COMPARISON OF VENOM COMPONENTS FROM FOUR TARANTULA SPECIES

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ABBREVIATIONS

A_{540} - absorbance at 540 nm

A_{280}/A_{260} - ratio of absorbance at 280 nm to that at 260 nm

AMP - adenosine-5'-phosphate

ADP - adenosine-5'-diphosphate

ATP - adenosine-5'-triphosphate

ATEE - N-acetyl-L-tyrosine ethylester

BAEE - N-benzoyl-L-arginine ethylester

BAPA - N-benzoyl-DL-arginine-p-nitroanilide

BSA - bovine serum albumin

CM - carboxymethyl

CMP - cytidine-5'-phosphate

CDP - cytidine-5'-diphosphate

CTP - cytidine-5'-triphosphate

DEAE - diethylaminoethyl

DNase - deoxyribonuclease

DTT - dithiothreitol

ECG - electrocardiograph

EDTA - ethylenediaminetetraacetic acid

EEG - electroencephalograph

GABA - γ-aminobutyric acid

GMP - guanosine-5'-phosphate

GDP - guanosine-5'-diphosphate

GTP - guanosine-5'-triphosphate

LD_{50} - lethal dose for 50 percent of the animals treated

ml - milliliter

µl - microliter

nm - nanometer

PSS - physiologic saline

RNase - ribonuclease

TAME - p-toluenesulphonyl-L-arginine methylester

TMP - thymidine-5'-phosphate

TDP - thymidine-5'-diphosphate

TTP - thymidine-5'-triphosphate

UV - ultraviolet

UMP - uridine-5'-phosphate

UDP - uridine-5'-diphosphate

UTP - uridine-5'-triphosphate

CHAPTER I

INTRODUCTION

The most extensively studied venoms are those which pose a danger to man. As a result, little is known about venomous secretions of other, assumingly less threatening creatures. In the class Arachnida, perhaps the most-studied venoms are those of the scorpions, the black-widow spider Latrodectus species, and the brown recluse or fiddleback spider Loxosceles reclusa.

Owing to their formidable appearance, members of the suborder Orthognatha, the tarantulas, are often greatly feared. Orthognath species of the United States, however, are generally considered to be innocuous to man, for despite the painful bite produced by the large fangs of these creatures, most species do not cause serious harm. However, one Australian orthognath, an Atrax species, is known to have caused the deaths of at least eleven people.

Regardless of any potential threat to man, little is known about most orthognath venoms. This study compared several venom components of four tarantula species: Dugesiella hentzi (Girard), Eurypelma panamense Simon, Aphonopelma emilia (White), and an undescribed Aphonopelma species from Arizona. The D. hentzi venom has already been extensively examined, and was used as a comparative model for the other venoms which have been only briefly examined or unstudied before. It

is hoped that the information obtained will contribute to a better overall understanding and characterization of the various components of all venoms.

CHAPTER II

LITERATURE REVIEW

A survey of tarantula venoms is complicated by what is meant by the term "tarantula." The true tarantula is actually a European wolf spider, Lycosa tarentula (a member of suborder Labidognatha). Because this spider is large and hairy, any eight-legged creature with a similar appearance is often loosely referred to as a tarantula. It has become acceptable in entomological terminology, however, to use this term for members of the suborder Orthognatha. Members of this group are not true spiders; there are some physiological differences, and the orthognaths are generally larger in size. The main physiological difference is that the chelicerae and fangs of the orthognaths articulate in a plane parallel to the median plane of the body (i.e., perpendicular to the ground). In the true spiders, suborder Labidognatha, the chelicerae and fangs are movable in a transverse plane, with the fangs pointed towards each other (1).

A further complication is that the taxonomy of the orthognaths is currently very disorganized. Consequently, a single species may have several names to designate it, and positive identification is difficult. With these nomenclature difficulties in mind, an attempt was made to review the chemistry of orthognath venoms.

Two species of Atrax, from Australia, are known to be dangerous to man. Atrax formidabilis (North Coast funnel-web spider) has caused

one known near-fatality (2) while Atrax robustus (Sydney funnel-web spider) has caused at least eleven fatalities. In those fatal cases where positive identification was made, the unusually agressive male of the species (A. robustus) was responsible. Studies have shown that its venom is more toxic than that of the female (3).

Despite numerous studies (usually of female venom), its toxic components eluded researchers for a number of years. The pH of the venom is within the range 4.5 to 5.0, containing 12 to 22% heat-coagulable proteins, which are considered non-toxic. Toxicity was stable to a temperature of 100°C for one hour in 0.1 M HCl, but not in 0.1 M NaOH. Toxicity was completely destroyed in 20 minutes at 120°C. Toxicity could be reduced by trypsin but not pepsin treatment (4). The whole venom had an absorption maximum near 275 nm, while a toxic dialysate had an absorption maximum at 265 to 270 nm. Alkali treatment yielded a peak at 330 nm (5). Injection of whole venom into horse, sheep, rabbit, and guinea pig failed to induce formation of antibodies capable of neutralizing toxic effects (antibodies were formed against non-toxic protein components). At least 16 ninhydrin-positive components were present after electrophoretic fractionation. Fractions with toxic effects similar to those of whole venom were found among the cathode-migrating components. In none of these fractions was toxicity (on a toxicity/mg dry weight basis when sufficient material was obtained to permit weighing) greater than that of the whole venom (6).

From a venom dialysate, a toxic, ninhydrin-positive component with an absorption maximum of 278 nm was obtained which was electrophoretically and chromatographically homogeneous. Although its lethal

dose for mice was of the same order as crude venom, markedly different toxic symptoms were observed. Acid hydrolysis of the material released spermine (a nephrotoxin) as the only ninhydrin-positive substance, along with a number of other UV-absorbing components (7).

In 1972, a principal toxic fraction with a molecular weight in the range of 15,000 to 25,000 was finally isolated by fractionation of whole venom with a series of ultrafiltration membranes (8). It very firmly adhered to chromatographic paper, cellulose acetate, and silica gel, absorbing little UV light. These properties had served to prevent its detection previously. The toxic fraction demonstrated no synergism with any of four other fractions obtained. The minimum lethal dose of the toxic fraction was similar to that of the whole venom.

Proteolytic activity towards BSA (5) and phosphodiesterase activity (9) have also been found in A. robustus venom. Free γ-aminobutyric acid (GABA) is a major component, and traces of free glycine, serine, and threonine are also present (7). Sodium, magnesium, phosphorus, and calcium were detected; trace metals chromium and titanium were detected exclusively in the venom (5). At least half of the female venom mass is composed of material of molecular weight less than 500 (8).

Studies of the venom of the Pterinochilus species from East Africa revealed the presence of at least eight compounds with molecular weights less than 1400, as well as at least sixteen compounds with larger molecular weights. Toxic properties were found in four basic components of similar size (10). Subsequent studies using gel filtration followed by acrylamide gel electrophoresis revealed 26 protein bands present. Three to five fractions were lethal to mice. These were characterized as basic polypeptides with molecular weights between

7000 and 13,000 (11).

Proteolytic activity was characterized in the venom of Pamphobeteus roseus of Brazil (12). Activity towards casein was high, but neither trypsin substrates (BAEE and TAME) nor chymotrypsin substrates (ATEE and BAPA) were affected. Activity was not affected by Trasylol, soybean or ovomucoid inhibitors, nor by trypsin or chymotrypsin inhibitors. The molecular weight of each of the three proteolytic fractions obtained was 10,840 ± 620. The proteolytic activity was thought to play a role in extracorporal digestion.

Phosphodiesterase activity was found in the venom of Aphonopelma cratuis (9). The venom of an Aphonopelma species from Arizona was found to contain 5% protein, and demonstrated protease and RNase activity (13). Neither L-amino oxidase nor DNase activity was present. Ten protein fractions were found in disc acrylamide gel electrophoresis. The venom was compared with that of the scorpion Centruroides sculpturatus, and similarities were found in the toxic symptoms, enzymatic activities, and electrophoretic mobilities of the protein fractions. Ratios of protein content and of lethality of the two venoms were similar, suggesting that the toxic properties were associated with protein content. An adequate titer of the tarantula rabbit antivenin could not be obtained. However, a guinea pig hypersensitized with the scorpion venom was triggered into anaphylaxis by the tarantula venom seven days later.

A number of Brazilian tarantula venoms have been examined. Basic proteins were predominant in electrophoretic separations of venom of Grammostola, Acanthoscurria, Lasiodora, and Pamphobeteus species (14). Five-hydroxytryptamine (serotonin) was detected in the venoms of

Acanthoscurria atrox, A. sternalis, and Pterinopelma vellutinum. Lasiodora klugii and Pamphobeteus roseus venoms were also assayed, but large amounts of interfering substances obscured detection of this compound (15). A protein-free fraction of Grammostola mollicoma venom had a mild depressant action on snail heart, but neither acetylcholine nor 5-hydroxytryptophan were detected in the venom (16).

It was postulated by Welsh and Batty (15) that 5-hydroxytryptamine may have a defensive role as a pain producer. However, they further postulated that since pain is produced by depolarization of nerve endings as a result of changes in permeability and ion movements, a more fundamental role for this compound (and other compounds commonly found in venoms such as histamine, kinins, and acetylcholine) may be to increase the absorption rate of the venom toxins.

An early study by Fischer and Bohn (17) examined the venoms of the Brazilian tarantulas Grammostola mollicoma, G. actaeon, G. pulchripes, Eurypelma vellutinum, Pamphobeteus sorocabae, P. roseus, P. tetracanthus, Acanthoscurria atrox, and Lasiodora klugii. All venoms displayed four protein fractions after paper electrophoresis, and contained appreciable amounts of free glutamic acid and GABA. A number of other free amino acids were also detected in the venoms. Spermine and trimethylenediamine were detected in the P. tetracanthus venom, apparently combined with several phenolic acids that were identified.

Perret (18) found proteolytic activity in venoms of Pterinochilus sp., Aphonopelma chalcodes, and Dugesiella hentzi as a result of contamination with saliva. He felt that inappropriate milking methods may have brought about its detection in other spider venoms in which proteolytic activity has been found, and recommended that its presence

in spider venoms should be held suspect until proven otherwise.

A considerable amount of information has been obtained from venom of Dugesiella hentzi, a tarantula of the midwestern United States. A Russian group (19) found that the venom contained 40-50% protein and a nucleotide cofactor; electrophoretic separation at pH 8.3 revealed ten anodic protein bands. This is in some contrast to other information that has been obtained from venom of this species.

D. hentzi venom as characterized by Schanbacher et al. (20) has a pH of 5.44, a protein concentration of 200 µg/µl, an absorbance maximum of 258 nm, and A_{280}/A_{260} of 0.376. Disc polyacrylamide gel electrophoresis at pH 8.9 (7% gel) revealed approximately eight acidic and seven basic proteinacious components. Major free amino acids identified were glutamic, aspartic, and GABA. Hyaluronidase activity was present; the molecular weight estimate of the enzyme was 37,000-39,000. Phospholipase A, chitinase, protease, and hemolytic activities were absent. A peak obtained from G-100 Sephadex chromatography which was toxic to cockroaches had a molecular weight of about 7300.

The venom hyaluronidase was characterized by Schanbacher et al. (21). Amino acid analysis showed a molecular weight of 39,600, the isoelectric pH was 6.8, and the pH optimum was 3.5-4.0.

Adenine nucleotides AMP, ADP, and ATP were also detected in D. hentzi venom as well as that of an Arizona Aphonopelma species (22). ATP was present in larger amounts than the other nucleotides. It was shown that ATP had synergistic toxic effects with the major toxin of D. hentzi venom.

The major toxin of the venom was isolated (23) and characterized as a necrotoxin with molecular weight 6700 (from amino acid analysis)

and isoelectric pH of 10.0. The protein had 16 lysine, 8 cysteine, and one tryptophan residues, while no tyrosine, methionine, alanine, arginine, or histidine residues were present. Modification of the tryptophan residue resulted in a loss of toxicity. In this study, the first fractionation of whole venom yielded three peaks, two of which were toxic to mice. All three were toxic to cockroaches.

Table I is presented to permit a comparison of lethalities of various tarantula venoms.

TABLE I

LETHALITY OF SOME TARANTULA VENOMS

Species	LD_{50}	Injection Route	Ref.
D. hentzi	60 µg/g mouse[1]	subcutaneous	20
	3200 µg/g cockroach[1]	intraabdominal	20
D. hentzi necrotoxin	8.5 µg/g mouse	intraperitoneal	22
Aphonopelma sp.	14.14 µg/g mouse	subcutaneous	13
Pterinochilus sp.	3.3 µg/g mouse	intravenous	11
	45.4 µg/g cockroach	intraabdominal	11
Atrax robustus	21 µg/g mouse[2]	intravenous	8

[1]Calculated from values given in (20) of $\frac{0.3\ \mu l\ \text{whole venom}}{\text{g mouse}}$ and $\frac{.31\ \mu l\ \text{whole venom}}{\text{cockroach}}$, using $\frac{200\ \mu g\ \text{protein}}{\mu l\ \text{whole venom}}$ (20) for conversion to µg/g values, and 19.5 mg as average weight of one cockroach.

[2]For female venom. Male venom known to be at least six times more toxic (8). The value shown in the table was calculated from a mean lethal dose (as given in (8)) of 0.4 mg for mice weighing 18-20 g.

Reports of the effects of tarantula venoms show a wide variation of syndromes.

Microscopic examination of tissues of mice killed by the D. hentzi necrotoxin showed that its primary site of action was the heart (23). Acute focal areas of myocardial necrosis were observed, most often associated with the endocardial or epicardial surface. These areas were composed of degenerating acidophilic shrunken cells with small pyknotic nuclei. The cells had often lost their usual polarity and were separated from one another, and small areas of hemorrhage were sometimes associated with the lesions. No histological differences were observed between mice injected with the toxin and those injected with whole venom. Further proof of muscle damage was obtained by showing a significant increase in serum creatine phosphokinase activity in mice injected with the toxin. (The intraperitoneal injection route was used in these studies.)

Subcutaneous injection of 10 μg of Aphonopelma sp. venom into an adult rat produced severe convulsions within about 20 minutes, followed by copious drooling and death in about one hour (13). Post mortem examinations revealed gastric hyperdistension, hepatic and nephric hyperemia, and pectechiae on the lungs. This syndrome is very similar to that caused by scorpion Centruroides sculpturatus venom.

Guinea pigs bitten with Pterinochilus sp. venom showed no local signs of the bite, but haemograms showed moderate leukocytosis with neutrophilia. ECG and EEG readings were affected, and it was shown that the venom penetrated the blood-brain barrier. Histological examination showed vacuolar and parenchymatous degeneration of renal tubules and liver, with diffuse fatty infiltration observed in some cases. The

brain showed degenerative alterations (24). According to Freyvogel et al. (10), mice died of respiratory paralysis when injected with this venom, while cockroaches (11) suffered cardiac arrhythmia followed by heartblock. According to Perret (11), the venom is 2 to 3 times more lethal for mice than that of Acanthoscurria musculosa, considered the most dangerous of the South American orthognaths (25).

