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COMPARATIVE BIOCHEMISTRY AND PHARMACOLOGY OF SALIVARY GLAND SECRETIONS

III*. CHROMATOGRAPHIC ISOLATION OF A MYOCARDIAL DEPRESSOR PROTEIN (MDP) FROM THE VENOM OF *CROTALUS ATROX*

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SUMMARY

A protein has been isolated from the venom of the western diamondback rattlesnake *(Crotalus atrox)* which induces acute myocardial depression when administered to experimental animals. Purification was achieved by gel filtration on Sephadex G-100, DEAE- and CM-cellulose ion-exchange chromatography, ultrafiltration, and adsorption chromatography on hydroxyapatite. Amino acid analysis of the highly purified protein indicated N-terminal isoleucine and C-terminal tyrosine residues, and the absence of free sulfhydryl groups.

Rabbits were immunized against the myocardial depressor protein (MDP) and a highly specific antiserum prepared which made it possible to study other snake venoms for the presence or absence of MDP. All of the North American Crotalid species of snakes contain MDP in varying degrees of concentration, but none of the Asiatic snake venoms tested reacted with the antiserum to the myocardial depressor protein. Intravenous administration of MDP to experimental animals (dogs, cats) produces an immediate and profound decrease in the cardiac output, the left ventricular systolic and mean pressures, the velocity of shortening of the contractile element, the systemic arterial pressure and an elevation in the left ventricular end-diastolic and pulmonary wedge pressures. These hemodynamic changes indicate that MDP administration induces an acute myocardial failure which is dose dependent. The potential use of this protein for the reproducible causation of left ventricular failure, obviating the need for the more commonly used surgical ligation of the coronary arteries, warrants a full investigation into its structure, active site and its mechanism of action on the myocardial cell.

INTRODUCTION

Snake venoms are complex glandular secretions known to contain a multitude

* For papers I and II in this series see refs. 4 and 5, respectively.

of biologically active proteins, peptides, and enzymes which are being increasingly and continually used to study physiological reactions in both the normal and pathological states. Such diverse activities as blood clotting, fibrinolysis, nerve impulse transmission, muscle contraction, and vasodilation are affected by specific components isolated from the venom gland secretions. Extensive reviews on the subject have been recently published[1–3]. During the course of our continued investigation of the comparative biochemistry and pharmacology of salivary gland secretions[4–6], the presence of a protein was detected in the venom of the western diamondback rattlesnake *(Crotalus atrox)* which produces acute and severe myocardial depression when administered to anesthetized experimental animals. This response is apparent within seconds following intravenous administration of the purified protein and is associated primarily with a drastic decrease in the cardiac output and the left ventricular and arterial pressure, which persists for periods of two hours or longer depending on the dose employed.

In the present report a method for obtaining this protein, henceforth named myocardial depressor protein (MDP), in highly purified form is outlined and some of its characteristics described. Preliminary accounts of portions of this work have been published[7,8].

EXPERIMENTAL

Materials

Crude venom from *Crotalus atrox* was collected from snakes housed in our serpentarium under standard conditions of light and humidity and prepared for chromatography as described previously[9]; Sephadex G-100 was obtained from Pharmacia (Piscataway, N.J., U.S.A.) and ion-exchange celluloses, DE-52 and CM-52, from Reeve Angel (Clifton, N.J., U.S.A.); hydroxyapatite was a product of Bio-Rad Labs. (Richmond, Calif., U.S.A.).

Gel filtration on Sephadex G-100

Venom (5–7 g) was dissolved in 50–75 ml of 0.12 *M* Tris–HCl–0.2 *M* NaCl (pH 8.6), centrifuged at 49,500 *g* for 30 min in a refrigerated centrifuge (4°) (Sorvall S-34 rotor). The supernatant fraction was then applied to a 5.0 × 100 cm column of Sephadex G-100 using upward flow adaptors (actual bed height, 94 cm) and elution carried out with the same buffer.

