphotidylcholine to the reaction mixture.

Lethality. For estimation of the toxicity of viriditoxin, a series of intravenous injections were made in 20-g mice. The quantity of toxin injected covered the range of 60–160 μg/mouse, with six mice injected at each dose. Mice were checked for lethality 24 h postinjection.

Immunodiffusion. Antiserum to hemorrhagic toxin *c* (Bjarnason & Tu, 1978) was prepared by injecting purified hemorrhagic toxin *c* (1.25 mg) mixed with Difco complete adjuvant into the lower hind legs of a New Zealand white rabbit. Three days later, 0.63 mg of toxin was injected, again with adjuvant. A final injection of 1.25 mg of toxin, in saline only, was then made 3 weeks after the original injection. About 1 week later, the rabbit was bled from the ear, and the serum was separated by centrifugation.

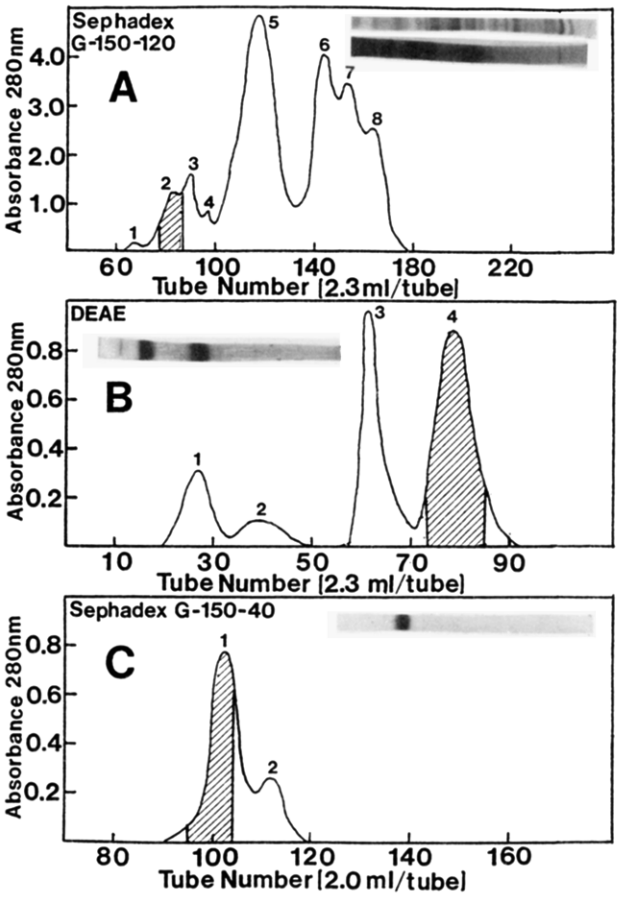

FIGURE 1: Isolation scheme of viriditoxin. The isolation procedure is described under Materials and Methods. Fractions containing viriditoxin are shaded. The electrophoretic patterns of each fractionation step are inset: crude venom (A, above); fraction A-2 (A, below); fraction B-4 (B); pure viriditoxin (C).

Immunodiffusion was performed in 1% Difco agar at 28 °C for 24 h. The concentrations of both hemorrhagic toxin *c* and viriditoxin were 2 mg/mL. After the experiment, the plate was first deproteinized with normal saline for 2 days; the saline was changed 2–3 times a day. The plate was then desalinated with distilled water for 8 h. The plate was stained with 0.02% Amido Black in 10% acetic acid for 25 min and then destained with 10% acetic acid for 2 days with 2–3 changes of destain per day.

Results

Isolation of Viriditoxin. The elution profile of crude prairie rattlesnake venom on the Sephadex G-150-120 column is shown in Figure 1A. Each of the eight fractions which resulted from this gel filtration step was tested for myotoxicity and hemorrhage. Fractions A-2, A-3, and A-4 showed both myotoxic and hemorrhagic activity, while fractions A-6 and A-7 induced myonecrosis but did not induce hemorrhage. All other fractions were devoid of both tissue effects. Since fraction A-2 exhibited the strongest myotoxicity, it was chosen for further purification. The myotoxicity associated with fractions A-6 and A-7 was assumed to be due to the actions of myotoxin *a* (Cameron & Tu, 1977).

