ISOLATION AND CHARACTERIZATION OF HEMORRHAGIC AND MYOTOXIC PROTEINS FROM VENOMS OF THE PRAIRIE RATTLESNAKE, *CROTALUS VIRIDIS VIRIDIS*

By

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NOMENCLATURE

AA	-	amino acid
A_{280}	-	absorbance at 280 nm
A_{340}	-	absorbance at 340 nm
A_{595}	-	absorbance at 595 nm
bis	-	N,N′-methylenebis(acrylamide)
°C	-	degrees Celius
CK	-	creatine kinase
cm	-	centimeter
conc.	-	concentration
CPD	-	cross-product diameter
CPY	-	cumulative percent yield
DEAE	-	diethylaminoethyl
disc	-	discontinuous
DMC	-	N,N′-dimethylated casein
DMHb	-	N,N′-dimethylated hemoglobin
DNP	-	dinitrophenyl
EDTA	-	ethylene diaminetetraacetic acid
gm	-	gram(s)
HPA	-	hide powder azure
hr	-	hour(s)
(i.d.)	-	intradermal (injection)
(i.m.)	-	intramuscular (injection)

(i.v.) - intravenous (injection)

K_{av} - in gel filtration, the ratio of the elution volume minus the void volume over the bed volume minus the void volume

kdal - kilodalton(s)

L - liter(s)

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

M - molar

mA - milliampere(s)

mg - milligram(s)

MHD - minimum hemorrhagic dose

min - minute(s)

mL - milliliter(s)

mM - millimolar

mol. wt. - molecular weight

mmol - millimole(s)

µg - microgram(s)

µL - microliter(s)

µm - micrometer(s)

nm - nanometer(s)

No. - number

PAGE - polyacrylamide gel electrophoresis

pI - isoelectric point

PSS - physiological saline

RPM - revolutions per minute

SDS - sodium dodecylsulfate

sec - second(s)

TEMED - N,N,N′,N′,tetramethylenediamine

Tris	-	tris(hydroxymethyl)-aminomethane
(v/v)	-	mL of material per 100 mL total volume
V_e	-	gel filtration elution volume
V_o	-	gel filtration void volume
V^t	-	gel filtration column bed volume
VTm	-	viriditoxin-like isolate or component from Miami Serpentatium Crotalus viridis viridis venom
VTwo	-	viriditoxin-like isolate or component from western Oklahoma Crotalus viridis viridis venom
(w/v)	-	grams of material per 100 ml total volume
x	-	fold, ie. x50 is fifty-fold magnification
x g	-	times the acceleration due to gravity

CHAPTER I

INTRODUCTION

Biochemical and pathogenetic analyses of snake venoms have provided insights into their composition, toxicity, mode of action, and the similarities between species. Venoms and their components have often been and continue to be useful clinical and investigative tools in science and medicine. A better understanding of the toxic nature of snake bite can be obtained from the investigation of specific venom components.

One of the hallmark effects of rattlesnake venom compared with other dangerous snake venoms is the severe local tissue damage that usually results from its injection. While rattlesnake bite rarely leads to death in larger animals, it often causes considerable trauma in the victim. The loss or diminished use of appendages is common, especially if treatment is not obtained. One recommended treatment for rattlesnake bite is neutralization by the administration of a specific antiserum. A commercially available antiserum exists and its administration can be crucial in saving the life of the victim in severe snakebite cases. However these antisera can induce life-threatening side effects and do not always fully protect against the often permanent and crippling local effects of many rattlesnake venoms. Hence, improved means of treatment for rattlesnake bite are sought. It is hoped by many that further understanding of the local

actions of the responsible rattlesnake venom components can lead to improved treatments. It is towards this end that the isolation and characterization of some hemorrhagic and myotoxic components in prairie rattlesnake venom was undertaken.

CHAPTER II

LITERATURE REVIEW

Taxonomy of Snakes

The taxonomic classification of snakes is complex. The original intent was to classify snakes as being poisonous or non-poisonous. In fact the most widely employed system of classification today still relies on this distinction as well as their dentition and some external features. Nevertheless there is much disagreement within the field as to which taxonomic classifications are proper. For instance, the number of families of snakes is desginated as three (Underwood, 1979), four (Kochva, 1987), or five (Tu, 1977) by differing authorities. Even the criteria for designating subspecies cannot be agreed upon as they are often too specific or too broad, leading to the trivial naming of local populations or the lumping together of genera and species.

The taxonomic system used by Underwood (1979) will be used in this thesis. Underwood defers to the works of Brattstrom (1964) and Klauber (1972) when addressing the taxonomy of pit vipers. Three families are described for the Caenophidia, higher snakes: Colubridae, Elapidae, and Viperidae. All three of these families contain poisonous snakes. Of these three only the Viperidae family is directly relevant to this work.

The Viperidae, or vipers, contain three subfamilies. One of these subfamilies is the Crotalinae. This subfamily is also known as the crotalids or pit vipers, whose snakes contain the characteristic pit organ between the eye and nostril. There are six genera of Crotalids. The genus Agkistrodon is considered to contain the more primitive crotalids, which are found both in Asia and the New World. Two genera which appear to be closely related are the genus Bothrops, occuring in Central and South America, and the genus Trimeresurus, occuring in the Orient (Maslin, 1942; Brattstrom, 1964). The rattlesnakes, named for their distinctive tail rattle, make up the genera Sistrusus and Crotalus; these two genera are found in the New World. Sistrurus may be more primitive than Crotalus (Brattstrom, 1964; Klauber, 1972). The genus Lachesis contains only one snake, L. muta, the bushmaster. The bushmaster is thought to be a primitive rattlesnake, whose range includes the regions from Central America to the Amazon Basin. Brattstrom (1964) believes rattlesnakes to be more closely related to Lachesis and Agkistrodon than to the other two pit viper genera.

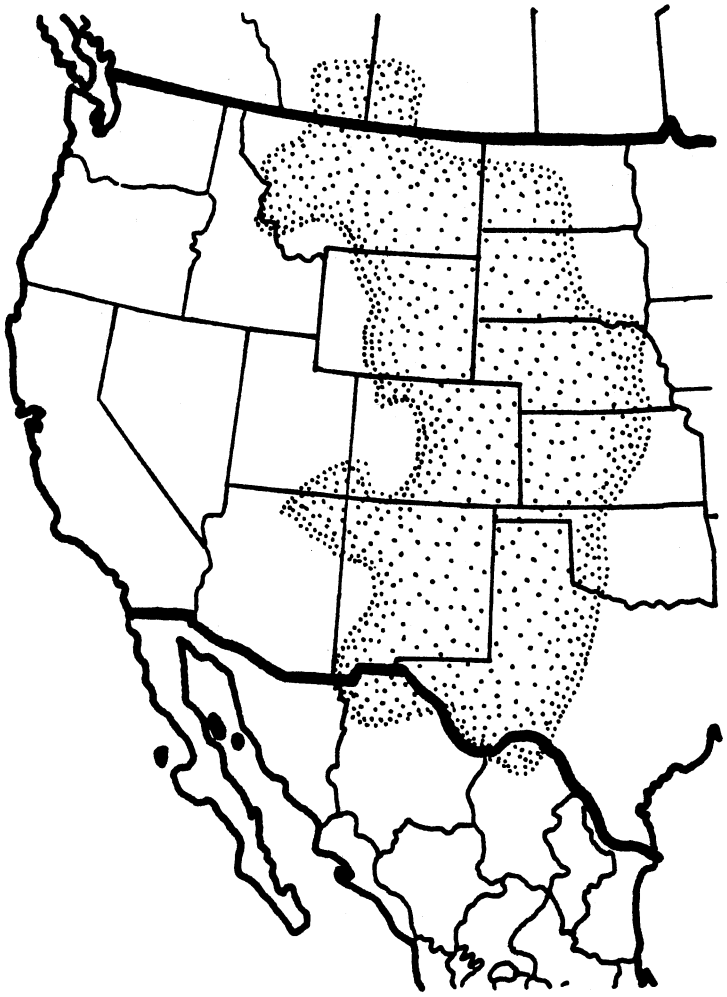
Crotalus viridis viridis, the prairie rattlesnake, is the species whose venom is the subject of this study. The geographical range of this snake is depicted in Figure 1. The extensive range of the prairie rattlesnake covers a large portion of the Great Plains of North America (Glenn and Straight, 1982).

Intraspecial Variation

Broad classification of snake species can often lead to differences within a species in characteristics not included in the

Figure 1. Geographical Range of the Prairie Rattlesnake, Crotalus viridis viridis.

Redrawn from Klauber (1972).



taxonomic classification of the species. Germane to this work are the many observations of intraspecial variation in snake venoms and their components. For instance, geographic intraspecial variation in protein composition and toxicity of crotalid venoms has been observed in Crotalus durissus terrificus (Lee, 1972), C. scutulatus scutalatus (Glenn and Straight, 1978), C. viridis viridis (Gleason et al., 1983), Agkistrodon contortrix (Moran and Geren, 1979a), Trimeresurus flavoviridis (Sadahiro and Omori-Satoh, 1980), Bothrops asper (Gutiérrez et al., 1980), and Crotalus atrox (Minton and Weinstein, 1986).

Description of Venom

Venom is a crude secretion, which after lyophilization is approximately 90% protein (Tu, 1977). The venom originates from the venom glands of the snake, although some small degree of intermixing with saliva, blood and other tissues is common. The structure and evolution of the venom gland has been extensively reviewed by Kochva (1987). The secretion of the proteins from the venom gland cells is thought to follow the classical mammalian secretory pathway discovered by Palade (1975), based on studies with the venom gland of Crotalus durissus terrificus (Marchi et al., 1978). A closer examination of the components of Crotalidae venoms follows.

Non-protein Components of Venom

Snake venom contains several categories of nonprotein components, including amino acids, nucleotides, and lipids. Carbohydrates, small peptides, biogenic amines and a variety of inorganic constituents are

also found in the venom (Tu, 1977).

Carbohydrates typical of the sugar moieties of glycoproteins are commonly found. These include neutral sugars, amino sugars and sialic acid. Their presence with an on venom proteins indicates that venom proteins are secretory proteins (Tu, 1977).

One of the principal biogenic amines is serotonin, 5-hydroxy-tryptamine, and its level in crotalid venom is typically less than 1 μg/ml (Tu, 1982). Serotonin is thought to play an important role in causing the pain associated with rattlesnake bite (Zarafonetis and Kalas, 1960). Endogenous serotonin can also be released by serotonin releasing proteins present in rattlesnake venom (Markwardt et al., 1966).

One small peptide common to many rattlesnake venoms, including Crotalus viridis viridis, is an angiotensin-converting enzyme inhibitor (Ferreira, 1966). These inhibitor peptides have an N-terminal pyroglutamic acid. The inhibitor blocks the enzymatic conversion of angiotensin I to angiotensin II. Angiotensin II is a hypertensive compound which can modulate the hypotensive effects of bradykinin, another autopharmacologically active peptide.

Some of the principal inorganic constituents of venom are salts of Zn^{++}, Ca^{++}, Mg^{++}, K^{+}, Na^{+}, SO_4^{--}, Cl^{-}, and phosphate (Tu, 1977). After extensive dialysis of eleven Crotalidae venoms against distilled water the metals most significantly retained were Na(I), Ca(II) and Zn(II) (Friedrich and Tu, 1971). The dialyzed Crotalus viridis viridis venom retained more than half of its Zn(II) and Ca(II).

Proteins of Venom

Many of the proteins of crotaline venoms have known enzymatic activities. Several of these proteins have been isolated and characterized, and have been found to range from high to low toxicity. Other toxic proteins which have no enzymatic activity have also been isolated from these venoms.

Enzymes commonly occurring in crotaline venom include phosphomonoesterase, phosphodiesterase, NAD-nucleosidase, L-amino acid oxidase, hyaluronidase, phospholipase A_2, small basic polypeptide myotoxins, and several varieties of proteases (Tu, 1982). These enzymes, with at least one exception, are hydrolytic enzymes. The phospholipases A_2, small basic polypeptides, and proteases are thought to be the most toxic proteins of crotalid snake venoms.

Low Toxicity Proteins

Phosphomonoesterase proteins are nucleotidases, the most common of which in rattlesnake venoms is 5′-nucleotidase. Phosphodiesterase, also known as nucleotide pyrophosphatase, liberates a 5′-nucleotide from the 3′-end of a polynucleotide and is known to work best on large substrates (Dolapchiev, 1980). "ATPase" activity in venom is due to phosphodiesterase and phosphomonoesterase. True ATPase activity in rattlesnake venom has not been found (Tu, 1982).

NAD nucleosidase is another protein commonly found in venoms. This protein catalyzes the hydrolysis of the nicotinamide N-ribosidic linkage of NAD. Its activity was found in the venom of six species of the genus Agkistrodon (Tatsuki et al., 1975). L-amino acid oxidase is

a protein which contains flavin adenine dinucleotide (FAD) and this moiety gives venom its distinctive yellow color. This protein is not hydrolytic but instead oxidatively deaminates L-amino acids. Several high molecular weight isozymes of this protein were isolated from the venom of Crotalus adamanateus (Wellner and Meister, 1960). Russell et al. (1963) have found that their lethal activity constitutes only one percent of the total lethal activity of the C. adamanateus venom.

Hyaluronidase is an enzyme which hydrolyzes the mucopolysaccharide hyaluronic acid, present in the intercellular matrix. Duran-Reynals (1936, 1939) stated that this enzyme was a "spreading factor", but in reality offered little experimental proof (Tu, 1982). Actually hyaluronic acid is important in attracting water, thus swelling the intercellular matrix. This is thought to facilitate cell migration and associated repair mechanisms (Toole, 1976, Rodén, 1980). Degradation by the enzyme hyaluronidase results in cessation of cellular migration (Toole, 1976).

Relatively Toxic Proteins

Phospholipase A_2 proteins constitute an important class of toxic proteins in crotaline snake venoms. This enzyme hydrolyzes the acyl bond 2 of lecithin. The enzymatic products resulting from the hydrolysis of lecithin (phosphatidylcholine) are a free fatty acid and lysophosphatidylcholine. Various phospholipase A_2 isolates exhibit neurotoxic and myotoxic activities (as will be discussed later), as well as hemolytic activities. Enzymatically active phospholipase A_2 is a dimer (Wells and Hanahan, 1969; Purdon et al., 1977), and has a close sequence homology to mammalian pancreatic phospholipase A_2.

Modification of the N-terminal residue of phospholipase A_2 does not interfere with its ability to bind to micelles, but does inhibit its ability to hydrolyze (Verheij et al., 1981). Lysophospholipase activity, which removes the last acyl chain from lysophosphatidylcholine may be a side reaction of the phospholipases A_2 (Fletcher et al., 1979).

A group of small basic polypeptides, notable for a high content of basic amino acids and molecular weights less than 10 kdal, have been isolated from a number of rattlesnake venoms. Since the direct relevance of these proteins to the literature review is their myotoxic activity, they will be discussed in depth later.

Proteases are extremely prevalent in crotaline venoms compared to the venoms of other families (Tu, 1982). The presence of multiple proteolytic activities in rattlesnake venoms is well known (Pfleiderer and Sumyk, 1961; Bjarnason and Tu, 1978; Kurecki et al., 1978). The venom of the western diamondback rattlesnake, Crotalus atrox, contains at least 15 distinct proteolytic enzymes. Seven hemorrhagic toxins have been isolated (Bjarnason and Tu, 1978, Nikai et al., 1984, 1985a), two kallikrein-like proteases (Fox and Bjarnason, 1984), four proteolytic anticomplementary factors (Man and Minta, 1977, Minta and Man, 1980), one non-hemorrhagic collagenolytic enzyme (Hong, 1982, Tsuchiya et al., 1974) and two fibrinolytic enzymes (Bajwa et al., 1981). Many of these proteolytic activities are commonly found in other crotalid venoms. For example only two of the rattlesnake venoms lack substantial hemorrhagic activity due to hemorrhagic protease(s) (Tu, 1982). Additional proteolytic activities to those described in C. atrox have been found in other snake venoms. Arginine esterases

thought to be proteases have been implicated in clotting, bradykinin release, and capillary-diameter-increasing activities. Arginine esterase activity found in three rattlesnake venoms was inhibited by protease inhibitors (Geiger and Kortmann, 1977). Thrombin-like serine proteases have also been isolated from venom. Crotalocytin, a thrombin-like serine protease from timber rattlesnake venom, induces platelet aggregration (Schmaier and Colman, 1980). Apparently three classes of platelet activating proteases exist. Crotalocytin typifies one class, and two other types of platelet activators are found in other crotalid venoms. Convulxin from the crude venom of C. durrisus cascavella and botrocetin from the crude venoms of many Bothrops species typify the other two classes (Vargaftig et al., 1980; Read et al., 1978). A fibrinogen degrading thrombin-like protease, crotalase, was isolated from Crotalus adamanteus venom and well characterized (Markland and Damus, 1971; Markland and Pirkle, 1976). Crotalase only cleaves the A peptide of fibrinogen, but slowly degrades the B(β) chain (Markland and Pirkle, 1977a,b), leaving degraded and soluble fibrin monomer which cannot polymerize.

Endopeptidases are much more common in snake venoms than are exopeptidases. Although some rattlesnake venoms have been shown to have exopeptidic activity, as measured by hydrolysis of L-leucyl-β-naphthylamide (Tu and Toom, 1967), no carboxypeptidase has ever been reported in any snake venom. The majority of endopeptidases which have been isolated have been described as metalloproteases and serine proteases.

Hemorrhagic Proteases

The proteases most relevant to this work are the hemorrhagic proteases. The importance of these proteases is underscored by the severe consequences of hemorrhage in local tissue damage as will be described in a later section.

As early as 1930 the connection between hemorrhagic and proteolytic activities in crotalid venoms was promoted (Houssay, 1930). In early isolations, purified hemorrhagins were shown to be proteolytic (Oshima et al., 1968). However, isolations by others seemed to indicate that the hemorrhagic and proteolytic activities of snake venom were distinct (Takahashi and Ohsaka, 1970b). Recently it has become clear that the lack of detectable proteolytic activity was due to the proteolytic substrates used (most often casein), which were not recognized by these special proteases (Fox and Bjarnason, 1984). In fact, the hemorrhagic proteases usually have better proteolytic activity towards connective tissue proteins (Fox and Bjarnason, 1984; Civello et al., 1983a).

At least thirty-four hemorrhagic toxins have been isolated from thirteen different species of crotalid snake venoms. Additionally, three hemorrhagic toxins, HR1, HR2, and HR3, have been isolated from the non-crotalid venom of Vipera palestinae (Ovadia, 1978) and recently a hemorrhagin has been isolated from the venom of Atractaspis engaddensis (Ovadia, 1987). The physicochemical and toxic properties of these hemorrhagic toxins are summarized in Table I. A brief discussion of these hemorrhagic toxins below will introduce many of these toxins' commonalities and differences.

TABLE I

SOME PROPERTIES OF HEMORRHAGIC COMPONENTS
ISOLATED FROM SNAKE VENOMS

Family	Crotalidae					Crotalidae			Crotalidae		Crotalidae	Crotalidae	
Genus	Agkistrodon					Agkistrodon			Agkistrodon		Bothrops	Bothrops	
Species	acutus					acutus			halys		jararaca	neuwiedi	
Subspecies									blomhoffii				
	Hemorrhagic Toxin					Aa-Hemorrhagic components			Hemorrhagic Factor		Hemorrhagic Factor	Hemorrhagic Factor	
Name	AC_1-proteinase	AC_2-	AC_3-	AC_4-	AC_5-	AaHI	AaHII	AaHIII	HRI	HRII	HF_2	NHF_a	NHF_b
Molecular Weight[1]	24.5	25	57	33	24	22	22	22	6.08S[2]	5.54S[2]	50	46	58
Multimeric Nature	monomer	monomer	N.D.[3,4]	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer
Zinc Content[5]	1.15	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Inhibited by EDTA	+[6]	+	+	+	+	+	+	+	+	+	+	+	+
Proteolytic	+	+	+	+	+	+	+	+	-[6]	+	+	+	+
Isoelectric Point	4.7	4.9	4.7	4.4	6.7	4.6	5.3	basic	4.70	4.18	N.D.	4.2-4.3	4.2-4.3
MHD	0.22[7]	0.431[7]	0.95[7]	0.31[7]	0.37[7]	0.4[7]	1.5[7]	10[7]	0.0012[7]	0.19[7]	N.D.	N.R[8]	N.R.
Myotoxic	N.D.	N.D.	+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Reference	Nikai et al. (1977,1982)	Sugihara et al. (1978)	Sugihara et al. (1979); Yagihashi et al. (1986)	Sugihara et al. (1980)	Mori et al. (1984)	Xu et al. (1981)			Oshima et al. (1972)	Oshima et al. (1968);Iwanaga et al. (1965)	Mandelbaum et al. (1975,1976)	Mandelbaum et al. (1984)	

TABLE I (CONTINUED)

Family	Crotalidae							Crotalidae	Crotalidae	Crotalidae		
Genus	Crotalus							Crotalus	Crotalus	Crotalus		
Species	atrox							adamanteus	horridus	ruber		
Subspecies									horridus	ruber		
	Hemorrhagic Toxin							Hemorrhagic Factor		Hemorrhagic Toxin		
Name	HTa	HTb	HTc	HTd	HTe	HTf	HTg	Proteinase H	HP-IV	HT-1	HT-2	HT-3
Molecular Weight	68	24	24	24	25.7	64	60	85.7	56	60	25	25.5
Multimeric Nature	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer	monomer
Zinc Content	0.99	0.82	0.86	0.86	1.03	1.15	N.D.	N.D.	1.0	0.73	1.1	1.0
Inhibited by EDTA	+	+	+	+	+	+	+	+	+	+	+	+
Proteolytic	+	+	+	+	+	+	+	+	+	+	+	+
Isoelectric Point	acidic	basic	6.2	6.1	5.6	7.7	6.8	6.1	5.1	5.8	5.2	9.6
MHD	0.04[a]	3[a]	8[a]	11[a]	1[a]	0.52[a]	1.39[a]	0.1[a]	0.2[a]	0.17[z]	0.27[z]	1.43[z]
Myotoxic	-[10]	+	N.D.	N.D.	-[10]	+	+	N.D.	N.D.	+	+	+
Reference	Bjarnason and Tu (1978) Ownby et al. (1978) Bjarnason and Fox (1987)					Nikai et al. (1984)	Nikai et al. (1985a)	Kurecki and Kress (1985)	Civello et al. (1983a)	Mori et al. (1987)		

TABLE I (CONTINUED)

Family	Crotalidae			Crotalidae	Crotalidae				Crotalidae	
Genus	Crotalus			Lachesis	Trimeresurus				Trimeresurus	
Species	viridis			muta	flavoviridis				gramineus	
Subspecies	viridis			muta						
				Hemorrhagic Factor	Hemorrhagic Principle					
Name	Viriditoxin	VTm	VTwo	LHF-I	HR-1A	HR-1B	HR-2a	HR-2b	HR_1	HR_2
Molecular weight	62,57	66,62	68.5,63.5	100	60	60	23.5	23.7	23.5	81.5
Multimeric Nature	mixed dimer	mixed dimer	mixed dimer	monomer	dimer[11]	monomer	monomer	monomer	monomer	monomer
Zinc Content	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	+[12]	N.D.
Inhibited by EDTA	N.D.	+	+	+	+	+	+	+	+	+
Proteolytic	+	+	+	+	+	+	+	+	+	+
Isoelectric Point	4.8	acidic	acidic	acidic	4.4	4.4	N.D.	N.D.	N.D.	N.D.
MHD	0.075[8]	0.015[13]	0.016[13]	0.6[7]	2.6[7]	3.8[7]	0.066[7]	0.066[7]	~3.8[7]	~1.9[7]
Myotoxic	+	+	+	N.D.	N.D.	N.D.	+	+	N.D.	N.D.
Reference	Fabiano and Tu (1981)	Gleason et al. (1983)		Sánchez et al. (1987)	Omori-Satoh and Sadahiro (1979)		Takahashi and Ohsaka (1970a); Nikai et al. (1987)		Ouyang and Huang (1979); Huang et al. (1984)	

TABLE I (CONTINUED)

Family	Crotalidae			Elapidae		Viperidae	
Genus	*Trimeresurus*			*Actractaspis*		*Vipera*	
Species	*mucrosquamatus*			*engaddensis*		*palestinae*	
Subspecies							
		Hemorrhagic Factor		Hemorrhagic Factor		Hemorrhagin	
Name	Mucrotoxin	HF_a	HF_b	"HF"	HR1	HR2	HR3
Molecular Weight	94	15	27	50	60	60	60
Multimeric Nature	monomer	monomer	monomer	monomer	monomer	monomer	monomer
Zinc Content	1.8	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Inhibited by EDTA	+	+	+	+	+	+	+
Proteolytic	+	+	+	+	+	+	-
Isoelectric Point	4.3	4.72	8.9	acidic	basic	weakly acidic	strongly acidic
MHD	2.31[7]	1.7[7]	2.3[7]	0.15-0.20[14]	0.2[14]	0.2[14]	0.4[14]
Myotoxic	+	+	+	N.D.	N.D.	N.D.	N.D.
Reference	Sugihara et al. (1983); Kishida et al. (1985)	Nikai et al. (1985b)		Ovadia (1987)		Ovadia (1978)	

TABLE I (CONTINUED)

[1]Molecular weights were determined by SDS-PAGE; values are given in kdal.