Determination of the physiological mode of action of the violent reactions produced in many humans after Atrax robustus envenomation has been more elusive than the identification of the toxic component(s) of the venom. Table I shows that the lethality of the venom in mice is not particularly impressive compared with some other orthognath venoms. In the mouse, envenomation produced immediate pain at the injection site followed by profuse salivation, lacrimation, breathing difficulties, paralysis, agitation, and incoordination leading to coma. Death was caused by a sudden stop in respiration, followed by cardiac arrest; hypothermia was usually present at this time (26). Human envenomation symptoms appear to come in two stages. In the first stage, soon after being bitten, symptoms are similar to those observed in mice, except paralysis is not observed, while muscle twitching may become quite violent. These symptoms subside, and hours later, victims have suddently died from asphyxiation or cardiac arrest (3). It has been reported that man and monkeys are more susceptible than other species to the toxic effects of the venom (27).

The 15,000-25,000 MW fraction isolated by Sutherland (8) was added to a rat phrenic nerve-diaphragm preparation and promoted an increase in tension, followed by long periods of irregular, brisk contractions. This material also caused minor contraction in ileum tissue, but the

lowest molecular weight material (less than 500) contained the strongest constrictor of smooth muscle. Several studies have been made to determine the venom's mode of action on vascular tissue (28, 29, 30), but none of the effects observed have been great enough to account fully for the venom's hypertensive effects.

In addition to biochemical and pharmacological studies of tarantula venoms, morphologic studies of the venom gland structures of D. hentzi (31), D. echina, Aphonopelma chalcodes, A. portala n. sp., and an Aphonopelma species (32) have been made.

It is interesting to note from Table I that the LD_{50} of Pterinochilus sp. venom to cockroaches was found to be much higher than it was to mice. Also, recall the three fractions of D. hentzi venom, two of which were toxic to mice, while all three were toxic to cockroaches. Venom of Atrax robustus also seems to demonstrate species specificity. Venoms of some parasitic wasps and conidae are toxic to only a single or a few species; in these cases specificity to the organisms's environment might be reflected (33). Several species-specific toxins have been found in venoms of the black widow spider, several scorpions, and some elapid snakes. It was speculated that in the case of a single venom which contains several species-specific toxins, a chemical adaptation to changes in food sources during evolution may be reflected.

The selectivity of some toxins, as well as the various physiological modes of action of all toxins, may make these compounds useful tools for biochemical analysis and for the understanding of other biochemical systems.

CHAPTER III

MATERIALS AND METHODS

Tarantulas

Specimens of Dugesiella hentzi were collected from areas surrounding Stillwater. Aphonopelma emilia, Eurypelma panamense, and Aphonopelma sp. specimens were obtained from private dealers. An indoor colony of approximately 30 D. hentzi, 25 A. emilia, 25 Aphonopelma sp., and 12 E. panamense tarantulas was maintained with a diet of crickets and water. This colony contained primarily females, since they are easier to maintain and handle than male tarantulas, and only venom collected from females was used in this study.

Venom Collection

Venom was collected by electrical stimulation, using a modification of the procedure described by Grothaus and Howell (34). Tarantulas were lightly anesthetized with CO_2 and placed, with fangs exposed, in a foam rubber restrainer. The chelicerae were wetted with a solution of 5% $NaHCO_3$ and 0.1% sodium dodecyl sulfate (SDS) to enhance electical contact. A piece of Tygon tubing was placed beneath the fangs to serve as a "trough" into which the venom could flow. This trough was positioned to avoid contamination with other body fluids or wetting solution. An electrical biting stimulus of 7 volts at 20 cycles per

second was applied across the chelicerae, using a Heathkit auto generator power supply, model IG-72. Venom flowed from the fangs directly onto the tubing, where it was immediately collected with a glass capillary tube and placed in an ice-bathed container. Venom samples from the same species of tarantula were pooled. All venoms were stored at -15°C until samples were needed for the research.

Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis was conducted using the pH 4.3 system of Reisfeld, Lewis, and Williams (35). This system was modified by allowing the gel to polymerize in a slab between two glass plates instead of inside a glass tube. This slab was made by pouring the gel between 2 6¼ X 10" glass plates, one with 5 X 1 1/8" notch cut out of the top. (The purpose of the notch was to permit entrance of electrophoresis buffer into the gel.) Strips of flexible plastic ruler (C-Thru Ruler Co. # M-109), cut to appropriate lengths, were placed between the plates as spacers. The strips were held in place with vaseline, and the glass plates were clamped together to form a thin mold in which the acrylamide polymerized to form a slab of gel. In order to form wells in which samples could be placed, a piece of the same ruler material was used in which 3/4 X ¼" notches were cut to form a comb which could be inserted between the glass plates into the acrylamide solution.

Using this apparatus, the separating gel, consisting of 15% acrylamide with .14% ammonium persulfate as catalyst, in pH 4.3 KOH-acetic acid buffer, was 170 X 140 X .8 mm. The stacking gel had a pH of 6.7 (KOH-acetic acid buffer), and was 6.7% acrylamide, with riboflavin as

catalyst. Samples applied to the ¼ X 3/4" wells traveled approximately 23 mm through the stacking gel before reaching the separating gel.

Crude venom samples and samples of purified components were subjected to electrophoresis at pH 5.0 at room temperature (Buchler Instruments model #3-1014A power supply), at 5 mamps overnight, then at 10 mamps until the tracking dye, pyronin red Y, was approximately 1½" from the bottom of the gel. The gels were stained with aniline blue-black.

In some cases, tube gels were used in place of the slab gels. The tubes had an inside diameter of 5 mm; stacking gels were 2 cm long and separating gels were 9 cm long. Samples in these gels were electrophoresed for 30 minutes at 2 mamps/gel, then for 2 hours at 4 mamps/gel.

This technique was used to obtain profiles of the components of the whole venoms, as well as to identify and monitor the purity of isolated components of the venoms.

SDS-acrylamide gel electrophoresis, used for the molecular weight determinations, was conducted according to the method of Weber and Osborn (36), again substituting the slab system for the tubes, as described previously. The gels contained 6% or 15% acrylamide with a pH of 7.2 (sodium phosphate buffer). Electrophoresis proceeded at 8 mamps overnight, then 10 mamps until the tracking dye (bromophenol blue) was about 2½" from the bottom of the gel.

Gel Filtration and Ion Exchange Chromatography

All columns were silanized by 3-hour treatment with 5% dimethyl dichlorosilane in toluene before use. Gel filtration media (Sephadex G-50 and G-25) were obtained from Pharmacia Fine Chemicals, Inc.

(Piscataway, N. J.), and ion exchange media (DEAE-Sephadex and CM-Sephadex) were obtained from Sigma Chemical Co. (St. Louis, Mo.).

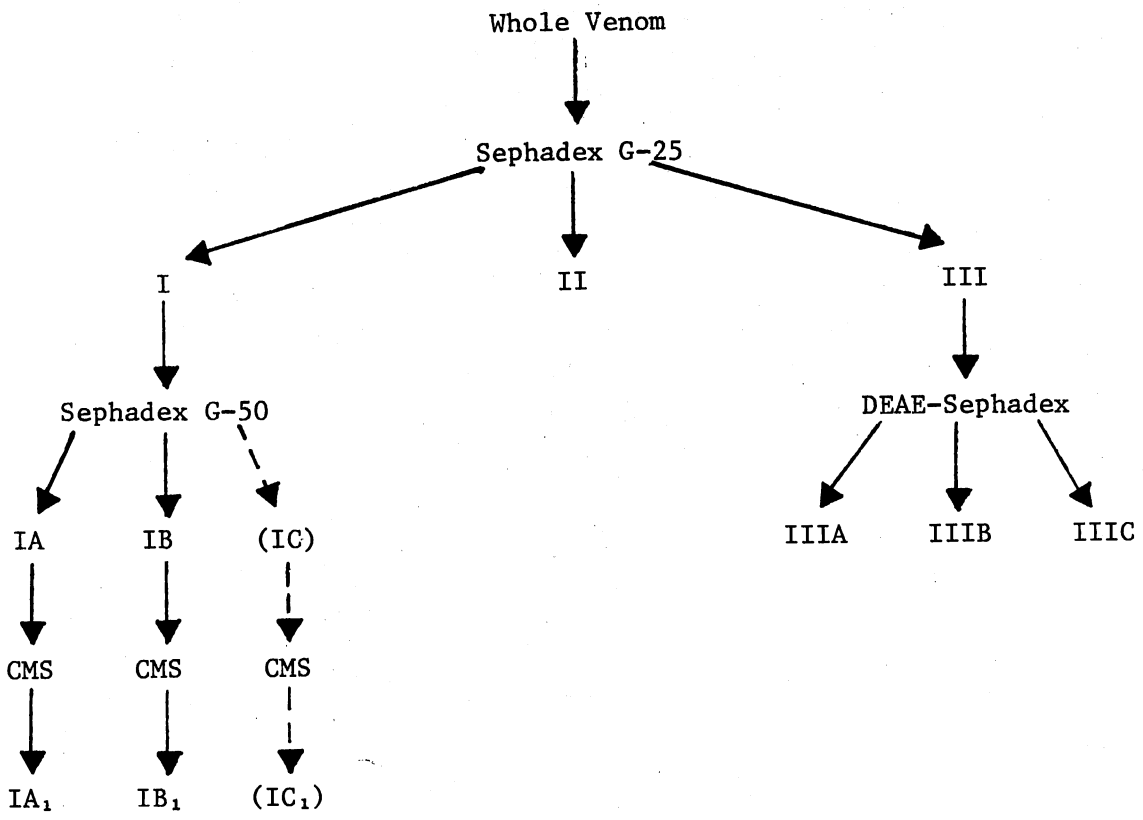
Each medium was allowed to swell in the buffer with which sample would be eluted. Where elution gradients were used, the medium was allowed to swell in the starting buffer. Gel filtration media were allowed to swell overnight, then deaerated with a water aspirator for one hour. Ion exchange media were allowed to swell for at least 1½ hours on a steam bath, then deaerated with a water aspirator for at least 30 minutes. Each column was packed by gravity flow, then allowed to equilibrate at the temperature at which it would be used. Gel filtration columns were calibrated with the appropriate molecular weight markers to ensure proper functioning before samples were applied. Salt gradients for ion exchange chromatography were made using a 500-ml Erlenmeyer flask with outlet (diameter at bottom approximately 9.5 cm) as reservoir for the more concentrated buffer, and a cylindrical bottle with inlet and outlet (diameter at bottom approximately 8 cm) as reservoir for the less concentrated buffer. All fractions collected were monitored at 260 and 280 nm using a Perkin-Elmer Coleman 101 Spectrophotometer. For the ion exchange chromatography, salt concentration was monitored with a Radiometer Copenhagen conductivity meter type CDM 2d. Fractions containing protein or nucleotide were pooled and lyophilized for further use.

Gel filtration and ion exchange methods described below for the separation and purification of venom components (methods not referenced or modifications of methods referenced) were developed previously in the laboratory of Dr. G. V. Odell.

Figure 1 shows the overall fractionation scheme used for the whole

Figure 1. Fractionation Scheme for Whole Venoms and Their Components.

Dotted lines indicate peaks obtained from some, but not all of the venoms. Solid lines indicate peaks obtained from all four venoms. CMS = carboxymethyl-Sephadex.


Whole Venom
Sephadex G-25
I
II
III
Sephadex G-50
DEAE-Sephadex
IA
IB
(IC)
IIIA
IIIB
IIIC
CMS
CMS
CMS
IA1
IB1
(IC1)

venoms and their components. These materials will be referred to by the designations used in Figure 1.

Fractionation of Whole Venoms

The method of Chan et al. (22) was used for initial separation of venom components. Two hundred fifty µl of pooled venom samples from each species were applied to a 1.6 X 84 cm column of Sephadex G-25 that was equilibrated at 4°C with a flow rate of 16 mls per hour. Sodium phosphate buffer, pH 6.8, was used as the eluent, and fractions of one ml were collected.

Fractionation of High Molecular Weight Components

Chromatography on Sephadex G-50 was used to fractionate further the high molecular weight components (peaks I) obtained from the initial separation. The column, 2 X 81 cm, was equilibrated at 4°C with a flow rate of 11 ml/hr. Ammonium bicarbonate, 0.02 M, was the eluent, and fractions of 2 ml were collected. The amount of sample applied was the equivalent of 500 µl of whole venom; that is, peak I from two G-25 separations for each venom.

Peaks obtained from the G-50 separation (IA, IB, IC) were further purified through a 1.5 x 64 cm CM-Sephadex A-25 column, using a modification of the method of Lee et al. (23). Because a salt gradient was used for elution, purification of each G-50 peak (IA, IB, IC) required a freshly packed column. The columns were packed and equilibrated at room temperature, and flow rates ranged from approximately 50 to 60 ml/hr. The gradient was made using 250 mls of 0.1 M ammonium bicarbonate and 25 mls of 0.5 M ammonium bicarbonate, and fractions of 3 ml

were collected.

Separation of Nucleotides

Separation of nucleotides (peaks III) was accomplished using a modification of the method of Chan et al. (22). Freshly packed 1.5 X 64 cm columns of DEAE-Sephadex A-25 were equilibrated at room temperature with flow rates ranging from 45 to 66 ml/hr. To avoid overloading the columns, only a portion of the material in peaks III was applied to each column. This portion was equivalent to 38 µl of whole venom. A salt gradient was used for elution, consisting of 200 ml of 0.1 M ammonium formate and 400 ml of 1.0 M ammonium formate. Fractions of 3.5 ml were collected.

Absorption Spectra

Absorption spectra for materials in peaks IIIA, IIIB, and IIIC were obtained using a Varian Techtron Model 635 Spectrophotometer equipped with a Varian 18-25 recorder. For each solution, the instrument was zeroed at 350 nm; the solution was then scanned from 350 nm to 200 nm. The pH of each solution was measured using Hydrion Papers (Micro Essential Laboratory, Brooklyn, N. Y.).

Hyaluronidase Assay

Whole venoms and peaks IA_1 were assayed for hyaluronidase activity according to the method based on those of Tolksdorf et al. (37) and Kass and Seastone (38), as described in the Worthington Enzyme Manual. In this procedure, hyaluronic acid concentration is measured by its ability to form turbidity with an acidic solution of albumin. Turbidity

is a function of hyaluronic acid concentration, and enzyme activity can be measured by a decrease in turbidity.