Ion-exchange chromatography

DEAE-cellulose. The active fractions from the Sephadex G-100 column were pooled, dialyzed for 24 h against distilled, deionized water at 4° and lyophilized.

The lyophilized fraction was dissolved in a minimal volume (5–10 ml) of 0.005 *M* Tris–HCl (pH 8.0), applied to a column of DEAE-cellulose (DE-52) prepared according to the method of Peterson and Sober[10], and eluted with the same buffer. Stepwise elution was then carried out with 0.005 *M* Tris–HCl–0.2 *M* NaCl (pH 8.0) and 1.0 *M* NaCl.

CM-cellulose. The pooled, active fraction from the DEAE-cellulose column was dialyzed (24 h, 4°, against 4000 vol. of water), lyophilized, redissolved in a small volume of 0.04 *M* sodium citrate (pH 3.7), dialyzed (6 h) against the same buffer and applied to a CM-cellulose column (2.5 × 45 cm) equilibrated with the starting buffer.

Stepwise elution was carried out as follows: 0.04 *M* sodium citrate (pH 3.7), 0.40 *M* sodium citrate (pH 3.7) and 1.0 *M* NaCl. The fractions containing the MDP were pooled, dialyzed against distilled water (24 h) in the cold (4°) and lyophilized.

Adsorption chromatography

A column (2.5 × 45 cm) was packed with hydroxyapatite gel and equilibrated with 1 m*M* potassium phosphate buffer (pH 7.4) at 4°. Flow-rate of the column was kept at about 14 ml/L and stepwise elution carried out with increasing molarity of the same buffer.

Gel electrophoresis

Electrophoresis in 12.8% polyacrylamide acidic gels containing 6 *M* urea was performed as described earlier[11] without modification.

Amino acid analysis

Amino acid analyses in duplicate were carried out on highly purified samples of the MDP according to the method originally described by Spackman, Stein, and Moore[12] using a Durrum analyzer Model D-500 with built-in computer. Salt-free samples were prepared by exhaustive dialysis against double-distilled deionized water. Aliquots were hydrolyzed in duplicate for 24 and 96 h at 110°, and the results averaged. No attempt was made to determine the presence or absence of carbohydrate in the purified protein.

Tryptophane determination

Tryptophane content was obtained from analysis of 94-h alkaline hydrolysis as described by Drèze[13].

Cystine + cysteine content

Approx. 1 mg of protein was oxidized with performic acid, hydrolyzed and analyzed for cysteic and aspartic acids as described by Hirs[14].

Cysteine determination

Approx. 5 mg of MDP were alkylated with iodoacetic acid according to the method of Crestfield *et al.*[15]. Following dialysis (to remove excess reagent and buffer) and lyophilization, an acid hydrolyzate was analyzed for the presence of carboxymethyl cysteine.

Assay for myocardial depressant activity

The purified protein was assayed for myocardial depressor activity using anesthetized, closed-chest beagle dogs as previously described[16]. MDP was administered intravenously at a dose of 0.5 mg per kg body weight; this dose, which was arrived at during earlier experimentation, produces an immediate and severe depression in the cardiac output (not less than 50%) and decreases the mean left ventricular and aortic arterial pressures by not less than 40% from control values.

Immunological methods

Five adult female New Zealand white rabbits were immunized with the highly

TABLE I

IMMUNIZATION SCHEDULE FOR PRODUCTION OF MDP-ANTISERUM

MDP = Myocardial depressor protein; FCA = Freund's complete adjuvant.

Days	*Treatment*
0, 10	1.8 mg of MDP in FCA (intramuscular)
20, 22, 32	0.4 mg MDP in saline solution (subcutaneously)
21, 33, 34	0.4 mg MDP in saline solution (intraperitoneal)
32	First blood collection after immunization
44	Second blood collection after immunization

purified myocardial depressor protein according to the schedule shown in Table I. Emulsions of Freund's complete adjuvant (FCA) and MDP were prepared by addition of 1 ml FCA to 1 ml of 0.85% NaCl containing 9 mg of MDP.