[2]Molecular weight determined by sedimentation. The molecular weights of HRI and HRII were calculated by the authors referenced to be 85 and 95 kdal respectively.

[3]N.D. = not determined; N.R. = not reported.

[4]A dimeric form similar to Trimeresurus flavoviridis venom hemorrhagic principle HR-1A may be present; see Figure 1 of Sugihara et al. (1979).

[5]Zinc content is given in moles of zinc per mole of protein.

[6]+ = tested positive; - = tested negative.

[7]MHD determined in rabbits as described by Kondo et al. (1960).

[8]MHD determined in 20 gm mice injected subcutaneously in the back and sacrificed 6 hr later. MHD is the dose in μg of toxin required to cause a 5 mm diameter hemorrhage on the inner surface of the skin.

[9]MHD determined in 25 gm mice injected subcutaneously in the back and sacrificed 4 hr later. MHD is the dose in μg of toxin required to cause a 1 cm diameter hemorrhage on the inner surface of the skin.

[10]Possibly insufficent time was allowed for myonecrosis to develop in muscle before sacrifice of mice.

[11]HR-1A undergoes a concentration dependent dimerization. See Discussion of Chapter III.

[12]Content of zinc was not determined, but its presence was implied. Removal of zinc by EDTA treatment of HR_1 caused a loss in the activities of HR_1, and these activities were restored upon addition of zinc to the apoenzyme.

[13]MHD determined by method of Ownby et al. (1984b). See Materials and Methods of Chapter III for details.

[14]MHD determined in mice: 0.1 ml of toxin solution was injected into the skin of the back and then mice were sacrificed 24 hr later. MHD is the dose in μg of toxin required to cause a 1 cm diameter hemorrhage on the inner surface of the skin.

Oshima et al. reported the isolation from the venom of Agkistrodon halys blomhoffii of hemorrhagic factor HRI, a 85 kdal toxin, in 1972, and that of hemorrhagic factor HRII, a 95 kdal toxin, in 1968. Their hemorrhagic activities were inhibited by EDTA treatment. In 1970 the purifications of three hemorrhagic principles, HR-1, HR-2a, and HR-2b from the venom of Trimeresurus flavoviridis, were reported (Omori-Satoh and Ohsaka, 1970). A few years later, HR-1 was further resolved into two 60 kdal immunologically related hemorrhagins, HR-1A and HR-1B (Omori-Satoh and Sadahiro, 1979). HR-1A apparently underwent a concentration-dependent polymerization, whereas HR-1B remained momomeric. A 50 kdal proteolytic hemorrhagic principle, HF_2, isolated from Bothrops jararaca venom, had both activities eliminated by EDTA treatment. A proteolytic hemorrhagin from the venom of Crotalus adamanteus, proteinase H, can be inhibited by EDTA treatment but not by treatment with phenylmethylsulfonyl flouride or human-α_2-macroglobulin (Kurecki and Kress, 1985). Five hemorrhagic toxins, AC_1-, AC_2-, AC_3-, AC_4-, and AC_5-proteinase, from the venom of Agkistrodon acutus have been isolated and characterized by Sugihara and coworkers (Nikai et al., 1977, 1982; Sugihara et al., 1978, 1979, 1980; Mori et al., 1984; Yakahashi et al., 1986). Ac_1-proteinase has a molecular weight of 24.5 kdal (Nikai et al., 1977) and apparently contains one mole of zinc per molecule, removal of which eliminates hemorrhagic and proteolytic activities (Nikai et al., 1982). Another report of hemorrhagic proteases isolated from this venom described the isolation of three similar hemorrhagic proteases, AaHI, AaHII, and AaHIII (Xu et al., 1981). At least, AaHI is apparently the same as Ac_1-proteinase. A 56 kdal hemorrhagin, HP-IV, from the venom of Crotalus horridus

horridus has also been purified and characterized (Civello et al., 1983a). It has weak collagenase activity and completely solubilizes glomerular basement membrane (Civello et al., 1983b). The peptide bond cleavage specificities of many of the proteolytic hemorrhagins have been elucidated on the β-chain of oxidized insulin. All six of the Zn^{++} containing hemorrhagic toxins from Crotalus atrox, HTa, HTb, HTc, HTd, HTe, and HTf, have been characterized in this manner (Bjarnason and Tu, 1978; Nikai et al., 1984). A seventh hemorrhagic toxin, HTg, has recently also been isolated from this venom (Nikai et al., 1985). When Zn^{++} was removed from the HTe enzyme, hemorrhagic and proteolytic activity were eliminated and then could be restored by the reintroduction of Zn^{++} (Bjarnason and Tu, 1978). Two of these hemorrhagic toxins, HTb and HTf, are basic, while the remainder of these toxins are acidic. Isolations and characterizations of other hemorrhagins have been reported, including hemorrhagins HR1 and HR2 from the venom of Trimeresurus gramineus (Ouyang and Huang, 1979; Huang et al., 1984), three hemorrhagins, mucrotoxin (Sugihara et al., 1983; Kishida et al., 1985), HF_a, and HF_b (Nikai et al., 1985b), from the venom of Trimeresurus mucrosquamatus, hemorrhagic factors NHF_a and NHF_b from the venom of Bothrops neuwiedi (Mandelbaum et al., 1984), and hemorrhagic toxins HT-1, HT-2, and HT-3 from the venom of Crotalus ruber ruber (Mori et al., 1987). The most recently isolated hemorrhagic factor, LHF-I from the venom of the bushmaster snake Lachesis muta muta, is apparently a monomer of 100 kdal as determined by SDS-PAGE (Sánchez et al., 1987). A summary of hemorrhagins and their properties is given in Table I.

An acidic (pI 4.8) high molecular weight (115 kdal) hemorrhagin,

dubbed viriditoxin, was isolated from the venom of Crotalus viridis viridis (Fabiano and Tu, 1981). Circular dichroism spectral analysis revealed that the protein probably has a high β-sheet content. Viriditoxin is somewhat similar to the hemorrhagic principle of Trimeresurus flavoviridis HR-1A (Omori-Satoh and Sadahiro, 1979) since it too exists as a dimer, although viriditoxin's two subunits are of different molecular weights and do not appear to monomerize under examined conditions (Fabiano and Tu, 1981).

Viriditoxin has low but detectable proteolytic activity against casein and cleaves the oxidized β-chain of insulin. This toxin was moderately lethal with an LD_{50} value of ~5.0 μg/gm in mice injected intravenously (i.v.). Interestingly viriditoxin was also found to be myotoxic by light microscopy and CK measurements 24 hours after injection into mice.

After a careful examination of the data in Table I it becomes clear that hemorrhagic toxins are proteolytic metalloenzymes which can be inhibited by divalent metal ion chelation. They apparently contain one mole of zinc per mole of protein, and can vary in size, toxicity, and pI. Closer examination reveals that there are two classes of hemmorrhagic toxins: high molecular weight (50-100 kdal) high activity hemorrhagins, and low molecular weight (20-30 kdal) relatively low activity toxins. This was proposed by Fox and Bjarnason (1983). Further it appears some members of 50-100 kdal class can occur as dimers.

Effects of Venom Toxins

The local and systemic toxic effects of venom are caused by

specific venom components, and some have been attributed to specific enzymatic or biochemically elucidated protein components. Systemic effects consist of alterations in the homeostasis of the whole organism. "Systemic effects of venom include changes in the cardiovascular, nervous, respiratory, and urinary systems, such as hemorrhage, hypotension, shock, coagulation, hemolysis, hemoconcentraton, lactacidemia and death" (Ownby, 1982). Local effects of toxins consist of changes in the immediate area which has been bitten and include all effects occuring only near the site of the bite, such as hemorrhage, myonecrosis, edema, and pain. In rattlesnake venoms the local effects can be so severe that they can lead to subsquent systemic impairments, failure, or in the worst cases death of the envenomated animal. The inherent danger of nearly all rattlesnake bites is the toxic local actions of venom components. Although toxins which have a direct systemic effect are common in rattlesnake and other crotalid venoms, their toxic importance is relatively minor in most cases. Lethal systemically acting toxins in rattlesnake venom are rare and in all but two cases clinically unimportant (Hardy, 1986).

Neurotoxic Crotalidae Venoms

A major category of systemic toxin in most families of snakes, the neurotoxins, are a relatively unimportant class of protein toxins in rattlesnake venoms. Isolated rattlesnake neurotoxins have been shown to act either postsynaptically or presynaptically. Only two rattlesnake venoms are reported to contain sufficient quantities of neurotoxins to make them the major factor in the venom's toxic action.

The principal neurotoxins found in the venoms of Crotalus durissus terrificus and Crotalus scutulatus scutulatus -- crotoxin and Mojave toxin, respectively -- have been extensively investigated and shown to act presynaptically. These venom neurotoxins can cause quick death in victims bitten by these snakes. The protein involved is nearly identical in both venoms. Both are dimers, consisting of a basic phospholipase subunit (B) and an acidic subunit (A). At least 15 isoforms of crotoxin are known to exist in the venom of Crotalus durissus terrificus (Faure and Bon, 1987). Differences in the amino acids found in positions of the B subunit have been previously documented (Fraenkel-Conrat et al., 1980). It was found in crotoxin that the A subunit increases the B subunit's neurotoxic activity (Habermann and Rubsamen, 1971). Later, it was shown that the A subunit increases the specificity of the binding of the dimer to the presynaptic membrane, accounting for the increased neurotoxicity of the B subunit in dimer form, even though its phospholipase A_2 activity was decreased with respect to that of the monomeric form (Breithaupt, 1976; Hendon and Tu, 1979). Blockage of motor nerve impulse transmission from the phrenic nerve to the diaphragm by crotoxin is thought to result in respiratory arrest followed by death (Gopalakrishnakone et al., 1980).

Lethality of Non-neurotoxic Crotalidae Venoms

Severe and lethal systemic consequences of poisoning by the other Crotalidae principally result from the local swelling, myonecrosis and hemorrhage which these venoms induce, in which neurotoxins probably do not play a part. Hemmorrhage is the most common and prominent effect

of rattlesnake poisoning. When poisoning is severe, the initial observed local effects can spread, with hemorrhage becoming systemic and present in nearly all the vital organs.

In contrast to the quick death caused by neurotoxins, death due to the severe consequences of local effects often requires two to three days. Prolonged hemorrhage with the resultant loss of a large volume of circulating blood (hypovolemia) may lead to death in some cases. Indeed Gopalakrishnakone et al. (1980) suggested in experiments with neurotoxic Crotalidae venom, under conditions where the onset of respiratory failure was slow, the more prominent cardiovascular changes may have resulted in death due to circulatory failure. Others feel death is due to a combination of venom-induced damage to the cardiovascular system, respiratory system, nervous system and hemostatic mechanism.

Hemorrhage

Hemorrhagic components of venom are thought to act directly upon the endothelial cells of blood vessels or on the connective tissue proteins surrounding them. When capillaries are disrupted, hemorrhage occurs and the blood, consisting of serum, erthyrocytes, neutrophils, eosinophils, basophils, and platelets, is released into the surrounding tissue. Capillaries are the most susceptible to hemorrhage, due to the simple structure of a one endothelial cell thick tube surrounded by a basement membrane of connective tissue proteins. The disruption of endothelial cells leading to hemorrhage may occur via two mechanisms: by diapedesis or by rhexis (Ownby et al., 1978). Hemorrhage per diapedesis opens the intercellular junctions of the

endothelial cells, allowing blood to pass through into the surrounding connective tissue (Tsuchiya et al., 1974; Ohsaka et al., 1975, Ohsaka, 1979). This is presumably caused by the disruption of the basement membrane beneath the endothelium. Hemorrhage per rhexis is due to a breakdown of endothelial cell plasma membranes and cell lysis, creating an intracellular fistula through which blood escapes (Ownby et al., 1978; Ownby, 1982, Ownby and Geren, 1987). Disruption of the basement membrane does not appear to be a prerequisite for initiation of hemorrhage per rhexis.

Hemorrhage per Diapedesis. Using the venom of Trimeresurus flavoviridis Ohsaka et al. (1971a) demonstrated hemorrhage occurred at "pinpoints" (implied intercellular junctions) along the capillaries of rat mesentary tissue without disruption of the endothelial cells. This study was done at the light microscopic level using microcinematography and its conclusions agreed with earlier findings of Fulton et al. (1956), who used the venom of Agkistrodon piscovorus on the cheek pouch of hamsters, that hemorrhage occurred per diapedesis. Ohsaka et al. (1975), using Trimeresurus flavoviridis venom, and Tsuchiya et al. (1974), using hemorrhagic principle HR-1, demonstrated erythrocytes within the intercellular junction of endothelial cells by electron microscopy and the adjacent basement membrane disrupted. Lysis of the endothelial cells apparently did not occur and platelet aggregrations were rare, but seemed to occur at the "pinpoints" of extravasation. Increased vascular permeability in the rat mesentary tissue treated with T. flavoviridis venom was seen to only occur at the "pinpoints" of extravasation by the use of a tracer dye (Ohsaka et al., 1971a). Treatment with HR-1 gave a time course

consistent with leakage only through these "pinpoints" by following the extravasation of labeled albumin and labeled erthryocytes.

Hemorrhage per Rhexis. Work early in this century, reviewed by Ownby (1982) strongly implied that crude snake venoms destroy the endothelial cells and create gaps through which blood may pass. McKay et al. (1970) demonstrated a "direct" lytic action on the endothelial cells due to a purified hemorrhagin of Vipera palestinae venom. However, it was Ownby and coworkers (1974, 1978) who conclusively demonstrated that hemorrhage can occur per rhexis in at least some cases of hemorrhage. Ownby et al. (1974) performed careful light and electron microscopic studies using the New World venom of Crotalus atrox injected into mouse skeletal muscle tissue. This study concluded that with time the endothelia of the capillaries were in various stages of degeneration. The sequence of events leading to cell lysis was described as dilation of the endoplasmic reticulum, perinuclear space, and often the cytoplasm, with subsequent vesicular release into the lumen of the capillary. In cells undergoing vesicular release the tight junctions were normal. In swollen cells there was an apparent decrease in the number of pinocytic vesicles. These changes were followed by rupture of the plasma membrane and then extravasation of blood. "Platelet aggregations were common, often plugging gaps in the capillary wall or occluding the lumen of the capillary." The basal lamina was mostly continuous but discontinuities did occur. In yet more severe cases only parts of cells, erthryocytes and platelets were seen in the endomysium and in these areas no intact capillaries were present. No diapedesis of blood was seen at any

stage examined, and hemorrhage was concluded to occur per rhexis.

In 1978, Ownby and coworkers then examined in a like manner three of the five isolated and characterized hemorrhagic toxins purified from Crotalus atrox venom (Bjarnason and Tu, 1978). Using the purified hemorrhagins HTa, HTb, and HTe, it was shown that the purified hemorrhagic components of this venom cause hemorrhage per rhexis in a manner much like that of crude venom. As before with crude venom, hemorrhage occured per rhexis and the intracellular junctions remained intact. Temporally HTa and HTe resulted in extensive hemorrhage by 5 min.; however HTb required a longer period of time, 3 hr, for the same amount of hemorrhage to occur. Although there was a temporal difference, the mechanism of hemorrhage elucidated by electron microscopy was indistinguishable. HTb also caused myonecrosis in addition to hemorrhage and the significance of this observation will be dealt with later. A recent study of the pathogenesis of hemorrhage induced by hemorrhagic proteinase IV from the venom of Crotalus horridus horridus again confirmed that hemorrhage can occur per rhexis (Ownby and Geren, 1987). This excellent study examined the pathogenesis of hemorrhage at both the light and electron microscopic levels, detailing the changes which occur ultrastructurally in endothelial cells in vivo.

Hemorrhage per Diapedesis vs. Hemorrhage per Rhexis. Two differences in the studies were the use of old versus new world venoms aand the different tissues employed. While the validity of the experimental designs employed by Ohsaka et al. (1971a, 1975) might be questioned, the venom and components studied were from an Old World

genus of Crotalidae and the hemorrhagins may cause a different sequence of events to occur during hemorrhage as recently suggested (Ownby and Geren, 1987). Additionally, the tissues examined might play a role in determining the mechanism of hemorrhage induced by a specific hemorrhagin. It is well known that there are different capillary types. For example, three hemorrhagins, Ac_1-, Ac_2-, and Ac_3-proteinase and the Agkistrodon acutus crude venom from which they were purified, gave differing patterns of hemorrhage in the internal organs when injected intravenously (Homma et al., 1980). The hemorrhage caused by A. acutus crude venom was predominantly in the lung and heart. The hemorrhage caused by the toxins was predominantly in various organs as follows: Ac_1-proteinase, kidney and stomach, Ac_2-proteinase, stomach and small intestine, and Ac_3-proteinase, lung.

Biochemical Mechanism of Hemorrhage. The biochemical mechanism of hemorrhage remains to be elucidated. It seems probable that the first event prior to hemorrhage is the proteolysis of some of the bite victim's proteins by the venom's zinc-containing metalloproteolytic hemorrhagins. One possible hypothesis is that the hemorrhagic toxin acts directly on the endothelial cells. This is somewhat supported by the observation that Crotalus atrox crude venom and HTa and HTe can cause substantial endothelial cell lysis within 2 to 5 minutes (Ownby et al., 1978; Ownby, 1982). However, a hypothetical indirect activation via secondary factors prior to endothelial cell lysis can not be ruled out solely on the basis of these in vivo experiments. For example, the rapid activation of the lysis of targeted cells by the complement system requires a concert of serum proteins (Reid and Porter, 1981). It is felt by some that proteolysis of the adjacent

basement membrane may initiate hemorrhage (Ohsaka, 1979). Electron microscopic studies (Tsuchiya et al., 1974) and *in vitro* studies on the proteolytic fragmentation caused by hemorrhagins on isolated glomerular basement membrane or other connective tissue proteins (Ohsaka et al., 1973a,b; Civello et al., 1983b) can be interpreted to support this hypothesis. Indeed, it has been observed that snake venom hemorrhagic-proteases cleave more bonds in connective tissue proteins than in typically tested protease substrates such as casein (Fox and Bjarnason, 1983). Suggestively, bacterial collagenase can cause hemorrhage (Just et al., 1970). However, the work of Ownby and co-workers clearly indicates that the disruption of the basement membrane is not a prerequisite for hemorrhage (Ownby et al., 1974, 1978; Ownby and Geren, 1987). This seeming contradiction can perhaps be resolved by considering that proteolytic hemorrhagins from different snake venoms may exploit different mechanisms for invalidating the endothelial cell barrier of the capillary. Another aspect of the hemorrhagic mechanism may be some alteration in the clotting process. Many hemorrhagins have been shown to cleave fibrinogen, but the pattern of cleavage is different than that obtained with thrombin (a partial citation of the relevant work is: Civello et al., 1983b; Huang et al., 1984; Kishida et al., 1984; Nikai et al., 1984, 1985a, 1987; Yagihashi et al., 1986; Mori et al., 1987). The pattern of fibrinogen cleavage is often different from one hemorrhagin to the next. For instance, sometimes no clot was formed by the hemorrhagin's cleavage of fibrinogen (Nikai et al., 1984, 1985a; Kishida et al., 1985; Mori et al., 1987) and other times an abberant clot was formed, but even so the hemorrhagin was shown to solubilize

fibrin (Civello et al., 1983b). Therefore, perhaps at least part of a hemorrhagin's function may be to alter the clotting mechanism. Whether the proteolysis caused by hemorrhagins is directed against the basement membrane, endothelial cell membrane, intercellular junction, serum proteins, and/or connective tissue proteins remains an unsolved question and a challenge for future research.

Myonecrosis

Muscle cell necrosis, or myonecrosis, is one of the severe toxic effects of rattlesnake envenomation. Further, the best available treatment, polyvalent antiserum, is only partially effective in neutralization of this effect (Homma and Tu, 1970; Minton, 1954; McCollough et al., 1970; Gutiérrez et al., 1981; Ownby et al., 1984a). Research on the pathological action of myotoxins has provided new insights into their pathogenetic mechanism (Ownby et al., 1976; Ownby et al., 1982; Cameron and Tu, 1978; Gleason et al., 1983). Such studies have shown that the myotoxic injury can be partially reversible or irreversible, depending on a variety of factors.

Several methods of analysis of myonecrosis have been employed. These include microscopy, biochemistry, electrophysiology, and immunology, to name a few. By far the most useful technique has been direct observation by microscopy at the light and ultrastructural levels. The other techniques, while often useful as independent measures, usually become powerful adjuncts to the histological methods in the study of myonecrosis. For instance, the release of creatine phosphokinase from muscle after treatment with myotoxins is easily monitored, and has been shown to be a reliable indicator of the onset

of myonecrosis (Lee et al., 1974; Ownby et al., 1982).

There are at least three distinguishable classes of myotoxins: phospholipases A_2, small basic polypeptides, and hemorrhaghic toxins that also also have myotoxic activity (such hemorrhagic toxins will be referred to as myotoxic-hemorrhagins.

The pathology and mode of action of a class of myotoxins comprised of small basic polypeptides which have no identifiable enzymatic activity is typified by myotoxin a from Crotalus viridis viridis venom (Cameron and Tu, 1977, 1978; Ownby et al., 1976, 1982). Other members of this class are crotamine from Crotalus durissus terrificus venom (Cheymol et al., 1971; Laure, 1975), myotoxins I and II from Crotalus durissus durissus venom (Eneff, 1982), and two myotoxins from the venom of Crotalus viridis concolor (Engle et al., 1983).

Studies with myotoxin a show that it causes a degeneration of muscle cells (Ownby et al., 1976), primarily a dilation of the sarcoplasmic reticulum similar to that caused by the crude venom (Stringer, 1972). Crotalus viridis viridis venom exhibits other myotoxic activities, such as marked mitochondrial changes and destruction of external lamina and the sarcolemma.

The dilation of the sarcoplasmic reticulum may be mediated directly or indirectly through changes in the permeability of the sarcolemma (Ownby et al., 1976). The hypothesized effect of myotoxin a activity on the sarcolemma would involve the transport of ions, causing swelling of the cell and cellular compartments. Studies with crotamine support the indirect mode of action (Brazil et al., 1979; Cheymol et al., 1971; Cameron and Tu, 1978). However, ultrastrucural

studies of thin sections treated with horseradish peroxidase-conjugated myotoxin a demonstrated significant binding at the sarcoplasmic reticulum (Tu and Morita, 1983). These data would tend to support a direct mode of action via the sarcoplasmic reticulum. Rabbit antiserum against myotoxin a, when incubated with myotoxin a before injection (i.m.) can neutralize the local effects of myonecrosis (Ownby et al., 1983). The effects of myotoxin a can also be neutralized *in vivo* if an injection (i.m.) of myotoxin a is followed by an injection (i.m.) of anti-myotoxin a serum after five minutes, but not after fifteen minutes (Ownby et al., 1984a).

Phospholipase A_2 can have myotoxic activities (Habermann and Breithaupt, 1978; Gopalakrishnakone, 1979; Gopalakrishnakone et al., 1980; Harris et al., 1975; Harris and MacDonell, 1981; Harris and Maltin, 1982; Gutiérrez et al., 1986; Mebs and Somejima, 1986). A myotoxic phospholipase A_2 from *Bothrops asper* venom causes a type of myonecrosis histologically distinct from that caused by myotoxin a (Gutiérrez et al., 1986). The resultant myonecrosis is typified by focal lesions of disrupted sarcolemma and swollen mitochondria. The isolated basic subunit of the neurotoxin crotoxin, also a phospholipase A_2, causes myonecrosis which can be potentiated by its acidic subunit (Kouyoumdjian et al., 1986). The onset of myonecrosis is also faster than for the other two classes of rattlesnake myotoxins. Monospecific antimyotoxin rabbit serum and polyvalent antiserum against the *Bothrops asper* myotoxic-phospholipase and the crude venom, respectively, neutralize myotoxic activity, phospholipase A_2 activity and lethality (Lomonte et al., 1987). At the concentration required for *in vitro* phospholipase A_2 activity

neutralization, the antisera do not neutralize myotoxicity (Lomonte et al., 1987; Gutiérrez et al., 1986). Noteworthy to mention for its possible implications on a mechanism of action for these phospholipase A_2 myotoxins is an *in vitro* study by Simpkins and coworkers (1971). When phopholipase A_2 was applied exogenously to human erythrocytes or lobster axonal membranes, significant decreases in membrane lipid mobility were observed by electron spin resonance. As will be discussed below in the section on ischemia, membrane fluidity seems to play a role in calcium loading in cells and their consequent coagulative necrosis.