The elution profile of fraction A-2 on DEAE Bio-Gel A is shown in Figure 1B. Fractions B-1 and B-2 eluted from the column prior to the initiation of the salt gradient which was used to elute fractions B-3 and B-4. Both fractions B-3 and B-4 induced myonecrosis. However, since fraction B-4 exhibited no proteolytic activity against hide powder azure, it was considered to be of far more potential interest than the

Table I: Yield, Biological Activity, and Isoelectric Point of Viriditoxin

prepn	yield[a] (%)	hemorrhagic act.	myotoxic act.	isoelectric point
1	0.5	+	+	4.9
2	0.6	+	+	4.8
3	0.6	+	+	4.8

[a] Purified from 2 g of crude venom/preparation.

Table II: Amino Acid Composition of Viriditoxin

amino acid	hydrolysis[a] 24 h	48 h	72 h	cor[b] value (μmol)	mol of AA/mol of protein	nearest integer
Asx	10.82	11.06	11.33	11.07	109.55	110
Thr	5.37	5.33	5.29	5.41	53.54	54
Ser	8.98	8.86	8.60	9.17	90.75	91
Glx	11.87	11.99	11.91	11.92	117.96	118
Pro	3.33	3.54	3.41	3.43	33.94	34
Gly	6.90	6.98	6.83	6.90	68.28	68
Ala	5.94	5.69	5.56	5.73	56.71	57
Val	5.64	5.82	5.88	5.78	57.20	57
Met	1.77	1.69	1.87	1.78	17.62	18
Ile	4.02	4.09	3.81	3.97	39.29	39
Leu	6.50	5.98	6.32	6.27	62.05	62
Tyr	3.53	3.47	3.42	3.59	35.53	36
Phe	5.64	5.52	5.61	5.59	55.32	55
His	2.55	2.50	2.52	2.57	25.43	25
Lys	8.81	8.28	8.45	8.51	84.22	84
Art	3.48	3.64	3.65	3.59	35.53	36
$^1/_2$-Cys	4.88	5.51	5.53	5.31	52.55	53
Trp[c]					21.35	21

[a] Values normalized to mol %. [b] The hydrolysis values for threonine, serine, tyrosine, and histidine were extrapolated to time zero. [c] Tryptophan content was determined by the spectroscopic method of Edelhoch (1967).

highly proteolytic fraction B-3 and was therefore subjected to further fractionation.

The elution profile of fraction B-4 on Sephadex G-150-40 is shown in Figure 1C. Although fraction C-2 showed no discernible myotoxicity or hemorrhagic activity, fraction C-1 induced both effects and was designated viriditoxin. A 2-g preparation of crude venom resulted in the purification of 10–12 mg of viriditoxin, for a final yield of ~0.6% (Table I).

Homogeneity. The progress of the purification process can be seen by the successive increases in electrophoretic homogeneity as evidenced by comparing the gels of crude venom (Figure 1A), fraction A-2 (Figure 1A), fraction B-4 (Figure 1B), and fraction C-1 (Figure 1C). As shown in Figure 1C (inset) viriditoxin was electrophoretically homogeneous in Tris–glycinate disc gel electrophoresis. The homogeneity of viriditoxin was also confirmed by isoelectric focusing gel electrophoresis. The isoelectric pH of the toxin was shown to be 4.8. The use of the glycoprotein stain with both gel systems failed to detect the presence of any carbohydrates in the toxin's chemical makeup.

Amino Acid Composition. The amino acid composition of viriditoxin is given in Table II. The values presented in Table II are the nearest integer to the corrected value multiplied by a constant which was used to correct for the difference between the minimum molecular weight calculated from the corrected values and the actual molecular weight of the toxin (which is discussed below). The corrected value represents an average of the three hydrolysis times of 24, 48, and 72 h, except for the amino acids threonine, serine, tyrosine, and histidine which were extrapolated to time zero. The most conspicuous aspect of the amino acid composition is the high content of acidic amino acids. The presence of a high concentration of aspartic acid and glutamic acid residues correlates rather well with the 4.8 isoelectric point obtained for viriditoxin.