The assay was modified by reducing volume to one-half of that described in the Worthington Manual. Solutions containing a known amount of hyaluronic acid were incubated for 10 minutes at 37°C with samples and reference enzyme (buffer: 0.1 M NaH_2PO_4 in 0.15 M NaCl, pH 5.3), then heated in a boiling water bath for 5 minutes to stop the reaction. After cooling, albumin reagent (2.5 g fraction V albumin powder/liter, in 0.5 M sodium acetate buffer, pH 4.2) was added and after 10 minutes turbidity was measured as A_{540} in a Turner model #350 spectrophotometer. Milligrams of hyaluronic acid remaining in these mixtures were determined from a standard curve of hyaluronic acid concentration.

Activity was measured as units per µl of whole venom, where one turbidity reducing unit (TRU) decreases the turbidity-producing capacity of 0.2 mg hyaluronic acid to that of 0.1 mg hyaluronic acid in 30 minutes at 37°C.

Hyaluronic acid, Grade 1, from human unbilical cord, hyaluronidase, from bovine testes, 3500 NF units/mg, and fraction V albumin powder were all obtained from Sigma Chemical Co.

Luciferase Assay for ATP

The firefly luciferase assay for ATP was used to detect its presence in whole venoms and in peaks IIIC. Optimum conditions developed for the assay (39) on the basis of a review by DeLuca (40) were used in the determinations.

The assay is based on the ATP-specific reaction of luciferin

(LH_2), catalyzed by luciferase (E).

$$E + LH_2 + ATP \xrightleftharpoons{Mg^{2+}} E\text{-}LH_2AMP + PP_i \quad (1)$$

$$E\text{-}LH_2AMP + O_2 \longrightarrow \text{oxyluciferin} + AMP + E + CO_2 + h\nu \quad (2)$$

Thus, ATP may be quantitated by measuring the light emitted from the reaction.

Reaction volumes were 1 ml, containing 100 µl Tricine buffer, pH 7.75, (0.025 M Tricine, 0.005 M Mg^{2+}, 5 X 10^{-4} M EDTA, 5 X 10^{-4} M DTT), 10 µl luciferase in Tricine, and sample. Light emissions were monitored with a JRB Integrating Photometer, Model 3000 (SAI Technology Co.). The enzyme-substrate preparation was Dupont Purified luciferase-luciferin, and all other chemicals were obtained from Sigma Chemical Co. or were of reagent grade.

Extraction of Gels for Amino Acid Analysis

Amino acid analysis was used to check for artifact formation from polyacrylamide gel electrophoresis. The following method, developed by Dr. Ta-Hsiu Liao, was used to extract proteins from the stained gels and prepare them for amino acid analysis.

The bands were cut out of the gel and soaked in water for 2 hours to remove acetic acid and isopropyl alcohol derived from the destaining solution. Protein material was then extracted by adding glacial acetic acid of a volume twice the weight of the piece of gel containing the band (this gives approximately 66% acetic acid for the first extraction). Two more extractions were made with 0.5 ml of 66% acetic acid; each of the 3 extractions was for six or more hours. Extracts were combined in

hydrolysis tubes and dried in a desiccator equipped with a vacuum pump.

Ten µl of 5% phenol, 2 µl of thioglycolic acid, and 0.2 ml of 6 N HCl were added to each dried extract, the tube was sealed under vacuum, and hydrolysis was allowed to proceed for 24 hours at 110°C. The hydrolysate was again dried under vacuum, then stored at -15°C until analysis.

Amino Acid Analysis

A microanalyzer using a single narrow bore column and an expanded scale recorder for measuring amino acids at the nanomole level was used for the analyses. This instrument, constructed by Dr. T. H. Liao (48), was an adaptation of the basic design of Spackman, Stein, and Moore (41).

Bioassays for Toxicity

Materials from peaks IB_1 were assayed for toxic activity. Amounts of protein present in these peaks were estimated using the A_{260}-A_{280} nomograph method, and solutions containing approximately 1 mg/ml protein in deionized water were prepared. A pair of white mice, each weighing 20-30 g, was used to assay each IB_1 venom fraction for toxic activity. Mice were injected intraperitoneally with 0.1 ml of solution (100 µg protein/mouse). A pair of control mice were each injected with 0.1 ml of 0.85% physiologic saline (PSS).

Heart tissue was obtained from each mouse 48 hours after injection or at death, whichever occurred first. (Mice still alive after 48 hours were killed by cervical dislocation.) The heart was removed, immediately rinsed with fixative, then placed in a volume of fixative (10%

buffered formalin) 20 times that of the heart.

Tissue samples were allowed to fix from 24-72 hours, then dehydrated and embedded in paraplast. Heart tissue sections 6 μm thick were obtained using an AO rotary microtome with steel knives. Sections were stained routinely with hematoxylin and eosin, then viewed with a Zeiss photomicroscope.

CHAPTER IV

RESULTS AND DISCUSSION

Polyacrylamide Gel Electrophoresis of Whole Venoms

The separations of the whole venoms obtained by polyacrylamide gel electrophoresis are shown in Figure 2 (tube system) and Figure 3 (slab system). An earlier study by Lee et al. (23) revealed only four broadly stained regions for the D. hentzi venom in this system (tubes), and identified a band observed approximately half-way down the gel as a single protein with necrotoxic activity. In this study, the results obtained from electrophoresis in the tube system appeared to demonstrate increased resolution. For the D. hentzi venom, a greater number of more distinct bands were observed, and two bands appeared that corresponded to the single necrotoxin of the previous study.

The slab gel system of Figure 3 appeared to resolve further the bands shown in Figure 2, particularly those near the cathode. The necrotoxin found by Lee et al. was identified as a small (MW 6700), very basic protein containing 16 lysine residues. Thus, it was expected that the toxic activity for each venom would be found in one or more of the farthest-migrating group of bands in the slab gel system, since this migration would be characteristic of small, basic proteins.

Figure 2. Polyacrylamide Gel Electrophoresis of Whole Venoms and Spermine, Tube System

(15% acrylamide gels, pH 4.3)

1. Aphonopelma emilia
2. Aphonopelma sp.
3. Dugesiella hentzi
4. Eurypelma panamense
5. Spermine

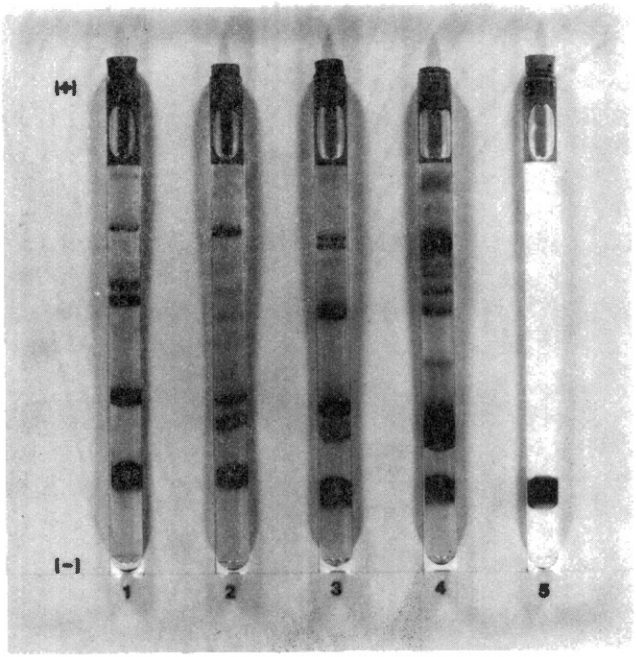
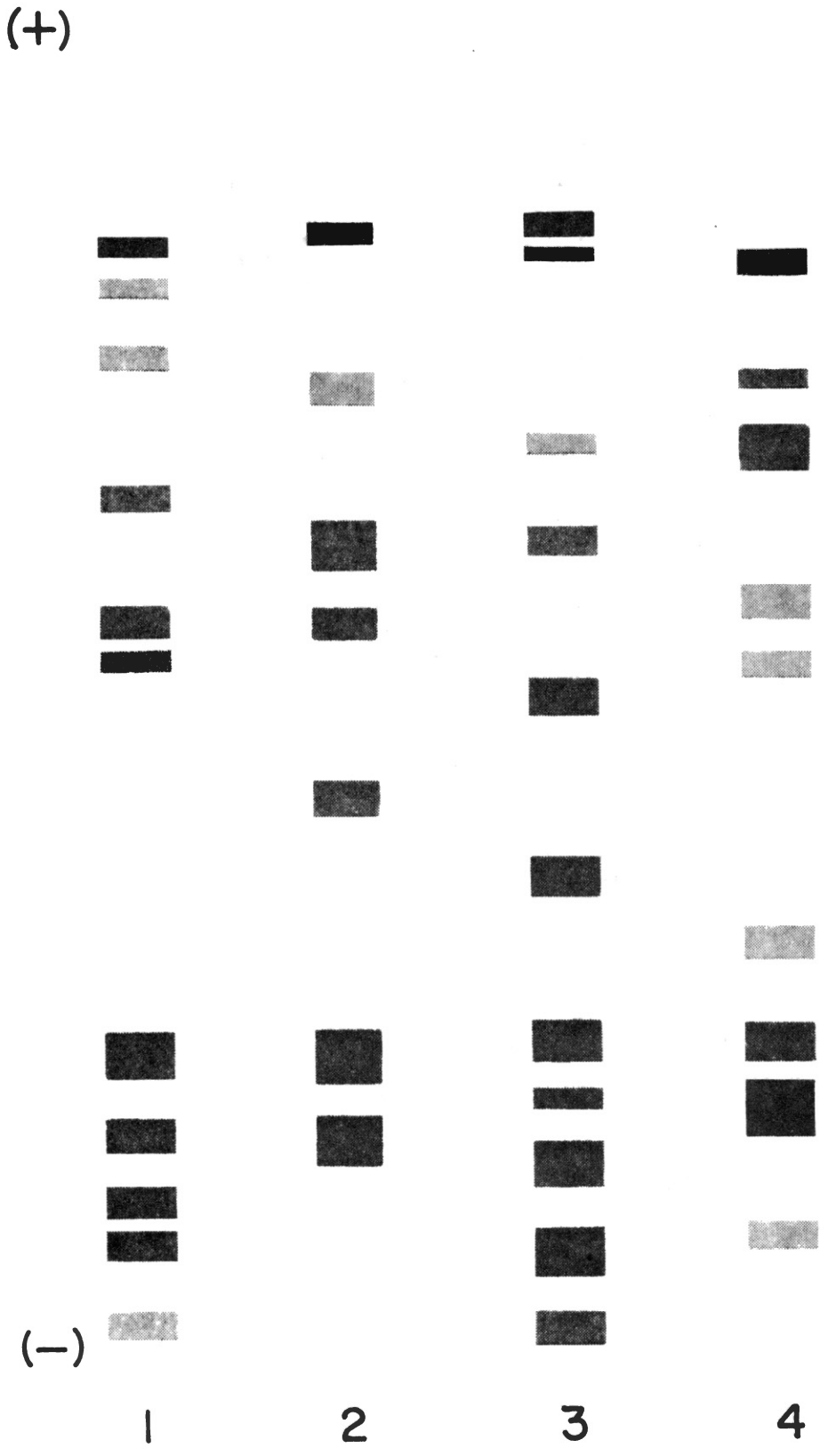

(+)
(−)
1
2
3
4
5

Figure 3. Polyacrylamide Gel Electrophoresis of Whole Venoms, Slab System

(15% acrylamide gels, pH 4.3)

1. Dugesiella hentzi
2. Aphonopelma emilia
3. Aphonopelma sp.
4. Eurypelma panamense

Shading shown in this representation indicates relative band intensities observed in the slab gels. The bands shown in Figure 2 which are closest to the cathode are not shown in Figure 3. In the slab system, electrophoresis was conducted such that these bands migrated out of the gel.


(+)
(−)
1
2
3
4

Although increased resolution of protein bands was apparent in the slab gel system, the possibility of artifact formation could not be ruled out since the gels, although acidic (which would help prevent oxidation), contained ammonium persulfate, which creates an oxidizing environment. Amino acid analysis of the necrotoxin characterized by Lee et al. revealed the presence of 8 cysteine residues, and no free sulfhydryl groups were detected. This implies that four disulfide bonds were present in the necrotoxin. Cleavage of these disulfide bonds during electrophoresis could produce artifact peptide fragments; cleavage followed by reformation of different disulfide bridges could produce a protein with a different mobility than that of the original. As a check for artifact formation, amino acid analyses were obtained for each of the bands in question. These analyses also served as a preliminary determination of the amino acid composition of each of the proteins.

After electrophoresis of each whole venom, each band from the group of bands migrating to the nearest cathode was extracted and analysed. In Figure 3, these were the last (bottom) 5 bands in D. hentzi venom, the last 2 in A. emilia venom, the last 5 in Aphonopelma sp. venom, and the last 4 in E. panamense venom. Two of the major bands from each of these groups were found in toxic fractions of the venoms, and these are discussed in more detail later.

Results of amino acid analyses of the remaining bands (Appendix) were for the most part inconclusive. Total amino acid material obtained from most of these bands was not large enough in comparison with a blank determination to yield reliable results.

The data obtained, however, showed that some amino acids detected

from these other bands were not present in the toxin bands. The minor bands appeared consistently in electrophoretic separations of the venom. These considerations would not indicate the occurrence of random fragmentation of the toxin bands during the electrophoresis process. No conclusive evidence was obtained from amino acid analyses to suggest the presence of artifacts, and the unidentified cathode-migrating bands may be peptides with unique identities.

Fractionation of Whole Venoms

Figure 4 shows typical elution patterns obtained for each whole venom by chromatography on Sephadex G-25. Fractions were pooled and lyophilized as shown. The peaks labelled I and III in each elution profile were subsequently purified and characterized as described below.

High Molecular Weight Components

Each peak I from Figure 4 was further fractionated by chromatography on Sephadex G-50. The equivalent of 500 µls of whole venom was applied to the G-50 column (i.e., peaks I from two G-25 fractionations of each whole venom). Resulting elution profiles are shown in Figure 5. Materials obtained from Sephadex G-50 chromatography (peaks IA, IB, IC) were further purified and characterized as hyaluronidases and toxins.

Hyaluronidases

Peaks IA from the G-50 fractionations were each applied to a CM-Sephadex column to complete their purification. Figure 6 shows the single major peak obtained from venom of each tarantula species.

Figure 4. Sephadex G-25 Fractionation of Whole Venoms

(Eluent: 0.02 M sodium phosphate, pH 6.8; one ml/fraction)

Markers	MW	V_e (mls)
blue dextran	2×10^6	66 = Vo
bacitracin	1.4×10^3	77
DNP-alanine	2.55×10^2	150

Solid lines beneath peaks indicate pooled fractions.