Hemorrhagic toxins can also have a myotoxic effect. Myonecrosis is known to be caused by members of both molecular weight classes of hemorrhagic toxins (Fox and Bjarnason, 1983). Until 1984, there had only been three reports of myotoxic-hemorrhagins: HTb was reported from *Crotalus atrox* venom (Ownby et al., 1978), and viriditoxin-like isolates were reported from *Crotalus viridis viridis* venom (Fabiano and Tu, 1981; Gleason et al., 1983). From 1984 until the present, eleven more myotoxic-hemorrhagins have been reported. A summary of the properties of the myotoxic-hemorrhagins is given in Table I. Three of the hemorrhagic toxins from *Crotalus atrox* venom have been properly investigated for myotoxic activity. HTb (Ownby et al., 1978), a member of the lower molecular weight class, and HTf (Nikai et al., 1984) and HTg (Nikai et al., 1985a), members of the higher molecular weight class, have all been shown to be myotoxic. Viriditoxin and the viriditoxin-like hemorrhagins (Gleason et al., 1983) from the venom of *Crotalus viridis viridis* have been shown to be myotoxic (Fabiano and Tu, 1981; Gleason et al., 1983). HTb, the most

thoroughly studied of the myotoxic-hemorrhagins, produced necrosis in mouse skeletal muscle, resulting in disorientation of myofibrils, erythrocytes within damaged muscle cells, increased glycogen deposition, swollen mitochondria severely damaged and stacked together, and lysis of the sarcolemma (Ownby et al., 1978). Polyvalent (Crotalidae) antiserum significantly neutralizes hemorrhage induced by Crotalus viridis viridis venom (Ownby et al., 1984b) and Crotalus horridus horridus venom (Smith and Ownby, 1985) when incubated with these venoms prior to injection (i.m.). The myotoxic effects of Crotalus viridis viridis crude venom can be neutralized in vivo if an injection (i.m.) of the venom is followed by a combined injection (i.m.) of antimyotoxin a serum and polyvalent (Crotalidae) antivenin after 30 minutes but not after 60 minutes (Ownby et al., 1986). The same treatment neutralizes the hemorrhagic effects of the venom if the antisera are injected (i.m.) after five minutes but not after 30 minutes.

Ischemia

Since hemorrhage impairs or interrupts the flow of nutrients to tissues, hemorrhaged tissue may develop ischemia. The effects of ischemic injury to myocardium are of obvious clinical importance. Consequently, ischemia's effects on myocardial tissues have been extensively studied. Hepatic tissue is also a model for the study of ischemia. In both tissues, Ca^{++} seems to be the mediator of the damage. Liver tissue provides an intermediate stage in ischemic damage, useful for study, that can not be found in muscle tissue because of the unusual regulatory chemical characteristics of muscle,

as will be seen shortly.

Intial Effects of Ischemia

The initial ischemic event in myocardial tissue is depolarization of the sarcolemma (Shine 1981) and a loss of contractility, even while the ATP level is still high (Neely and Feuvray, 1981). Since the oxygen supply is less than the oxygen demand required for function (Jennings and Reimer, 1981), oxidative respiration is inhibited and glycolysis becomes the only significant source of ATP. However, glycolysis is inhibited by NADH, H^+, and lactate accumulation in unperfused ischemic myocardial tissue (Neely and Feuvray, 1981). Ischemia causes within minutes a reduction in cAMP levels in myocardial tissue (Schulze and Will-Shahab, 1984). The percentage of activated glycogen phosphorylase declines threefold in 40 min after the onset of ischemia in skeletal muscle (Chasiotis and Hultman, 1983), reflecting decreased cAMP levels and the blockage of glycolysis. Accumulation of glycogen granules as a consequence of blockage of glycolysis is well known, and has been found to occur in skeletal muscle necrosis (Ownby et al., 1974).

Ischemic Calcium Loading in Myocardial Tissue. By 15-20 minutes of ischemia, the myocardial sarcolemma is leaky to Ca^{++} (Shine, 1981), as shown by sarcoplasmic accumulation of non-sequesterable Ca^{++} analogs. However, the sarcoplasmic reticulum is still sequestering Ca^{++} at this time, as shown by the uptake of sequesterable Ca^{++} analogs. By 45 minutes sequesterable analogs are also building up in the sarcoplasm, implying either that the sarcoplasmic reticulum has

become leaky as well, or that the reticular Ca^{++} pumps lack the energy to function (Shine, 1981). Indeed Grong et al. (1984) found that ATP levels decreased five-fold in myocardial tissue after 45 minutes of ischemia. Myocardial mitochondria also became damaged during Ca^{++} loading since they also sequester Ca^{++}. Swelling of mitochondria can occur by 15 minutes of ischemia and is reversible by reperfusion at 30 minutes, through restoration of the osmotic balance of the mitochondria. However, permanent disruptive damage to mitochondrial cristae occurs by 60 minutes (Neely and Feuvray, 1981).

Calcium Loading in Skeletal Muscle. Ca^{++} leakage through the plasma membrane causes many types of damage. Drugs which increase sarcoplasmic Ca^{++} cause disruption of the sarcolemma, sarcoplasmic reticulum, mitochondria, and myofibrils in skeletal muscle (Benoit et al., 1980; Duncan, 1978). Ca^{++} at extracellular concentrations stimulates autolytic activity in skeletal muscle homogenates (Stauber et al., 1977). Organelles which actively sequester Ca^{++}, such as the sarcoplasmic reticulum and mitochondria, become Ca^{++}-loaded in damaged skeletal muscle (Oberc and Engel, 1977).

Calcium Loading in Hepatocytes. Experimentally it has been observed that liver cells accumulate Ca^{++} only upon reperfusion with Ca^{++}-containing, oxygenated media (Farber et al., 1981). The result is a loss of cell volume regulation, accompanied by increased tissue water, increased Na^{+} content and membrane dysfunction (Farber, 1982). Mitochondria, which have become permeabilized before reperfusion, never regain normal function and become extremely swollen (Farber et al., 1981).

Summary of Calcium Loading. Ca^{++}-mediated injury is characterized by an early, reversible phase followed by a later, irreversible phase. The loss of reversibility of the injury closely parallels a massive Ca^{++} uptake with accompanying fluid uptake. This is followed by the appearance of coagulative necrosis. In muscle tissue, Ca^{++}-loading appears to be concommitant with ATP loss as substantial energy-dependent Ca^{++} stores are released from the sarcoplasmic reticulum, with loss of reversibility occurring by one hour. However, in liver tissue the Ca^{++} content appears to be insufficient for Ca^{++}-loading, such that only the effects of ATP loss and mild Ca^{++} exposure occur during ischmia, and Ca^{++}-loading occurs only upon reperfusion. The link between Ca^{++} loading and irreversible injury appears to be membrane phospholipid degradation (Farber, 1982).

Calcium-Activated Phospholipases

There are cytoplasmically oriented Ca^{++}-activated phospholipases A_2 in most membranes of almost all cells (Farber, 1982; Colard-Torquebiau et al., 1976). It has been shown that Ca^{++}-activated degradation of membranes results in a decrease in the arachidonic acid content, and thereby the double bond index, of the membranes (Storch and Schachter, 1985). This can be as much as a 33% decrease in arachidonic acid content, yielding an 18% decrease in the double bond index, in hepatocyte membranes incubated in Ca^{++} solutions for 2 hours. Removal of Ca^{++} by EDTA cannot reverse this permanent loss in membrane fluidity, suggesting that an indirect mechanism of action by Ca^{++} is involved. A reasonable working hypothesis for the action of Ca^{++} is stimulation of intracellular phospholipases A_2,

leading to cleavage and loss of arachidonic acid residues from membrane phospholipids (Storch and Schachter, 1985). Indeed, lysophosphatide production alone cannot account for this fluidity loss. In experimental studies observing fluorescence anisotropy of a probe in artificial bilayers, it has been found that acyl bond 2 cleavage alone has no significant effect on membrane fluidity. Cleavage and loss of a palmitic acid likewise has no significant effect. However cleavage and loss of arachidonic acid significantly decreases the lipid fluidity. This study agrees well with previous studies which suggest that fluidity changes do not occur, as measured by phase transition temperature, even when lysophosphatidyl choline in dipalmitoylphosphatidyl choline vesicles is in a 1:1 molar ratio (Klopfenstein et al., 1974). In fact 1-acyl lysophosphatidyl choline and free fatty acids in an equimolar ratio form bilayers rather than micelles, thereby ruling out a detergent effect of the lysophosphatidyl choline on the membrane (Jain et al., 1980; Jain and deHaas, 1981). That these studies on artificial systems are related to <u>in vivo</u> processes is demonstrated by studies in which exogenous phospholipase A_2 applied to normal membranes decreased lipid mobility (Simpkins et al., 1971).

Farber et al. (1981) found that liver cells lose 40% of their phospholipid content in three hours of ischemia. They demonstrated that the phospholipid bilayer had become crystalline and the membrane proteins had aggregated. They suggest that the loss of bilayer fluidity is the actual injury, irreversible and leading to leakiness and Ca^{++}-loading, and that small molecules and ions can leak through the boundaries between the crystal membrane and the protein aggregates.

In summary, with loss of energy in the form of ATP, calcium is lost from energy-dependent cellular stores. The amount lost is apparently sufficient, as described above, to activate cellular phospholipases A_2. Membranes then lose arachidonic acid and fluidity. In liver cells, which have no large energy-dependent calcium stores, there is insufficient intracellular calcium to cause calcium loading of organelles. Swelling and membrane disruption will occur when the liver tissue is reperfused in calcium-containing solutions, after the plasma membranes of cells have lost fluidity and become leaky. On the other hand, muscle tissue contains sufficient intracellular calcium stores to cause calcium loading to occur simultaneously with membrane fluidity loss. In either case, increased water uptake, due to osmosis, in cells whose membranes have lost fluidity may fracture cell membranes after calcium loading has occurred, leading to cell lysis.

Eicosanoid Enhancement of Ischemic Necrosis

A secondary effect of Ca^{++}-induced phospholipase A_2 activity is due to the arachidonic acid released from the membrane, as reviewed by Lefer (1985). This release activates prostaglandin synthase, resulting in the production of eicosanoids. One of these eicosanoids, thromboxane A_2, is considered to be a mediator of circulatory shock, myocardial ischemia, and sudden cardiac death. It is considered to dramatically enhance cellular damage in the ischemic myocardium through positive feedback loops. Ischemia causes thromboxane A_2 to be produced, and thromboxane A_2 causes vasospasm and clotting, which enhance ischemia. Thromboxane A_2 causes the release of lysosomal

enzymes, which further damage ischemic cells and release more arachidonic acid from membranes, thus further enhancing thromboxane A_2 production. Another eicosanoid, leukotriene B_4, is chemotactic for macrophages and eosinophils, and increases microvascular permeability. Infiltration of these blood cells into necrotic tissue to retrieve cellular debris is a well known process. Hence eicosanoids appear to be involved in many aspects of ischemic myonecrosis.

CHAPTER III

PURIFICATION AND CHARACTERIZATION OF HEMORRHAGIC AND MYOTOXIC PROTEINS FROM *CROTALUS VIRIDIS VIRIDIS* VENOMS

Introduction

Rattlesnake venom rarely kills the human bite victim, but often leads to severe local mutilation or loss of the bitten part (Clement and Pietrusko, 1979). Two principal locally damaging activities are hemorrhage and myonecrosis. The venom from the prairie rattlesnake *Crotalus viridis viridis*, which contains toxins exhibiting these activities, was chosen for this study.

The first such toxin to be isolated from this venom was the myotoxic component myotoxin *a*, a small basic polypeptide (Cameron and Tu, 1977). During the isolation of myotoxin *a*, Cameron and Tu observed high molecular weight fractions in the venom that were hemorrhagic and myotoxic. In subsequent work, viriditoxin, a myotoxic and hemorrhagic venom component with weak proteolytic activity, was isolated from one of these high molecular weight fractions (Fabiano and Tu, 1981).

In this study, in part reported previously (Gleason et al., 1983), isolations were developed for viriditoxin-like components from the venoms of two different populations of *Crotalus viridis viridis*. The

isolates, other venom components and the venoms were found to be intraspecially variant upon comparison of the two populations. The proteolytic, hemorrhagic and myotoxic activities of these venoms, the viriditoxin-like isolates and other venom components were further characterized and some of their properties profiled during isolation.[1]

Materials and Methods

Venoms

Crude Crotalus viridis viridis venom was obtained from two sources. Crude, lyophilyzed C. viridis viridis venom was obtained from the Miami Serpentarium, Miami, Florida. Crude C. viridis viridis venom was also obtained from prairie rattlesnakes captured in western Oklahoma. Other venoms used were obtained from the Miami Serpentarium unless otherwise indicated. The collection, handling and milking of the snakes and the subsequent preparation of the venom is described below.

Prairie rattlesnakes were collected in western Oklahoma and then

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[1]The viriditoxin-like component of Crotalus viridis viridis venom from the Miami Serpentarium will hereafter be referred to as VTm. The viriditoxin-like component of Crotalus viridis viridis venom from a population collected in western Oklahoma, previously referred to as viriditoxin-variant (Gleason et al., 1983) will only be referred to as VTwo. Often it will be necessary to refer to viriditoxin, its isolation, or its characterization as previously reported (Fabiano and Tu, 1981) and as such it will only be referred to as viriditoxin. On occasion it will be necessary to discuss this study's isolates together and in this case they will be referred to as viriditoxin-like isolates, components, etc. Rarely, it will be necessary to include viriditoxin in reference to all work with these toxins as viriditoxin-like isolates, etc., but this will be easily understood by the context of the sentence and the inclusion of viriditoxin (Fabiano and Tu, 1981) in such cases will be referenced for additional clarity.

maintained for a period of 9 months. Venom was collected on three different occasions over the last 4 months of this period. The rattlesnakes were kept sheltered in a clean, dry and quiet environment, provided with water and bedding which was changed periodically, and fed live young mice and live or frozen 3 day old chicks. The rattlesnakes were milked from one fang and then the other. The fang was protracted from the roof of the snake's open mouth with a slender metal rod and placed inside a clean small plastic centrifuge tube clamped to a ringstand. To aid the flow of venom gentle pressure was applied to the occipital region of the snake's head, forcing the snake to bite the tube and thereby contracting the venom gland. Individual yields were highly variable, from 2-3 drops per fang to approximately 0.5 mL in one case. Generally, larger snakes gave more venom. Typically, venom from four snakes was milked into one plastic centrifuge tube and was quickly frozen at -78 °C. After all the venom was collected, the tubes were quickly thawed, kept at 0 °C and centrifuged at 2500 x g for 30 min at four °C to remove cellular debris. Supernatants were pooled, frozen at -78 °C in 15 ml glass scintillation vials and lyophilyzed. All lypohilized crude venoms were kept desiccated and stored below 0 °C. Any subsequent resuspension of dry venom or resulting fractionation was either kept frozen or at four °C unless otherwise indicated. Methods of maintaining snakes and obtaining venom have been reviewed by Kochva (1987).

Chemicals

Sephadexes G150-120 and G150-40, lactate dehydrogenase, bovine serum albumin, ovalbumin, chymotrypsinogen A, cytochrome C,

blue-dextran, dinitrophenyl(DNP)-alanine, benzoylated dialysis tubing, bromophenol blue, aniline blue black, Coomassie brillant blue R-250, hide powder azure, hemoglobin (type 1), picryl sulfonic acid, and sodium borohydride were obtained from Sigma Chemical Co., St. Louis, MO. tris-(hydroxymethyl)aminomethane (Tris), KCl, formaldehyde and ammonium persulfate were obtained from Fisher Scientific Co., Fair Lawn, N.J. Vitamin-free Hammerstein quality casein was purchased from Nutritional Biochemicals. Sodium dodecyl sulfate (SDS), acrylamide, N,N′-methylenebis(acrylamide) (bis), N,N,N′,N′-tetramethylenediamine (TEMED), high and low molecular weight electrophoresis protein standards and Biogel A diethylaminoethyl (DEAE) were obtained from Bio-Rad Laboratories. Monoethanolamine was obtained from Eastman Organic, Chemical Distillation Product Ind., Rochester, NY. Glutaraldehyde, OsO_4, Polybed 812, nadic methyl anhydride, and Mallory's Trichrome stain were obtained from Polysciences Inc., Warrington, PA.

Sephadex G150-120 Gel Filtration

Four hundred to 550 mg of dry crude Crotalus viridis viridis venom was normally dissolved in 4 to 8 mL of buffer A: 0.05 M Tris-HCl, 0.10 M KCl, pH 9.0. Resuspended crude venom was kept at 4 °C, the solution was centrifuged for 5 min at 2500 x g, and the supernatant was loaded on a Sephadex G150-120 column. Samples were 5-12% (w/v) protein (A_{280} measurement or Bradford Protein Assay).

The G150-120 column was nominally 2.5 x 100 cm. Sample was loaded directly to the top of the column or layered on to the bed underneath the column buffer. Sample volume was never more than 2% of the column

volume. Proteins were eluted at 10 mL/hr and collected in glass tubes in nominally 2.5 mL fractions. Protein was monitored in collected fractions by measuring their absorbance at 280 nm. Aliquots from the individual fractions or from pooled fractions were then analyzed by standard Tris-glycinate discontinuous (disc) electrophoresis, SDS-polyacrylamide gel electrophoresis (PAGE), bio-assays and proteolytic assays as described below. In most cases fractions enriched for VTm or VTwo were selected on the basis of the elution profile, SDS-PAGE analysis of column fractions and hemorrhagic and myotoxic activity profiles and prepared for further purification. Pools of fractions were dialyzed against quartz-distilled water. Dialysis was carried out in benzoylated dialysis tubing with a molecular weight cutoff of 2 kdal, typically with three 1:100 volume changes. Dialyzed pools of fractions were lyophilized and weighed.

Anion Exchange Chromatography

The pool to be fractionated was equilibrated in buffer B: 4 mM monoethanolamine, pH 9.0, 2 mM KCl, and was loaded onto a 1.5 x 40 cm Biogel A DEAE column in Buffer B. The column was then washed with 100 mL of Buffer B, and eluted with a 100 to 250 mL linear KCl gradient from 2 mM to 300 mM KCl, usually at a slope 1/10 the steepness previously reported (Fabiano and Tu, 1981). Measurement of the conductivity of the KCl-eluted fractions allowed calculation of the KCl gradient. The flow rate was 30 mL/hr. Nominally 2.5 mL fractions were collected throughout the procedure. Analysis and pooling was as described above.

Histology

Purified toxins, crude venom fractions or crude venom were injected (i.m.) at a given dose (ie., 2.5 μg of toxin per gram of mouse; μg/gm) into the dorsolateral aspect of the right hind thigh of 20-30 gm CD-1 Charles Rivers white mice. Fractions or toxins tested were desalted prior to injection and dissolved in physiological saline (PSS): 0.85% NaCl (w/v). Volumes injected were always 5 μL per gram of mouse. After 24 hr mice were sacrified by cervical dislocation or by an overdose of diethyl ether. The injected muscle was immediately excised and fixed in 2% glutaraldehyde in 0.27 M cacodylate buffer, pH 7.4. Tissue was secondarily fixed in 1% OsO_4 and dehydrated in a graded series of acetone solutions. Muscle tissue was embedded in Polybed 812 and polymerized at 60 ℃ for 72 hr. Blocks were sectioned into 1 μm thick sections using a Sorvall ultramicrotome, mounted, stained with Mallory's trichrome and examined by light microscopy. Photomicrographs of tissue sections were taken using a Zeiss photomicroscope and Kodak Pan-X film.

Kondo's Method of Hemorrhagic Assay

Kondo's method of hemorrhagic assay (Kondo et al., 1960) as modified by Ownby and coworkers (1984b), is based on the reproducibility of intradermal (i.d.) injection to produce a measurable hemorrhagic area. Twenty-four to 31 gm white mice were prepared for injection by shaving fur from their backs with electric shears. The toxin or fraction tested was dissolved in PSS at varying concentrations as required and was injected (i.d.) into the center of

the depilated area. The volume injected was always 5 μL per gram of mouse. After 90 min the mice were sacrificed by cervical dislocation or an overdose of diethyl ether. The hemorrhagic area of skin and surrounding unaffected tissue was removed with scissors, placed so the inside surface of the skin was visible, pressed flat between two plates of glass and the cross-product diameter (CPD) determined. The CPD is the product of two measurements of the diameter of the hemorrhagic area, which are made at right angles to each other. The measurements cross through the center of the hemorrhagic area. This compensates for irregularities and ellipticity in the hemorrhagic area. A minimum hemorrhagic dose (MHD) is defined as the μg of toxin per gram of mouse which yield a CPD of 25 mm^2. This allows smaller as well as larger CPD measurements to be made so that the MHD can be accurately determined. Measurement of CPDs greater than 200 is common. Plotting the mean CPDs obtained from various doses of injections (i.d.) versus the log(dose) for those injections gives a linear curve which can be interpolated to give a MHD or compared to other such curves.

Proteolytic Assays

N,N′-dimethylated Protein Substrate Method. "N,N′-dimethylated" refers to one or more side groups of lysine in a protein which are monomethylated or dimethylated (Lin et al., 1969; Fields, 1971). The methylation of proteins significantly lowers the background signal of an assay detecting the appearance of free primary amines during proteolytic cleavage of the protein. To make these substrates, 1.5 grams of casein or hemoglobin were warmed to dissolve in 150 mL of 0.2

M sodium borate, pH 9.0. This was then cooled to 0 °C and kept at that temperature until the procedure was complete. To this rapidly stirring solution was added 300 mg of $NaBH_4$ with a few drops of 2-octanol present to prevent foaming. Then 27% formaldehyde was added in 100 µL increments until a total of 3 mL was added over a period of 30 min. After a few more minutes to allow complete reaction, the solution was acidified to pH 6.0 with 50% acetic acid. This was then exhaustively dialyzed against deionized water, centrifuged for 30 min at 15,000 x g, frozen at -78 °C, lyophilized, and stored dry at -10 °C.

Glass tubes containing 1.0 mL of 0.1% (w/v) N,N′-dimethylated casein or N,N′-dimethylated hemoglobin in 0.05 M Tris, pH 7.2 were incubated with 50 µL of various protein samples to be tested for proteolytic activity. Protein samples were added to the reaction mixtures while they were on ice. Incubations were typically for 2 hr at 37 °C. The reactions were stopped by placing them in boiling water for 1 min. Next 4.0 mL of 4% (w/v) sodium bicarbonate, pH 8.5, and 1.0 mL of 0.1% (w/v) picrylsulfonic acid (freshly made) were added. These were then incubated in the dark for 30 min at 50 °C and then 1.0 mL of 10% (w/v) SDS and 0.5 mL 1.0 N HCl were added. Prior to the addition of the picrylsulfonic acid and until after the HCl was added, the operations were either performed under lights not emitting wavelengths around 340 nm (Westinghouse F96T12/GO gold flourescent bulbs) or in the dark. These precautions were necessary to prevent the photochemical hydrolysis of picrylsulfonic acid into picric acid, which absorbs light at 340 nm and reduces the sensitivity of the assay. The addition of the acid precipitates the unreacted picrylsulfonic acid; however, the presence of SDS keeps the picryl peptidyl sulfonamides

solublized. The absorbance at 340 nm was then measured using a Hitachi Model 100-80 spectrophotometer.

Hide Powder Azure Method. This assay is based on the release of soluble proteolytic fragments containing covalently linked Remazolbrillant blue dye from an insoluble dye-linked protein matrix (Rinderknecht et al., 1968). Hide powder is a complex mixture of bovine hide proteins, keratin and collagen. In this assay, 20 mg of the dye-linked protein was suspended in 2.5 mL of 50 mM Tris-HCl, pH 7.8 and kept at 0 °C. To this was added a small quantity of protein to be evaluated for proteolytic activity. Incubation was typically performed at 37 °C for 30 to 120 min with gentle vortexing every 5 min to resuspend the insoluble dye-linked proteolytic substrate. For longer times, assays were placed on a 37 °C shaker during incubation. At the end of the incubation the reactions were stopped by placing the tubes on ice. After settling, approximately 2 mL of partially cleared supernatant was removed and placed into a small plastic centrifuge tube kept on ice. These were then centrifuged at 2000 RPM for 5 min in a Sorvall GLC-2 centrifuge at 4 °C. Supernatants were then individually collected, warmed to room temperature and their absorbance at 595 nm determined on a Hitachi Model 100-80 spectrophotometer. Elevated absorbance over that of background indicated proteolytic release of soluble dye-linked protein fragments into the recovered supernatant.

Phospholipase A_2 Assay

The method of phospholipase A_2 detection was as described

previously (Habermann and Hardt, 1972). An egg yolk emulsion was prepared by stirring one separated egg yolk to homogeneity in 100 mL of quartz distilled water for 10 min. Six mL of 2.5% $CaCl_2$ was added to this emulsion, which was then filtered through cheesecloth to remove the yolk sac. The pH was adjusted to 8.0 with NaOH, and the emulsion was kept on ice. The emulsion of egg yolk was then incorporated into 0.6% agarose and poured into 60 mm plastic petri dishes. Cylindrical holes were then punched into the plate. Toxins or venom fractions, along with phospholipase A_2 positive and negative controls and a protease control, were added to separate wells in the plate. The plate was then covered, kept moist and incubated at 50 °C for 20 hr. The phospholipase A_2 activity was quantitated by measuring the zone of clarified egg yolk around positive wells. The incorporation of a protease control ensured no proteolytic activity was assigned a phospholipase A_2 activity since proteases only give diffuse clearing of the cloudy egg yolk emulsion. The zones of clearing were visualized by dark-field underlighting.

