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# COMPARATIVE BIOCHEMISTRY AND PHARMACOLOGY OF SALIVARY GLAND SECRETIONS

## II. CHROMATOGRAPHIC SEPARATION OF THE BASIC PROTEINS FROM SOME NORTH AMERICAN RATTLESNAKE VENOMS

CARLOS A. BONILLA*

*Department of Physiology and Biophysics, School of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colo. 80521 (U.S.A.)*

AND

M. KATIE FIERO

*Department of Biochemistry, University of Texas, Southwestern Medical School, Dallas, Texas 75206 (U.S.A.)*

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SUMMARY

The highly basic proteins from three North American rattlesnake venoms have been isolated by a simple combination of Bio-Gel P-2 recycling adsorption chromatography and ion exchange on carboxymethyl-cellulose. These basic proteins constitute less than 10% of the total protein in the crude venoms and are characterized by having small molecular weights, isoelectric points above ~ pH 10.8 and pharmacological activities (*in vivo* and *in vitro*) resembling those of crotamine, the neurotoxin from *Crotalus durrisus terrificus*, the South American tropical rattlesnake.

## INTRODUCTION

The lethal action of venoms is undoubtedly due to interaction of each venom's specific mixture of components. Separation and bioassay of these components shows the majority of toxic activity in fractions distinct from the enzymes, which frequently possess neurotoxic activity. Isolation and purification of many of these toxic elements has shown them to be low-molecular-weight polypeptides. Of the venoms examined, neurotoxins of this nature have been demonstrated in all elapid snakes (cobras and kraits), all Australian venomous snakes, all hydrophid snakes, and in some viperid and crotalid species. Extensive reviews on the subject have been published[1-3]. Scorpion venom neurotoxins have also been recently isolated[4,5] and shown to be

* To whom reprint requests should be directed.

highly basic, small-molecular-weight polypeptides not unlike those found in snake venoms.

With the exception of crotamine[6], the basic protein neurotoxin from *Crotalus durrisus terrificus*, nothing is known about the basic proteins from American rattlesnakes. The absence of highly basic proteins in said venoms has, in fact, been reported by several authors[7,8]. It is of interest to note, however, that small-molecular-weight basic proteins having neurotoxic activity have already been obtained in a high degree of purity from *C. v. helleri*[9] and *C. adamanteus*[10]. The purification and some properties of the highly basic proteins from *C. h. horridus* (timber rattlesnake), *C. h. atricaudatus* (canebrake rattlesnake), and *C. v. viridis* (prairie rattlesnake) are the subject of this report.

## EXPERIMENTAL

### *Materials*

Bio-Gel P-2 (200–400 mesh) and carboxymethyl-cellulose (Cellex-CM) were purchased from Bio-Rad Laboratories. The reagents for polyacrylamide electrophoresis were obtained from the E-C Corporation. Cellophane tubing purchased from the Union Carbide Corporation was gently boiled for about 40 min in $10^{-4}$ *M* EDTA and stored in distilled water at 2° until used. Lyophilized *C. h. horridus*, *C. h. atricaudatus*, and *Naja naja* venoms were obtained from Sigma Chemical Co., St. Louis, Mo. *C. v. viridis* venom was collected in our serpentarium.

Venoms were prepared for chromatography in the following manner: A given amount of venom was dissolved in a minimal amount of distilled water cleared by centrifugation at 27,500 × *g* for 30 min and the supernatant fluid used as starting material. All other chemicals used were reagent grade; distilled water was passed through a large column of Amberlite MB-1 resin (Mallinckrodt, analytical reagent grade) and had a resistance of approximately $2 \cdot 10^6$ Ω. Unless otherwise stated, all analytical procedures were carried out at 2°.

### *Methods*

*Purification of basic proteins.* All effluents were continuously monitored at 254 mμ with an ISCO Model UA ultraviolet analyzer. Protein in the effluent fractions was determined by measuring the absorbance at 280 mμ, appropriate dilutions of the fractions being made when absorbance was too high to allow accurate measurement. Individual peaks were pooled and concentrated by lyophilization following dialysis against distilled water. The length of dialysis varied with each chromatographic procedure, as follows: adsorbed fractions on Bio-Gel P-2 (5 h) and CM-cellulose fractions (4 h). Protein concentration was determined by the procedure of LOWRY *et al.*[11], using bovine serum albumin as the standard.

*Adsorption chromatography on polyacrylamide gel.* Dry Bio-Gel P-2 was suspended in an excess of water for at least 98 h, the fine particles removed by decantation and the slurry packed into a column (2.5 × 100 cm) by gravity flow, followed by equilibration with distilled water for 48 h. Recycling chromatography was performed exactly as previously described[12] using high-precision chromatronix valves for sample injection and fraction retrieval.

*CM-cellulose chromatography.* Carboxymethyl-cellulose (Cellex-CM) was further

purified and prepared for column chromatography by the method of PETERSON AND SOBER[13]. Following equilibration in 0.04 *M* sodium citrate (pH 3.48) the material was packed to a bed height of 22 cm in 1.5 × 30 cm columns. This was followed by further washing with 1 l of the same buffer and the samples added to the resin surface. A constant flow rate was maintained with peristaltic LKB pumps (LKB Instruments, Inc., Maryland) with fractions of approximately 3.0 ml collected by means of an automatic fraction collector (ISCO).