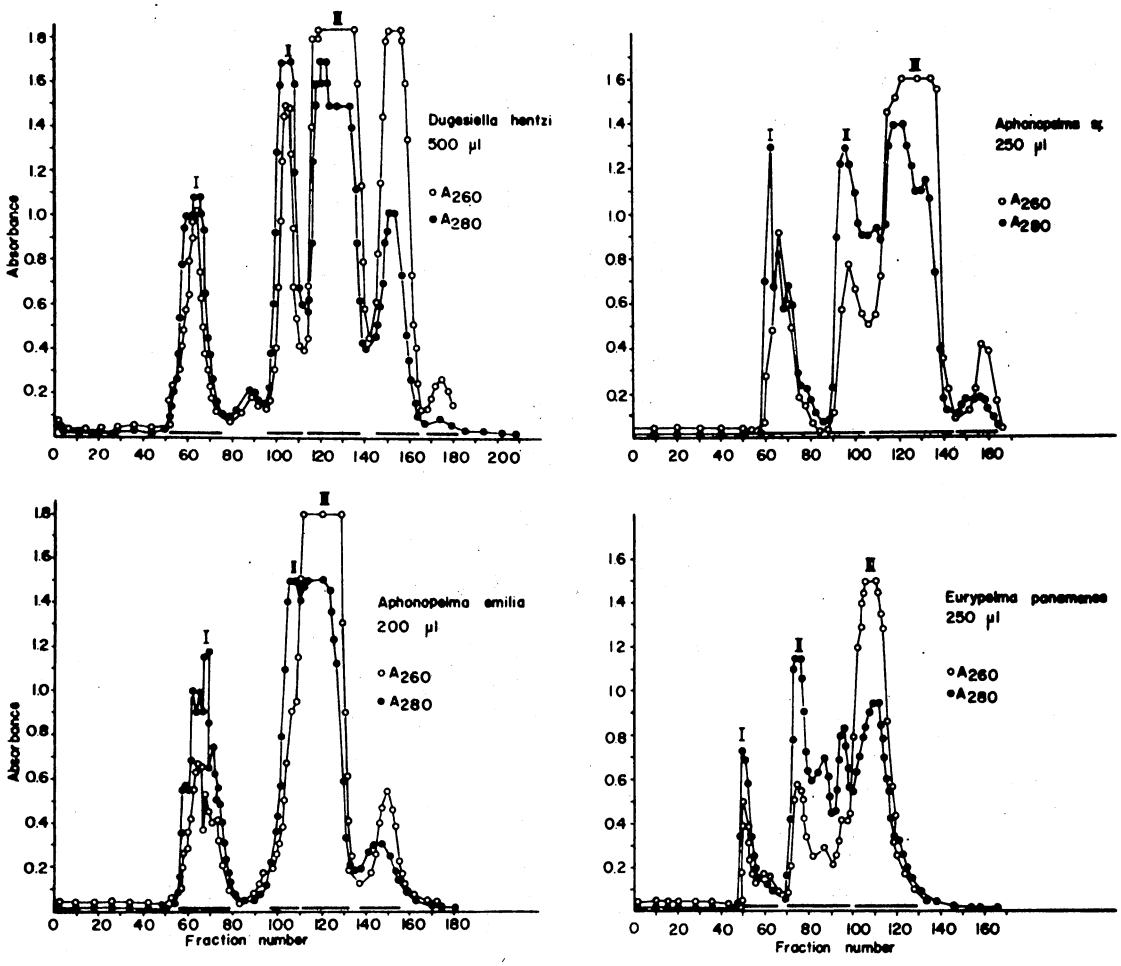

Dugesiella hentzi
500 µl
o A260
• A280
Aphonopelma sp.
250 µl
o A260
• A280
Aphonopelma emilia
200 µl
o A260
• A280
Eurypelma panamanus
250 µl
o A260
• A280
Absorbance
Fraction number
I
II

Figure 5. Sephadex G-50 Fractionation of Peaks I (High Molecular Weight Components)

(Eluent: 0.02 M ammonium bicarbonate, pH 7.7; two mls/ fraction)

Markers	MW	V_e (mls)
blue dextran	2×10^6	66 = Vo
chymotrypsinogen	2.5×10^4	78
cytochrome C	1.24×10^4	92
DNP-alanine	2.55×10^2	198

Solid lines beneath unresolved peaks indicate pooled fractions.

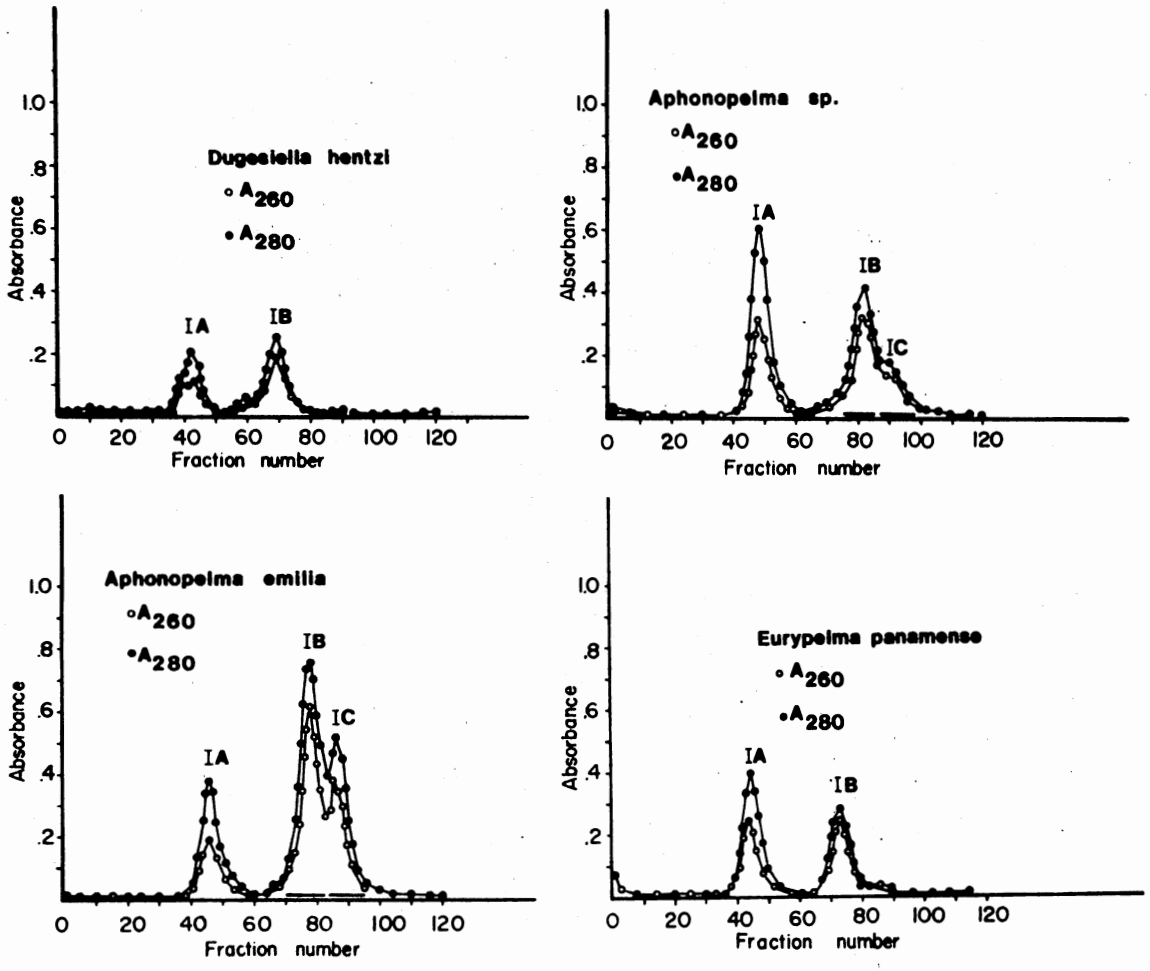
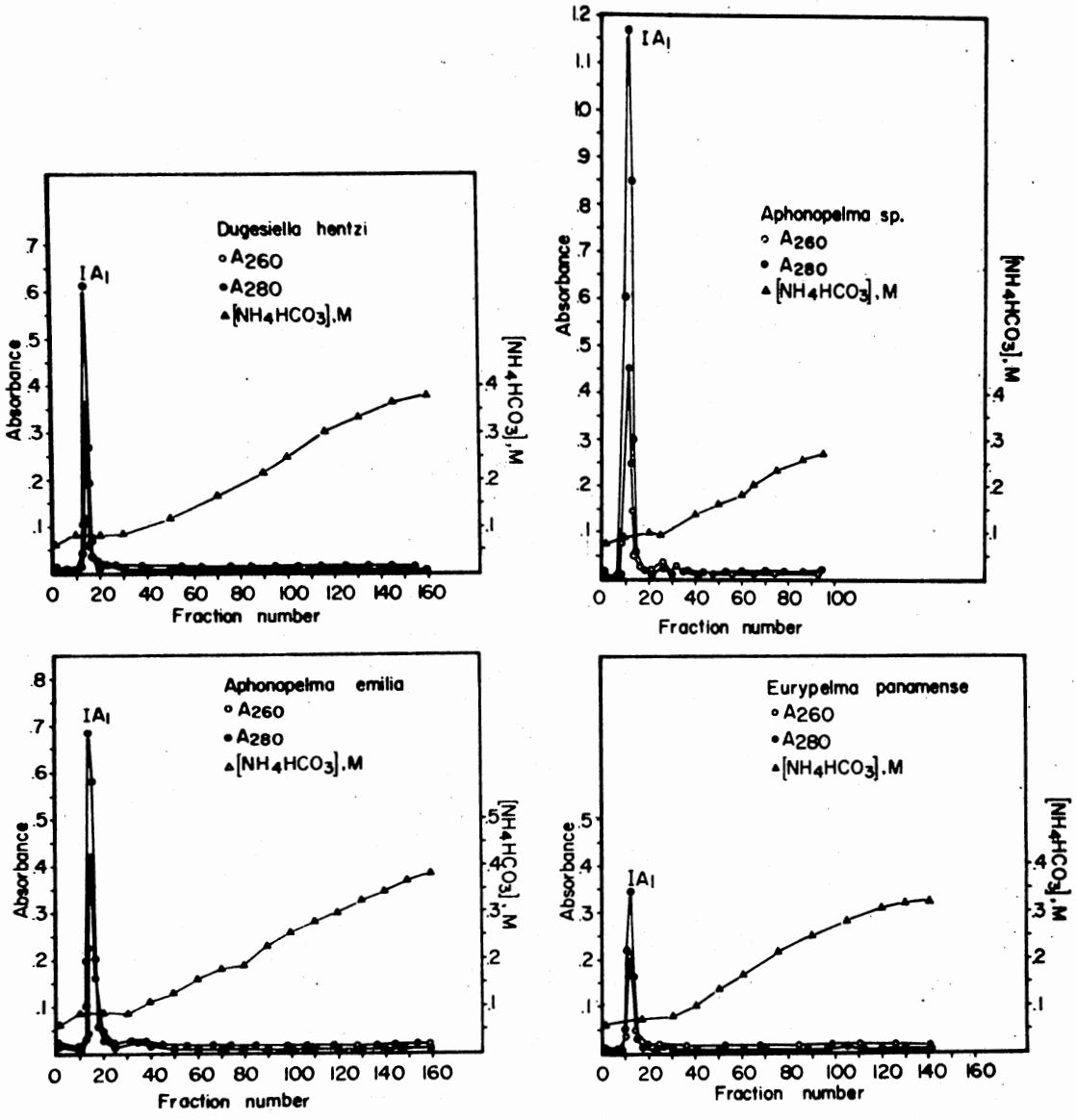

Dugesiella hentzi
°A260
•A280
IA
IB
Absorbance
Fraction number
Aphonopelma sp.
°A260
•A280
IA
IB
IC
Absorbance
Fraction number
Aphonopelma emilia
°A260
•A280
IA
IB
IC
Absorbance
Fraction number
Eurypelma panamense
°A260
•A280
IA
IB
Absorbance
Fraction number

Figure 6. CM-Sephadex Elution Profiles of Peaks IA (Hyaluronidases)

(Eluent: 0.1 M-0.5 M ammonium bicarbonate salt gradient; 3 mls/fraction)


Dugesiella hentzi
°A260
•A280
▲[NH4HCO3],M
IA1
Absorbance
[NH4HCO3],M
Fraction number
Aphonopelma sp.
°A260
•A280
▲[NH4HCO3],M
IA1
Absorbance
[NH4HCO3],M
Fraction number
Aphonopelma emilia
°A260
•A280
▲[NH4HCO3],M
IA1
Absorbance
[NH4HCO3],M
Fraction number
Eurypelma panamense
°A260
•A280
▲[NH4HCO3],M
IA1
Absorbance
[NH4HCO3],M
Fraction number

Assays for hyaluronidase activity confirmed its presence in the material from each peak (IA_1).

In order to identify the corresponding bands in the whole venoms and check for purity, the pooled and lyophilized material from each peak IA_1 was dissolved in 1 ml of distilled water (0.7 ml for E. panamense) and applied to 15% polyacrylamide gels along with the corresponding whole venoms. Composite results are shown in Figure 7. Samples of the purified materials were applied in amounts equivalent to 10 µls and 20 µls of whole venom (for E. panamense, equivalent to 15 µls and 30 µls of whole venom), overloading intentionally to check for minor contaminants. For each purified material a single band was observed which corresponded to the uppermost band of the whole venom from which it was isolated.

Molecular weights of the hyaluronidases were determined using SDS-acrylamide gel electrophoresis. Standard molecular weight markers were selected on the basis of an earlier study by Schanbacher et al. (20), in which the molecular weight of the D. hentzi enzyme was estimated as 39,000 using this technique. As shown in Figure 8, the molecular weights obtained in this study ranged from 42,000 to 46,000 for the venom hyaluronidases.