Blood was collected from the marginal ear vein, centrifuged and the serum harvested and stored at −20°. High titers of MDP antiserum were maintained by continued challenge of each animal with 0.4 mg of MDP in saline by the intradermal route at 10–20-day intervals.

Microimmunodiffusion tests were done essentially as described by Crowle[17] using agarose-coated microscope slides (1% Calbiochem agarose) in phosphate buffer at pH 7.4 using the technique of Pineda *et al.*[18] without modification.

Enzyme analysis

Pooled fractions from the Sephadex G-100 column were analyzed for L-amino acid oxidase[19], 5-nucleotidase, phosphodiesterase[20], and phospholipase-A[21].

RESULTS

Gel filtration

Sephadex G-100 gel filtration (Fig. 1) resolved the crude venom from *C. atrox* into five subfractions which have been partially identified as follows: fraction IA, a large-molecular-weight fraction excluded from the column contains most of the 5′-nucleotidase activity and other unidentified components; fraction IB contains the L-amino acid oxidase, phosphodiesterase, some remnant 5′-nucleotidase activity and other components.

Subfractions IIA and IIB contain all of the phospholipase-A activity, the bulk of the proteolytically active proteins, the hemorrhagic components of the venom and the myocardial depressor protein. The ratio of these two subfractions to the total protein in the starting material varies drastically in different batches of the crude venom and is correlatable, at least to some extent, to the geographical region from which the snakes were collected[22]. Fraction III contains the small-molecular-weight components of the venom including a peptide (M.W., 2000) responsible for some of the severe hypotension observed following the bite by the western diamondback rattlesnake[23].

Ion-exchange chromatography

DEAE-cellulose. Chromatography of the pooled, dialyzed, lyophilized frac-

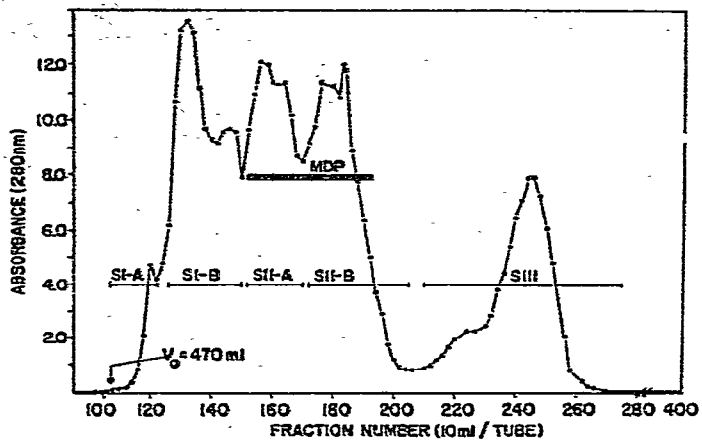

Fig. 1. Elution profile of the gel filtration step (100 × 5 cm Sephadex G-100) used in the purification of MDP from *C. atrox* venom. Load: 5 g venom in 80 ml 0.12 *M* Tris–HCl–0.20 *M* NaCl buffer (pH 8.6). The fractions pooled for further purification are indicated by the solid bar. Further details in text.

tion II (A and B) on DE-52 yields three major peaks (Fig. 2) of which fraction (DII) contains most of the MDP.

CM-cellulose. The elution profile obtained from ion exchange chromatography of fraction DII on CM-cellulose is shown in Fig. 3. Most of the MDP is found in the retarded fraction (Cc) but some activity is also normally found in fraction Cd and the ratio for the two peaks varies for different lots of crude venom.