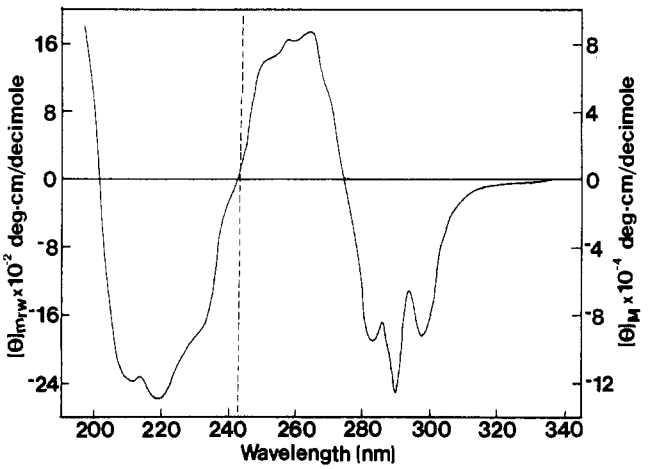

FIGURE 2: Circular dichroic spectrum of viriditoxin. Mean residue ellipticities ($[\theta]_{mrw}$) for the 200–242-nm region are based on a mean residue weight of 115. Molar ellipticities ($[\theta]_M$) for the 242–345-nm region are based on a molecular weight of 115 000.

Molecular Weight. The molecular weight of reduced and alkylated viriditoxin was determined on Tris–glycinate–Na-DodSO$_4$ gels of 10, 12, and 14% acrylamide. Gel electrophoresis revealed two bands which corresponded to molecular weights of approximately 57 000 and 62 000. From these data it was concluded that viriditoxin is a protein, consisting of two subunits, with a total molecular weight of ~119 000. This molecular weight estimate is in good agreement with viriditoxin's retention on gel filtration (unpublished data).

CD Spectroscopy. The CD spectrum of viriditoxin is shown in Figure 2. The spectrum has a double minimum at 219 nm ($[\theta]_{mrw} = 2.60 \times 10^3$ deg cm^2/dmol) and 212 nm ($[\theta]_{mrw} = 2.37 \times 10^3$ deg cm^2/dmol). Three additional negative bands are apparent in the 270–310-nm portion of the spectra. These three local minima are located at 283 nm ($[\theta]_M = 9.37 \times 10^4$), 290 nm ($[\theta]_M = 1.25 \times 10^5$), and 298 nm ($[\theta]_M = 9.20 \times 10^4$). The only positive CD band is a broad band extending from approximately 245 to 275 nm. This positive band has a maximum of $[\theta]_M = 8.71 \times 10^4$ at 265 nm.

Interpretation of the bands in the 200–240-nm region is made quite difficult by the apparent overlap between the optically active transitions which arise from aromatic side-chain contributions. The positive contributions from these aromatic amino acid residues are superimposed upon the negative CD bands generated by the peptide linkages, thus greatly complicating the CD spectra in this region and resulting in low band intensities. The amino acid residue which is most likely responsible for the greatest positive contribution in this region is tryptophan which has been shown to have a strong positive CD band at 223 nm (Legrand & Viennet, 1965). Tyrosyl residues may also be responsible for some of the cancellation in this region.

The broad positive band spanning the 245–275-nm region of the spectra quite probably arises from cystine transitions. The fine structure associated with this band is interpreted to result from phenylalanine contributions. The negative portion of the spectra in the 275–310-nm region have two possible explanations: These bands may arise from the cumulative effect of a negative tryptophanyl L_b band superimposed onto a negative cystinyl and/or tryptophanyl L_a band. The gradual return to the base line in the 310–340-nm region of the spectra is particularly suggestive of a negative cystinyl band. A second valid interpretation is that a positive tryptophanyl L_b band is superimposed on a stronger negative cystinyl and/or trypto-

Table III: Serum Creatine Kinase Levels

injection type	units/mL[a]
uninjected mice	31 ± 3.8[b]
injected with saline	46 ± 4.2
injected with crude venom	84 ± 6.9
injected with myotoxin *a*	133 ± 10.6
injected with viriditoxin	126 ± 9.4

[a] One unit of activity was defined as the amount of enzyme needed to phosphorylate 1 nmol of creatine/min at 25 °C. [b] Sample variation determined at 95% confidence.

phanyl L_a band. In this case the two troughs at 286 and 294 nm would actually correspond to the positive tryptophanyl L_b band. Assignments of the bands in the aromatic region of the spectra were based upon the review of Strickland (1974).