Standard Tris-glycinate Disc Polyacrylamide Gel Electrophoresis

Standard Tris-glycinate disc PAGE at pH 8.9 was performed by the method of Brewer and Ashworth (1969), with compositions of the gel components as follows:

STACKING GEL: 7% acrylamide, 0.18% bis, 0.373 M Tris, pH 8.9, 0.03% TEMED, 0.07% ammonium persulfate.

RESOLVING GEL: 5% acrylamide, 1.25% bis, 0.0625 M Tris, pH 6.8, 1% riboflavin, 20% sucrose.

SAMPLE SOLUTION: 10% sucrose, 0.0625 M Tris, pH 6.8, 0.0006% bromophenol blue.

Gels were either in tubes, 1.5 mm internal diameter, or in slabs, 0.75 mm or 1.5 mm thickness. Proteins in tube gels were electrophoresed at 2 mA per tube for 0.5 hr and then at 4 mA per tube until the dye front was 0.5 cm from the bottom of the tube. Proteins in slabs were electrophoresed, using a Protean Slab Electrophoresis Cell by BioRad, at 5 mA for 1 hr and then at 5-20 mA until the dye front was 1 cm from the bottom of the slab. Gels were run in 0.3% Tris, 1.44% glycine, pH 8.3. The power supply used for all electrophoresis was a Buchler Model 3-1500 set for constant current. Gels were then stained by one of three methods.

METHOD 1: Stained with 0.5% aniline blue black in 7% (v/v) acetic acid for 30 min; destained in 7% acetic acid.

METHOD 2: Stained with 0.25% (w/v) Coomassie brillant blue R-250 in 10% (v/v) acetic acid, 50% (v/v) methanol; destained in 10% acetic acid, 50% methanol.

METHOD 3: Stained using BioRad Silver Stain as described in BioRad bulletin #1089.

Gels were scanned on a Helena Laboratories Auto Scanner.

SDS Polyacrylamide Gel Electrophoresis

SDS slab gel electrophoresis was performed by the method of Weber and Osborn (1975). In some cases the resolving gel was poured as a gradient gel, eg. 7% acrylamide at the top which increased linearily in concentration to 15% acrylamide at the bottom. Gels were run in 0.6% Tris, 2.88% glycine, 0.1% SDS, pH 8.3. The composition of

gel components was as follows:

STACKING GEL: 5.5% acrylamide, 0.15% bis, 0.125 M Tris-HCl, pH 6.8, 0.1% ammonium persulfate, 0.1% SDS, 0.1% TEMED.

RESOLVING GEL: 7-15% acrylamide, 0.19-0.4% bis, 0.375 M Tris, pH 8.8, 0.1% ammonium persulfate, 0.1% SDS, 0.1% TEMED.

SAMPLE SOLUTION: 12.5% glycerol, 0.125 M Tris, pH 6.8, 1% SDS, 0.0125% bromophenol blue, 10% β-mercaptoethanol.

Analytical Chromatography Molecular Weight Estimation by Gel Filtration

Gel filtration columns were calibrated by the method of Andrews et al (1964). Proteins used for calibration were lactate dehydrogenase (140 Kdal), bovine serum albumin (66.5 Kdal), ovalbumin (45 Kdal), chymotrypsinogen A (24.5 Kdal) and cytochrome C (12.4 Kdal). The column void volume (V_o) was determined by dextran blue. The molecular weights of the standards were plotted as the $\log_{mw}$ versus the elution volume (V_e) or versus V_e/V_o and the molecular weight of the unknown protein(s) calculated. In order to obtain approximate estimates of the molecular weights of fractions from uncalibrated columns for which the void volume, bed volume (V_t), and elution volume were known, the K_{av} was calculated. The K_{av} is defined to be $(V_e-V_o)/(V_t-V_o)$. Molecular weight estimates were made using published selectivity curves for given Sephadex G-types (Pharmacia handbook: Gel Filtration, Theory and Practice) where a selectivity curve plots the K_{av} versus the $\log_{mw}$.

Amino Acid Analysis

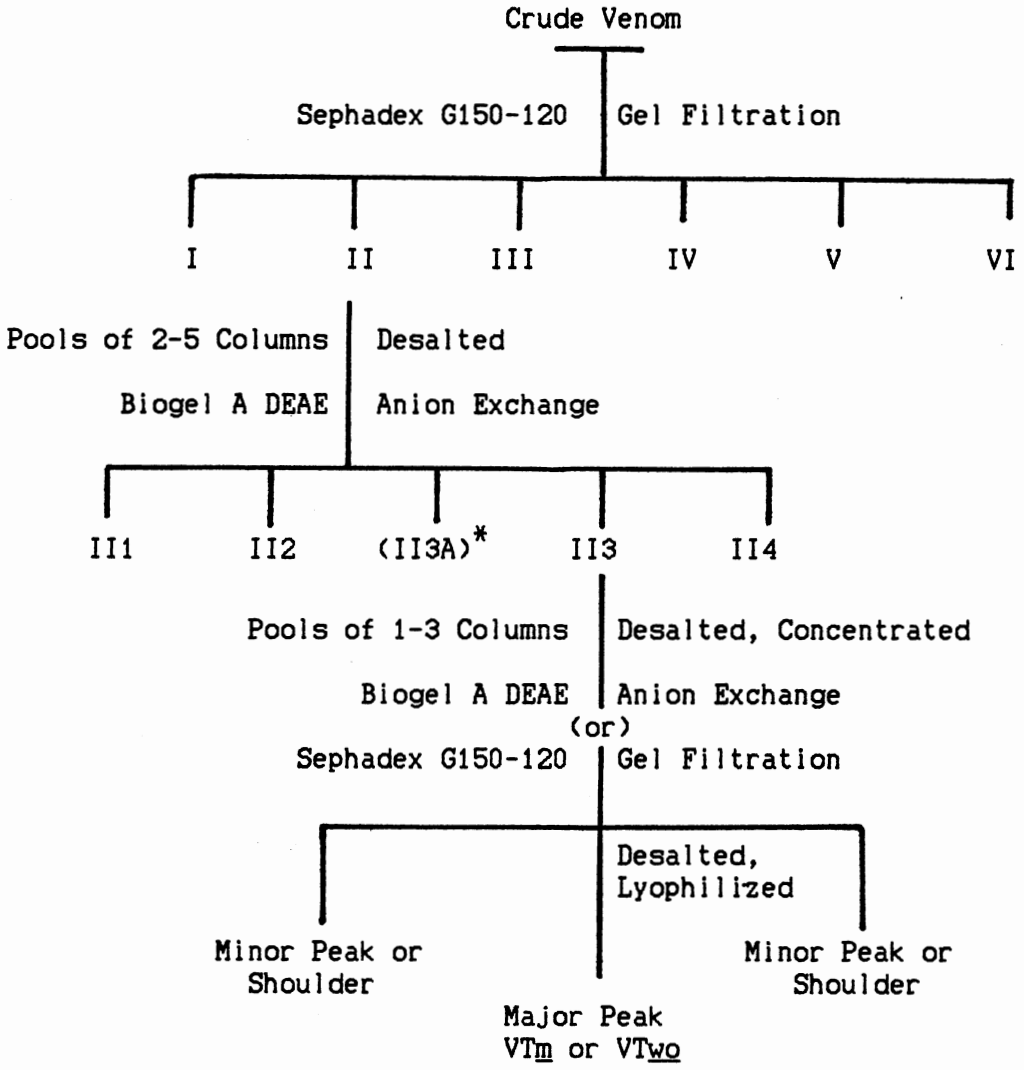
Amino acid analysis was performed as described by Moore and Stein (1957) as modified by Liao et al. (1973). Hydrolysis of viriditoxin-like isolates was performed in 6.0 N HCl under vacuum in sealed glass at 110 °C for 24, 48, and 72 hrs. Analysis was accomplished using a Beckman model 120 C automatic amino acid analyzer interfaced with an Autolab computing integrater.

Results

The Outline of Isolation

The schemes for the isolation of the viriditoxin-like components from both prairie rattlesnake venom sources were similar and were a modification of a previously developed procedure (Fabiano and Tu, 1981). The general scheme was relatively simple and is outlined here for clarity. The similarites and differences will be fully described below. A generalized scheme is shown in Figure 2. Gel filtration and DEAE chromatography were the only two separation techniques needed to obtain the viriditoxin-like isolates. Typically the venom was first fractionated by gel filtration chromatography over Sephadex G150-120. Appropriate pools were then made. The pools enriched for the viriditoxin-like components from two to five gel filtrations of crude venom were usually combined in pairs and then fractionated by Biogel A DEAE chromatography. The ion-exchange chromatography pool enriched for the viriditoxin-like components was then purified to homogeneity by a second sizing step or another Biogel A DEAE step. Isolates were identified by SDS-PAGE and biological activity, and homogeneity was

Figure 2. Generalized Scheme of Isolation for VT$_m$ and VT$_{wo}$.



*Found only in the Isolation of VTwo.

determined by standard Tris-glycinate disc PAGE and by SDS-PAGE.[2]

Isolation of Viriditoxin-like VTm from Crotalus viridis viridis (Miami) Venom

Crotalus viridis viridis (Miami) venom was fractionated by Sephadex G150-120 chromatography. The elution profile of this fractionation is seen in Figure 3. The sizing column was calibrated as described in the Materials and Methods. In a manner like that previously reported (Fabiano and Tu, 1981), the fractions comprising pools Im-VIm were pooled on the basis of elution profile.[3] The fractions which were pooled are indicated in Figure 3. Pools Im-VIm were desalted by dialysis, lyophilized and weighed as seen in Table II. The range of molecular weights of proteins for each pool given in this table by calibration of the G150-120 column agrees well with values based on K_{av} calculations. Calibration of gel filtration columns and calculation of molecular weights by this method and K_{av} were as described in the Materials and Methods. Pool VIm contained small proteins and non-protein components retained after dialysis.

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[2]For the sake of comparison of these isolates, the pools from the first gel filtration column will be designated by a Roman numeral, eg. II. The pools from the next step, DEAE chromatography, will be designated by an arabic number, eg. 2. Furthermore, since two sources of crude venom were used, there will be a further designation for the source of the pool. Pools derived from Miami Serpentarium and western Oklahoma venoms will be designated by an "m" and a "wo", respectively. The order of designations will be first, gel filtration pool number, second, crude venom source, and third, DEAE chromatography pool number, eg. IIm3 or IIwo3. The viriditoxin-like components will be referred to as VTm and VTwo after they have been purified to homogeneity or when referring to bands of these proteins in polyacrylamide gels.

[3]IIm pooling in the final months of this study was optimized by SDS-PAGE analysis across the initial portion of the eluted fractions.

Figure 3. Sephadex G150-120 Elution Profile of Crotalus viridis viridis (Miami) Venom.

500 mg of venom was dissolved in 6 mL of Buffer A and was loaded onto a 2 x 86.5 cm column. Fractions collected were 2.2 mL/tube.

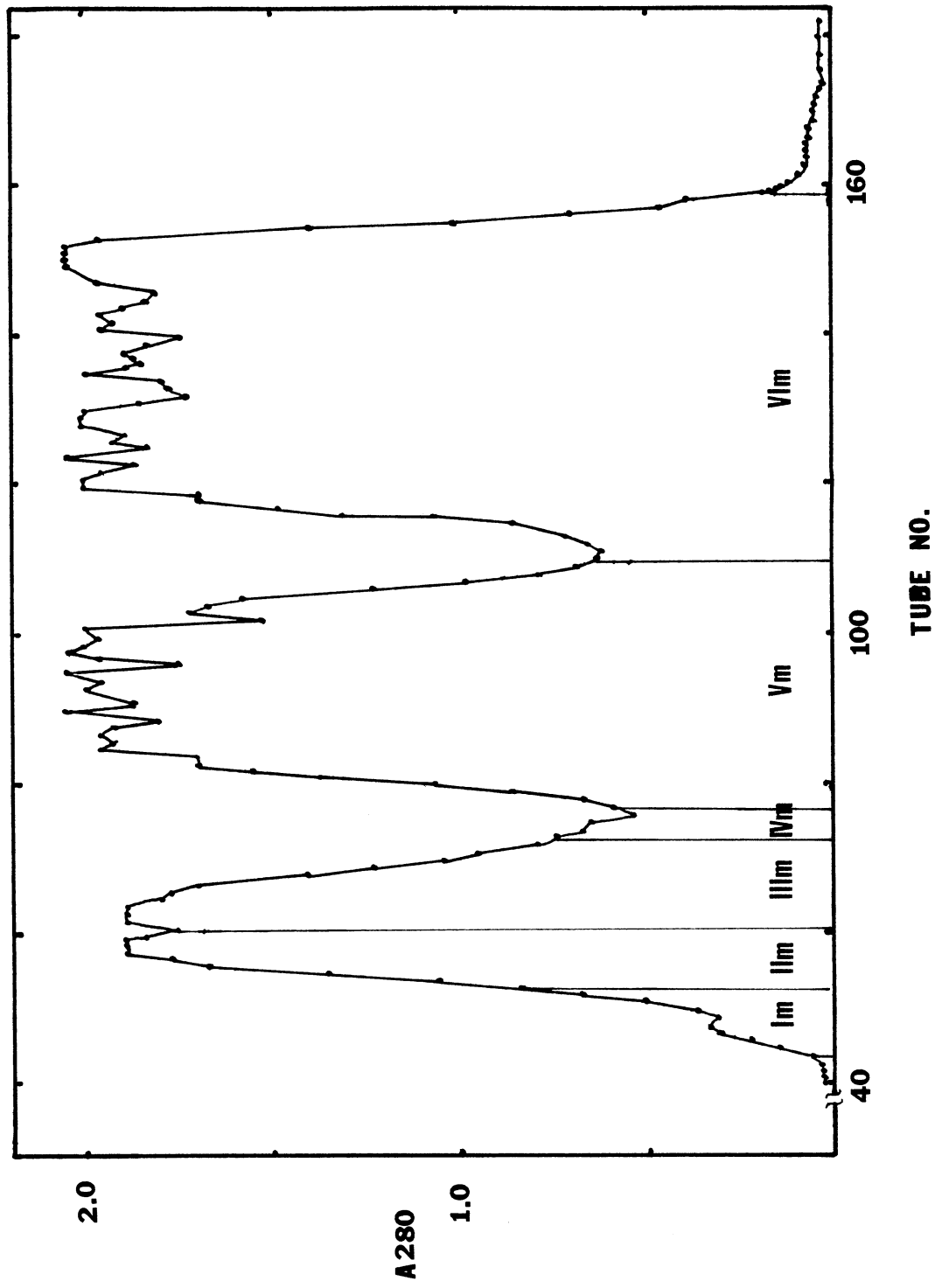
2.0
1.0
A280
40
100
160
TUBE NO.
Im
IIm
IIIm
IVm
Vm
VIm

TABLE II

DISTRIBUTION OF PROTEIN, HEMORRHAGE AND MYOTOXICITY IN POOLS FROM SEPHADEX G150-120 FRACTIONATION OF CROTALUS VIRIDIS VIRIDIS (MIAMI) VENOM AS SEEN IN FIGURE 3

Pool	Calcuated K_{av} mol. wt. range (kdal)	Hemorrhage[1]	Myotoxicity[2]	% of Proteins[3] in Crude Venom
Im	> 250 - 168	+	+	2.1
IIm	168 - 101	++	+++	7.1
IIIm	101 - 53	+++	++	6.9
IVm	53 - 39	-	-	0.8
Vm	39 - 10	-	-	38.0
VIm	< 10	-	++	45.0

[1](-) = no evidence of hemorrhage; (+) = mild hemorrhage; (++) = moderate hemorrhage; (+++) = severe hemorrhage.

[2](-) = no evidence of myotoxicity; (+) = mild myotoxicity; (++) = moderate myotoxicity; (+++) = severe myotoxicity.

[3]Total protein recovered from column was 100%.

Pool IIm comprised 7.0 percent of the total weight of venom components recovered and was further resolved by anion-exchange chromatography as described below.

Histological assay of the gel filtration fractions identified hemorrhagic and myonecrotic activities. Pools were dissolved in PSS at a protein concentration of 0.5 mg/mL. The samples were injected (i.m.) into the dorsolateral aspect of the right hind thigh of 20 to 30 gram mice. The volume injected was 5 μL per gram of mouse. After 24 hr the mice were sacrificed, and the injected skeletal muscle was prepared for light microscopy as described in the Materials and Methods. Control mice were injected (i.m.) with PSS, and further handled, exactly as described above. The stained sections were examined by light microscopy and scored for the severity of hemorrhage and myonecrosis.[4] These results are presented in Table II. Pool Im was mildly hemorrhagic and myotoxic. Pools IIm-IVm caused moderate to severe hemorrhage and moderate to severe myotoxcity. Pool Vm had no discernable hemorrhagic or myotoxic activity, although an egg emulsion plate assay demonstrated weak phospholipase A_2 activity in this fraction (results not shown). Pool VIm caused no hemorrhage but did result in moderate myotoxicity as evidenced by vacuolization of the muscle cells. This myotoxic activity is likely due to myotoxin a, a basic low-molecular weight protein, previously characterized by Cameron and Tu (1977) and Ownby et al. (1976).

Pool IIm was fractionated by Biogel A DEAE chromatography. Two

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[4]This was a single-blind scoring with the assessment performed by an experienced technician, Terry Colberg.

gel filtration IIm pools similar to that seen in Figure 3 were combined and loaded in buffer B onto a Biogel A DEAE column equilibriated with Buffer B. The non-retained proteins were collected and the bound fractions were eluted with an increasing gradient of KCl as seen in Figure 4. The KCl concentration of the fractions within the gradient is shown in this figure. Peaks IIm3 and IIm4 began to elute at 20 mM and 50 mM KCl, respectively. IIm(1-4) were pooled as indicated, desalted and lyophilized. The weights of these pools are shown in Table III.

Histological assay of the ion-exchange pools demonstrated two pools caused severe hemorrhage and myonecrosis. Methods employed were as before. The severity of hemorrhage and myonecrosis caused by pools IIm(1-4) are given in Table III. No myotoxic activity was seen in pools IIm1 and IIm2 and no hemorrhage was seen in pool IIm1, with only mild hemorrhage present in pool IIm2. Pools IIm3 and IIm4 caused severe hemorrhage and severe myonecrosis.

VTm was purified from two IIm3 pools by a second Biogel A DEAE chromatography. This final purification step is seen in Figure 5A. The main peak was resolved from small peaks of protein eluting before and after this peak. The fractions pooled to obtain the VTm isolate are shown by the shaded area in this figure. Total yield after desalting by dialysis and lyophilization was 36 mg, corresponding to a 1.6 percent yield from 2200 mg of crude venom.[5]

Additionally, two IIm4 pools were fractionated over a Sephadex

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[5]Rabbit antiserum was made using this isolation. Ouchterlony analysis of the antiserum is included in Appendix A.

Figure 4. Biogel A DEAE Elution Profile of Pool II<u>m</u>.

95.7 mg of desalted and lyophilized II<u>m</u> pools similar to that in Figure 3 were resuspended in Buffer B and loaded. A linear gradient of increasing KCl in 4 mM monoethanolamine-HCl, pH 9.0, is indicated (solid triangles). The concentration of KCl increases by 1.1 mM for every mL of gradient; thus the slope equals 1.1 mM/mL.

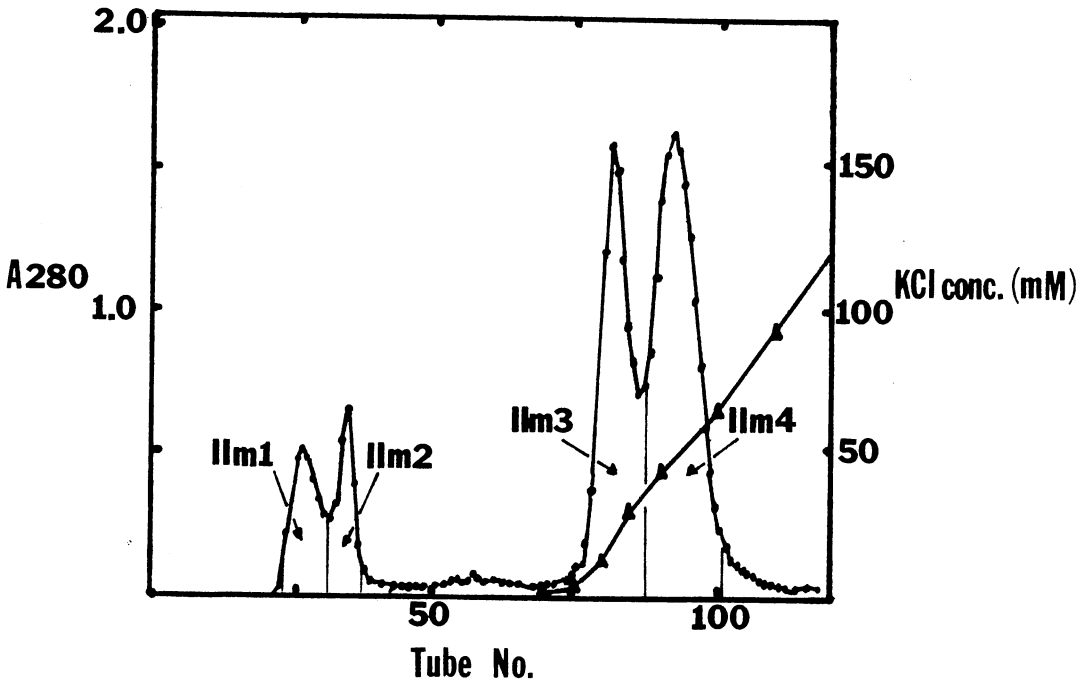

2.0
A280
1.0
150
100
50
KCl conc. (mM)
IIm1
IIm2
IIm3
IIm4
50
100
Tube No.

TABLE III

DISTRIBUTION OF PROTEIN, HEMORRHAGE AND MYOTOXICITY IN POOLS FROM BIOGEL A DEAE FRACTIONATION OF SEPHADEX G150-120 POOL IIm AS SEEN IN FIGURE 4

Pool	Hemorrhage[1]	Myotoxicity[2]	% of Proteins[3] in Crude Venom
IIm1	-	-	0.6
IIm2	+	-	0.5
IIm3	+++	+++	2.6
IIm4	+++	+++	1.7

[1](-) = no evidence of hemorrhage; (+) = mild hemorrhage; (++) = moderate hemorrhage; (+++) = severe hemorrhage.

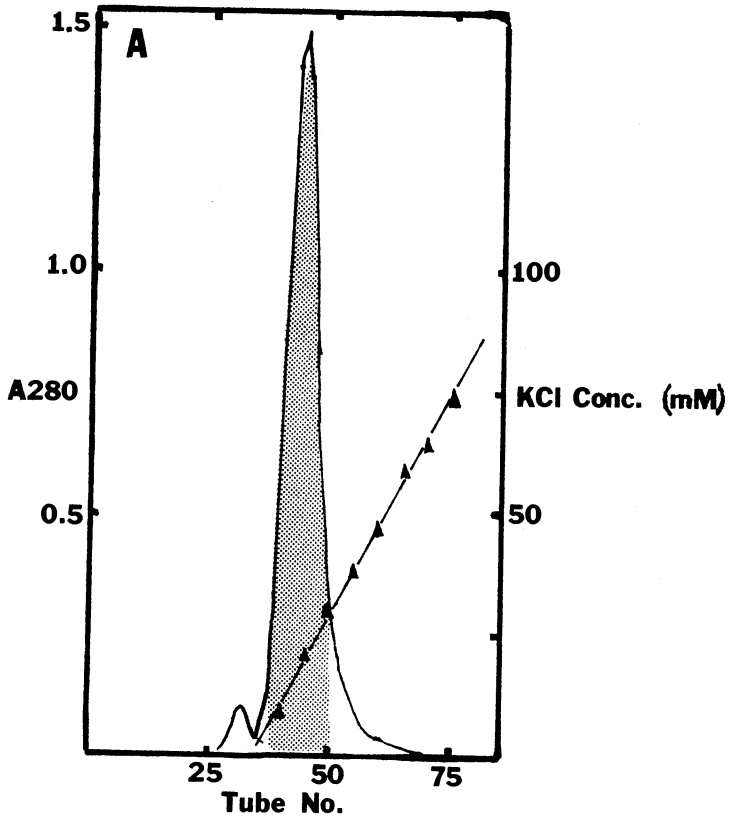
[2](-) = no evidence of myotoxicity; (+) = mild myotoxicity; (++) = moderate myotoxicity; (+++) = severe myotoxicity.