Adsorption chromatography

Several batches of venom were processed up to and including the CM-cellulose step and the resulting fractions Cc pooled prior to the final purification step on

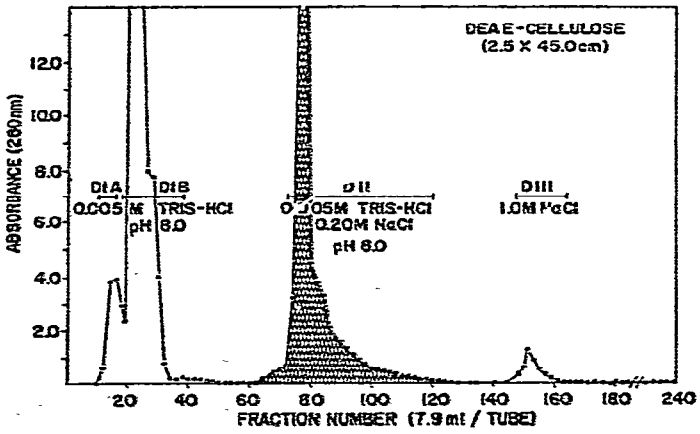

Fig. 2. DEAE-cellulose chromatography of partially purified MDP from the gel filtration step. Fraction DII containing the MDP was eluted with 5 m*M* Tris–HCl buffer containing 0.2 *M* NaCl and further purified by cation-exchange chromatography on CM-cellulose.

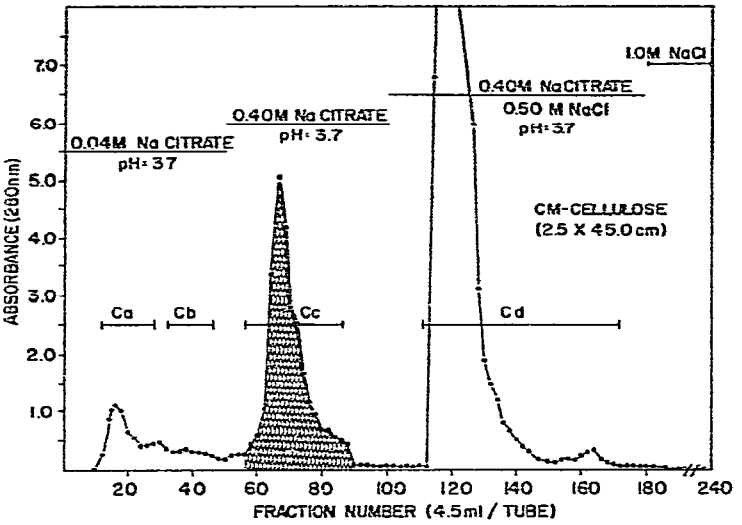

Fig. 3. Chromatography of DEAE-cellulose purified MDP on CM-cellulose. Stepwise elution was carried out as shown in the figure and the fractions marked by the cross-hatched area pooled, concentrated by ultrafiltration and lyophilized.

Bio-Gel HTP. For the elution profile, shown in Fig. 4, the resulting fractions Cc from several batches of venom were pooled, dissolved in distilled water and percolated through a column of Bio-Gel HTP (2.5 × 45 cm) previously equilibrated with 0.001 *M* potassium phosphate buffer (pH 6.8). Pooled fraction HA-I containing the highly purified MDP was then re-chromatographed on hydroxyapatite and the resulting fraction (see Fig. 5) used for further chemical and biological analysis.

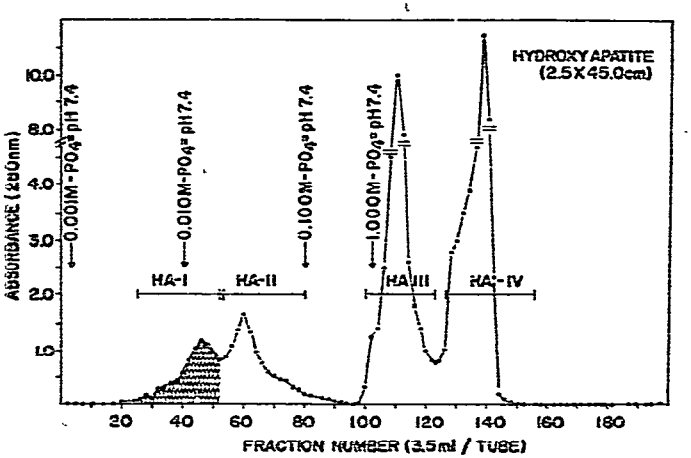

Fig. 4. Hydroxyapatite column chromatography of highly purified MDP following purification by gel filtration and ion exchange. Several batches of venom were fractionated through the CM-cellulose step and the resulting fractions Cc pooled, concentrated and applied to a Bio-gel HTP column. Fraction HA-I (indicated by the shaded area) contained the bulk of the MDP and appeared homogeneous as shown by re-chromatography on hydroxyapatite.