The double minimum of the CD spectra of viriditoxin is somewhat suggestive of an α helix; however, the low intensity of these minima clearly indicates that the CD contributions to this portion of the spectra are more complex than patterns that commonly arise from helix, β structure, or random coil. Consequently, a satisfactory conformational analysis of viriditoxin is not possible at this time by using conventional, quantitative methods of spectral analysis. There are, however, indications that viriditoxin may contain a significant portion of β structure: Since α-helical segments elicit high-intensity bands in the 205–225-nm region of the spectra (frequently $[\Theta]_{mrw} = 2 \times 10^4$ deg cm^2/dmol or greater), the low amplitude of the viriditoxin spectra effectively precludes the possible existence of any significant portions of α helix within the protein. Similarly, the presence of a high content of random coil can be considered unlikely by virtue of the positive ellipticity at 200 nm, rather than a strong negative band in this region as is characteristic of random coil. Consequently, the existence of a significant degree of β structure, perhaps highly distorted, can be inferred to be present in viriditoxin. This postulated β structure can be supported by a comparison with other proteins, such as the immunoglobulins which have similar low-intensity bands in the far-ultraviolet region (Bjork & Lindh, 1974) and have been demonstrated to contain prominent portions of β and β-like structure by X-ray crystallographic studies (Poljak et al., 1973; Poljak, 1975).

Proteolytic Activity. The proteolytic activity of viriditoxin was tested against three different substrates: dimethylcasein, B chain insulin, and a protein–dye complex. Detectable levels of proteolytic activity were found to occur only when dimethylcasein was used as a substrate. The proteolytic activity of viriditoxin, per microgram, was found to be significantly less than 1% that of trypsin on dimethylcasein. When compared to the proteolytic activity of hemorrhagic toxins *a*, *b*, *c*, and *d* (Bjarnason & Tu, 1978), the activity of viriditoxin per microgram was ~3% as active as these toxins.

Biological Activity. Serum Creatine Kinase Levels. The elevation of serum creatine kinase levels in laboratory mice following envenomation with prairie rattlesnake crude venom, myotoxin *a*, and viriditoxin can be seen in Table III. Injections of viriditoxin and myotoxin *a* both induced significantly higher serum creatine kinase levels than that of crude prairie rattlesnake venom. The increase in the serum creatine kinase levels of saline-injected mice over uninjected mice can be attributed to damage resulting from the intramuscular injection.

Myonecrosis. The histological effects of viriditoxin appear to be very similar to the effects of prairie rattlesnake crude venom, as previously described (Stringer et al., 1972; Ownby et al., 1976). The prevalent pathological effects of viriditoxin on muscle tissue, apparent under the light microscope, are a central migration of nuclei, loss of muscle fiber striations, and a severe vacuolization of muscle cells which ultimately results in cell lysis.

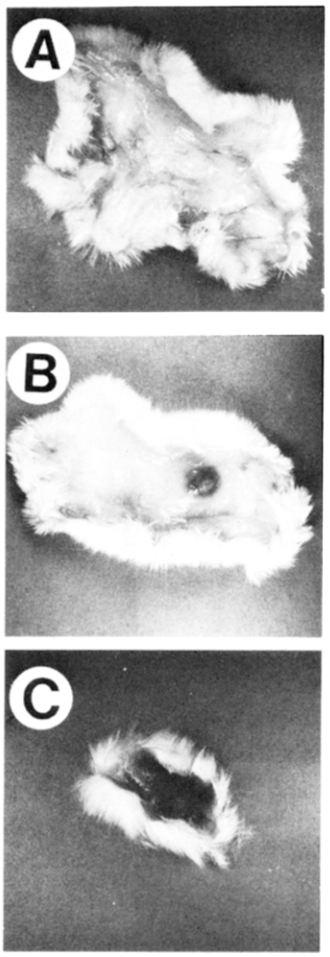

FIGURE 3: Example of viriditoxin-induced hemorrhage 6 h after subcutaneous injection into 20-g mice. (A) Interior of the skin of a mouse which received a control injection of 0.1 mL of 0.9% NaCl. (B) Skin of a mouse which received a 3-μg injection of viriditoxin in 0.1 mL of saline (the diameter of the hemorrhagic circle is 6–7 mm). (C) Portion of a skin from a mouse injected with 15 μg of viriditoxin.

Hemorrhagic Activity. Following subcutaneous injections, viriditoxin was demonstrated to be a hemorrhagic toxin with a minimum hemorrhagic dose of 1.5 μg in 20-g mice. Examples of viriditoxin-induced hemorrhage can be seen in Figure 3. Examination under the light microscope also revealed hemorrhage in the muscle tissue of those mice which received intramuscular injections of viriditoxin.