*Analytical procedures*

*Polyacrylamide electrophoresis.* Fractions at the final stage of purification were identified and compared to whole venom by means of gel electrophoresis (E-C Apparatus Corp.). This procedure was carried out in acidic, 6 *M* urea polyacrylamide gels by a modification of the method of JORDAN AND RAYMOND[14] as previously described[15].

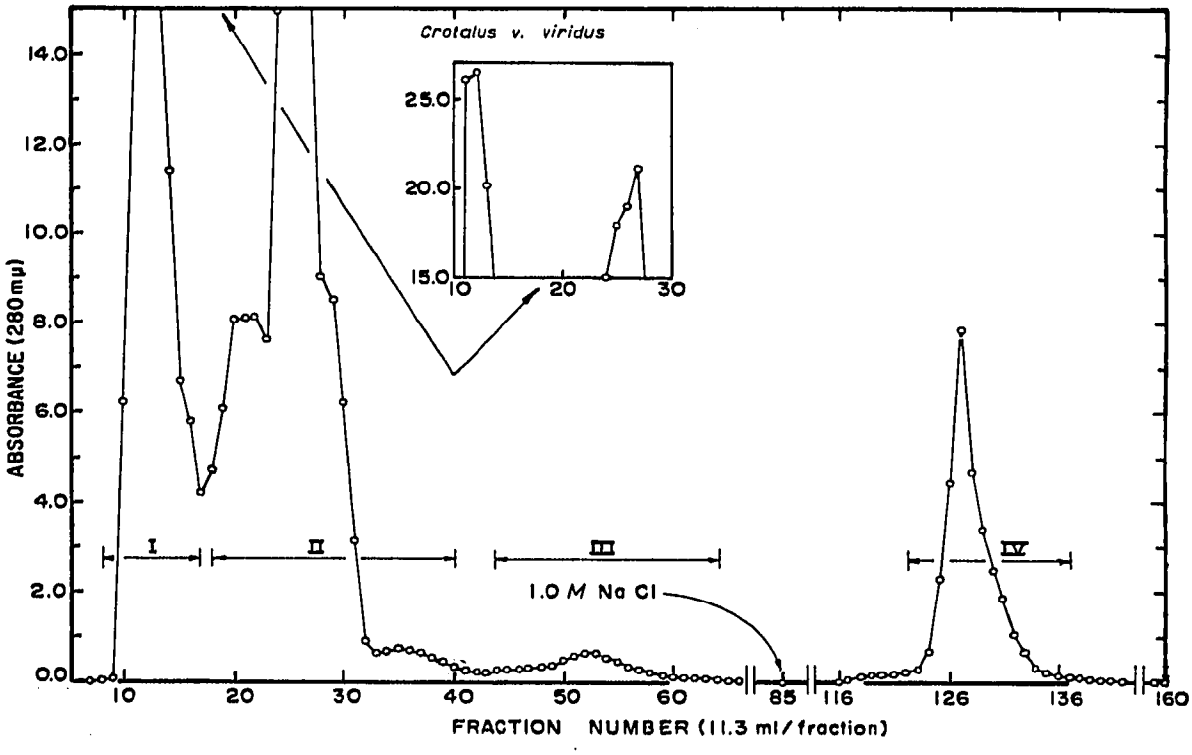
*Amino acid composition.* Duplicate samples of electrophoretically homogeneous basic proteins were prepared for amino acid analysis by dialysis against two changes of 4000 volumes of distilled water at 2° for a total of 2 h. Aliquots (1.40 mg of protein) were hydrolyzed for 28 h by the procedure of MOORE AND STEIN[16]. The hydrolysates were analyzed on a Beckman-Spinco 120C amino acid analyzer. Tryptophan was determined by the colorimetric methods of SPIES AND CHAMBERS[17] and BARMAN AND KOSHLAND[18].