Elution behaviors of all four enzymes were quite similar in CM-Sephadex as well as G-50 chromatography; migrations in acrylamide gels (with and without SDS) were quite similar also. This implies that the size and net charge of the proteins were generally similar; structural characteristics might have been similar also. Although the general value of 40,000 for the molecular weight of these enzymes was rather high in comparison to those of other hyaluronidases, gel

Figure 7. Polyacrylamide Gel Electrophoresis of Whole Venoms and Venom Hyaluronidases

(15% acrylamide, pH 4.3)

1A. *Dugesiella hentzi* whole venom
1B. *Dugesiella hentzi* hyaluronidase (peak IA_1)

2A. *Aphonopelma emilia* whole venom
2B. *Aphonopelma emilia* hyaluronidase (peak IA_1)

3A. *Aphonopelma* sp. whole venom
3B. *Aphonopelma* sp. hyaluronidase (peak IA_1)

4A. *Eurypelma panamense* whole venom
4B. *Eurypelma panamense* hyaluronidase (peak IA_1)

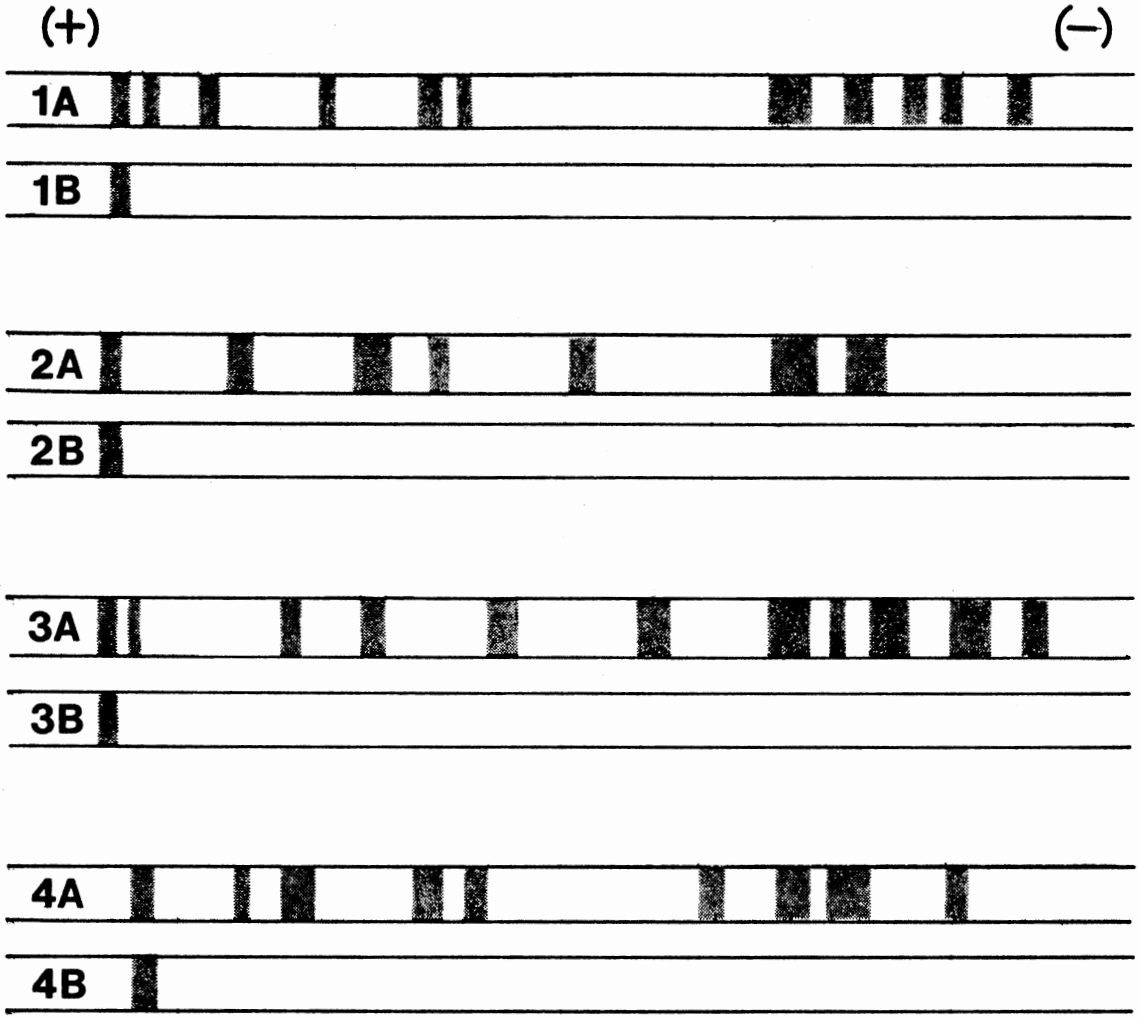

(+)
(−)
1A
1B
2A
2B
3A
3B
4A
4B

Figure 8. Molecular Weight Determination of Purified Venom Hyaluronidases

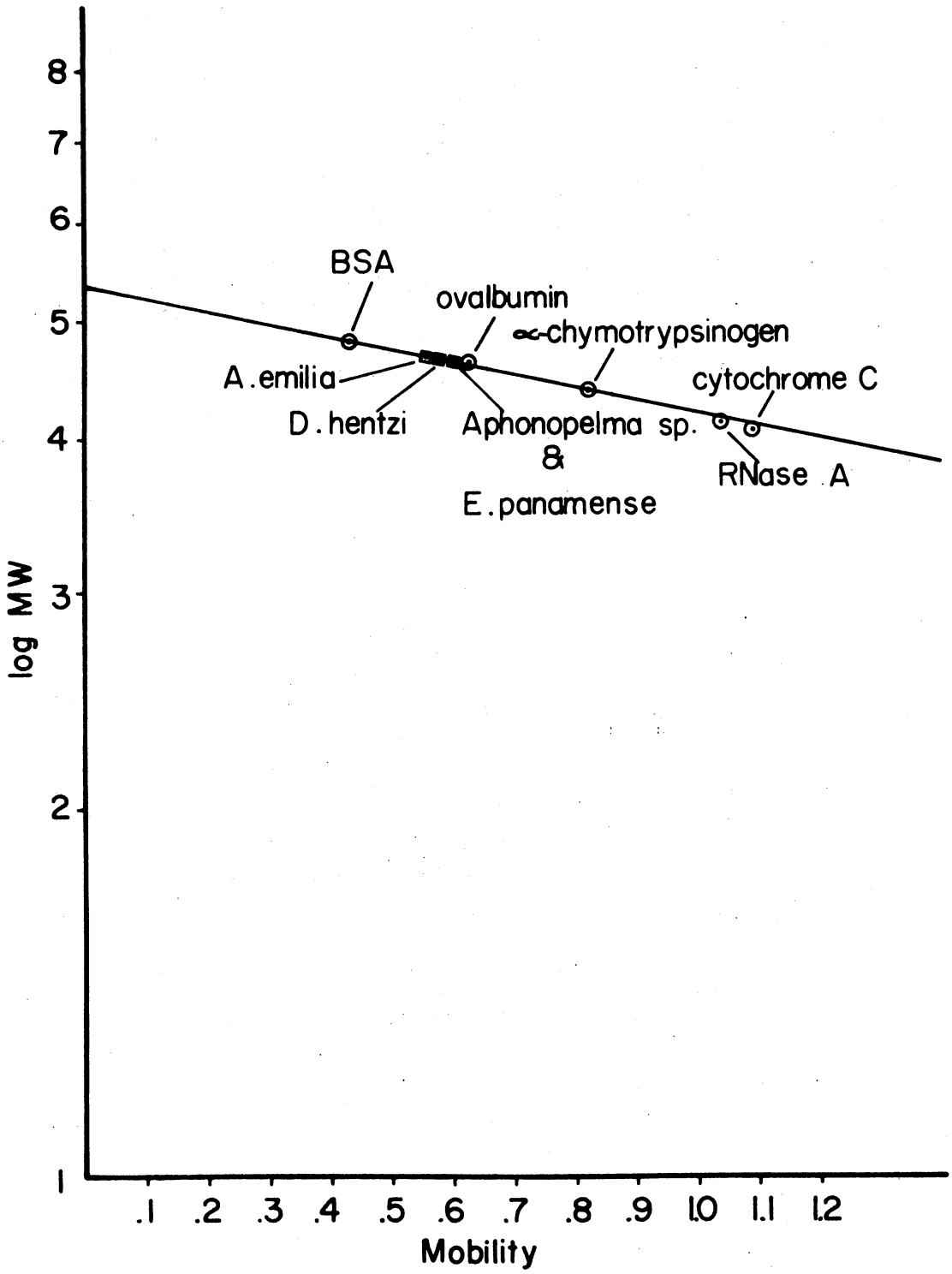
(6% acrylamide, pH 7.2)

Molecular Weights of Markers:

bovine serum albumin - 67,000
ovalbumin - 45,000
α-chymotrypsinogen - 25,000
ribonuclease A - 13,700
cytochrome C - 12,400

Molecular Weights of Hyaluronidases:

Dugesiella hentzi - 45,000
Aphonopelma emilia - 46,000
Aphonopelma sp. - 42,000
Eurypelma panamense - 42,000


8
7
6
5
4
3
2
1
log MW
BSA
ovalbumin
∝-chymotrypsinogen
A. emilia
D. hentzi
Aphonopelma sp.
&
E. panamense
cytochrome C
RNase A
.1
.2
.3
.4
.5
.6
.7
.8
.9
1.0
1.1
1.2
Mobility

electrophoresis did not suggest the presence of subunits, and the values obtained here were in fair agreement with the previous report of Schanbacher et al.

Hyaluronidase hydrolyzes the endo-N-acetylhexosaminic bonds of hyaluronic acid to produce tetrasaccharides as the major product. Hyaluronic acid is an intracellular mucopolysaccharide of connective tissue, and it is generally accepted that the venom hyaluronidase functions as a spreading factor, enhancing the invasive power of the venom. The enzyme has been detected in venoms of a number of snakes, as well as in those of ants, bees, Gila monster, hornets, scorpions, spiders, and wasps (42).

Hyaluronidase activity in the whole venoms was measured, and the following results were obtained:

Species	Activity, TRU/µl whole venom
D. hentzi	138
A. emilia	222
Aphonopelma sp.	624
E. panamense	40.8

Schanbacher et al. (21) found a value of 225 TRU/µl whole venom for D. hentzi venom.

The wide variations in enzyme activity observed were somewhat unexpected, since other physical properties for the enzymes appeared to be so similar. These variations in activity may simply reflect differences in the concentration of the enzyme in each of the four venoms. It should be noted, however, that seasonal variations in concentration of tarantula venom components have been observed in previous studies in this laboratory (Dr. Tak K. Chan, personal communi-

cation), higher concentrations being obtained during spring and summer months. It has been observed in this study as well as in earlier studies in this laboratory that venom quantity gradually decreases with successive milkings. These phenomena have been observed in snake venoms as well (52). Rapid successive milkings (1-7 days) during winter months were attempted at one point in this research, and a dramatic decrease in venom concentration (as compared with that obtained during the summer from less frequently milked tarantulas) was observed. Seasonal variations in tarantula venom composition and the effects of multiple milkings have also been observed by Perret (43).

Thus, although a number of factors may affect hyaluronidase activity, seasonal variations and the effects of multiple milkings may be significant in determining the amount of activity observed. Neither of these variables were controlled in this study. For venom used in the hyaluronidase assays, there appears to be a correspondence between observed enzyme activity in the venoms and the milking dates and number of times specimens had been milked. Consequently, a comparison of hyaluronidase enzyme activity may have little meaning unless these factors are controlled. If the enzyme activities observed do not necessarily reflect a static concentration level of the enzyme in each of the venoms, the broad range of values obtained here at least serves to demonstrate the potentials of activity that the venoms may possess.

Toxins

Peaks IB (and IC) from the G-50 fractionation (Figure 5) were each applied to a CM-Sephadex column as described above. Resulting elution

profiles are shown in Figure 9. The small peak observed in each of these profiles which elutes near fractions 10 to 20 appears to be residual hyaluronidase from the previous G-50 fractionation. Peak IC from Aphonopelma sp. venom was apparently lost during this purification step, or it may correspond to the minor peak eluting near fractions 30-40 in the second CM-Sephadex elution profile shown for this species in Figure 9. This peak and other minor ones obtained from this purification step were retained but not used in further studies of the toxins, since their elution behavior did not appear to correspond to the elution behavior expected from a small basic necrotoxin such as that characterized by Lee et al.

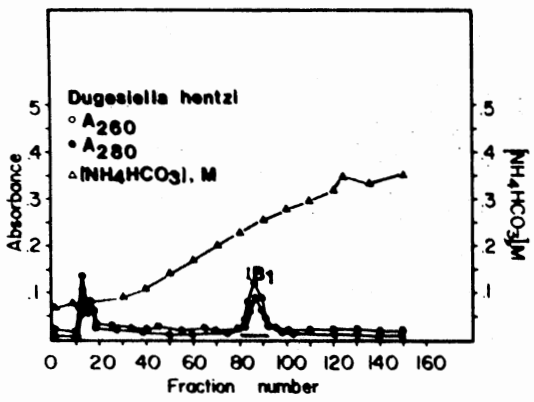
Materials from the peaks indicated in Figure 9 were pooled and concentrated, then small portions were applied to 15% polyacrylamide gels to monitor purity. Results are summarized in Figure 10. None of the materials isolated from the CMS purification step appeared to be homogeneous (with one exception that showed dissimilar migration to the gel).

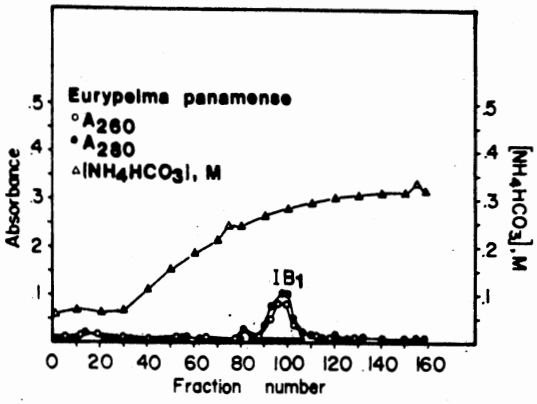
Amino Acid Analyses. Amino acid analyses were obtained for each of the bands comprising peak IB_1 from each of the venoms. For E. panamense, materials obtained from disc gel electrophoresis of whole venom which corresponded to bands of peak IB_1 were analyzed. Protein materials analyzed for amino acid content were extracted from bands cut from the slab gels. Determination of cysteine content (by performic acid oxidation) of such materials has not been tested, so cysteine content was not obtained. Tryptophan content was not determined. Table II presents results obtained from 24-hour hydrolysates of the

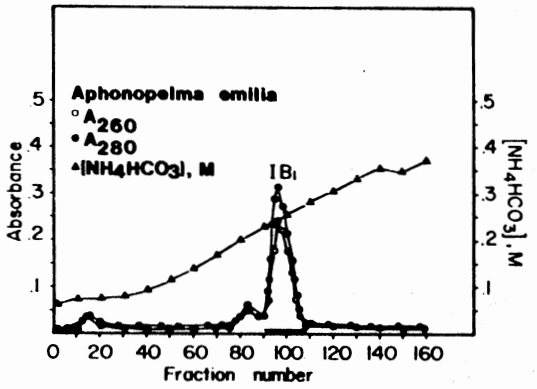
Figure 9. CM-Sephadex Elution Profiles of Peaks IB and IC (Toxins)

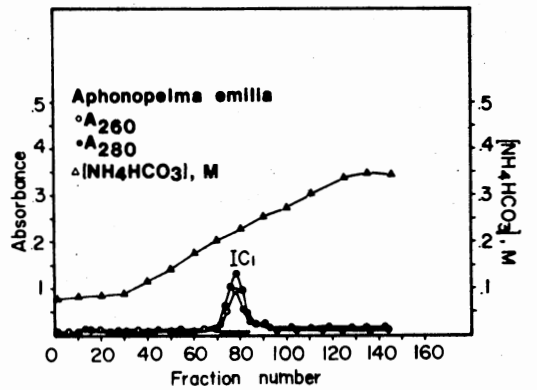
(Eluent: 0.1 M-0.5 M ammonium bicarbonate salt gradient; 3 mls/fraction)

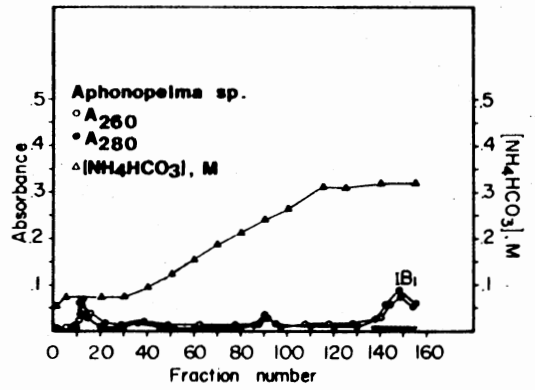
Solid lines beneath peaks indicate fractions pooled and concentrated.