Hemolytic Activity. The hemolytic activity of viriditoxin was tested in the presence and absence of phosphatidylcholine. It was found that phosphatidylcholine was required for hemolysis. Even in the presence of phosphatidylcholine the demonstrated hemolytic action of viriditoxin was very weak, with a toxin concentration of >400 μg/mL needed in order to obtain 50% hemolysis.

Lethality. A series of intravenous injections indicated that viriditoxin was less toxic than prairie rattlesnake crude venom. An LD_{50} estimate of ~5.0 μg/g was obtained for viriditoxin as compared to 1.01 μg/g for *C. viridis viridis* crude venom (Friederich & Tu, 1971). Viriditoxin demonstrated 100% lethality at doses of 8 μg/g or greater, and doses of 3 μg/g or less demonstrated no lethality.

Immunological Cross-Reactivity. As can be seen in Figure 4, the reaction of hemorrhagic toxin *c* with its own antiserum

Table IV: Comparison of Tissue Damaging Toxins Isolated from Rattlesnake Venom

genus:	*Crotalus*		*Crotalus*				*Crotalus*
species:	*viridis*		*atrox*				*durissus*
subspecies:	*viridis*						*durissus*
			hemorrhagic toxins				
	viriditoxin	myotoxin *a*	*a*	*b*	*c*	*e*	crotamine
hemorrhage[a]	+	–	+	+	+	+	–
myonecrosis[a]	+	+	–	+[b]	–	–	+[c]
proteolytic[a]	+	–	+	+	+	+	–
total residues	1018	42[d]	636	200	213	219	42
isoelectric pH	4.8	9.6	acidic	basic	acidic	5.6	basic
reference	present investigation	Cameron & Tu (1977)	Bjarnason & Tu (1978)				Laure (1975)

[a] A (+) indicates that the biological activity has been detected for that toxin; a (–) indicates that the activity has not been observed. [b] Ownby et al. (1978). [c] Cameron & Tu (1978). [d] Cameron and Tu originally reported 39 residues; however, when myotoxin *a* was sequenced, 42 residues were found (Fox et al., 1979).

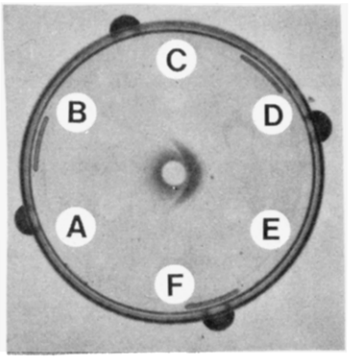

FIGURE 4: Immunodiffusion experiment of viriditoxin (*C. viridis viridis*) against the antisera for hemorrhagic toxin *c* (*C. atrox*). (Center well) Antisera for hemorrhagic toxin *c*; (A and B) viriditoxin; (C and F) saline control; (D and E) hemorrhagic toxin *c*.

produced a precipitin line. However, viriditoxin did not form a precipitin line against hemorrhagic toxin *c* antisera. This lack of immunological cross-reactivity indicates that there is no common antigenicity for viriditoxin and hemorrhagic toxin *c*. This is clear evidence that these two toxins are totally distinct from one another.

Discussion

Venom components have been extensively studied by numerous investigators and have been the subject of several reviews (Devi, 1968; Tu, 1973, 1977). However, the majority of these studies have concentrated upon the venoms of cobras, kraits, and sea snakes. The venoms of these snakes have proven to be of particular interest due to the presence of neurotoxins within these venoms and the high lethality associated with these toxins. In contrast, the toxic components of rattlesnake venoms, especially those components responsible for local tissue damage, have not been well studied. Myonecrosis is one of the most serious local tissue effects which result from rattlesnake bites. To date, the only report of an isolated myotoxin, myotoxin *a*, is from prairie rattlesnake (*C. viridis viridis*) venom (Cameron & Tu, 1977) (see Table IV). However, crotamine (Table IV), a paralytic polypeptide isolated from *Crotalus durissus terrificus* venom (Laure, 1975), has recently been shown to induce muscle necrosis (Cameron & Tu, 1978). Another tissue damaging effect of rattlesnake venoms is hemorrhage (Ownby et al., 1974). Those components of rattlesnake venom which are responsible for the hemorrhagic activity have been somewhat better studied than the myotoxins. The isolation of hemorrhagic toxins from the venoms of Asian snakes has been reported (Oshima et al., 1968; Takahashi & Ohsaka, 1970; Grotto et al., 1967), and hemorrhagic toxins have also been isolated from venoms of South American and Japanese snakes (Mandelbaum et al., 1975; Nikai et al., 1977). More recently, five hemorrhagic toxins were isolated from the venom of the western diamondback rattlesnake (*C. atrox*) (Bjarnason & Tu, 1978) (Table IV).