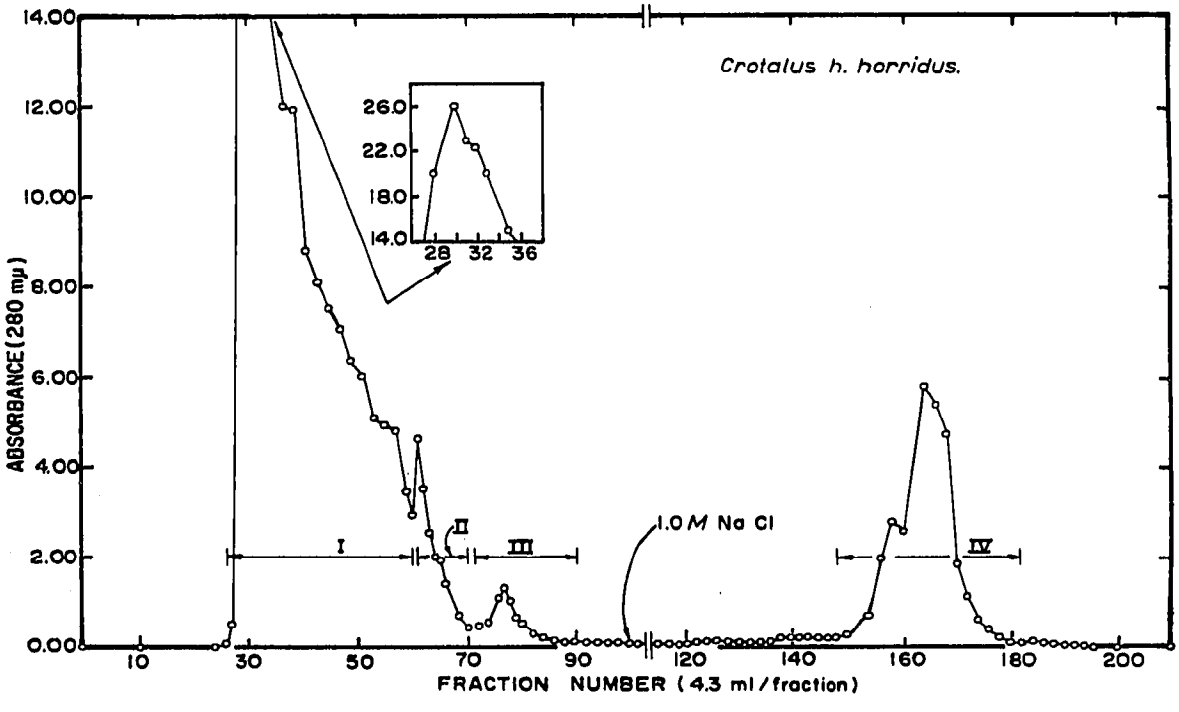
*Biological assay procedures.* Adult (over 30 days old) male Swiss albino mice (*Mus musculus*) from a homogeneous stock and weighing 25–35 g were used in all experiments. The diet consisted of Purina Laboratory Chow and tap water *ad libitum*.

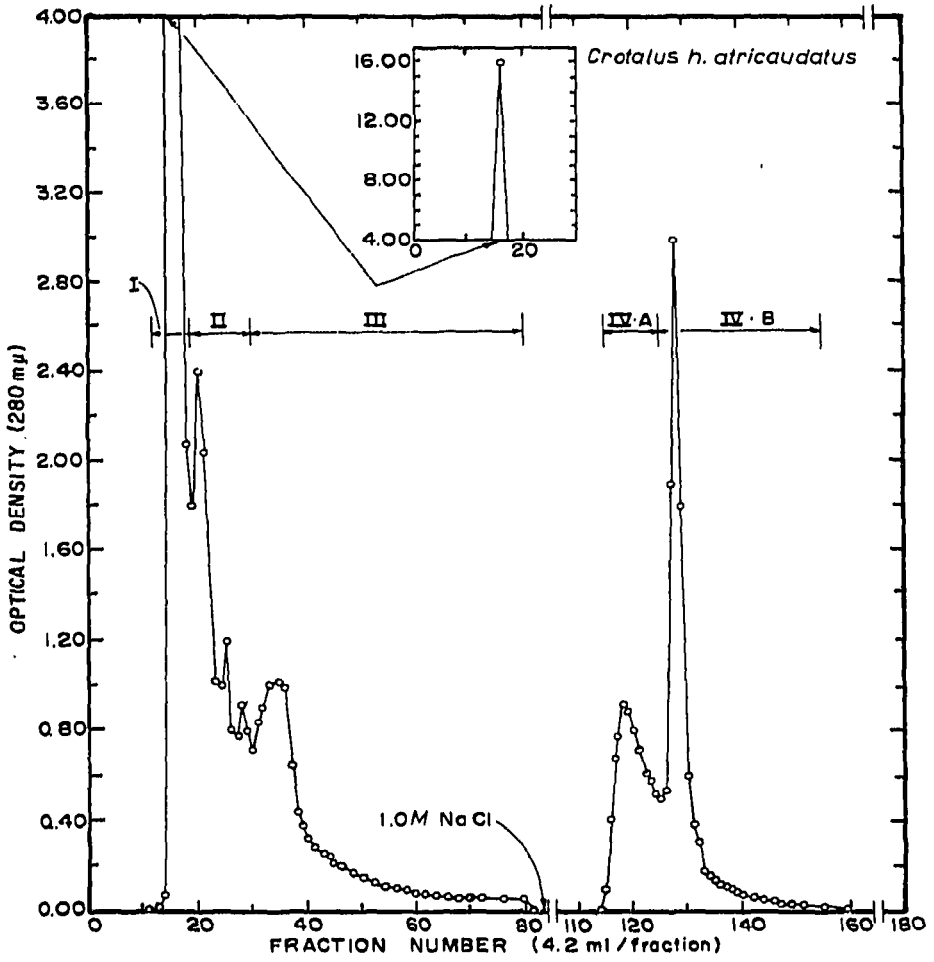
Varied amounts of material from the different stages of purification of the venom were administered to groups of ten mice. The dose was administered intraperitoneally, as so many mg per kg of body weight, carried in a minimal amount (not exceeding 0.2 ml) of physiologic saline solution. The effects on neuromuscular transmission were studied using a modified BÜLBRING[19] phrenic nerve-diaphragm preparation as described by RUSSELL AND LONG[20]. (Detailed results of this facet of the study will be reported separately.)