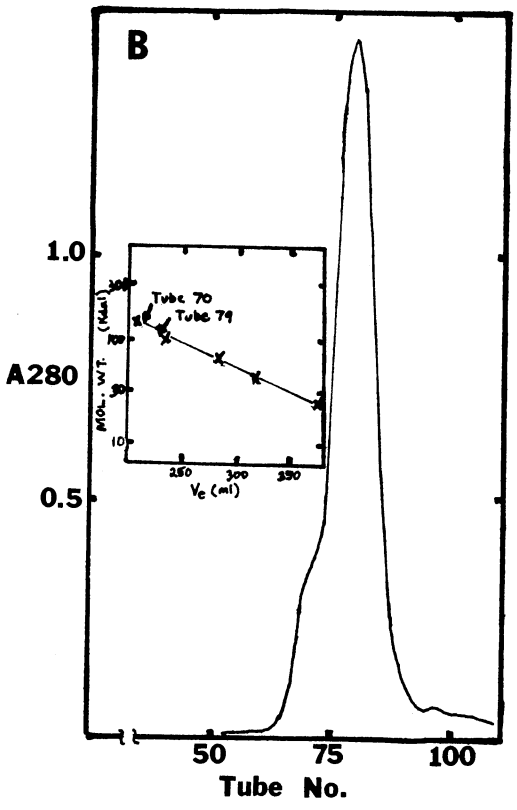
[3]Total protein recovered from column was 68%.

Figure 5. Final Purification of Pool II$_m$3 to VT$_m$ and Estimation of Molecular Weight of Pool II$_m$4.

A. Biogel A DEAE fractionation of two combined II$_m$3 pools. Fractions collected were 2.5 mL/tube. A linear gradient of increasing KCl is indicated (solid triangles). The shaded area corresponds to fractions pooled as VT$_m$.

B. Sephadex G150-120 fractionation of two combined II$_m$4 pools. Fractions collected were 2.9 mL/tube. Inset is a calibration curve of the column determined with molecular weight standards as described in the Materials and Methods.


A
1.5
1.0
0.5
A280
100
50
KCl Conc. (mM)
25
50
75
Tube No.


B
1.0
0.5
A280
Tube 70
Tube 79
Mol. Wt. (Kdal)
250
300
350
Ve (ml)
50
75
100
Tube No.

G150-40 column as seen in Figure 5B. The calibration of this column is shown in the inset of Figure 5B. The estimated molecular weight of the inflection at tube 70, probably due to VTm, was 135 kdal, while the main peak eluted maximally at tube 79, giving an estimated molecular weight of 104 kdal. Tubes 75 to 90 were pooled, desalted and lyophilized.

Crude venom, pools IIm, IIm3, and IIm4 and purified VTm were examined by standard Tris-glycinate disc PAGE. Gel scans of stained tube gels of the crude Crotalus viridis viridis (Miami) venom and pools IIm, IIm3 and IIm4 are compared in Figure 6A. Crude venom contained numerous protein bands, four of which were seen to be major protein bands in sizing pool IIm. Pool IIm3 was nearly homogenous. Pool IIm4 was largely comprised of one major band, although a minor band of greater electrophoretic mobility was visible. The VTm isolate (Figure 5A) appears homogeneous by standard Tris-glycinate disc PAGE as shown in Figure 6B.

VTm and the IIm4 pool after refractionation by gel filtration were analyzed by SDS-PAGE. 7.5% SDS-PAGE of reduced, alkylated VTm and the Sephadex G150-40 gel filtration pool of IIm4 is seen in Figure 7.[4] Sample preparation and electrophoresis were as described in the Materials and Methods. VTm contains two polypeptides with molecular weights of 60 and 56 kdal. These values correspond well with the reported molecular weights of the heterogeneous viriditoxin dimer (Fabiano and Tu, 1981). No gel scan of the subunits was performed, but they bound nearly equal amounts of dye. The gel filtration pool

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[4]SDS-PAGE kindly provided by Tak Chan at Langston College.

Figure 6. Standard Tris-Glycinate Disc PAGE Tube Gels of Crude *Crotalus viridis viridis* (Miami) Venom, Selected Pools, and VTm.

A. Comparative gel scans are shown of crude venom, pool IIm as in Figure 3, and pools IIm3 and IIm4 as in Figure 4, separated on 7.5% acrylamide standard Tris-glycinate disc PAGE tube gels, using pH 8.8 resolving gels. Gels were stained with analine blue black. Gels were scanned from the top of the resolving gel to the tracking dye.

B. The VTm isolate is confirmed to be homogeneous and is compared to crude venom by standard Tris-glycinate disc PAGE tube gel.

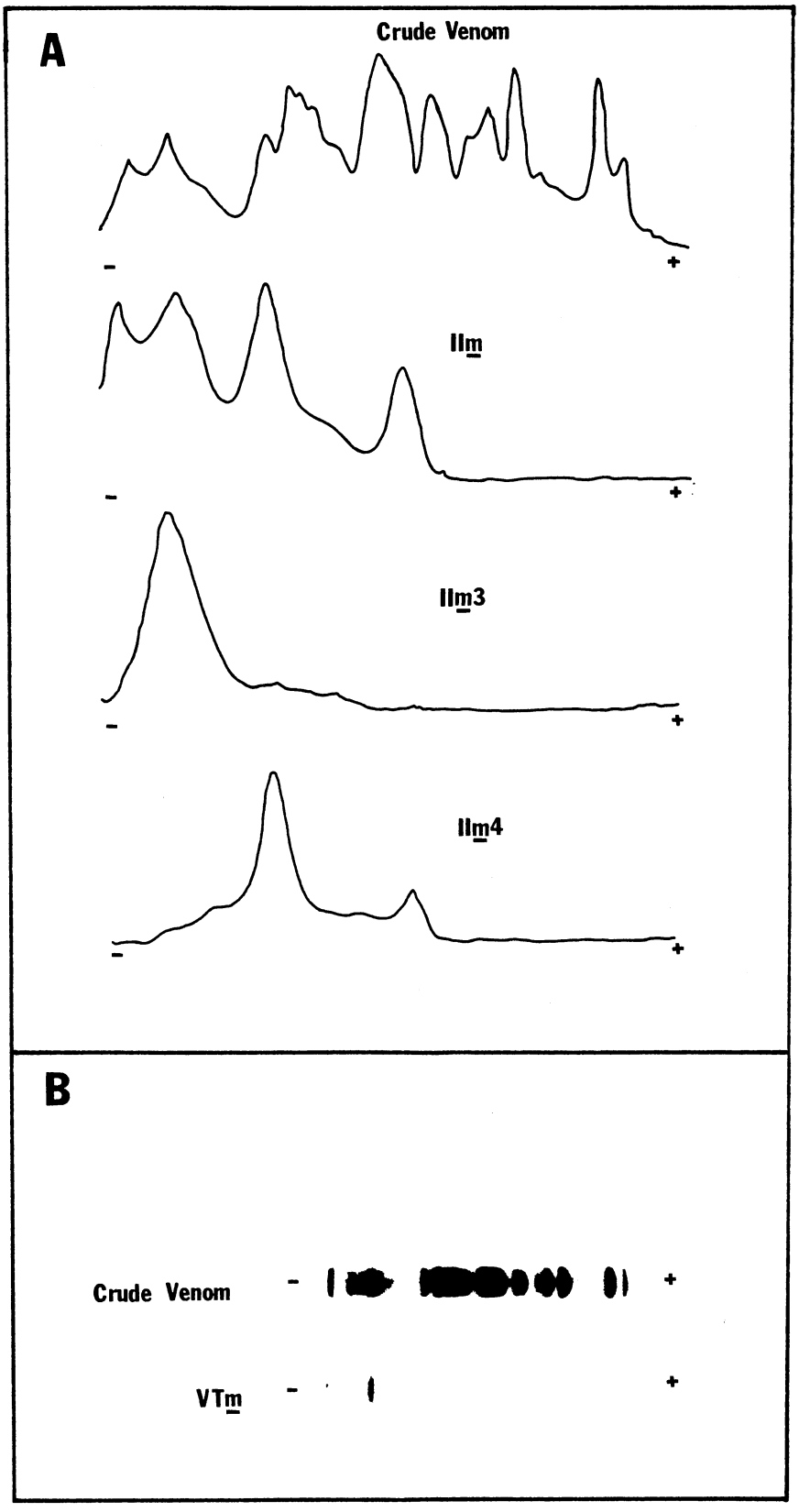
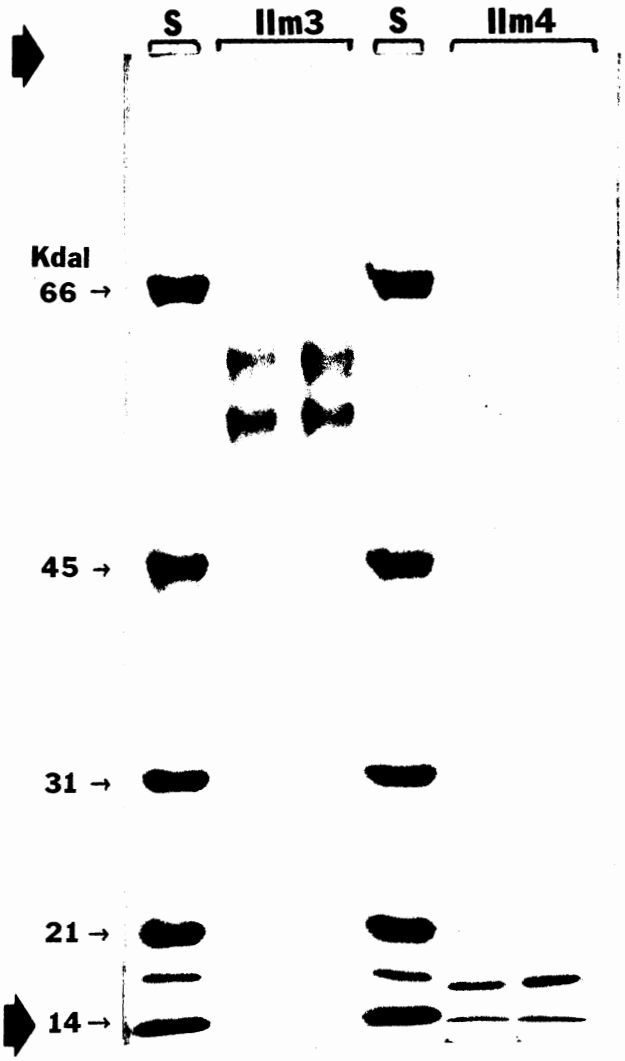

A
Crude Venom
-
+
IIm
-
+
IIm3
-
+
IIm4
-
+
B
Crude Venom
-
+
VTm
-
+

Figure 7. 7.5% SDS-PAGE Analysis of Pools II$_m$3 and II$_m$4.

Samples were reduced and alkylated prior to electrophoresis. The molecular weights of standard proteins (S) are indicated to the left of the gel. The bold arrows at the top and bottom of the gel indicate the positions of the top of the resolving gel and the tracking dye, respectively.


S
IIm3
S
IIm4
Kdal
66 →
45 →
31 →
21 →
14 →

from IIm4 was largely comprised of lower molecular weight proteins, less than 20 kdal, even though it had a gel filtration calibrated molecular weight of 104 kdal.

VTm caused hemorrhage and myonecrosis 24 hr after injection (i.m.) into mouse skeletal muscle. Methods were as before. Photomicrographs of stained sections from mice injected (i.m.) with PSS and VTm are shown in Figure 8. Plate A shows that 24 hr after injection (i.m.) with PSS muscle cells were normal, capillaries were intact, and no extravasated erythrocytes were present in the connective tissue. Plate B shows that 24 hr after injection (i.m.) with VTm, hemorrhage and myonecrosis were evident. Hemorrhage is evidenced by the erythrocytes in the connective tissue, and toxicity by the presence of vacuoles within damaged muscle cells.

Isolation of viriditoxin-like VTwo from Crotalus viridis viridis (western Oklahoma) venom

Approximately one year into the research, a local source of crude Crotalus viridis viridis venom became available, consisting of snakes collected in western Oklahoma. This source of starting material was highly attractive and was utilized since the cost of the venom was less, and the milking of the snakes and the quality of the product could be controlled. Venom obtained by milking these snakes was immediately processed as described in the Materials and Methods.

Crude venom was fractionated by Sephadex G150-120 chromatography. Lyophilized crude Crotalus viridis viridis (western Oklahoma) venom was fractionated as seen in Figure 9. The indicated pool IIwo corresponds to pool IIm in Figure 4. Pooling was based solely on

Figure 8. Light Micrographs of Normal Mouse Skeletal Muscle and Skeletal Muscle 24 Hours after Injection of VTm.

Light micrographs of thick (1 μm) plastic sections stained with Mallory's trichrome.

A. Normal muscle (NM) and intact capillaries (C) 24 hr after injection (i.m.) with PSS. Magnification is x200.

B. Experimental hemorrhage and myonecrosis in muscle 24 hr after injection (i.m.) with VTm. Erythrocytes are visible within the connective tissue and muscle cells, which appear damaged. Vacuolization (V) in three muscle cells is indicated. VTm was injected at a dose of 2.5 μg/gm. Magnification is x200.

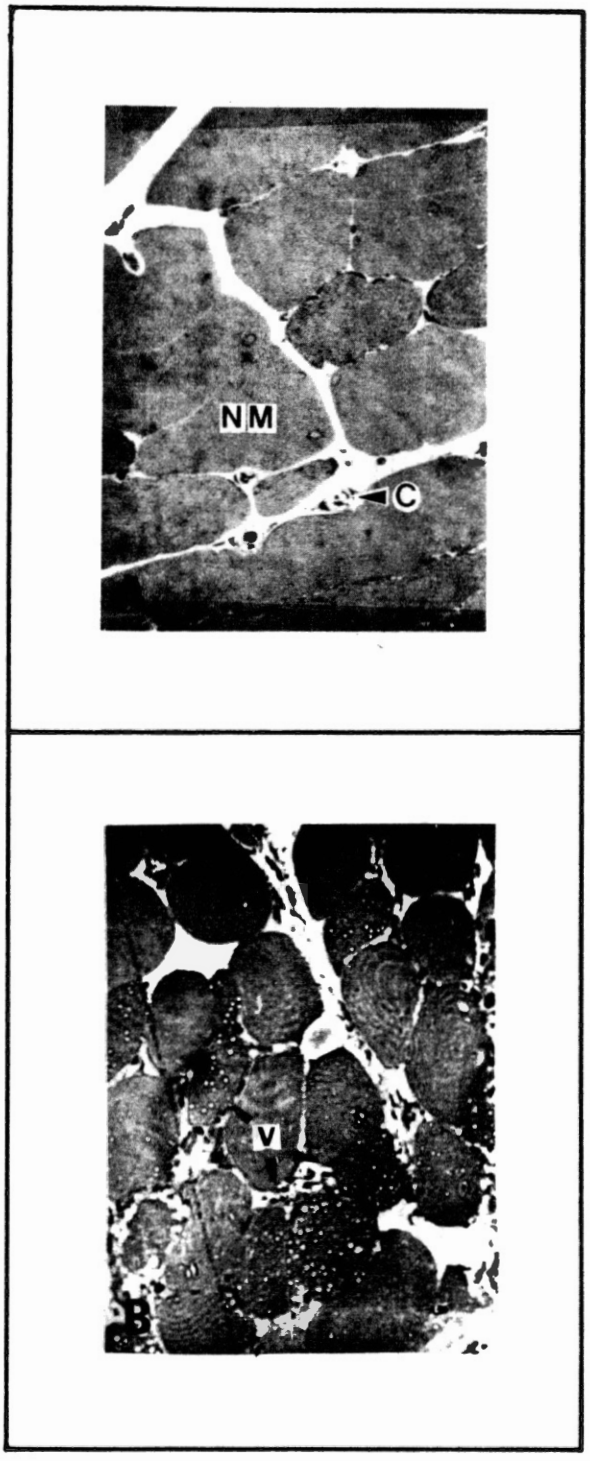
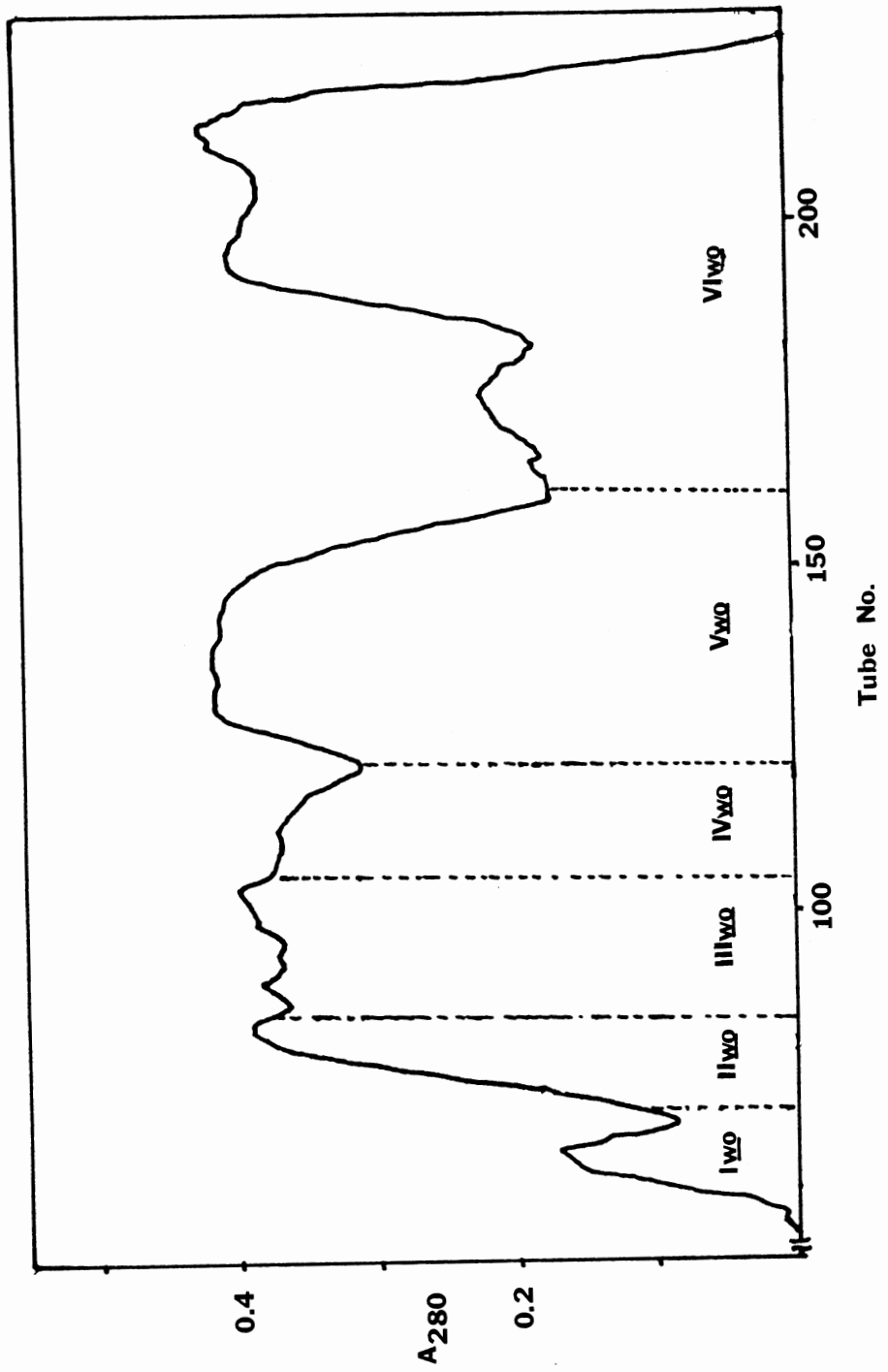

NM
C
V

Figure 9. Typical Fractionation of Crude Crotalus viridis viridis (Western Oklahoma) Venom by Sephadex G150-120 Gel Filtration.

Four hundred mg of venom was dissolved in Buffer A and loaded onto a 2.0 x 145 cm Sephadex G150-120 column. Fractions collected were 2.5 mL/tube.

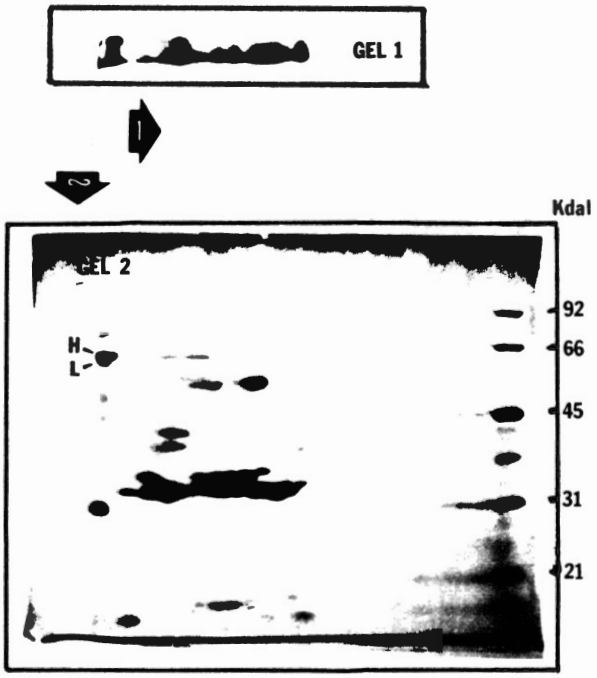

0.4
0.2
A_{280}
100
150
200
Tube No.
Iwo
IIwo
IIIwo
IVwo
Vwo
VIwo

elution profile as before.

Pool II$_{wo}$ and crude venom (western Oklahoma) were analyzed by two dimensional polyacrylamide gel electrophoresis. 6.5% slab Tris-glycinate disc PAGE was used as the first dimension of separation of crude venom and pool II$_{wo}$. The separation of crude venom is shown in Figure 10, GEL 1. The separation of pool II$_{wo}$ is not shown. Protein was visualized with silver stain. Identical unstained gel lanes of crude venom and II$_{wo}$ were incubated with SDS sample buffer and electrophoresed into separate 9% SDS-PAGE gels simultaneously using a double Protean slab gel system as described in the Materials and Methods. Figure 10, GEL 2, shows the visualization of the two dimensionally separated crude venom proteins by silver staining; the two dimensional separation of pool II$_{wo}$ is not available and therefore is not shown. Only two protein groupings had molecular weights similar to that observed for the VT$_m$ heterogenous dimer. The left grouping of proteins consisted of two polypeptides designated H and L. Polypeptide H was of greater molecular weight and more heavily stained than polypeptide L, which was located directly below it. The left grouping was also seen in the two dimensionally separated II$_{wo}$ pool and had an electrophoretic mobility in the first dimension characterisitic of VT$_{wo}$ (see below). The right grouping in GEL 2 had two discernable polypeptides of the wrong electrophoretic mobility in the first dimension. This right grouping was not seen in the two dimensionally separated II$_{wo}$ pool. Thus, the H and L polypeptide grouping is the only viriditoxin-like component present in *Crotalus viridis viridis* (western Oklahoma) venom. Scanning of GEL 2 as described in the Materials and Methods revealed that the H and L

Figure 10. Two Dimensional Separation of Crude Crotalus viridis viridis Venom Proteins by Electrophoresis.

The venom proteins were first separated in a 6.5% standard Tris-glycinate disc PAGE slab gel in the direction indicated by arrow 1. After treatment, a lane of this slab gel was then laid edgewise on top of a 9% SDS-PAGE slab gel. The proteins in the disc PAGE slab gel lane, already separated into many bands according to mass-to-charge ratio, were then electrophoresed in the direction of arrow 2 and separated by molecular weight. The separation of the crude venom proteins in the first dimension is demonstrated by GEL 1, a lane of the 6.5% disc PAGE slab gel identical to the one used in the preparation of GEL 2 in all respects (except that the bands have been visualized by silver staining). The second dimension of separation, which took place in GEL 2 at a right angle to the first gel, separates each disc PAGE band into its components by their molecular weights. Molecular weights of protein standards separated at the right edge of GEL 2 calibrate GEL 2, allowing the estimation of the molecular weights of the venom proteins to the left. The visible upper edge of GEL 2 is the top of the resolving gel. The tracking dye front is visible at the bottom of GEL 2. Proteins in GEL 2 were visualized by silver staining.


GEL 1
1
2
Kdal
GEL 2
H
L
92
66
45
31
21

polypeptide grouping, which is most probably VTwo as described below, comprised less than two percent of the stained protein.

Pool IIwo was fractionated by Biogel A DEAE chromatography using conditions equivalent to those discussed earlier for the IIm pool. Three peaks of protein were resolved in the KCl gradient as seen in Figure 11. The nomenclature of the peaks in Figures 4 and 11 corresponds as follows. Peaks IIm(1,2) are similar to peaks IIwo(1,2). This was the material which did not bind to the anion exchanger in the presence of 4 mM KCl and sometimes eluted as only one peak. Peak IIwo3A, which began to elute at approximately 10 mM KCl, had no counterpart in the IIm salt gradient in Figure 4, but did in Figure 5A where the steepness of the salt gradient was reduced. Peak IIm3 corresponds to IIwo3 and IIm4 corresponds to IIwo4, as these two pairs of equivalent peaks began to elute at approximately 20 and 50 mM KCl, respectively. Further discussion of the relationship of the peaks in Figure 11 to those seen in Figure 4 will be dealt with in a later section. The fractions were pooled as shown in Figure 11, desalted by dialysis, lyophilized and weighed. Yields of pools IIwo3A, IIwo3, and IIwo4 were 10 mg, 15 mg, and 15.5 mg, respectively. Measurement of the proteolytic activity of 1 μg samples of these pools revealed a descending order of proteolytic activity against hide powder azure: IIwo3A > IIwo4 > IIwo3. Protease assay was as described in the Materials and Methods.