Dugesiella hentzi
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
IB1
Fraction number


Eurypelma panamense
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
IB1
Fraction number


Aphonopelma emilia
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
IB1
Fraction number


Aphonopelma emilia
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
IC1
Fraction number


Aphonopelma sp.
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
IB1
Fraction number

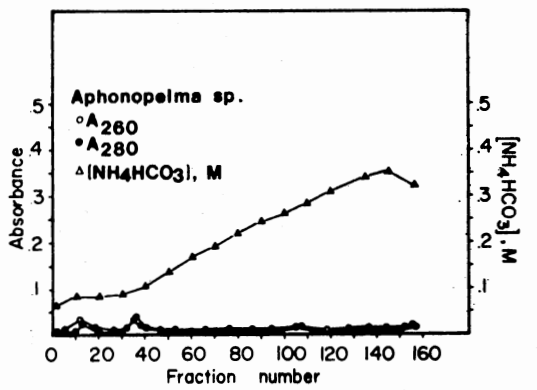

Aphonopelma sp.
°A260
•A280
△[NH4HCO3], M
Absorbance
[NH4HCO3], M
Fraction number

Figure 10. Polyacrylamide Gel Electrophoresis of Whole Venoms and Toxin Fractions

(15% acrylamide, pH 4.3)

1A. Dugesiella hentzi whole venom
1B. Dugesiella hentzi toxin fraction (peak IB_1)

2A. Aphonopelma emilia whole venom
2B. Aphonopelma emilia toxin fraction (peak IB_1)
2C. Aphonopelma emilia peak IC_1 (not assayed for toxicity)

3A. Aphonopelma sp. whole venom
3B. Aphonopelma sp. toxin fraction (peak IB_1)

4A. Eurypelma panamense whole venom
4B. Eurypelma panamense toxin fraction (peak IB_1)

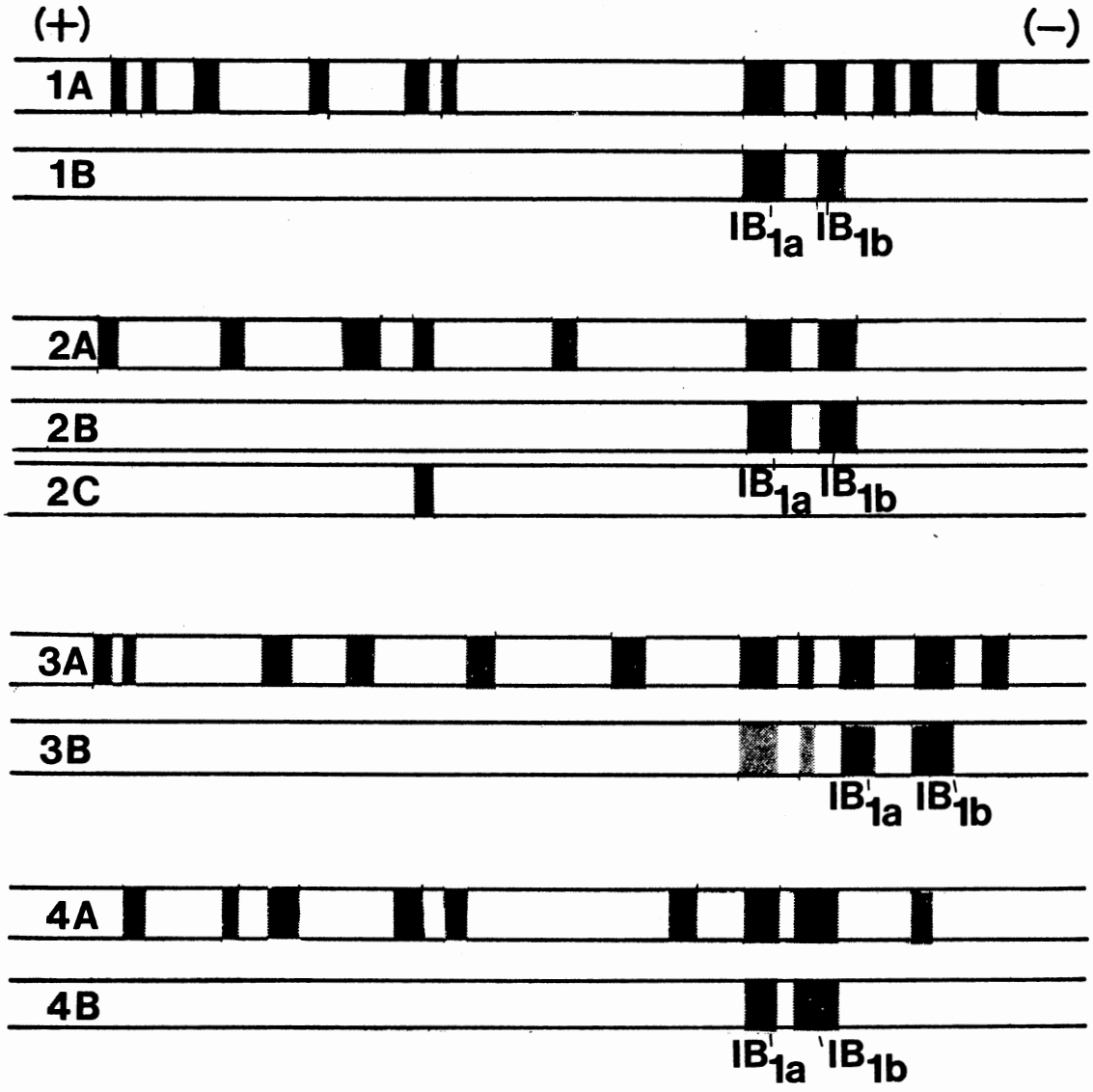

(+)
(–)
1A
1B
IB'1a IB'1b
2A
2B
2C
IB'1a IB'1b
3A
3B
IB'1a IB'1b
4A
4B
IB'1a IB'1b

TABLE II

AMINO ACID COMPOSITION OF BANDS IN FRACTIONS IB_1[1]

Amino Acid	D. hentzi		A. emilia		Aphonopelma sp.		E. panamense		D. hentzi (23)[4]
	IB_{1a}	IB_{1b}	IB_{1a}	IB_{1b}	IB_{1a}	IB_{1b}	IB_{1a}	IB_{1b}	
Aspartate	3.72	3.34	3.17	3.49	8.78	10.91	8.78	7.32	4.0
Threonine	2.14	1.95	ND	ND	ND	0.78	ND	ND	2.0
Serine	3.10	3.02	4.63	4.51	3.77	7.33	4.04	3.76	4.0
Glutamate	12.29	12.01	12.19	12.42	6.18	6.80	11.73	11.64	12.0
Proline	9.45	11.70	11.65	10.59	7.01	6.07	9.00	9.15	8.0
Glycine	12.47	12.20	11.96	12.20	13.73	13.32	13.96	14.00	12.0
Alanine	ND[2]	ND	ND	ND	2.77	2.83	ND	ND	ND
Valine	3.08	2.95	3.11	3.15	1.26	1.70	1.73	1.29	2.0
Methionine	ND	ND	ND	ND	ND	ND	ND	ND	ND
Isoleucine	11.04	10.69	11.44	11.64	8.49	7.89	6.16	6.43	10.0
Leucine	6.64	6.41	7.30	7.59	9.78	8.80	5.33	5.63	6.0
Tyrosine	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phenylalanine	5.69	5.65	4.72	4.58	5.88	5.09	6.09	6.52	8.0
Histidine	ND	ND	ND	(ND)[3]	ND	ND	ND	ND	ND
Lysine	30.38	30.07	29.83	(29.83)	29.76	25.32	30.81	31.74	32.0
Arginine	ND	ND	ND	(ND)	3.19	3.16	2.37	2.53	ND
Total Nanomoles Detected	2270	2680	4566	3227	1438	1215	754	1289	

[1]Underlined member of each pair was present in the larger amount, based on observed intensity of bands after staining. Quantities of each amino acid present are expressed as percentage of total amino acids detected from band extract: nmoles AA/total nmoles detected.

[2]ND = Not Detected, or detected at levels less than 0.5% of total nmoles detected.

[3]Parentheses indicate hypothetical values.

[4]Calculated from data given in (23). Values were converted to nmole % after corrections for cysteine and tryptophan: Total of 59 residues - 8 cysteine - 1 tryptophan = 50 residues or 50 nmoles of amino acids. Then, nmole % = number of residues (nmoles) of AA/50 nmoles.

described materials.

Date in Table II show that members of each IB_1 pair appeared to be very similar. For the D. hentzi proteins, the greatest difference in composition was in proline content. During these analyses, cysteine that has been destroyed by thioglycollic acid sometimes elutes from the column at the same point that proline elutes (this depends on conditions present during the preparation procedure). Thus, an observed difference in proline content for these 2 proteins may not be a real one. Data obtained here agreed reasonably well with the amino acid composition of the necrotoxin studied by Lee et al.

An instrument malfunction occurred during analysis of A. emilia protein IB_{1_b} which obscured detection and measurement of histidine, lysine, and arginine residues. By the recommendation of Dr. T. H. Liao, the amounts of these residues present in IB_{1_b} were assumed to be the same as those of IB_{1_a}, and percentages of the other residues were calculated on this basis. Under these assumptions, the amino acid compositions of both proteins were quite similar.

The greatest differences observed in composition of the Aphonopelma sp. proteins were in aspartate, serine, and lysine content. Serine (and glycine) residues often appear in blank determinations, so differences observed here may not be real. Likewise, the differences in lysine and aspartate content are not consistent with the observed migrations of the proteins in the gel.

Differences were observed in aspartate and lysine content in the E. panamense proteins, but differences were small.

Since only single determinations were made for amino acid composition for each of these proteins, the small differences noted here

that were observed between members of each pair probably are not significant.

A comparison of all IB_1 proteins showed many similarities. All contained about the same relative amount of lysine. Data obtained here for the D. hentzi and A. emilia proteins were nearly identical. E. panamense and Aphonopelma sp. proteins contained small (and similar) amounts of arginine, which were not present in proteins from the other two venoms. Histidine, tyrosine, and methionine were detected in none of the proteins. Relative percentages of each amino acid present in each of the proteins differed by less than 6% between any of the species.

Differences in amino acid composition which might account for the observed differences in migration between IB_{1_a} and IB_{1_b} proteins were not detectable. Migration differences might be explained by differences in the location of disulfide bonds. During electrophoresis, disulfide bonds of some of the material might be cleaved, then reformed in a different arrangement. If this was occurring, however, the relative intensities of the pair of bands would probably vary in different gels containing the same venoms. This did not occur. The pair of proteins could be 2 isotoxins, differing only in arrangement of disulfide bonds. If this is the case, one isotoxin is present in larger amounts than the other. In electrophoresis of whole venoms and of IB_1 proteins, the larger and/or darker band was consistently in the same position relative to the other member of the pair. There could be slight differences in amino acid composition that were not detectable from the data obtained.

In the molecular weight determination (SDS-acrylamide gel electro-

phoresis) of materials from peaks IB_1, single bands were observed, thus the presence of dimers was not evident. Molecular weights obtained for the IB_1 proteins ranged from 5,500 to 8,900, as shown in Figure 11.

Toxicity tests. Since no significant differences were evident in amino acid composition of the IB_1 proteins from each venom, no further purification of the materials obtained after CMS chromatography was attempted. Also, the amounts of materials obtained after this step and the amounts of venom required for their isolation limited the feasibility of further purification. Consequently, bioassays for toxic activity were made using the products obtained from the CMS purification step (peaks IB_1). These consisted of a pair of apparently very similar proteins (IB_{1_a} and IB_{1_b} of Figure 10) isolated from each venom. (In the material obtained from Aphonopelma sp. venom, trace amounts of 2 other cathode-migrating components were present (Figure 10).)

Each of the mice injected with IB_1 proteins received a dose of approximately 100 µg protein. Both mice injected with the material from A. emilia venom died within 2 hours after injection; both mice injected with the fraction from E. panamense venom died within 2½ hours after injection. Mice injected with fractions IB_1 from the other 2 venoms were still alive after 48 hours; these were sacrificed and tissue samples were obtained at this time.

Microscopic examination of heart tissue samples from the injected mice revealed focal areas of necrosis in the ventricular wall. Swollen and vacuolated cells were observed, and cell lysis was evident. These phenomena were observed in tissue samples from all mice injected with IB_1 venom fractions. Figure 12 shows typical results obtained.