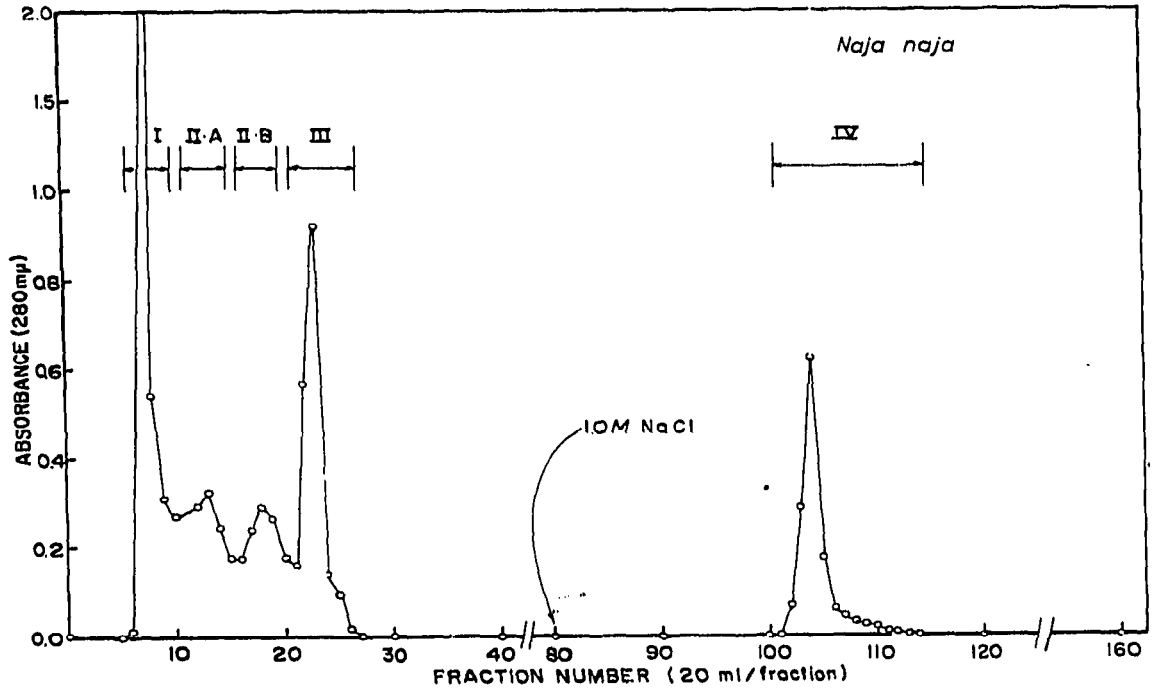
## RESULTS

Recycling adsorption chromatograms of *C. v. viridis* (Fig. 1), *C. h. horridus* (Fig. 2) and *C. h. atricaudatus* (Fig. 3) venoms do not differ significantly from each other or from those obtained by the same method during the isolation of *C. adamanteus* basic protein[12,21]. In each case, those proteins having isoelectric points of ~ pH 10.8 or higher are adsorbed unto the polyacrylamide gel and can be selectively desorbed by increasing the ionic strength of the eluting medium (*i.e.* 1.0 *M* NaCl). Even when very high sample loads are applied to the column, excellent recoveries of basic protein can still be affected by increasing the number of cycles through which the crude venoms are subjected. (Figs. 1–3 represent second-cycle elution profiles.) Cobra (*Naja naja*) venom contains a very high percentage of basic protein (over 40%) which can be rapidly isolated to a very high degree of homogeneity after five continuous cycles on Bio-Gel P-2. A representative elution curve is shown (Fig. 4) for comparative purposes.