Pools IIwo3A, IIwo3 and IIwo4 were analyzed by SDS-PAGE. 11% SDS-PAGE of boiled and reduced samples from these pools is seen in Figure 12. The gel was first stained with Coomassie blue and then overstained with silver in order to visualize any contaminants that

Figure 11. Biogel A DEAE Elution Profile of Pool II<u>wo</u> and Repurification of Pool II<u>wo</u>3.

63.0 mg of protein from two II<u>wo</u> pools was dissolved in Buffer B, loaded onto a Biogel A DEAE column, washed and eluted. A linear gradient of increasing KCl in Buffer B is indicated (open circles). The KCl concentration increased by 0.4 mM for every mL of gradient; thus the slope is 0.4 mM/mL. The alternate shading indicates pools II<u>wo</u>3A, II<u>wo</u>3 and II<u>wo</u>4.

Repurification of pool II<u>wo</u>3 by a second Biogel A DEAE fractionation is shown in the inset. Fifteen mg of protein from the II<u>wo</u>3 pool was dissolved in Buffer B, loaded onto a Biogel A DEAE column, washed, and eluted. A linear gradient of KCl in Buffer B is indicated (open circles). The fractions indicated by shading were pooled as VT<u>wo</u>.

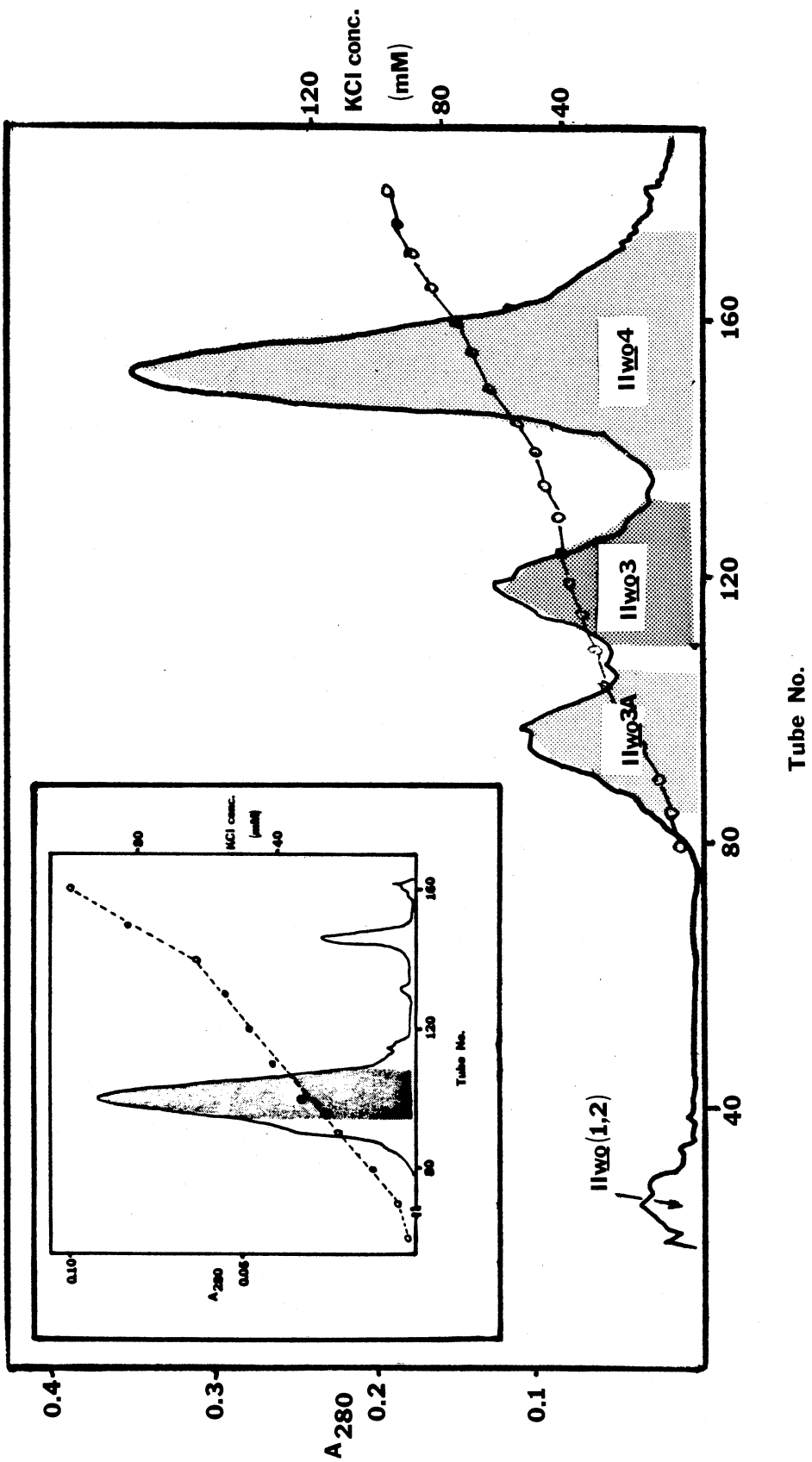
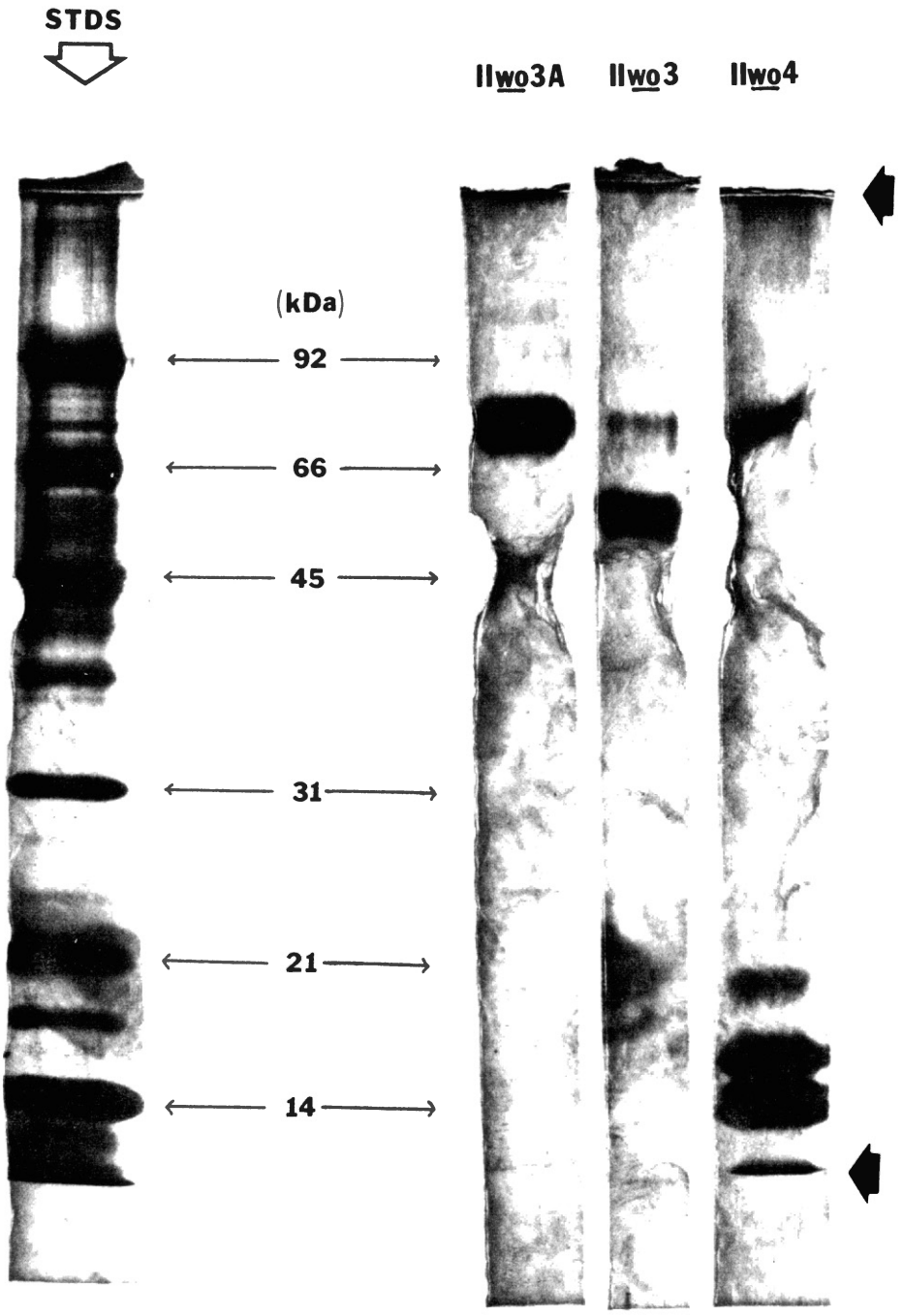

A280
0.4
0.3
0.2
0.1
KCl conc. (mM)
120
80
40
40
80
120
160
Tube No.
IIwo(1,2)
IIwo3A
IIwo3
IIwo4

Figure 12. SDS-PAGE Analysis of Pools from Figure 11.

Lanes containing pools II<u>wo</u>3A, II<u>wo</u>3 and II<u>wo</u>4 are indicated. A lane to the left indicates molecular weights of standard proteins separated in the same gel. Solid bold arrows at the right top and right bottom indicate the positions of the top of the resolving gel and the tracking dye, respectively.


STDS
IIwo3A
IIwo3
IIwo4
(kDa)
92
66
45
31
21
14

might be present in the pools. The silver stained gel is presented in Figure 12. Pool II$_{wo}$3A consisted of a 77 kdal polypeptide. In another SDS-PAGE gel a molecular weight of 73 kdal was obtained for this protein (results not shown). This hemorrhagic protein has proteolytic activity and will be referred to as the 77 kdal protease. Peak II$_{wo}$3 was enriched for a protein similar to VT$_m$, designated VT$_{wo}$, which was apparent as a dimer with Coomassie blue staining but can only be seen as one band after the silver staining. The silver staining revealed a minor contaminant at 77 kdal in this pool. A pattern of low molecular weight polypeptides similar to that seen in Figure 7 was apparent in the II$_{wo}$4 pool, and the estimated molecular weights were 18.5 kdal, 15.5 kdal, 14.5 kdal, and 13.5 kdal. Other minor bands of higher molecular weight were also present in this pool.

Pools II$_{wo}$3A, II$_{wo}$3, and II$_{wo}$4 caused hemorrhage and myonecrosis 24 hr after injection (i.m.) into mouse skeletal muscle using procedures described in the Materials and Methods. Plates A, B, and C in Figure 13 correspond to the tissue taken from mice 24 hr after injection (i.m.) with pools II$_{wo}$3A, II$_{wo}$3 and II$_{wo}$4, respectively. The necrotic muscle cells and extravasated erythrocytes present in all the plates demonstrate that each of the pools tested caused hemorrhage and myonecrosis in mouse skeletal muscle. Muscle cells and endothelial cells in mice injected (i.m.) with PSS were normal after 24 hr.

Pool II$_{wo}$3 was subsequently purified by rechromatography on Biogel A DEAE as before. The elution profile in this purification was similar to that seen in Figure 5A, and is shown in the inset in Figure 11. The final yield of VT$_{wo}$ was 7 mg, corresponding to a 0.9 percent

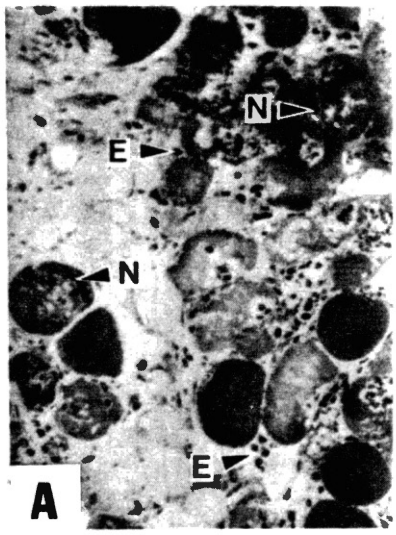
Figure 13. Light Micrographs of Mouse Skeletal Muscle Injected with Biogel A DEAE Pools Seen in Figure 11.

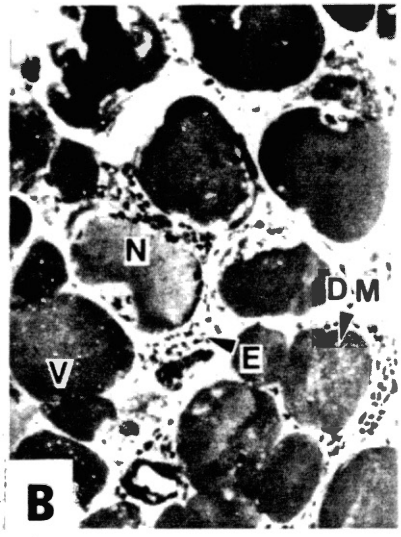
Pools dissolved in PSS were injected (i.m.) at a dose of 2.5 μg/gm as described in the Materials and Methods. Light micrographs of thick (1 μm) plastic sections stained with Mallory's trichrome are shown.

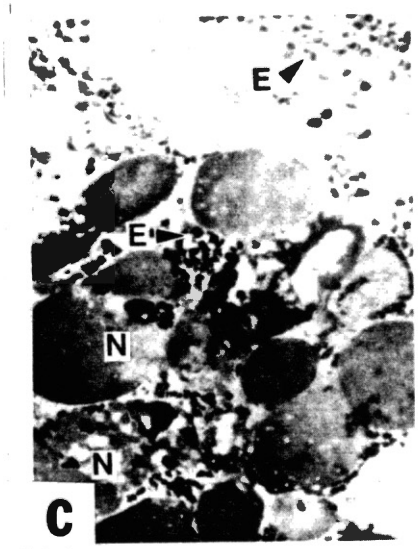
A. Tissue was taken 24 hr after injection with pool II*wo*3A. Note necrotic muscle cells (N) and erythrocytes (E) in connective tissue. Magnification is x200.

B. Tissue was taken 24 hr after injection with pool II*wo*3. Cells in different stages of necrosis are evident: vacuolated cells (V), necrotic cells (N), and cells with dispersed myofibrils (DM). Note erythrocytes (E) in the connective tissue. Magnification is x250.

C. Tissue was taken 24 hr after injection of pool II*wo*4. Note necrotic cells (N) and erythrocytes (E) in connective tissue. Magnification is x300.


N
E
N
E
A


N
D M
E
V
B


E
E
N
N
C

yield of the total protein from 0.8 gm of crude venom. The homogeneity of the VTwo isolate was confirmed by standard Tris-glycinate disc PAGE as will be seen in a later figure.[7]

The minimum hemorrhagic dose of VTwo was determined. VTwo, as seen in Figure 11, was injected (i.d.) in PSS at different doses into the backs of mice as described in the Materials and Methods.[8] A MHD value of 0.015 µg/gm was obtained for VTwo.[9]

Amino acid analysis of VTwo was performed. The amino acid composition of VTwo is given in Table IV and has been previously reported elsewhere (Gleason et al., 1983). The analysis was as described in the Materials and Methods. Using a molecular weight estimate for VTwo determined by SDS-PAGE as 132 kdal, the residue values were determined as described in the Materials and Methods. The average residue number for a protein of this size would be 1148 residues, using an average amino acid molecular weight of 115 gm/mol and assuming that each amino acid constitutes 1/20 of the protein's total composition. The analysis revealed that VTwo contained a relatively large amount of acidic residues which correlated rather well with its elution characteristics during anion exchange chromatography. VTwo also was found to contain a relatively small amount of the aromatic residues tyrosine and phenylalanine. The content of tryptophan was not determined.

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[7]This preparation was used in the pathogenetic studies described in Chapter IV.

[8]This data along with other MHD data is presented in Appendix B.

[9]This value was erroneously reported as 0.15 µg/gm in a previous report (Gleason et al., 1983).

TABLE IV

AMINO ACID ANALYSIS OF VT$_{wo}$

Amino Acid	Hydrolysis[1]			Corrected Value[2]	Mole of AA/Mole of Protein[3]
	24 hr	48 hr	72 hr		
Asx	14.81	14.53	14.76	14.70	168.76
Thr	5.70	5.57	5.54	5.78	66.35
Ser	6.35	5.99	5.36	6.91	79.33
Glx	8.97	8.89	8.92	8.93	102.52
Pro	4.64	4.64	4.70	4.66	53.51
Gly	7.62	7.62	7.63	7.62	87.48
Ala	6.69	6.71	6.73	6.71	77.03
Val	5.92	6.21	6.48	6.20	71.18
Met	1.70	1.70	1.82	1.74	19.98
Ile	5.12	5.34	5.68	5.38	61.76
Leu	7.24	7.27	7.21	7.24	83.12
Tyr	2.79	2.91	2.62	2.77	31.80
Phe	3.45	3.51	3.46	3.47	39.84
Lys	2.35	2.34	2.43	2.37	27.21
His	3.40	3.35	3.40	3.38	38.80
Arg	5.84	5.85	5.90	5.86	67.27
$\frac{1}{2}$-Cys	7.41	7.58	7.37	7.45	85.53

[1]Values were normalized to mole percent.

[2]Mean values, except for Thr and Ser, which were extrapolated to zero time.

[3]Values were calculated based on the K_{av} calculated molecular weight of 132 kdal.

Elution profile characterization by SDS-PAGE.

SDS-PAGE analysis of aliquots from fractions in the high molecular weight fractions from gel filtration of crude venom is seen in Figure 14. This method permitted the localization of the fractions most enriched for VTm. The protein was seen to elute with a constant ratio between the staining intensities of the two subunits. Since VTm did not emerge as a well resolved peak in the fractionation by gel filtration, it had not been previously possible to precisely locate the fractions containing VTm. SDS-PAGE analysis made it possible to pool precisely the VTm-enriched fractions, thereby minimizing the co-pooling of other proteins. The pooled fractions from this column are shown in Figure 14. VTm eluted maximally at fraction 77, and the estimated molecular weight based on K_{av} is 147 kdal. The IIm4 major protein eluted maximally at fraction 84, and the estimated molecular weight based on K_{av} is 103 kdal.

Purification of Proteolytic and Hemorrhagic Activities During the Isolation of VTm

Hemorrhagic and proteolytic activities in the initial fractionation step were assayed. The purification of these activities during the isolation of VTm was investigated since viriditoxin (Fabiano and Tu, 1981) and VTm are hemorrhagic and proteolytic toxins. Viriditoxin was reported to have a weak proteolytic activity against N,N′-dimethylcasein but not against hide powder azure. Figures 15 and 16 show a typical gel filtration of crude venom (Miami). The

Figure 14. SDS-PAGE Analysis of the Distribution of Venom Proteins in Fractions Across a Sephadex G150-120 Gel Filtration Column.

A typical fracionation by gel filtration of crude Crotalus viridis viridis (Miami) venom on a 2.5 x 100 cm Sephadex G150-120 column is shown. Above this is a gel in which aliquots withdrawn from the tube no. indicated were tested by SDS-PAGE. The arrows drawn from the gel indicate the gel lanes in which protein from the tubes below was tested. VTm is the major protein present in the lanes between the arrows. Pooling was as indicated. Molecular weight standards (S) were run on the same gel. The gel was stained with silver.

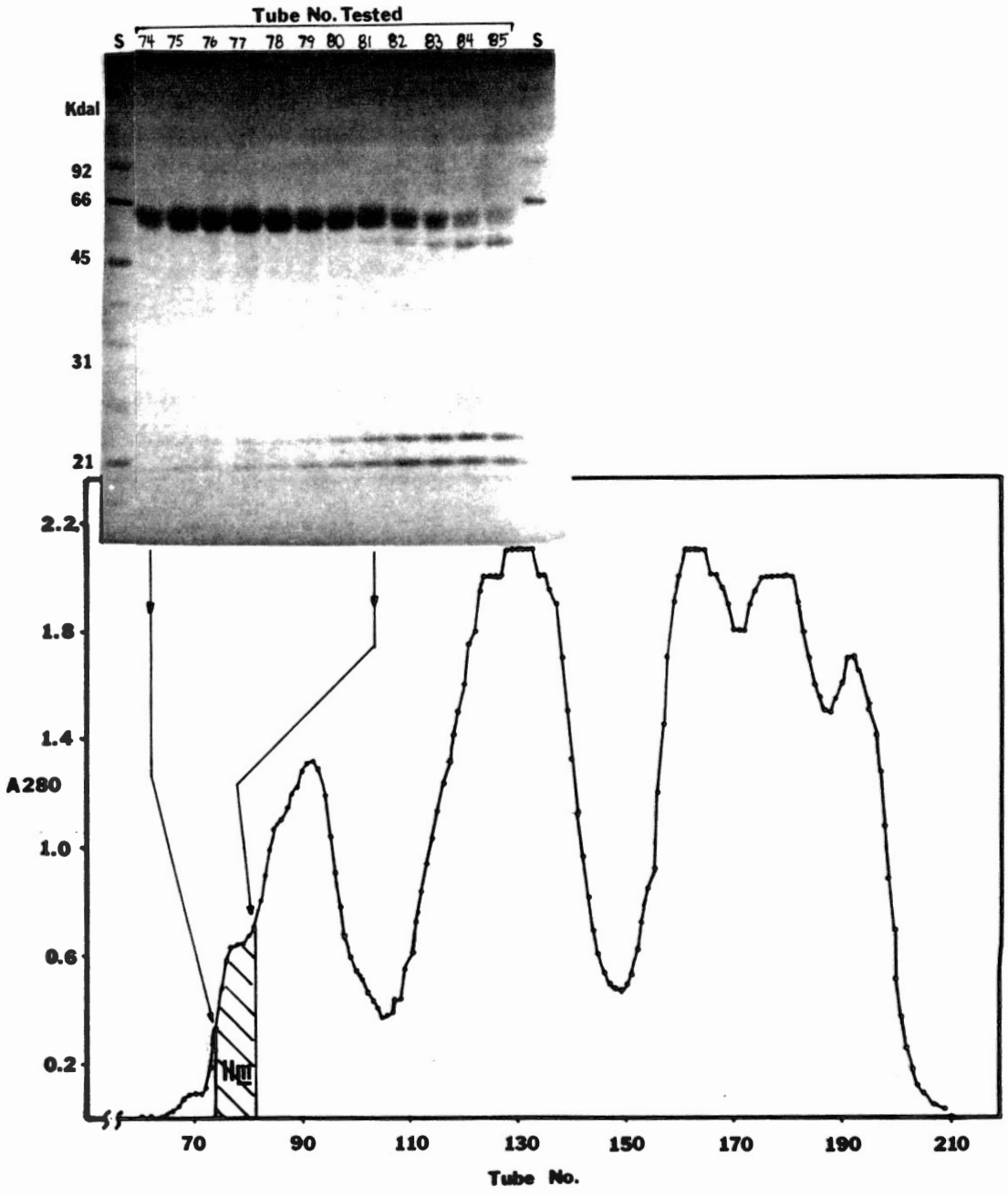
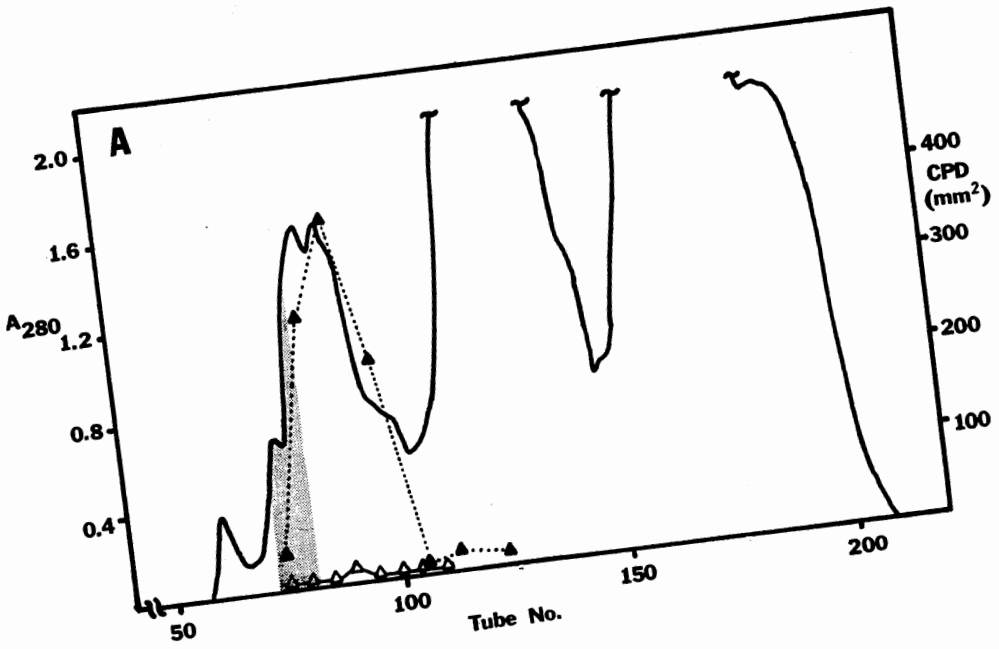

Tube No. Tested
S 74 75 76 77 78 79 80 81 82 83 84 85 S
Kdal
92
66
45
31
21
2.2
1.8
1.4
A280
1.0
0.6
0.2
Hgt
70
90
110
130
150
170
190
210
Tube No.