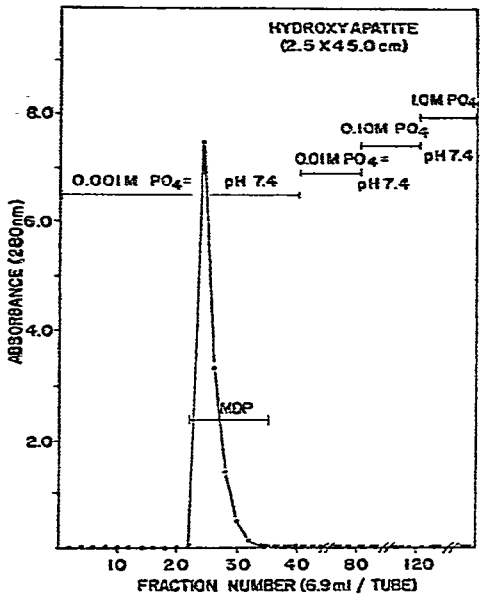

Fig. 5. Re-chromatography of highly purified MDP on hydroxyapatite. Fraction HA-I (see Fig. 4) was dissolved in 5 ml of 0.001 *M* potassium phosphate buffer (pH 7.4) and carefully layered on the column which had been equilibrated with the same buffer. Other details as in Fig. 4.

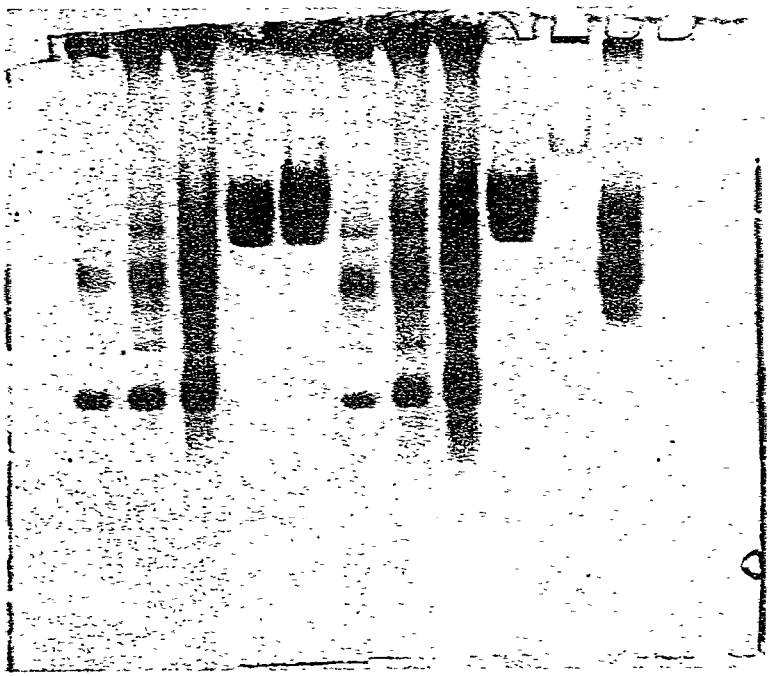

Fig. 6. Polyacrylamide gel electrophoresis of (from left to right): pooled *C. atrox* venom from Odessa, Texas (10, 20 and 40 μl; myocardial depressor protein (20, 40 μl); pooled *C. atrox* venom from Big Springs, Texas; myocardial depressor protein (40 μl); hypotensive peptide from *C. atrox* venom (20 μl); fraction Cd (see Fig. 2) from DEAE-cellulose chromatography. Conditions for electrophoresis: voltage, 250 V (constant); current, 100 mA; prerun, 4 h; electrolyte, 0.37 *M* glycine–0.1 *M* citric acid buffer (pH 2.9); separation, 4 h (3°). Gel concentration, 12.8% Cyanogum-41. Migration is from top (+) to bottom (−).