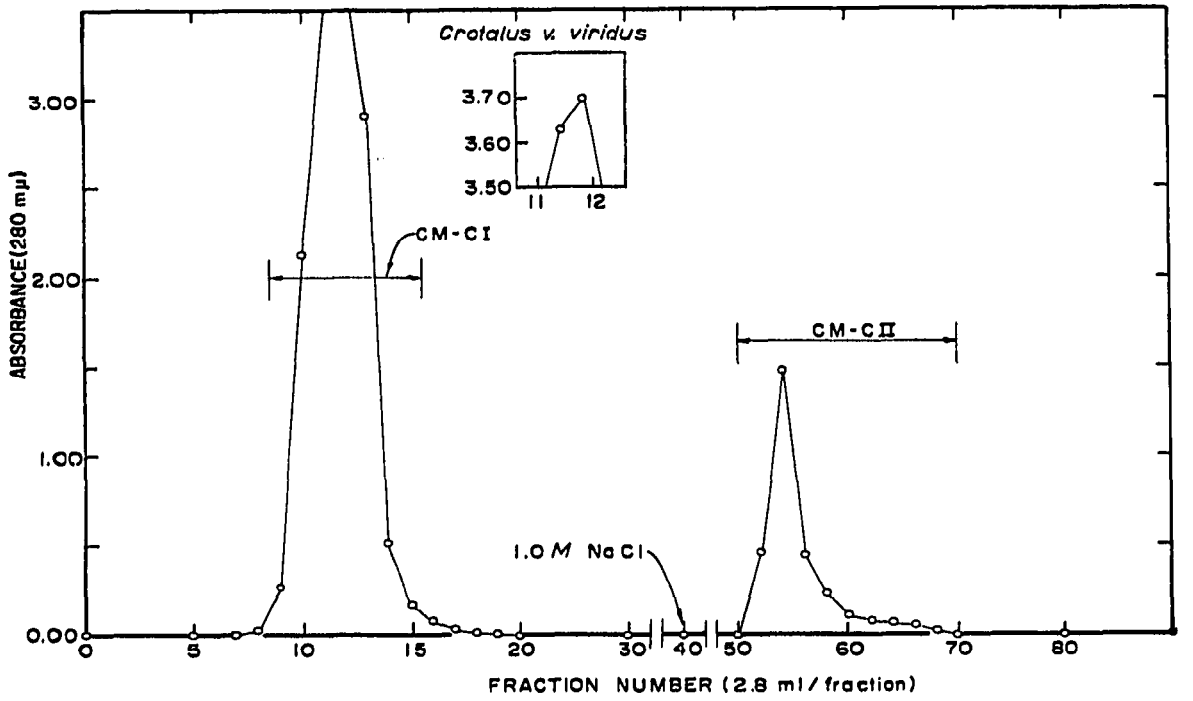

Crotalus v. viridus
ABSORBANCE (280 mμ)
FRACTION NUMBER (11.3 ml/fraction)
I
II
III
IV
1.0 M Na Cl

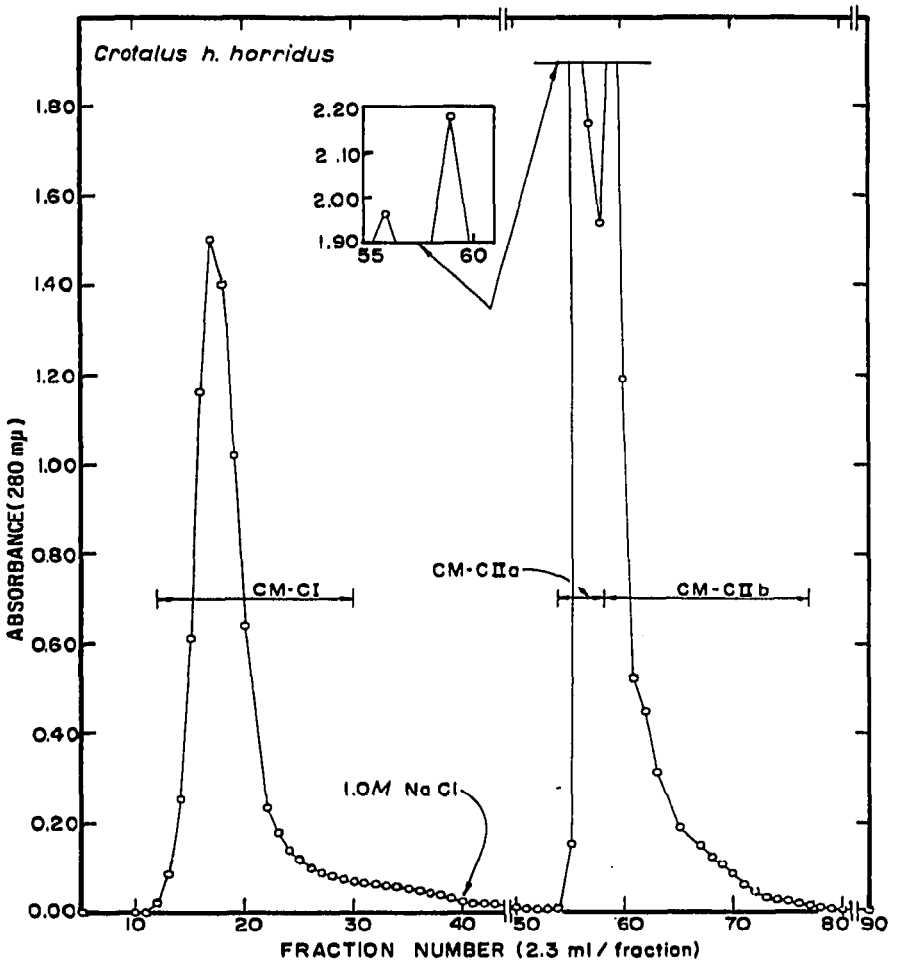

Crotalus h. horridus.
ABSORBANCE (280 mμ)
FRACTION NUMBER (4.3 ml/fraction)
I
II
III
IV
1.0 M Na Cl