Figure 15. Assay for Hemorrhagic and Caseinolytic Activity with and without Inhibition by Metal Chelation Across a Typical Sephadex G150-120 Gel Filtration of Crude Venom (Miami).

Five hundred mg of crude Crotalus viridis viridis (Miami) venom was loaded onto a 2.5 x 90 cm Sephadex G150-120 column. 2.5 mL fractions were collected.

A. Hemorrhagic Activity. Non-EDTA treated samples (▲) were diluted 1:5 with PSS prior to injection into mice. EDTA-treated samples (△) were made 12.5 mM with 0.05 M EDTA and treated for 24 hr at 4 °C. EDTA-treated samples were then diluted in PSS to give a final dilution of 1:5 prior to injection into mice. Assays were done in duplicate. The shaded area represents pool IIm.

B. Proteolytic Activity Against N,N′-Dimethylated Casein. Fractions were treated with EDTA as above. 10 µL of non-EDTA-treated fractions (●) and 13.3 µL of EDTA-treated (○) fractions were added to 1 mL of substrate solution and incubated for 2 hr at 37 °C. Assays were done in duplicate.


A
2.0
1.6
A280 1.2
0.8
0.4
400
CPD
(mm2)
300
200
100
50
100
150
200
Tube No.

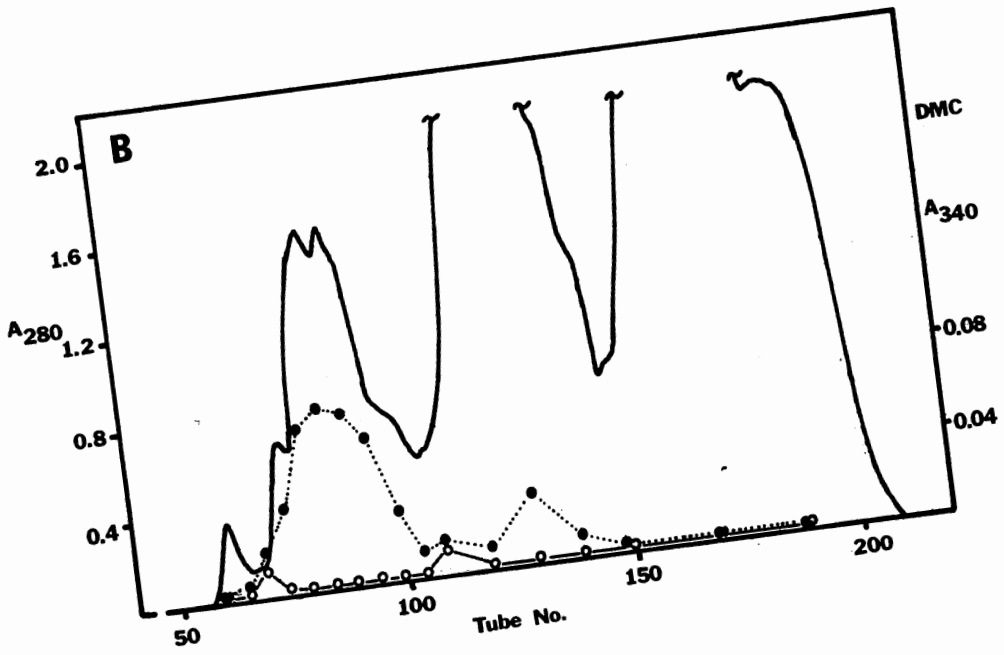
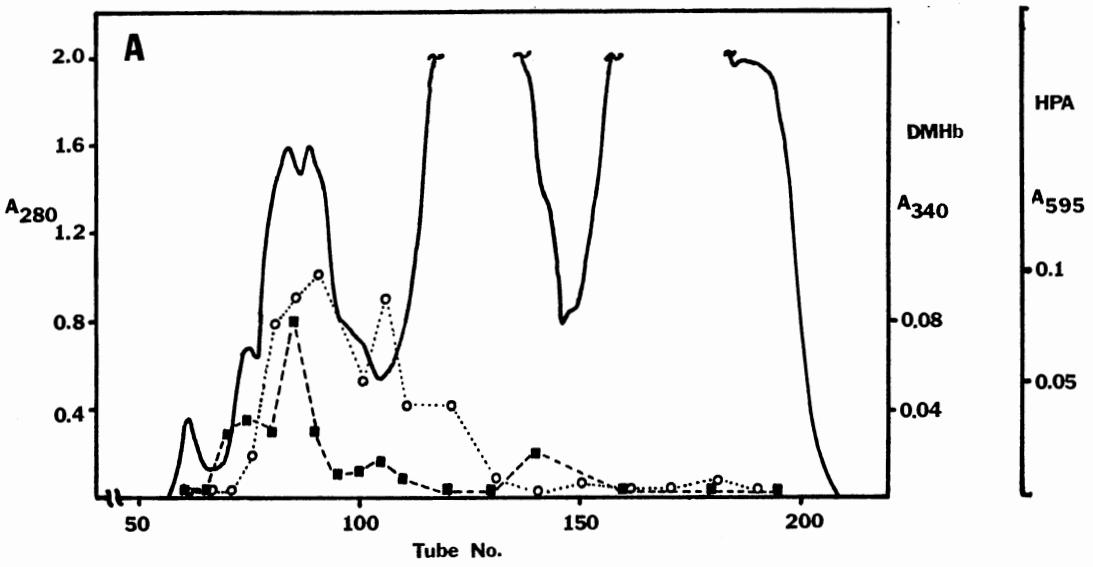

B
2.0
1.6
A280 1.2
0.8
0.4
DMC
A340
0.08
0.04
50
100
150
200
Tube No.

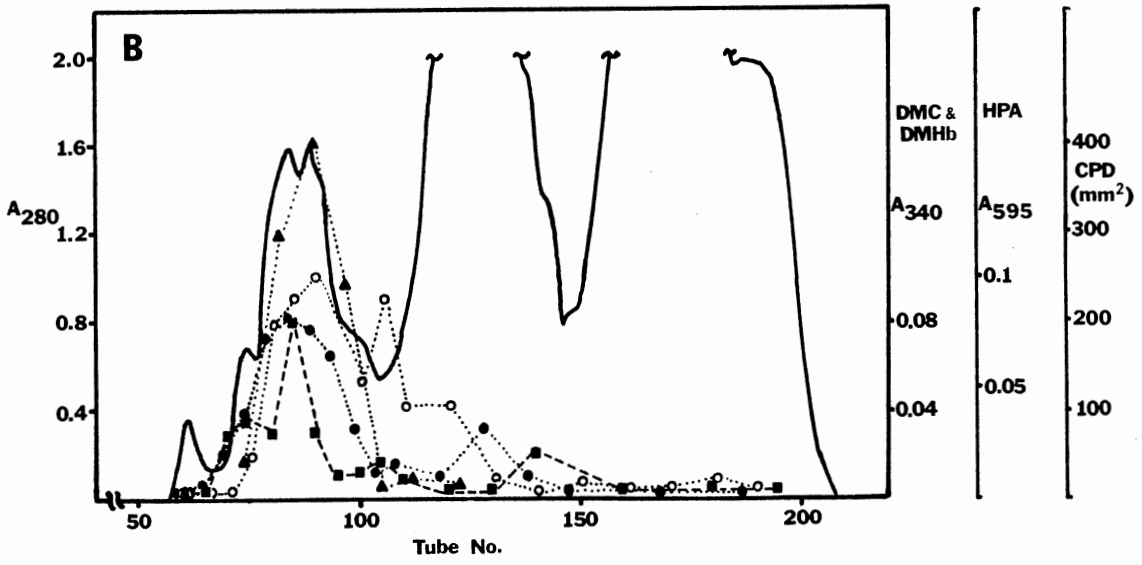
Figure 16. Assay for Proteolytic Activity Against N,N′-dimethylated Hemoglobin and Hide Powder Azure Across the Fractionation seen in Figure 15 and Comparison of All Four Activities.

Crude Crotalus viridis viridis (Miami) venom was fractionated by gel filtration as seen in Figure 15.

A. Proteolytic activity against HPA (O) and DMHb (■). In the HPA assays, 50 µL of fraction tested was added to 20 mg of HPA in 5.0 mL of buffer and incubated for 2 hr at 37 °C. In the DMHb assays, 10 µL of fraction tested was incubated with 1 mL of substrate for 2 hr at 37 °C. All assays were done in duplicate.

B. All proteolytic activities are shown together with hemorrhagic activity for comparison. Hemorrhagic activity (▲) and proteolytic against DMC (●) are represented as in Figure 15.


A
2.0
1.6
1.2
0.8
0.4
A280
DMHb
A340
0.08
0.04
HPA
A595
0.1
0.05
50
100
150
200
Tube No.


B
2.0
1.6
1.2
0.8
0.4
A280
DMC & DMHb
A340
0.08
0.04
HPA
A595
0.1
0.05
400
CPD (mm²)
300
200
100
50
100
150
200
Tube No.

resultant pool IIm, the first step in purification of VTm, is shown by the shaded area in Figure 15A only. The elution profile is shown by the heavy line.

Hemorrhagic activity was determined in duplicate using the modified Kondo's method described in the Materials and Methods in duplicate, and values obtained either agreed or were reassayed. Aliquots across the column were assayed with or without EDTA treatment as described in the Materials and Methods. The results of the hemorrhagic assay expressed as cross product diameter (CPD) are shown in Figures 15A and 16B. Treatment with EDTA clearly eliminated the hemorrhagic activity. It can also be seen that the majority of hemorrhagic activity elutes after the IIm pool.

In Figure 15B proteolytic activity of fractions against N,N'-dimethylated casein (DMC) was examined with or without EDTA treatment of the fractions as above. EDTA treatment of the aliquots eliminated the proteolytic activity against DMC in the same EDTA-labile hemorrhage-causing fractions seen above.

Aliquots from the fractionation were also tested for proteolytic activity against hide powder azure (HPA) and N,N'-dimethylated hemoglobin (DMHb). These proteolytic activities are presented in Figure 16A. Figure 16B summarizes most of the hemorrhagic and proteolytic measurements seen in these two figures. Several interesting relationships between the three proteolytic activity curves and the hemorrhage curve can be discerned in Figure 16B. First, there is a small peak of proteolytic activity against DMHb in the center of the IIm pool region. This peak coincides with a significant shoulder in the proteolytic activity against DMC. No significant

relationship between the IIm pool region and proteolytic activity against HPA could be seen. Second, ten fractions after the peak of proteolytic activity in the IIm pool region, the proteolytic activities against DMC and DMHb both peak together. Third, sixteen fractions later, a peak of proteolytic activity against HPA occurs. Thus, three clusters of proteolytic activity peaks occur within the peak of hemorrhagic activity. Proteolytic activities eluting after the peak of hemorrhage were also observed as shown in Figure 16B.

Hemorrhagic and proteolytic activities in the second purification step were assayed. One half of pool IIm from the column shown above was dialyzed directly against Buffer B without prior lyophilization, loaded onto a DEAE Biogel A column, and then fractionated as previously described. The elution profile of this fractionation, denoted by the heavy solid line, is seen in Figures 17 and 18, and the shaded area represents the IIm3 pool. The hemorrhagic and proteolytic activities of these fractions were determined as before. In Figure 17A, the hemorrhagic activity is shown along with the KCl gradient determined as before and denoted by a dotted line. In Figure 17B the proteolytic activities against DMC and HPA are shown. In Figure 18 all the activities are shown together for comparison.

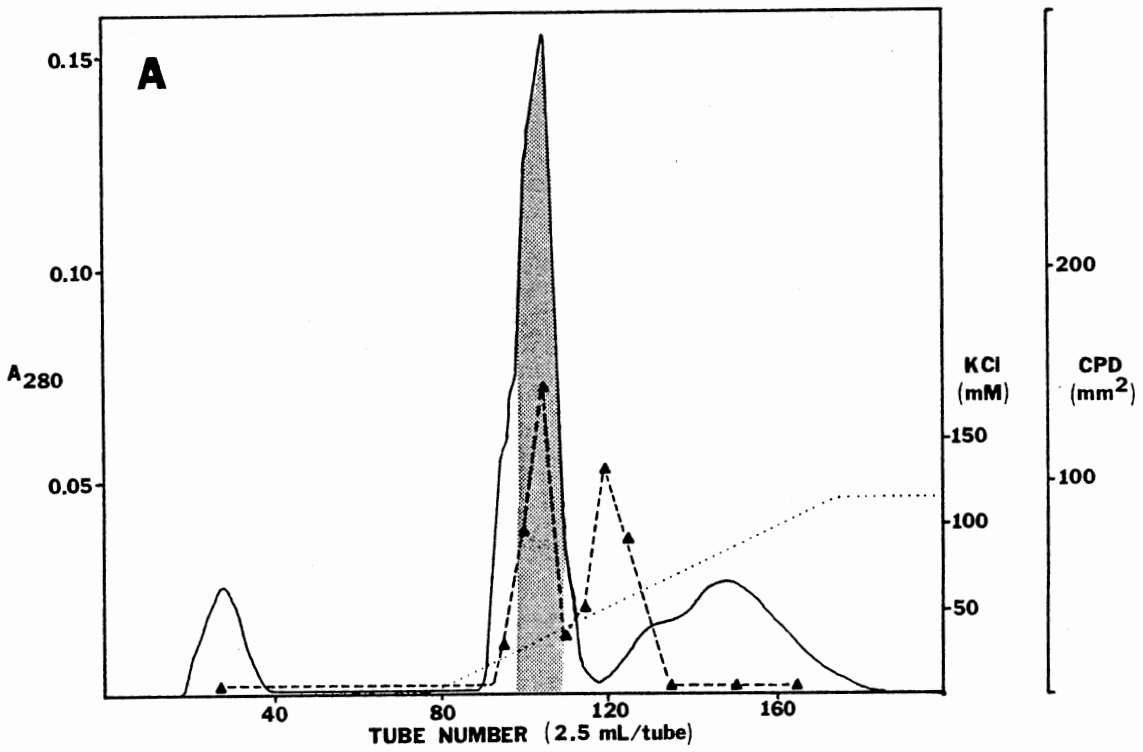
Aliquots from this fractionation were analyzed by 5-20% gradient SDS-PAGE with silver staining as seen in the Figure 18 inset. Analysis of the fractions comprising the IIm3 pool indicated that it was nearly pure VTm. However, there was a shoulder on the leading edge of the peak. SDS-PAGE analysis demonstrated that the shoulder, centered at fraction 97, was due to a small amount of the 77 kdal protease. This shoulder due to the 77 kdal protease eluted just prior

Figure 17. Assay for Hemorrhagic and Proteolytic Activity Across a Biogel A DEAE Fractionation of Pool II<u>m</u> from Figure 15.

One-half of the fractions pooled for VT<u>m</u> isolation as indicated in Figure 15 were prepared, loaded onto a Biogel A DEAE column and eluted as described. The KCl concentration in the gradient increases by 0.5 mM for every mL of gradient (slope = 0.5 mM/mL).

A. The hemorrhagic activity (▲) measured as cross-product diameter is indicated. Fractions were diluted 1:3 with PSS prior to injection into mice. Yield of the pool by lyophilized dry weight was 5.0 mg. The shaded area represents pool II<u>m</u>3.

B. The proteolytic activities of selected fractions against N,N′-dimethylated casein (●) and hide powder azure (○) are shown. In the DMC assays, 10 µL of fraction tested was added to 1 mL of substrate solution and incubated for 10 hr at 37 °C. In the HPA assays, 75 µL of fraction tested was added to 20 mg of HPA in 5.0 mL of buffer and incubated for 20 hr at 37 °C. All assays were done in duplicate.


A
0.15
0.10
0.05
A_{280}
KCl
(mM)
150
100
50
CPD
(mm^2)
200
100
40
80
120
160
TUBE NUMBER (2.5 mL/tube)

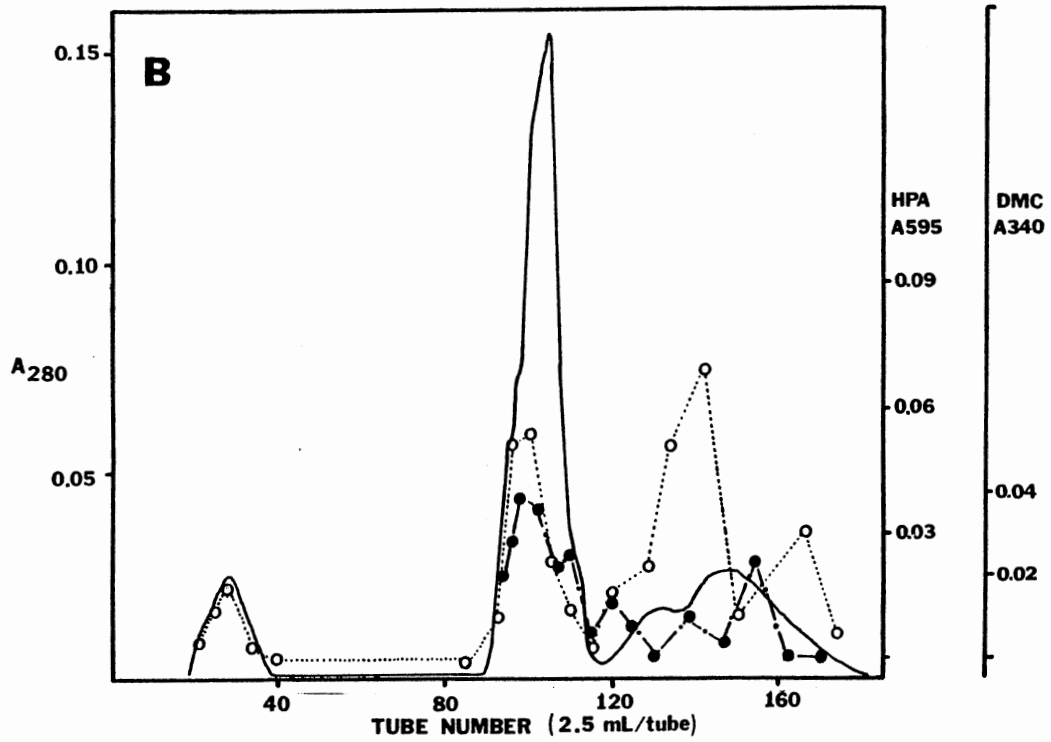
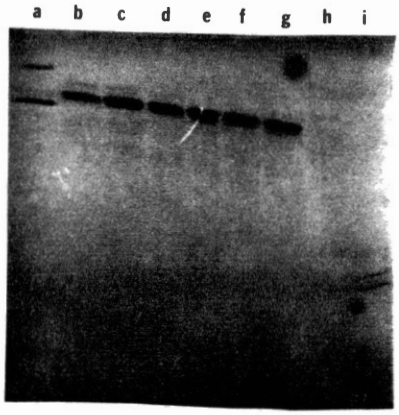
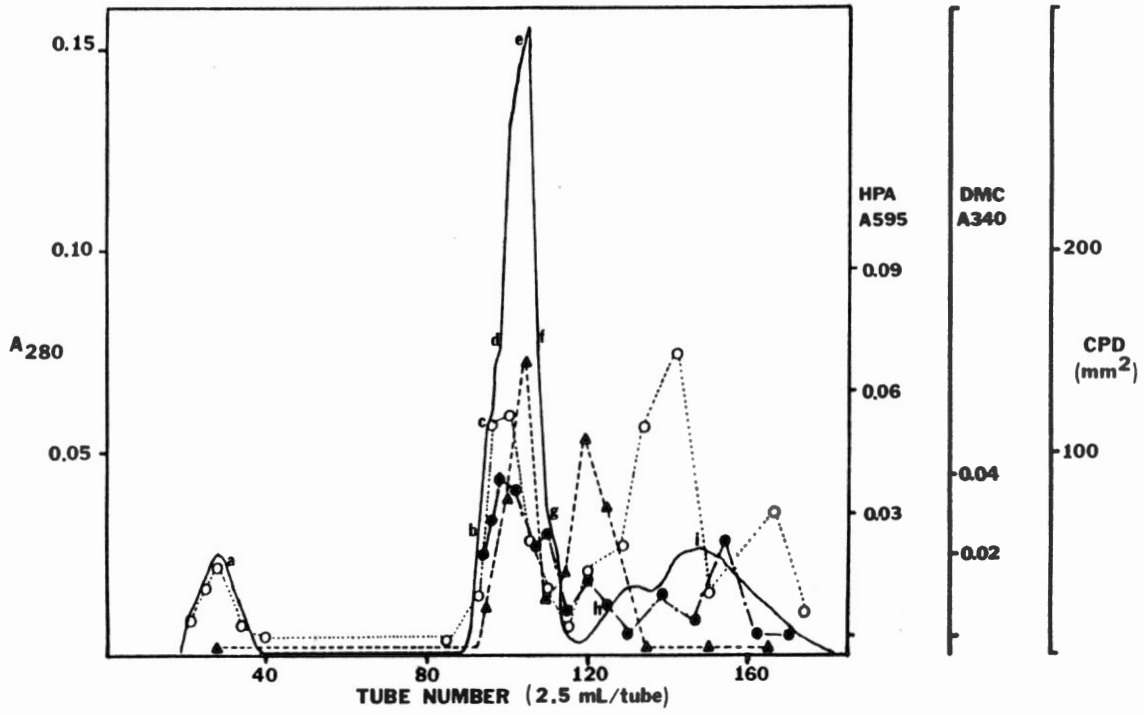

B
0.15
0.10
0.05
A_{280}
HPA
A595
0.09
0.06
0.03
DMC
A340
0.04
0.02
40
80
120
160
TUBE NUMBER (2.5 mL/tube)

Figure 18. Comparison of All Activities in Figure 17 and SDS-PAGE Profiling of the Eluted Fractions.

The same fractionation is shown as in Figure 17. The proteolytic activities of fractions against DMC (●) and HPA (○) and the hemorrhagic activities (▲) of fractions measured as cross-product diameter are shown.

Protein seen in gel lane corresponds to the protein present at that point in the elution profile as indicated by small letters.


a b c d e f g h i


0.15
0.10
0.05
A280
HPA
A595
0.09
0.06
0.03
DMC
A340
0.04
0.02
200
100
CPD
(mm2)
40
80
120
160
TUBE NUMBER (2.5 mL/tube)

to the main bulk of the VTm containing peak. In fact, gel scanning reveals that less than 10% of the protein in fraction 95, near the center of the shoulder, was the 77 kdal protease. The third KCl eluted peak, not clearly separated from the second, was determined to be principally the IIm4 major protein by this technique.

The hemorrhagic activity was separated into two distinct peaks as seen in Figure 17A. Substantial hemorrhagic activity was observed across the IIm3 fraction and matched its elution profile. Another peak of hemorrhagic activity was found over the front edge of the second KCl eluted peak. This activity was either due to a minor component, or this hemorrhagic component simply contained a limited number of aromatic residues such that A_{280} was low in comparison to the amount present.

A strong peak of proteolytic activity against DMC and HPA was present in the front edge of the first KCl eluted protein peak as seen in Figure 17B. This peak of proteolytic activity coincided with the protein profile shoulder contaminated with the 77 kdal protease centered at fraction 97. Proteolytic activity against DMC was broadly spaced across the first hemorrhagic activity peak as seen in Figure 18, while proteolytic activity against HPA occurred mainly in the leading edge. A shoulder of proteolytic activity against DMC and a peak of proteolytic activity against HPA both coincided with a second peak of hemorrhagic activity. After the second hemorrhagic peak there were two more peaks of eluted proteolytic activity against both substrates, but these were not associated with any hemorrhagic activity.

The 77 kdal protease is similar to the 73-77 kdal fraction seen

in the SDS-PAGE analysis of II*wo*3A (Figure 7) and also elutes prior to the viriditoxin-like component. The peak in II*m*3 of proteolytic activity against hide powder azure had a specific activity of approximately 0.7 $mg^{-1}hr^{-1}$. The specific activity determined for the homogeneous 77 kdal protease of fraction II*wo*3A (Figure 6) was approximately 3-4 $mg^{-1}hr^{-1}$. The specific activity of the VT*m*, the major protein present in the peak, can account for roughly 10% of the observed specific activity present in the leading edge of II*m*3 (Figure 17). The 77 kdal protease contaminant can account for at least 50 to 60 percent of the proteolytic activity against HPA, based on the specific activity of the 77 kdal protease from western Oklahoma venom. Using the data available, the 77 kdal protease has at least a 50 times greater specific proteolytic activity against HPA than either VT*m* or VT*wo*.