Gel electrophoresis

The electrophoretic patterns obtained with crude venom and the purified MDP are shown in Fig. 6 and indicate that, using the procedure herein described, this protein can be recovered in homogeneous or nearly homogeneous form.

Amino acid analysis

The results of the amino acid analysis of the highly purified MDP are shown in Table II. These values were derived from duplicate hydrolyzates at 24 and 96 h. Values for serine and threonine were extrapolated to zero time hydrolysis and that reported for isoleucine was the maximum value obtained.

TABLE II

AMINO ACID COMPOSITION OF MDP

Amino acid	*Hydrolysis time*		*Adjusted value*	*Residues*	
	24 h	*96 h*			
Alanine	0.052	0.056	0.054	4.5	4
Arginine	0.034	0.035	0.035	2.9	3
Aspartic acid	0.121	0.118	0.120	10.0	10
Glutamic acid	0.098	0.100	0.099	8.2	8
Glycine	0.099	0.095	0.097	8.0	8
Histidine	0.018	0.013	0.015	1.2	1
Isoleucine	0.025	0.028	0.028*	2.3	2
Leucine	0.042	0.041	0.041	3.4	3
Lysine	0.059	0.058	0.058	4.8	5
Methionine	0.011	0.014	0.012	1.0	1
Phenylalanine	0.024	0.021	0.022	1.8	2
Proline	0.056	0.059	0.058	4.8	5
Serine	0.052	0.038	0.058**	4.8	5
Threonine	0.043	0.036	0.045**	3.8	4
Tryptophan***	—	0.004	0.004	—	—
Tyrosine	0.029	0.023	0.026	2.2	2
Valine	0.044	0.050	0.047	3.9	4
Cysteine§	—	—	—	—	—
Cystine§	—	—	0.053	4.4	4
Total minimal residues					71

* Maximum value.
** Extrapolated to zero time hydrolysis.
*** Alkaline hydrolysis.
§ For methods of determination: see text.

No carboxymethyl cysteine was found after acid hydrolysis of the alkylated protein and, thus, it has been deduced that MDP contains no free sulfhydryl groups.

Since the ratio cysteic–aspartic acid was 0.88 following hydrolysis of the performic acid oxidized protein and the amount of aspartic acid 0.120 μmoles per mg protein, the combined cystine plus cysteine is reported as 0.106 μmoles/mg. Therefore, MDP contains 0.053 μmoles per mg of cystine based on its complete lack of free sulfhydryl amino acid residue.

Molecular weight estimation

In the absence of ultracentrifugal analysis the molecular weight for MDP was estimated from amino acid analysis and by differential ultrafiltration using Amicon membranes. With the number of methionine residues taken as unity, the minimal molecular weight for MDP was estimated to be approximately 10,000 daltons (9379), with tryptophan excluded from the calculation. An attempt to corroborate this molecular weight was made by passage of a solution of MDP (10 mg/ml) through Amicon ultrafiltration membranes. While 96% of the MDP could be recovered in the ultrafiltrate (after three consecutive washes), from a PM30 membrane 100% of MDP remained in the retentate after ultrafiltration on a UM10 membrane.

Bioassay of MDP

Table III shows the myocardial depressant activity of this highly purified protein from the venom of *Crotalus atrox*. Intravenous administration of MDP to anesthetized, closed chest beagle dogs induces a severe and immediate depression in the cardiac output, the arterial pressure and other dependent variables. These effects appear to be a direct effect of MDP on myocardial function and will be described in detail in a more appropriate journal[24].