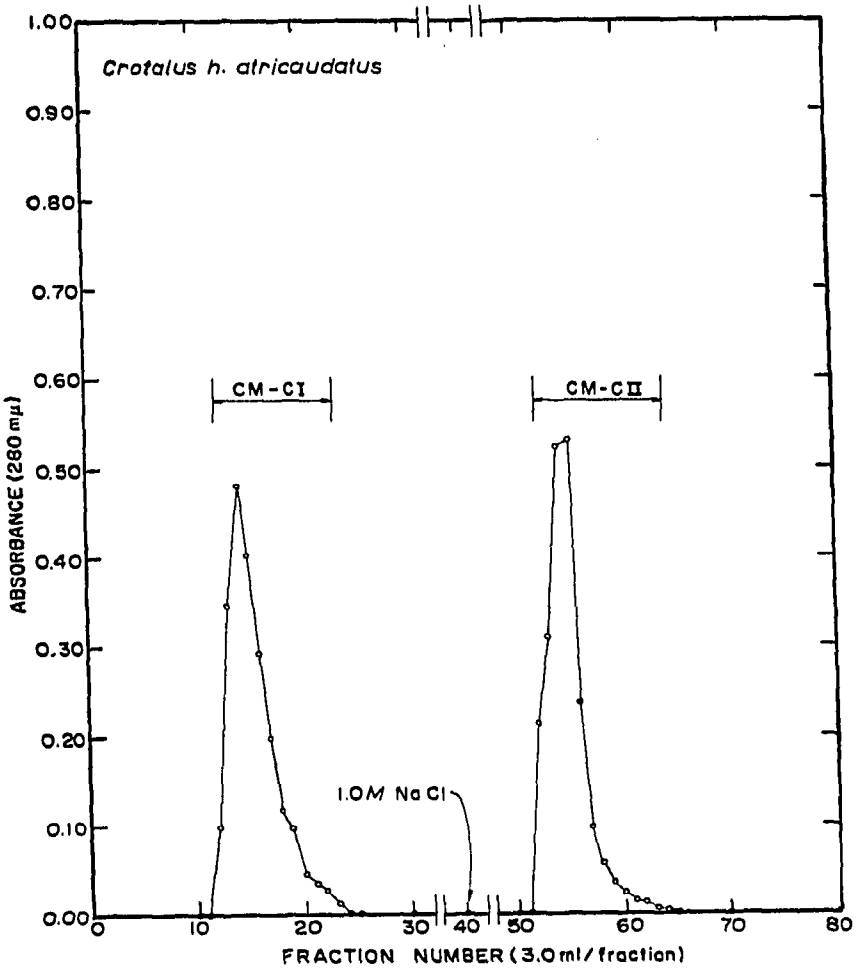



Figs. 1–4. Bio-Gel P-2 adsorption chromatography of *C. v. viridis* (2.2 g), *C. h. horridus* (1.5 g), *C. h. atricaudatus* (2.8 g) and *Naja naja* (0.6 g) venoms. Figs. 1–3 represent two-cycle fractionations while Fig. 4 (*Naja naja*) represents a five-cycle elution profile. Column dimensions and conditions for elution are described in the text.


Crotalus v. viridus
3.70
3.60
3.50
11
12
CM-CI
CM-CII
1.0 M NaCl
ABSORBANCE (280 mμ)
3.00
2.00
1.00
0.00
0
5
10
15
20
30
40
50
60
70
80
FRACTION NUMBER (2.8 ml / fraction)


Crotalus h. horridus
2.20
2.10
2.00
1.90
55
60
CM-CI
CM-CIIa
CM-CIIb
1.0M NaCl
ABSORBANCE (280 mμ)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
10
20
30
40
50
60
70
80
90
FRACTION NUMBER (2.3 ml / fraction)




Figs. 5–7. Ion-exchange chromatography of Bio-Gel P-2 purified basic proteins from *C. v. viridis* (278 mg), *C. h. horridus* (85 mg) and *C. h. atricaudatus* (25 mg) venoms. The adsorbed fractions (IV; Figs. 1–3) were pooled, dialyzed (3–6 h), concentrated by lyophilization and applied to 1.5 × 30 cm columns of CM-cellulose. Fractions CM-CII represent the small-molecular-weight, highly basic protein toxins from each venom.

*Ion-exchange chromatography.* Results of ion-exchange chromatography of the adsorbed fractions from Bio-Gel P-2 are shown in Figs. 5–7. In each case, two major peaks can be discerned. On bioassay, both fractions were highly toxic but by different modes of action. While CM-CI fractions had anticoagulant activity, fractions CM-CII were neurotoxic.

*Analytic procedures*

*Polyacrylamide gel.* It can be seen from the results obtained after gel electrophoresis (Fig. 8) that the basic proteins from Crotalid venoms can be purified by the procedures described in this report and that the final fractions (CM-CII) are consistent with a high degree of homogeneity.