The tissue damaging toxins which have been isolated from the venoms of snakes in the family Crotalidae are summarized in Table IV. As can be seen from this table, although viriditoxin exhibits similar biological activities to other toxins isolated from Crotaline venoms, it is a chemically unique toxin. Viriditoxin, which contains a high number of acidic amino acid residues, is the first reported nonbasic protein capable of inducing myonecrosis. The biological activity of hemorrhagic toxin *b* isolated from *C. atrox* venom appears to have the greatest similarity to viriditoxin, with both proteins having myonecrotic and hemorrhagic activities. However, these two proteins have quite different amino acid compositions and molecular weights.

Both viriditoxin (*C. viridis viridis*) and hemorrhagic toxin *c* (*C. atrox*) are acidic proteins capable of inducing hemorrhage. An immunological comparison was made in order to detect any similarities between these two toxins. Immunological tests are very sensitive to similarities and differences between proteins. For instance, crotoxin isolated from the venom of *C. durissus terrificus* (South America) and Mojave toxin isolated from the venom of *Crotalus scutulatus* (North America) cross-reacted immunologically (Gopalakrishnakone et al., 1980). This is as would be expected since these two toxins are very similar chemically (Cate & Bieber, 1978). However, viriditoxin and hemorrhagic toxin *c* did not cross-react, thus confirming that these two proteins are quite different.

The present investigation emphasizes that snake venom toxins may have more than one biological activity. Viriditoxin has both hemorrhagic and myotoxic activities. It is not uncommon for a toxin to exhibit more than one biological activity; for example, some cytotoxins have been shown to also have cardiotoxic activity. Additionally, both myotoxin *a* and crotamine demonstrate myotoxic as well as hemolytic activity (Tu, 1977). The biological versatility of these toxins is probably a function of the toxin's membrane specificity. Those toxins which are capable of attacking both erythrocyte and muscle cell membrane, such as myotoxin *a* (*C. viridis viridis*), are apparently less restricted by membrane structure than those toxins which appear to be active only against the endothelial cells of capillary tubes, such as hemorrhagic toxins *a* and *c* (*C. atrox*).

Of all the snake venom components, neurotoxins from the venoms of Elapidae and Hydrophiidae are the most extensively

investigated. Neurotoxins consist of either 60–62 or 70–74 amino acid residues and are highly basic proteins. It is clear from the large size, acidic nature, and biological activities of viriditoxin that this toxin is completely different from the well-studied neurotoxins. Chemically, viriditoxin is most similar to hemorrhagic principle HR-1 isolated from *Trimeresurus flavoviridis* venom (Omori-Satoh & Ohsaka, 1970). Hemorrhagic principle HR-1 is an acid protein with a molecular weight of 100000. This toxin has been shown to induce hemorrhage and has not been found to exhibit any proteolytic activity.

Serum creatine kinase levels are frequently used to monitor the cellular damage to heart tissue following a myocardial infarction. This method utilizes the release of creatine kinase into the circulatory system as a result of damage to the muscle cells of the cardiac or skeletal muscles. By use of experimentally envenomated mice, serum creatine kinase levels have previously been used to isolate a muscle damaging toxin from tarantula venom (Lee et al., 1974); however, the present study represents the first application of serum creatine kinase assays to snake venom induced myonecrosis. The utilization of serum creatinine kinase levels as an indicator of myotoxicity has three distinct advantages over the use of conventional histological methods. First, elevated serum creatine kinase levels can be detected very rapidly (2–3 h after injection), while the histological assay often requires several days. Second (as reported in Table III), serum creatine kinase assays are sensitive to lower injection doses (10 μg, as opposed to 50 μg, with the histological assay). Finally, serum creatine kinase assays allow for quantification, thus facilitating comparative studies of the tissue damaging effects of various venoms and venom components. It can be concluded that the use of both serum creatine kinase assays and histological assays, in conjunction with one another, is the most effective means of studying the myotoxicity of snake venom.

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