Figure 11. Molecular Weight Determination of Purified Venom Toxins

(15% acrylamide, pH 7.2)

Molecular Weights of Markers:

myoglobin - 17,800
cytochrome C - 12,400
glucagon - 3,550

Molecular Weights of Toxins:

Dugesiella hentzi - 5,800
Aphonopelma emilia - 6,500
Aphonopelma sp. - 5,500
Eurypelma panamense - 8,900

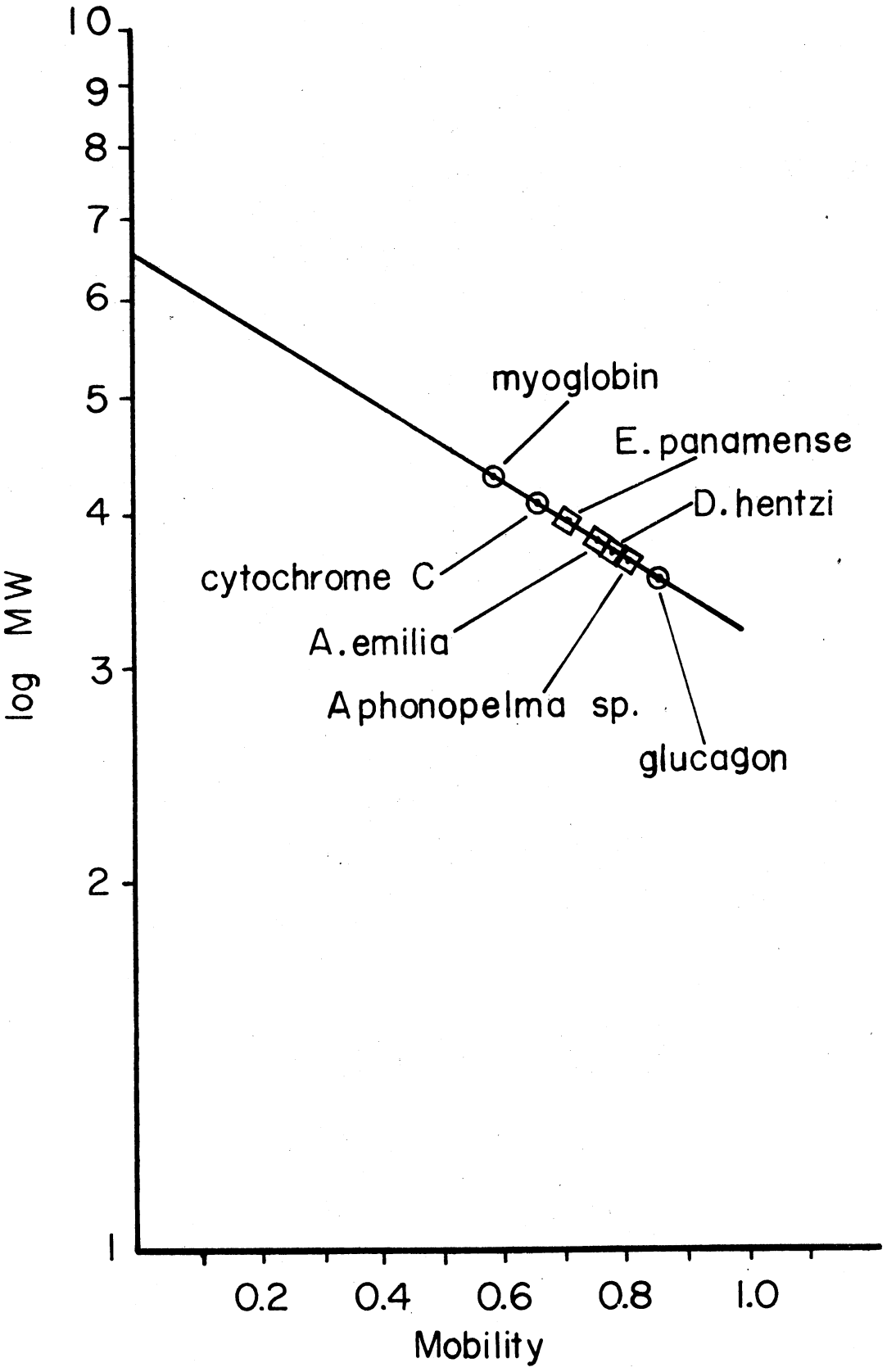
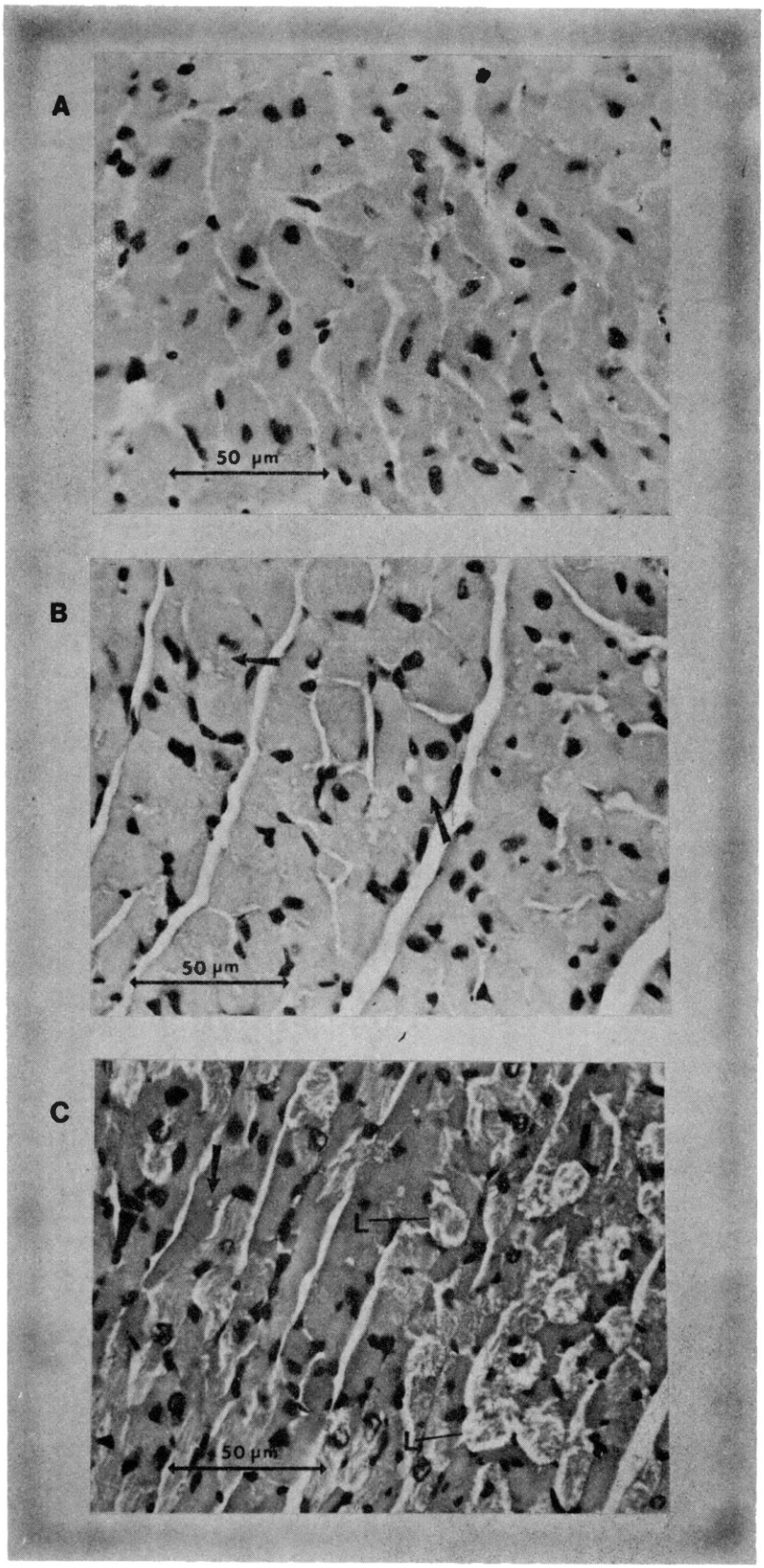

10
9
8
7
6
5
4
3
2
1
log MW
myoglobin
E. panamense
D. hentzi
cytochrome C
A. emilia
Aphonopelma sp.
glucagon
0.2
0.4
0.6
0.8
1.0
Mobility

Figure 12. Photomicrographs of Cardiac Tissue Samples Obtained from Mice Injected with Fractions IB_1

A. Photomicrograph of cardiac muscle from control mouse (injected with 0.1 ml PSS).

B. Photomicrograph of cardiac muscle from experimental mouse (injected with 0.1 ml D. hentzi fraction IB_1, approximately 100 µg/mouse). Note intracellular vacuoles (arrows).

C. Photomicrograph of cardiac muscle from experimental mouse (injected with 0.1 ml A. emilia fraction IB_1, approximately 100 µg/mouse). Note intracellular vacuoles (arrows) and cells undergoing lysis (L).


A
50 µm
B
50 µm
C
L
L
50 µm

Results of the bioassays showed that necrotoxic activity was present in each of the IB_1 venom fractions. One or both of the proteins present in each of the venom fractions may be responsible for this activity. A more detailed description of the toxins' mode(s) of action would be obtained by the use of electron microscopy techniques. The studies done here were only preliminary from a physiological standpoint, their purpose being primarily to determine if toxic activity was present.

Nucleotides

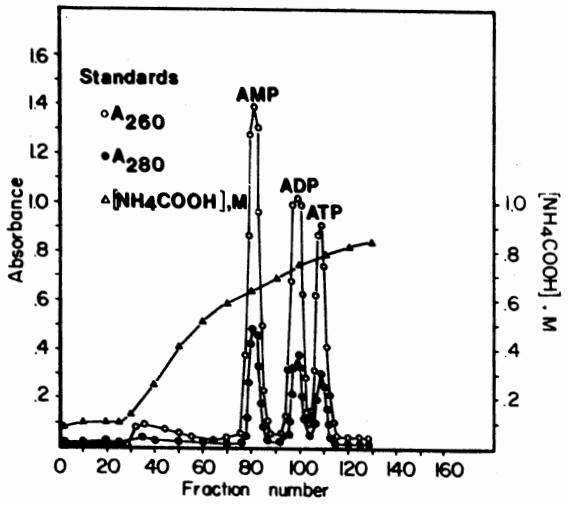
A portion of each peak III from Figure 4, equivalent to 38 µls of whole venom, was applied to a DEAE-Sephadex column as previously described. Resulting elution profiles are shown in Figure 13, along with the profile obtained from a reference sample containing 1 mg each of AMP, ADP, and ATP. These adenine nucleotides were identified in the venom samples by comparing their retention volumes with those of the reference compounds.

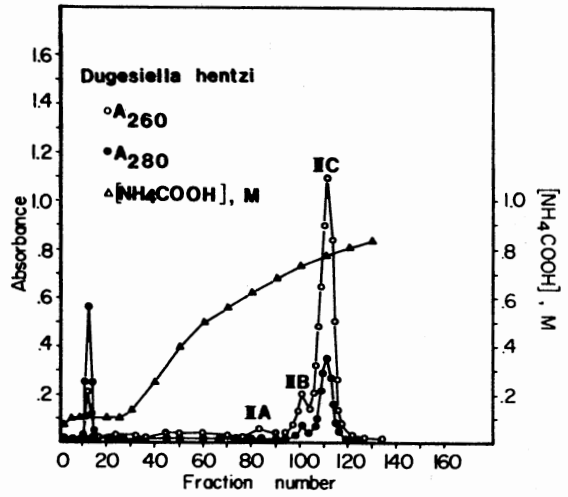
Attempts were made to confirm the identification of materials present in peaks IIIA, IIIB, and IIIC by use of the high voltage paper electrophoresis method of Smith (49). Results were inconclusive due to salt interference in the samples.

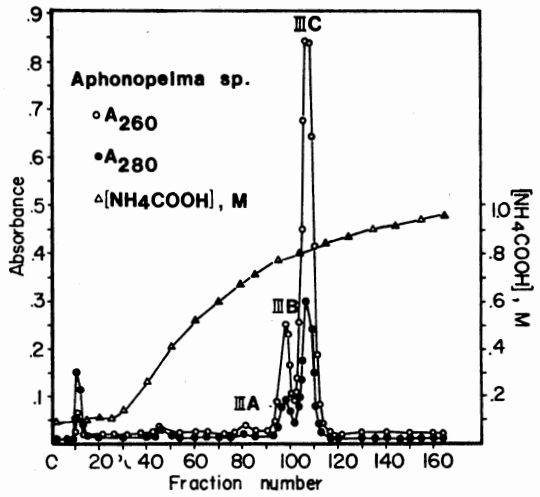
Absorption spectra were obtained from the pooled, lyophilized and redissolved materials in peaks IIIA, IIIB, and IIIC. Absorption maxima were obtained, but interference due to high salt concentration obscured detection of absorption minima. Table III summarizes the information obtained from the absorption spectra. The pH of each of the sample solutions was measured with pH paper. Absorption spectra show that

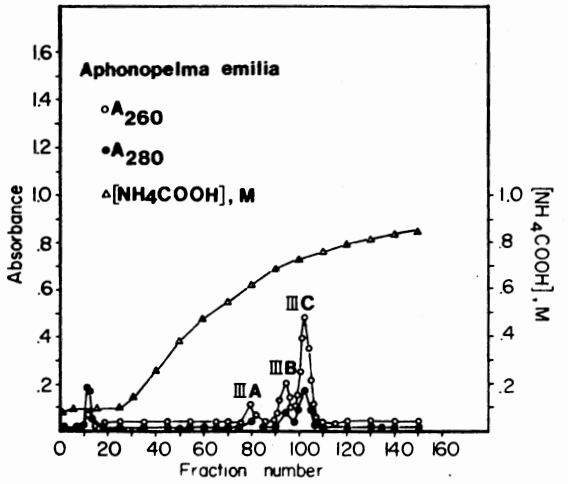
Figure 13. DEAE-Sephadex Elution Profiles of Peaks III (Nucleotides)

(Eluent: 0.1 M-1.0 M ammonium formate salt gradient; 3.5 mls/fraction)


Standards
°A260
•A280
△[NH4COOH],M
AMP
ADP
ATP
Absorbance
[NH4COOH], M
Fraction number


Dugesiella hentzi
°A260
•A280
△[NH4COOH], M
IIA
IIB
IIC
Absorbance
[NH4COOH], M
Fraction number


Aphonopelma sp.
°A260
•A280
△[NH4COOH], M
IIIA
IIIB
IIIC
Absorbance
[NH4COOH], M
Fraction number


Aphonopelma emilia
°A260
•A280
△[NH4COOH], M
IIIA
IIIB
IIIC
Absorbance
[NH4COOH], M
Fraction number

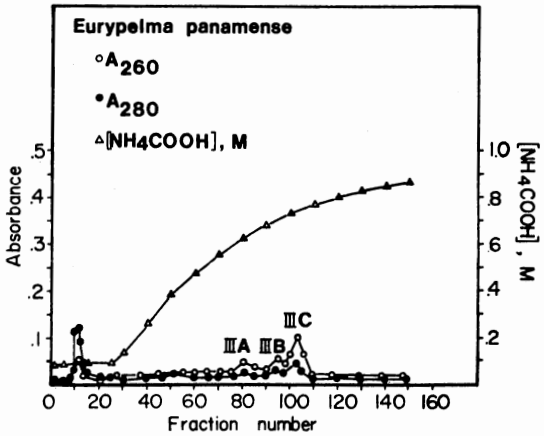

Eurypelma panamense
°A260
•A280
△[NH4COOH], M
IIIA IIIB
IIIC
Absorbance
[NH4COOH], M
Fraction number

TABLE III

ABSORPTION MAXIMA FOR PEAKS IIIA, IIIB, AND IIIC AND COMPARISON WITH LITERATURE VALUES GIVEN FOR NUCLEOTIDES

Species	Peak	λmax, nm	pH	Literature Values for λmax of Common 5'-ribonucleotides (50)		
				Nucleotide	λmax, nm pH 7	pH 2
D. hentzi	IIIA	257	3-4	AMP	259	257
	IIIB	258	3-4	ADP	259	257
	IIIC	259	6-7	ATP	259	257
				CMP	271	280
A. emilia	IIIA	259	6-7	CDP	271	280
	IIIB	256	3-4	CTP	271	280
	IIIC	259	6-7	GMP	252	256 (pH 1)
				GDP	252	256 (pH 1)
Aphonopelma sp.	IIIA	258	3-4	GTP	252	256 (pH 1)
	IIIB	259	6-7	UMP	262	262
	IIIC	259	6-7	UDP	262	262
E. panamense	IIIA	257	3-4	TMP[1]	267	267
	IIIB	258	3-4	TDP	267	267
	IIIC	259	6-7	TTP	267	267

[1]From (51). Value given only for TMP, but those of TDP and TTP should be the same. Thymidine is a deoxyribonucleoside.

the major components present in peaks IIIA, IIIB, and IIIC were adenine nucleotides.