*Amino acid analysis.* Table I shows the results of amino acid analyses as determined from duplicate 28-h hydrolysates. No tryptophan residues were found by the methods described. The minimal molecular weights based on amino acid composition

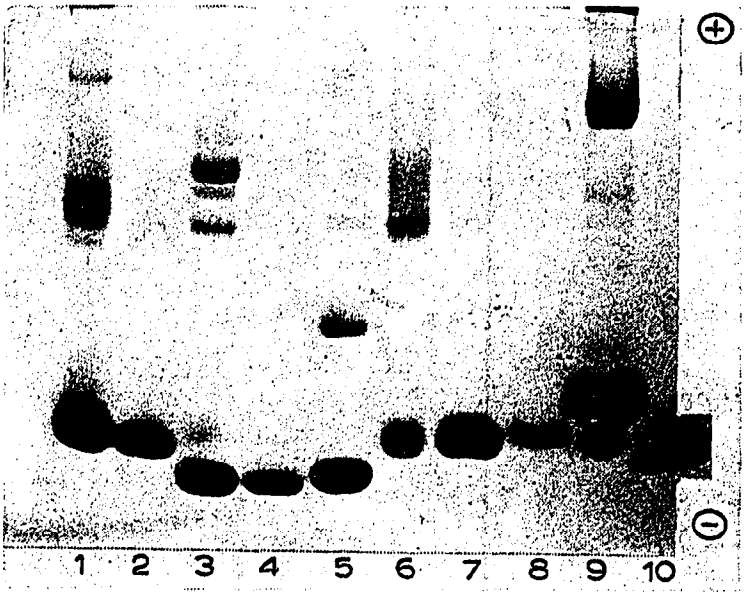



Fig. 8. Polyacrylamide electrophoresis of crude venoms and their corresponding purified basic protein toxins. Conditions for electrophoresis: voltage, 250 V (constant); current, 100 mA; pre-run, 3.5 h; electrolyte, 0.37 *M* glycine–citric acid buffer, pH 2.9; load, 20–40 µl (14–28 mg/ml); length of separation, 5 h (4°); gel concentration, 14% Cyanogum-41. 1 = *C. h. atricaudatus* crude venom; 2 = *C. h. atricaudatus* basic protein; 3 = *C. v. viridis* crude venom; 4 = *C. v. viridis* basic protein; 5 = *C. d. terrificus* crude venom, shown here for comparative purposes; 6 = *C. h. horridus* crude venom; 7 = *C. h. horridus* basic protein; 8 = *Naja naja* basic protein (see fraction IV, Fig. 4); 9 = *Naja naja* crude venom; 10 = *C. adamanteus* basic protein. The gels were purposely over-destained (80 min) in order to show the purified basic proteins in better contrast to the crude venoms.

of the basic proteins are as follows: *C. v. viridis*, 6308–6952; *C. h. atricaudatus*, 6310–6777; *C. h. horridus*, 4808–5178.

*Isoelectric points.* Isoelectric points for the purified basic proteins were obtained by comparison with the electrophoretic behavior of lysozyme, cytochrome *c* and ribonuclease whose isoelectric points decrease in that order[22]. The venom basic proteins and lysozyme migrated unidirectionally to the cathode during electrophoresis for 6 h, at pH 10.6 in 8 *M* urea polyacrylamide gels. The relative positions of the standard proteins were in accord with their respective isoelectric points. Since all the venom basic proteins migrated ahead of lysozyme, their isoelectric point exceeded 10.8, the isoelectric point of lysozyme[22].

*Biological assay procedures*

The $LD_{50}$ values and generalized symptomatology following administration of the purified basic proteins to Swiss albino mice are shown in Table II. On the modified BÜLBRING preparation, the basic proteins stimulate the indirectly elicited contraction (muscle through nerve), while depressing the directly elicited contraction (muscle directly). These changes occur concurrently and appear to be initiated simultaneously at concentrations as low as $2 \times 10^{-5}$ mg. Further details on the neurotoxic activity of all the basic proteins will be reported separately.