TABLE III

HEMODYNAMIC EFFECTS OF MDP

AAP, Mean aortic arterial pressure (mmHg); SVR, systemic vascular resistance (dyne·cm·sec^{-5}); Q, cardiac output (l/min); CI, cardiac index (ml/kg/min); LVEDP, left ventricular end-diastolic pressure (mmHg); LVP, mean left ventricular pressure (mmHg); PWP, pulmonary wedge (capillary) pressure (mmHg). The number of animals studied is given in parentheses under the control values. Each value represents the mean ± standard error and its probability value by Student's t test analysis; NS = not significant. Studies were carried out in male beagle dogs (10–17 kg), anesthetized with pentobarbital sodium (30 mg/kg) and respiration maintained with a Bennett respirator. Catheters were advanced with ECG monitoring via the neck and femoral vessels to the pulmonary artery, right atrium, aortic root and left ventricle. Pressure transducers (Statham) placed at midthoracic level were used to measure left ventricular (p23Gb) and other pressures (p23Db): aortic, left ventricular end-diastolic (LVEDP), pulmonary artery, and pulmonary wedge pressures. Central venous pressure was monitored with a water manometer and cardiac output determined with indocyanine green. All pressures and dye curves were recorded on a 16-channel photographic oscilloscopic system (Electronics for Medicine). Once a post-catheterization hemodynamic steady-state was reached, MDP was administered intravenously in a bolus (0.50 mg/kg in 2 ml slaine) via the right femoral vein and the ensuing hemodynamic changes analyzed and compared to control values.

Time (min)	*AAP*	*SVR*	*Q*	*CI*	*LVEDP*	*LVP*	*PWP*
Control	124 ± 7 (8)	7984 ± 512 (8)	1.22 ± 0.09 (8)	110 ± 5 (8)	9.1 ± 1.1 (8)	66 ± 4 (8)	8.3 ± 1 (5)
3		11966 ± 2521	0.44 ± 0.10	39 ± 8			
P value		NS	<0.001	<0.001			
5	53 ± 7		0.40 ± 0.12	37 ± 10	13.5 ± 0.8	33 ± 7	8.2 ± 2.4
P value	<0.001		<0.001	<0.001	NS	<0.005	NS
10	50 ± 8	13468 ± 3465			19.7 ± 3.6	33 ± 5	12.4 ± 4.2
P value	< 0.001	NS			< 0.02	< 0.005	NS
20	47 ± 6	14211 ± 4220	0.39 ± 0.11	34 ± 10	13.6 ± 2.9	31 ± 5	5.5 ± 0.7
P value	< 0.001	NS	< 0.001	< 0.001	NS	< 0.001	< 0.10
30	43 ± 5	8412 ± 1841	0.57 ± 0.20	52 ± 16	15.1 ± 3.3	31 ± 5	11.5 ± 3.5
P value	< 0.001	NS	< 0.02	< 0.005	NS	< 0.001	NS
45	49 ± 11	7939 ± 1921	0.58 ± 0.12	52 ± 10	11.3 ± 3.3	29 ± 8	16.2 ± 4.1
P value	< 0.001	NS	< 0.005	< 0.001	NS	< 0.005	NS

Immunological characterization of MDP

The results of immunodiffusion analysis are shown in Fig. 7. MDP is highly antigenic in the rabbit when the immunization schedule herein described is followed.