The luciferase assay for ATP confirmed its presence in peaks III of Figure 12 for each of the venom fractions.

The luciferase assay was also used to obtain the concentration of ATP in each of the whole venoms. Results were as follows:

Species	[ATP], µg/µl whole venom
D. hentzi	2.23
A. emilia	1.38
Aphonopelma sp.	2.95
E. panamense	2.00

The concentrations of ATP were similar in all four venoms. A previous study by Chan et al. (22) determined ATP concentrations in the D. hentzi and Aphonopelma sp. venoms to be 28.1 µg/µl and 56.6 µg/µl whole venom, respectively. However, assay conditions used in their study were not as sensitive as those used here, and other nucleotides may have interfered to yield the higher concentrations their study obtained. Their study did not specify the purity of the lantern extracts used, but according to DeLuca (40), crude firefly lantern extracts contain transphosphorylases that are capable of catalyzing ATP synthesis from other nucleotide tri- and diphosphates. It was shown by Perret (43) that levels of free amino acids, proteins, and hyaluronidase activity were subject to seasonal variations as well as effects of multiple milkings; it is probable that nucleotide concentrations are subject to these variations and effects also.

Prior to the study by Chan et al. the presence of nucleotides in spider venom had not been reported. It was suggested in that study

that ATP, as an energy-rich compound, might be required for venom release. Their study demonstrated that ATP had synergistic toxic effects with the major toxin of D. hentzi venom. In consideration of the concentrations of ATP in the venoms that were obtained by their study, they suggested that ATP might have a special function in enhancing the toxicity of the venom. The lower concentrations of ATP determined by this study did not discredit the hypothesis of a synergistic role of ATP with the toxin, but the data obtained here did not reinforce that hypothesis.

Polyamines

Unpublished results of Dr. T. K. Chan gave evidence for the presence of spermine in the D. hentzi venom. This compound was identified by mass spectrometry by comparing the spectrum of the venom sample with a reference sample of spermine. The compound identified as spermine was obtained after purification of material corresponding to that present in peak II of this study (Figure 4).

Relative mobilities of the farthest cathode-migrating bands and a reference sample (Figure 2) suggest that the venoms of D. hentzi and E. panamense contain spermine. The other two venoms contain a similar, somewhat less basic compound that possibly is spermidine.[1]

During this study steps were taken to purify the materials present

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[1]In the slab gel system, if electrophoresis was stopped soon enough, a thin, very darkly staining band was observed across the bottom of the gel. This may have been polyamines of the various venoms. The slab gel system was not appropriate for checking this with reference compounds, however, since too much diffusion occurred to permit identification of reference or venom samples.

in peaks II in an attempt to identify polyamine compounds. Use of thin-layer chromatography followed by mass spectrometry was unsuccessful in identifying such compounds.

Spermine has demonstrated nephrotoxic activity in mice, rats, guinea pigs, rabbits, and dogs (45). The LD_{50} for mice was determined as approximately 25 μg/g (46). Toxicity in mice has also been demonstrated by spermidine (47). The concentration of these polyamines in the tarantula venoms is unknown, but if they are present, it is conceivable that they could have additive or synergistic toxic activity with the protein toxins, or that the polyamine complexes possess a unique toxicity of their own. The spermine-phenolic acid complexes from A. robustus venom demonstrated toxicity in mice at levels of approximately 20 μg/g (7).

CHAPTER V

SUMMARY

A comparative study of the four tarantula venoms revealed a number of similarities.

Hyaluronidase activity was present in each of the venoms, and the enzyme from each venom was purified. Chromatographic and electrophoretic behaviors were similar, and molecular weight estimates for the enzymes ranged from 42,000 to 46,000. Levels of hyaluronidase activity in the whole venoms were determined.

Adenine nucleotides AMP, ADP, and ATP were identified as the major nucleotide components present in each of the venoms. The concentrations of ATP in each venom were determined, and ranged from 1.38 to 2.95 μg/μl whole venom.

The concentrations of venom components may be subject to seasonal variations and effects of multiple milkings. Since these factors were not controlled in this study, the levels of hyaluronidase activity obtained here may not reflect the relative activities of the venoms in natural situations. Concentrations of ATP in the venoms appeared to be similar, but this component may not be present at static levels.

A fraction containing necrotoxic activity was isolated from each venom. Each of these fractions was shown to contain two apparently very similar proteins. One or both of the proteins in each fraction may be responsible for the toxic activity. Molecular weight estimations for

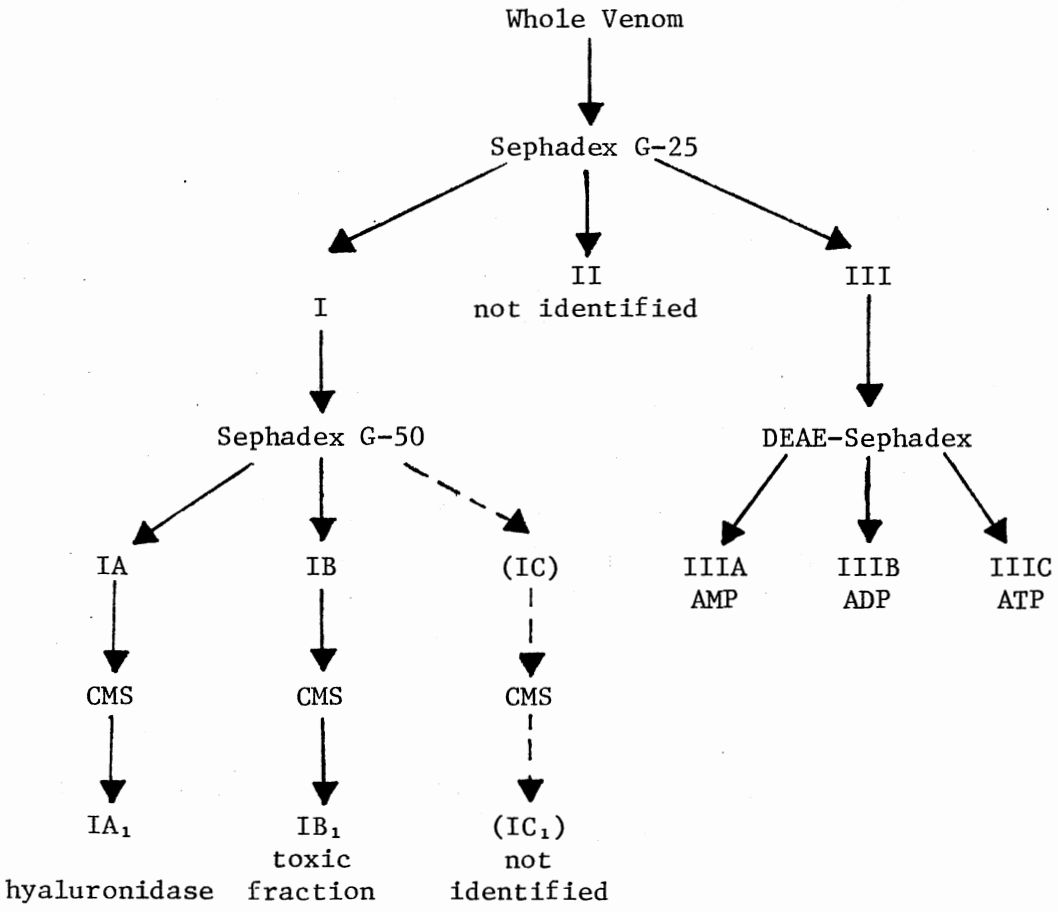
the toxins ranged from 5,500 to 8,900. Amino acid compositions (on the basis of relative amounts present) for the proteins present in the toxic fractions were similar for all four venoms. Most notable similarities were a lysine content of around 30% (calculated excluding cysteine and tryptophan residues), and the absence of histidine, tyrosine, and methionine residues. Figure 14 summarizes venom fractions identified.

Much of the information obtained from this study was only preliminary in nature. The role of nucleotides present in the venoms needs a clearer definition. Complete amino acid analyses of the toxins are needed, and further structural information such as amino acid sequence and location of disulfide bonds would be most interesting from a structure-function standpoint. Studies which identify structure-toxic action relationships, such as residue modification studies, are needed. More evidence is needed to confirm the presence of two toxins in each venom, and to identify which of these proteins (if not both) possesses toxic activity. A more detailed description of the toxic activities of the proteins could be obtained with electron microscopy studies. Relative toxicities of each venom and toxin might be interesting. Presently unidentified components of the venoms could demonstrate other interesting effects towards the overall activities of the whole venoms. The major limitation of this study was the shortage of material.

This study was the first to examine the venoms of Aphonopelma emilia and Eurypelma panamense. Further information was obtained from the venom of the Aphonopelma sp. The similarities found in venom content are interesting in terms of the understanding of the activities of all venoms. Hyaluronidase is an enzyme detected in numerous

Figure 14. Fractionation Scheme for Whole Venoms with Components Identified

Dotted lines indicate peaks obtained from some, but not all of the venoms. Solid lines indicate peaks obtained from all four venoms. CMS = carboxymethyl-Sephadex.


Whole Venom
Sephadex G-25
I
II
not identified
III
Sephadex G-50
DEAE-Sephadex
IA
IB
(IC)
IIIA
AMP
IIIB
ADP
IIIC
ATP
CMS
CMS
CMS
IA1
hyaluronidase
IB1
toxic
fraction
(IC1)
not
identified

arthropod venoms. Studies of toxic components of orthognath venoms to date consistently indicate that basic proteins of low molecular weight are responsible for toxic activity. Such comparative knowledge provides basic information on which studies of other orthognath venoms can proceed. Species similarities found here might also be found in venoms of other groups of related species.

The chemical characterization of venom components provides valuable information for the understanding of the biochemical mechanisms which operate living systems.

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APPENDIXES

TABLE IV

AMINO ACID ANALYSES OF CATHODE-MIGRATING PROTEINS[1]

Amino Acid	*D. hentzi*					*A. emilia*		*Aphonopelma* sp.					*E. panamense*				Blank
	IB_{1a}	IB_{1b}	3	4	5	IB_{1a}	IB_{1b}	1	2	IB_{1a}	IB_{1b}	5	1	IB_{1a}	IB_{1b}	4	
Asp	3.72	3.34	10.19	?[4]	10.99	3.17	3.49	3.93	11.78	8.78	10.91	6.27	9.56	8.78	7.32	5.25	12.50
Thr	2.14	1.95	6.45	5.36	5.62	ND	ND	6.10	ND	ND	0.78	7.83	ND	ND	ND	6.44	5.63
Ser	3.10	3.02	21.13	25.01	8.33	4.63	4.51	6.25	11.71	3.77	7.33	5.78	4.67	4.04	3.76	9.29	13.24
Glu	12.29	12.01	9.58	6.33	8.79	12.19	12.42	8.92	7.47	6.18	6.80	5.77	11.84	11.73	11.64	10.06	16.98
Pro	9.45	11.70	ND	ND	9.61	11.65	10.59	9.49	5.09	7.01	6.07	14.13	8.57	9.00	9.15	9.34	ND
Gly	12.47	12.20	28.61	30.40	17.21	11.96	12.20	10.28	9.67	13.73	13.32	14.82	15.36	13.96	14.00	9.85	22.81
Ala	ND[2]	ND	10.03	13.39	7.46	ND	ND	1.62	3.09	2.77	2.83	8.40	0.99	ND	ND	5.53	10.55
Val	3.08	2.95	ND	ND	ND	3.11	3.15	6.03	5.13	1.26	1.70	4.37	ND	1.73	1.29	ND	ND
Met	ND	ND	ND	ND	3.27	ND	ND	4.49	2.85	ND	ND	1.12	1.85	ND	ND	7.10	1.93
Ile	11.04	10.69	2.30	3.37	5.59	11.44	11.64	5.29	2.52	8.49	7.89	5.36	7.28	6.16	6.43	5.41	3.95
Leu	6.64	6.41	4.48	5.50	6.02	7.30	7.59	7.93	5.23	9.78	8.80	5.88	6.09	5.33	5.63	6.07	7.02
Tyr	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Phe	5.69	5.65	ND	ND	ND	4.72	4.58	2.03	6.19	5.88	5.09	ND	5.44	6.09	6.52	5.84	ND
His	ND	ND	ND	ND	ND	ND	(ND)[3]	3.95	ND	ND	ND	3.50	ND	ND	ND	ND	ND
Lys	30.38	30.07	7.23	10.64	11.17	29.83	(29.83)	16.28	23.33	29.76	25.32	16.76	28.35	30.81	31.74	9.29	5.39
Arg	ND	ND	ND	ND	5.95	ND	(ND)	6.93	5.92	3.19	3.16	+[5]	+[5]	2.37	2.53	10.52	ND
Total Nanomoles Detected	2270	2680	24.33	27.63	113.87	4566	3227	696	409	1438	1215	302	192	754	1289	115	40.47

[1] Analyses given for each group of bands in order of migration: 1 = farthest from cathode, 5 = nearest cathode. Bands in toxic fractions are designated by the names used in the text. Quantities of each amino acid present are expressed as percentage of total amino acids detected from band extract: nmoles AA/total nmoles detected.

[2] ND = Not detected, or detected at levels less than 0.5% of the total nmoles detected.

[3] Parentheses indicate hypothetical values.

[4] Interference by other substances prevented detection and measurement.

[5] Present, but not measured by integrator.

VITA

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