TABLE I

AMINO ACID COMPOSITION OF SOME RATTLESNAKE VENOM BASIC PROTEINS

| Amino acid | *C. v. viridis* | | | *C. h. atricaudatus* | | | *C. h. horridus* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Average or extrapolated*[a] *(μmole)* | *Residues* | | *Average or extrapolated*[c] *(μmole)* | *Residues* | | *Average or extrapolated*[d] *(μmole)* | *Residues* | |
| | | *Calculated*[b] | *Assumed* | | *Calculated*[b] | *Assumed* | | *Calculated*[b] | *Assumed* |
| Lysine | 0.165 | 6.1 | 6 | 1.24 | 5.6 | 5–6 | 0.79 | 8.7 | 9 |
| Histidine | 0.056 | 2.1 | 2 | 0.72 | 3.3 | 3 | 0.31 | 3.4 | 3–4 |
| Ammonia | 0.261 | 9.7 | 10 | 0.69 | 3.1 | 3 | 0.30 | 3.3 | 3 |
| Arginine | 0.058 | 2.2 | 2 | 0.41 | 1.9 | 2 | 0.28 | 3.1 | 3 |
| Aspartic acid | 0.129 | 4.8 | 5 | 1.29 | 5.9 | 6 | 0.30 | 3.3 | 3 |
| Threonine | 0.042 | 1.6 | 1–2 | 0.59 | 2.9 | 3 | 0.11 | 1.2 | 1 |
| Serine | 0.078 | 2.9 | 3 | 0.71 | 3.2 | 3 | 0.14 | 1.6 | 1–2 |
| Glutamic acid | 0.095 | 3.5 | 3–4 | 0.81 | 3.7 | 4 | 0.22 | 2.4 | 2–3 |
| Proline | 0.070 | 2.6 | 2–3 | 0.00 | 0.0 | 0 | 0.00 | 0.0 | 0 |
| Glycine | 0.128 | 4.7 | 5 | 1.54 | 7.0 | 7 | 0.43 | 4.8 | 5 |
| Alanine | 0.053 | 2.0 | 2 | 0.50 | 2.3 | 2 | 0.07 | 1.0 | 1 |
| Half cystine | 0.105 | 3.9 | 4 | 0.66 | 3.0 | 3 | 0.11 | 1.2 | 1 |
| Valine | 0.045 | 1.7 | 2 | 0.61 | 2.8 | 3 | 0.14 | 1.6 | 1–2 |
| Methionine | 0.027 | 1.0 | 1 | 0.22 | 1.0 | 1 | 0.09 | 1.3 | 1 |
| Isoleucine | 0.065 | 2.4 | 2–3 | 0.26 | 1.2 | 1 | 0.09 | 1.3 | 1 |
| Leucine | 0.070 | 2.6 | 2–3 | 0.39 | 1.8 | 2 | 0.10 | 1.1 | 1 |
| Tyrosine | 0.050 | 1.8 | 2 | 0.38 | 1.7 | 2 | 0.09 | 1.3 | 1 |
| Phenylalanine | 0.051 | 1.9 | 2 | 0.53 | 2.4 | 2–3 | 0.12 | 1.3 | 1 |
| Tryptophan[e] | 0.000 | — | 0 | 0.00 | — | 0 | 0.00 | — | 0 |
| Total | | | 56–61 | | | 52–54 | | | 38–42 |

[a] By extrapolation based on duplicate 28- and 56-h hydrolysates.
[b] Taking methionine = 1.0. No corrections have been made for hydrolytic losses.
[c] Average values of duplicate 28-h hydrolysates.
[d] Values obtained from a single 28-h hydrolysate.
[e] Determined by colorimetric[17] and spectrophometric[18] methods.

TABLE II

*In vivo* PHARMACOLOGICAL EFFECTS OF PURIFIED RATTLESNAKE VENOM BASIC PROTEINS

Subcutaneous $LD_{50}$ in mice: *C. v. viridis*, 41 mg/kg; *C. h. horridus*, 43 mg/kg; and *C. h. atricaudatus*, 32 mg/kg body weight.

| *Time (min)* | $LD_{99}$ | $LD_{50}$ |
|---|---|---|
| 1 | Immediate prostration, acute exophthalmus, blanching and flattening of ears, marked extension of hindlimbs, motor twitches and death within 30 sec. | Immediate prostration, exophthalmus, some blanching and flattening of ears, extensor paresis of hindlimbs with curling of forelimbs under body. |
| 2–3 | | Continued hindlimb paresis or paralysis, other signs improved. Righting reflex intact. |
| 3–5 | | Weakness of hindlimbs. |
| 5–8 | | Marked muscular weakness, motor twitches, irregular respirations, prostration → death; or some muscular weakness, chiefly in hindlimbs → survival |

## DISCUSSION

A procedure for the isolation of the basic proteins from *C. v. viridis*, *C. h. horridus* and *C. h. atricaudatus* has been described in this report. This procedure involves repeated adsorption chromatography on highly cross-linked polyacrylamide gel and ion exchange on CM-cellulose.

Within a few minutes of administration of the basic proteins, the animals could be observed with marked neurotoxic symptoms. They showed ptosis, were ataxic, and had jerky body movements with a stiff-legged, shuffling gait. Sometimes a transient hyperexcitability was observed, with the animal rapidly losing the ability to respond to any stimuli. The most striking single syndrome, occurring to some degree in all animals observed, was extreme tonic hyperextension of the hind limbs, often accompanied by rigidity of the tail, which was maintained for long periods (6 or more hours in animals with a sublethal dose). There were frequent convulsive episodes, generally preceding death. Irregular, often audible, gasping respiration was observed. Death was of rapid onset, if occurring at all. As was the case for *C. adamanteus* basic protein, the relative toxicity of diluted solutions has been found to devease as the purity of the toxins increased. This effect can be explained by the denaturation of the toxins at very low concentrations and it will be worthwhile to see if the effect can, in fact, be reversed by the addition of proteins (*i.e.* serum albumin). Rapid onset of neurotoxic symptoms follows even when very small doses are administered to the assay animals. In the case of *C. v. viridis* and *C. h. horridus* basic proteins 0.51 mg/kg body weight, or approximately 1/80 of the $LD_{50}$ provoked marked neurotoxic symptoms which were sustained for several hours although no deaths were recorded.

Necropsy of these animals revealed no hemorrhagic sites or other gross pathology, and complete absence of necrosis at the site of injection was observed.

Now that low-molecular-weight neurotoxins have been isolated from *C. adamanteus*, *C. h. atricaudatus*, *C. h. horridus* and *C. v. viridis* venoms, further research is indicated to test their immunologic properties. It may be therapeutically advantageous to bind these materials to CM-cellulose in order to enhance their antigenicity in the preparation of antivenin.

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