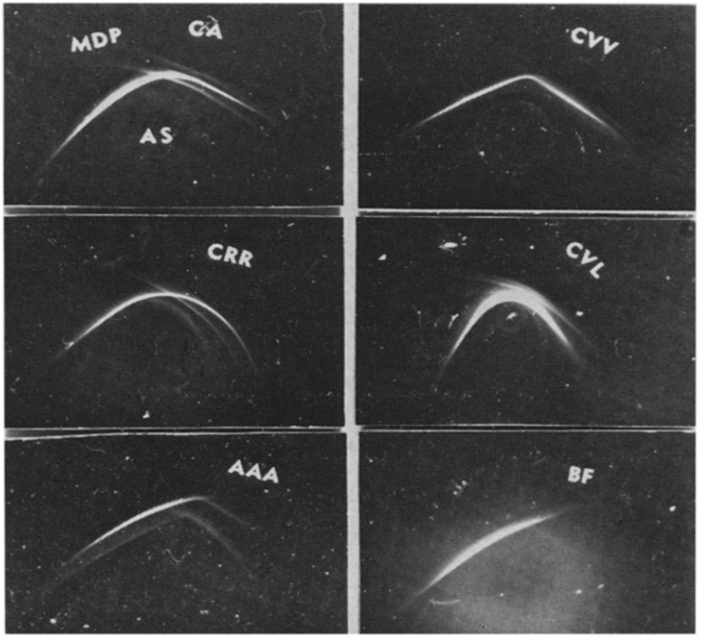

Fig. 7. Microimmunodiffusion analysis for identity lines of myocardial depressor protein (MDP) in various snake venoms. In each case undiluted specific antiserum (AS) to MDP was placed in the central well, MDP (0.01 mg/ml) in the left and crude venom (5 mg/ml) in the adjacent (right) well. The following venoms were tested and found to form identical lines: CA (*C. atrox*, western diamondback rattler); CVV (*C. viridis viridis*, prairie rattler); CRR (*C. ruber ruber*, red diamondback rattler); CVL (*C. viridis lutosis*, Great Basin rattler); AAA (*Agkistrodon acutus acutus*, sharp-nosed pit viper) showed a partial line of identity while BF (*Bungarus fasciatus*, banded krait) had complete non-identity.

DISCUSSION

That the venom secretions from all species of rattlesnakes are similar has been a widely believed misconception which, at least in part, arose from the fact that some of the enzymatically active proteins, *i.e.*, phospholipase A, phosphodiesterase, and 5′-nucleotidase are indeed present, in varying concentrations, in all of these venoms.

As early as 1966, Bertke *et al.*[25] advanced the idea that "Venoms represent body fluids whose compositions are characteristic for a given species". This idea has been confirmed by the results of extensive investigations in our laboratory of the non-enzymatic components of venoms from different rattlesnake species and subspecies. Thus, four of these venoms *(C. adamanteus, C.h. horridus, C.v. viridis,* and *C.h. atricaudatus)* contain a small-molecular-weight toxin which has a presynaptic action that can be blocked by d-tubocurarine or β-bungarotoxin[26]. These toxins are, however, absent in most of the other rattlesnake venoms so far studied in our labora-

tory. On the other hand, two of the venoms *(C. adamanteus* and *C.h. horridus)* possess a thrombin-like enzyme[27,28] which is not present in most of the others. These differences exist for other components of rattlesnake venoms, *i.e.*, bradykinin releasing enzyme, α-protease, etc., and serve to reiterate the fact that these secretions are highly specialized and species specific.

The methods for the isolation and purification of a protein with myocardial depressor activity from the venom of *Crotalus atrox* are described in this report. This so-called myocardial depressor protein or MDP can be obtained in highly purified form using standard techniques of separation: gel filtration, ion-exchange and adsorption chromatography on hydroxyapatite. The final preparation is a protein with a minimal molecular weight of approx. 10,000 daltons, which does not contain free sulfhydryl groups, sulfur being present as the disulfide, cystine. MDP is devoid of the phospholipase A, and the proteolytic and esterolytic activities known to exist in the crude venom of *C. atrox*. When administered intravenously to anesthetized beagle dogs, MDP induces a severe depression of myocardial activity which is dose dependent. Because of these unique biological effects, MDP should prove a very useful tool for probing further into the cellular mechanisms of myocardial contractility, without toxic side effects to other organs, and without having to resort to surgical ligation of the coronary arteries. This, however, will require a completely homogeneous protein preparation which, up to this point, we have not been able to obtain. In spite of the very high degree of purification so far reached, the present MDP preparation appears to have at least one and possibly two minor contaminants which are still detectable by polyacrylamide electrophoresis and immunological techniques. It is our primary interest to achieve a homogeneous MDP preparation in order to investigate its mechanism of action on the mammalian myocardium.

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