Hemorrhagic and proteolytic activities in the final purification step were assayed. The II*m*3 pool above and two similar II*m*3 pools were dialyzed against buffer B, and their protein content was determined by A_{280} measurement. These pools were then concentrated on three one mL Biogel A DEAE pasteur pipet columns equilibrated with Buffer B. These concentrating columns were then eluted with 2 x 1.2 mL of 300 mM KCl in Buffer B. The eluates were then combined, loaded onto a Sephadex G150-120 column and fractionated as seen in Figure 19. The elution profile is shown by the heavy line and the fractions that were eventually pooled are indicated by the shaded area. Aliquots from each fraction in the pool were combined and analyzed on 7.5% SDS-PAGE tube gels, with standards run simulataneously in an identical gel, as shown in Figure 20. The proposed pool of VT*m* was seen to be

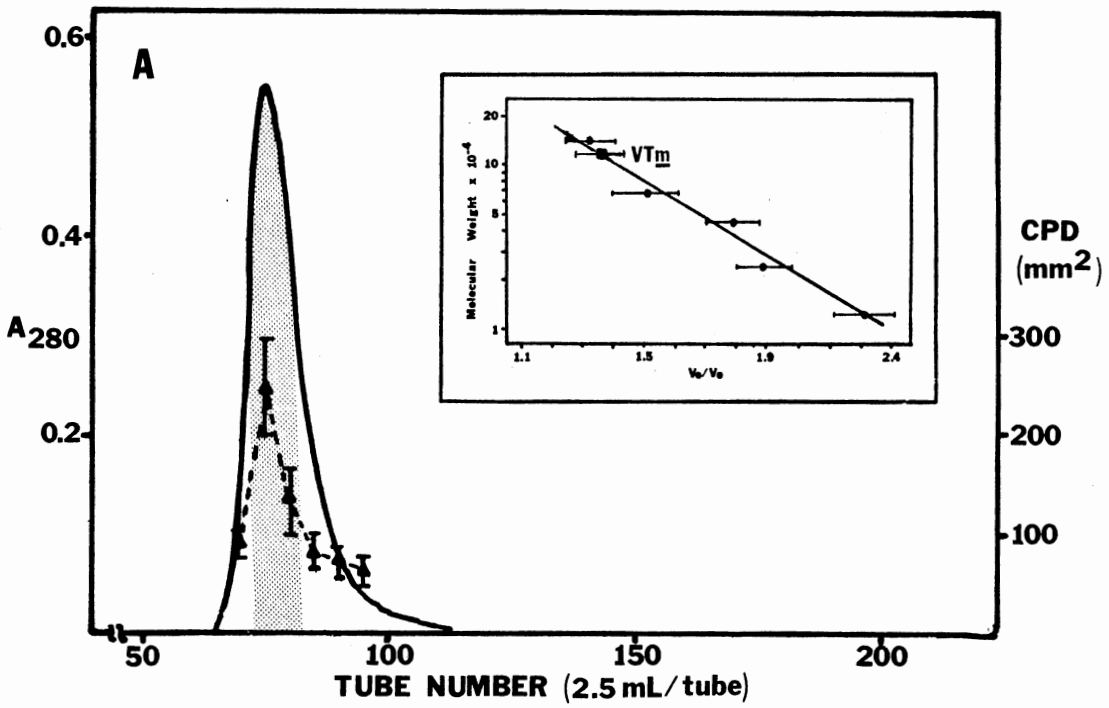
Figure 19. Fractionation Through Sephadex G150-120 of Combined II$\underline{m}$3 Pools and Assay of the Hemorrhagic and Proteolytic Activities Across the Fractions.

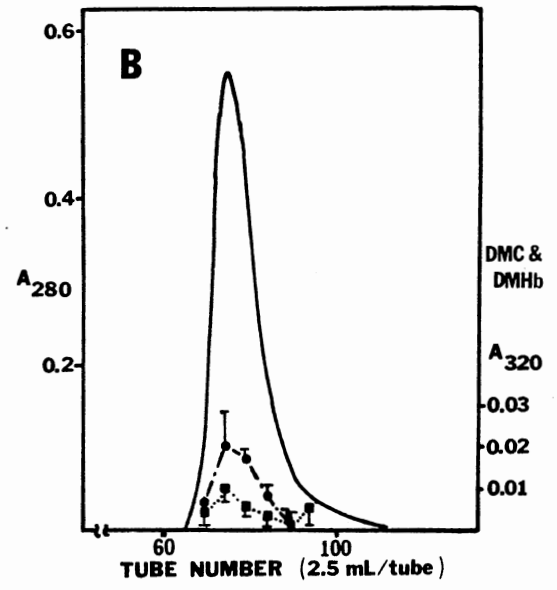
Combined Biogel A DEAE II$\underline{m}$3 pools originating from 2450 mg of venom fractionated on 5 Sephadex G150-120 columns were rechromatographed on a 2.5 x 90 cm Sephadex G150-120 column. 2.5 mL fractions were collected. All assays were done in triplicate, and the error bars give the standard deviation.

A. The complete elution profile obtained from gel filtration is shown. The hemorrhagic activity (▲) of tested fractions elutes in the fractions pooled. Fractions were diluted 1:10 with PSS and injected into mice. The resultant cross-product diameters are shown. The shaded area indicates the fractions pooled as VT$\underline{m}$. Inset shows calibration of the column with molecular weight standards as described in the Materials and Methods (solid circles). The solid square denotes the elution position of VT$\underline{m}$.

B. The proteolytic activity of fractions against N,N′-dimethylated casein (●) and N,N′-dimethylated hemoglobin (■) is shown. 20 µL of column fractions tested was incubated with 1 mL of substrate for 4 hr at 37 °C in either case.

C. The proteolytic activity of fractions against hide powder azure (O) is shown. 50 µL of fractions tested was incubated with 20 mg of HPA in 5.0 mL of buffer at 37 °C for 6 hr.


A
0.6
0.4
0.2
A280
CPD
(mm2)
300
200
100
50
100
150
200
TUBE NUMBER (2.5 mL/tube)
VTm
Molecular Weight x 10-4
20
10
5
1
1.1
1.5
1.9
2.4
Ve/Vo


B
0.6
0.4
0.2
A280
DMC &
DMHb
A320
0.03
0.02
0.01
60
100
TUBE NUMBER (2.5 mL/tube)

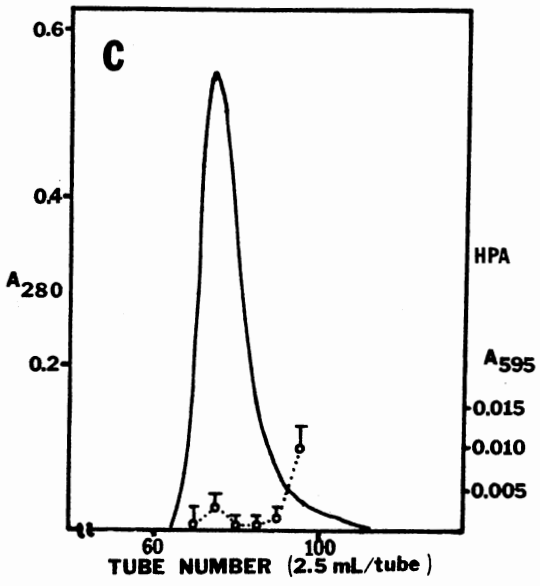
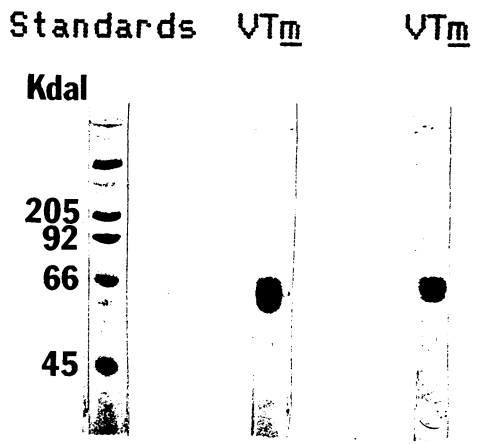

C
0.6
0.4
0.2
A280
HPA
A595
0.015
0.010
0.005
60
100
TUBE NUMBER (2.5 mL/tube)

Figure 20. SDS-PAGE of VTm Pool Seen in Figure 19.

Migration of VTm after treatment with SDS, heat denaturation and reducing agents is the same as when only detergent is present. Molecular weights estimated from gel are given in text. Gels were stained with Coomassie blue.



Sample Treatment	Standards	VTm	VTm
SDS	+	+	+
100 °C	+	+	–
2-Mercaptoethanol	+	+	–

homogeneous by this method. The gel filtration molecular weight of VTm was estimated to be 134 kdal based on calibration of the gel filtration column. The fractions were then pooled and their volume and absorbance at 280 nm determined. The final pool was desalted by dialysis, lyophilized and weighed.

Before pooling of the fractions the hemorrhagic and proteolytic activities across this column were determined. Hemorrhagic activity is plotted in 19A. Proteolytic activities against DMC and DMHb are shown in Figure 19B. Proteolytic activity against HPA is shown in Figure 19C. Hemorrhagic activity coeluted with the major peak. Proteolytic activities against DMC and DMHb also peaked with the hemorrhagic activity. Proteolytic activity against HPA was slight but substantially increased with an unknown contaminant fraction eluting in the tail of the hemorrhagic activity.

The yields and hemorrhagic and proteolytic activities of the purification steps are summarized. Table V assesses the purification of VTm from crude venom seen in Figures 15 through 19. The yields of the pools were calculated from the pool volumes and absorbance measurements, using the experimentally determined absorptivity for VTm, 0.47 $ml*mg^{-1}*cm^{-1}$. The absorptivity was determined using the absorbance at 280 nm of the VTm pool, the volume of the pool and the actual weight of the lyophilized pool after desalting by dialysis. The amount of starting material and the yields of the pools and VTm isolate are given in Table V. Five initial Sephadex G150-120 fractionations were used. Four and one half IIm pools from these columns were then either fractionated separately or combined and fractionated on three separate Biogel A DEAE columns. The three

TABLE V

PURIFICATION OF VTm HEMORRHAGIC AND PROTEOLYTIC ACTIVITIES

Venom, Pool, Isolate	Total Yield (protein) (mg)	Percent Yield at Each step	Cumulative Percent Yield (CPY)	Specific Activity MHD μg/gm	DMC A_{340} mg*hr	DMHb	HPA A_{595} mg*hr
Venom (Miami)	2200	100	100	0.165	--	--	0.69
IIm	179	8.1	8.1	0.055	1.3	0.38	0.19
IIm3	57.2	42	3.4	--	1.08	--	0.082
VTm	20.6	36	0.94	0.016	0.39	0.18	0.003

Venom, Pool, Isolate	Relative Activity MHD	DMC	DMHb	HPA	Purification Number Rel. DMC / Rel. HPA*CPY
Venom (Miami)	1	--	--	3.6	--
IIm	3	1.0	1.0	1.0	1
IIm3	--	0.83	--	0.082	4.6
VTm	10.3	0.30	0.47	0.02	99

combined IIm3 pools were then fractionated on one final Sephadex G150-120 column. The percent yields of each step and the cumulative percent yields after each step are also given in the table. The final yield of VTm was 20.6 mg, which was a 0.94% cumulative yield (CPY) of initial protein.

The minimum hemorrhagic dose values were determined for crude venom, pool IIm, and the final pool of VTm. The data on which these values are based is presented in Appendix B. Small amounts of the venom, the IIm pool, and the VTm pool were resuspended in PSS and the MHD was determined as described in the Materials and Methods. The MHD for the IIm3 pool was not determined. The relative MHD is the ratio of the MHD of the venom, pool or isolate to the MHD of crude venom. The MHD of VTm was determined to be 0.016 μg/gm, which represents a 10.3 fold increase in specific hemorrhagic activity relative to crude venom. From Figures 15 through 19, it is clear that the viriditoxin-like component is responsible for only a small part of the total hemorrhagic activity of the venom. Assuming that all the hemorrhagic activities of crude venom are additive, which may well not be the case, VTm represents roughly six percent of the hemorrhagic activity of the venom.[10] Assuming no loss of VTm during the purification, a

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[10]The percent of hemorrhagic activity in the crude venom due to VTm was calculated by integration of the areas under the peaks of hemorrhagic activity seen in Figures 15 and 17. In each figure the area of the shaded activity area pooled for VTm isolation was divided by the total activity area under the hemorrhagic activity peak. Sixteen percent of he hemorrhagic activity in Figure 15 was pooled as IIm, and 40% of the hemorrhagic activity was pooled as IIm3 as seen in Figure 17. This results in a cumulative percent yield of 6% of the hemorrhagic activity in the crude venom being fractionated and pooled as VTm. This represents a lower limit on VTm content in the crude venom, as losses almost certainly occurred during the purification.

purification of VTm of 170-fold would have been achieved.[11] This represents an upper limit, as losses almost certainly occurred and any loss would tend to artificially enlarge this purification number.

The proteolytic activity of the fractions tested within a pool was used to determine the average proteolytic activity of the pool. The absorbance of the pool fractions at 280 nm was used to determine the average weight of the aliquots tested, using the experimentally determined absorptivity of VTm. The calculation of the average and specific proteolytic activities was as described in the Materials and Methods.[12] The specific proteolytic activities of the pools and isolates against DMC, DMHb and HPA are given in Table V. The relative proteolytic activity is the ratio of the specific proteolytic activity of the venom, pool or isolate to the specific proteolytic activity of the IIm pool. The loss of specific and relative proteolytic activities approximates what would have been expected by cursory examination of the purification in Figures 15 through 19. In Figure 19 the emergence of a proteolytic fraction subsquent to VTm was indicated. This activity, well resolved from the bulk of the sole protein peak, was in the drawn out tail of the protein peak and had a significant proteolytic activity against HPA and DMHb. Proteolytic activity against DMC was not determined in this region. The relative

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[11]The purification number is the hemorrhagic activity of VTm relative to crude venom, 10.3 (see Table V) divided by the cumulative yield of hemorrhagic activity, 0.06 (see text; cumulative yield x 100 = cumulative percent yield).

[12]Average proteolytic activity is a unitless measurement of absorbance as in the figures, while specific proteolytic activity additionally takes into account the length of assay in hours and the average aliquot weight of the fractions tested in milligrams.

proteolytic activity against DMHb and DMC only decreased approximately two-fold and three-fold, respectively, while there was a 50-fold decrease in relative proteolytic activity against HPA as seen in Table V. Hence, the purification of VTm proteolytic activity against DMC away from the proteolytic activity against HPA can also serve as a measure of VTm purification. Such a purification number is presented in Table V as the ratio of activity against DMC to activity against HPA divided by the cumulative percent yield starting with the IIm pool. The isolate was purified nearly 100-fold from the IIm pool in the last two purification steps according to this projection.

Comparison of VTm and VTwo and Their Crude Venoms

The VTm and VTwo isolates were directly compared by SDS-PAGE analysis. In Table VI the results of this analysis are presented. Reduced and heated samples of VTm and VTwo were run side by side on 10 and 12 percent SDS-PAGE slab gels. Protein standards were also included in the gels and the molecular weights of the VTm and VTwo polypeptides were estimated as described in the Materials and Methods. Four molecular weight estimates were available from these two gels for each polypeptide of each isolate, and the average values are presented the in the table. Although the values obtained were high compared to other molecular weight estimates previously determined in this work, the variation in these values was no more than ± 0.5 kdal in each case and as such allows comparison of the isolates. It can thus be seen that the subunits of the VTm isolate were approximately 2 kdal smaller than the corresponding subunits of the VTwo isolate. For both

TABLE VI

MOLECULAR WEIGHT ESTIMATES OF VIRIDITOXIN, VTm AND VTwo

Toxin, Researcher, Sample Treatment	Molecular Weight Estimate large polypeptide (kdal)	small polypeptide (kdal)	Coomassie Blue R250[1] bound: large polypeptide / small polypeptide
Viriditoxin Fabiano and Tu[2] Reduced and alkylated	62	57	not reported
VTm Chan and Gleason[3] Reduced and alkylated	60	56	~1:1
VTm Gleason et al.[4] Reduced only	66	62	1.4:1[1]
VTwo Gleason et al.[4] Reduced only	68.5	63.5	5.3:1[1]

[1]Apparent large to small polypeptide stoichiometry based on the amount of Coomassie blue bound. Absorbance rations determined by gel scanning were 1.5:1 for VTm and 6:1 for VTwo. Gel scanning values were then corrected for mass of polypeptides and reported in the above table.

[2]Fabiano and Tu, 1981; proteins were reduced and alkylated prior to electrophoresis in SDS-PAGE tube gels which had 10%, 12%, and 14% acrylamide separating gels.

[3]Proteins were reduced and alkylated prior to electrophoresis in a SDS-PAGE slab gel with a 7.5% acrylamide separating gel.

[4]Gleason et al., 1983; proteins were only reduced prior to electrophoresis in SDS-PAGE slab gels with 10% and 12% acrylamide separating gels.

isolates the difference between the larger polypeptide and the smaller polypeptide is 4 to 5 kdal. This difference is the same as was obtained for the previous SDS-PAGE molecular weight estimates determined in this work.

The intensity of Coomassie blue R250 staining of the high and low molecular weight polypeptides was determined by gel scanning as described in the Materials and Methods. The amount of dye bound by the lower molecular weight polypeptide of VTm was much greater than the amount of dye bound by the lower molecular weight polypeptide of VTwo. However, the high molecular weight polypeptides of VTm and VTwo seemed to bind equivalent amounts of dye. Quantitatively, the ratio of the high-molecular-weight polypeptide to the low-molecular-weight polypeptide based on Coomassie blue binding is 1.4:1 for VTm and 5.3:1 for VTwo.

There are three probable causes for the observed dye binding difference between the two lower molecular weight polypeptides of the viriditoxin-like isolates. The isolates could consist of a variety of multimeric forms; of varying mixtures of homo-and heterodimers; or of subunits with differing dye binding affinities. The first possibility can be excluded on the basis of gel filtration molecular weights. No multiplicity of subunit association beyond that of a dimer was observed when the molecular weight of either viriditoxin-like component was calculated by gel filtration column calibration or by K_{av} value calculation. The second possibility is contradicted by SDS-PAGE analysis of gel filtration column fractions. The ratio of the high to low molecular weight polypeptide staining intensity remained constant during sizing, which could mean that no high:high or

low:low associations occurred. Hence, the dye binding ratio differences may be due to differences in subunit dye binding affinities, and the isolates would therefore be heterogeneous dimers. The efficiency of Coomassie blue R250 binding may vary as much as 2-fold in serum proteins alone (Fazekas de St. Groth et al., 1963) or even more depending on a protein's amino acid composition or glycosylation (Wong et al., 1985), much in the same way as Coomassie blue G250 used in the Bradford (1976) protein assay can vary in its binding to protein.

To further investigate the relationship between the two subunits, samples of VTm were treated with sample buffer lacking β-mercapto-ethanol and were not denatured by heating. Other VTm samples were reduced and heated as usual. The samples were analyzed by 10% SDS-PAGE and the Commassie blue stained slab gel is shown in Figure 20. Two polypeptides were obtained at essentially the same molecular weights whether or not the VTm samples were heated and reduced. This indicates that there are no disulfide bridges covalently linking the subunits together, and that treatment with room temperature SDS was sufficient to dissociate the dimer.

The two crude venoms and the viriditoxin-like isolates were analyzed by standard Tris-glycinate disc PAGE. The VTm isolate from Figure 6 and crude Crotalus viridis viridis (Miami) venom were electrophoresed in separate tube gels as described in the Materials and Methods. In a separate experiment VTwo, purified by a second DEAE chromatography from the IIwo3 pool seen in Figure 11, was electrophoresed along with crude Crotalus viridis viridis (western Oklahoma) venom in separate tube gels. These four tube gels are

presented in Figure 21. First, as expected, the VTwo isolate is seen to be homogeneous. The VTm isolate is also homogeneous. The mobilities of the two isolates are very similar, as would be expected from their molecular sizing data and from their DEAE elution charactersitics. The molecules have nearly the same molecular weights and both begin to elute at 20 mM KCl in the salt gradient of DEAE fractionations. The viriditoxin-like protein band can be located in the tube gel of the crude venom by direct comparison with the tube gel of the viriditoxin-like isolate from that venom. The two venoms have several proteins in common, but some common protein bands are present in different levels in the two crude venoms.

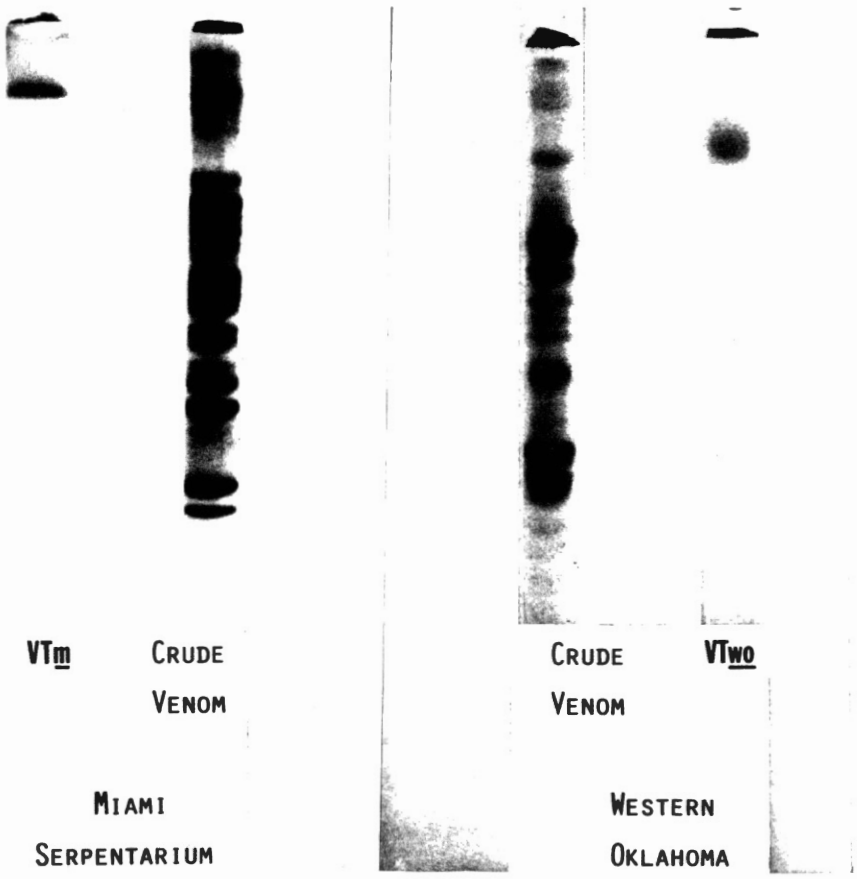
Comparison of the Biogel A DEAE fractionations of VTm, VTwo, and viriditoxin

Biogel A DEAE chromatography of the IIm and IIwo pools presented a complex pattern of KCl eluted protein peaks. Comparison of the Biogel A DEAE fractionations of pools IIm and IIwo can unify this data into a clear picture. Since the isolation reported by Fabiano and Tu (1981) for viriditoxin was the basic outline for our isolations it is prudent and central to this comparison to include relevant portions of that work in this discussion.

Two underlying factors determined the differences observed in the protein peaks eluted in the salt gradients: the position of the sizing pool and the steepness of the salt gradient used. Initially, pooling of IIm and IIwo was based on the elution profile of venom fractionated by Sephadex G150-120 in a manner like that previously reported for viriditoxin (Fabiano and Tu, 1981). However, these workers did not

Figure 21. Standard Tris-Glycinate Disc PAGE of Crotalus viridis viridis Crude Venom (Miami), VTm, Crude Venom (western Oklahoma), and VTwo.

Samples were analyzed by 7% acrylamide standard Tris-glycinate disc PAGE tube gel. These preparations of VTm and VTwo were shown to be homogeneous. The two isolates were separated simulataneously with their respective crude venoms. Gels were stained with analine blue black.


VTm
CRUDE
VENOM
MIAMI
SERPENTARIUM
CRUDE
VENOM
VTwo
WESTERN
OKLAHOMA

determine where viriditoxin maximally eluted in their fractionation of crude venom (Miami). Figure 15 shows that VTm maximally elutes somewhat ahead of the pool previously suggested (Fabiano and Tu, 1981) and used in the earlier isolations of VTm and VTwo, as shown in Figures 4 and 11, respectively. The elution position of the IIm4 major protein seen in Figure 15 clearly shows why the IIm4 peak was enlarged in DEAE fractionation of the IIm or IIwo pools selected solely on the basis of elution profile. When pool selection was aided by SDS-PAGE the IIm4 peak was seen to actually consist of two poorly separated sets of protein, most likely because the quantity of the IIm4 major protein had been reduced by proper IIm pooling. The ratio of the IIm4 peak area to the IIm3 peak area was 1.8 in the earlier fractionation and 0.4 in the later fractionation. Altered pooling may also explain the absence of a IIm2 peak in later fractionations.

In addition to the selection of the IIm or IIwo pool, the steepness of the salt gradient used to elute proteins retained on the DEAE Biogel A column also determined the character of the eluted protein peaks. When the salt gradient conditions recommended by Fabiano and Tu (1981) were used only one peak was obtained in the salt gradient (results not shown). Resolution of this peak into two or more peaks was only possible when the steepness of the salt gradient was lowered as in Figure 4. In later fractionations, the salt gradient was made even shallower, resulting in the better separation of the IIm3 and IIm4 peaks and the resolution of the IIm4 peak into two peaklets as seen in Figure 17. The slope of the earlier gradient was 2.5 times greater than that used in the later gradient and 0.37

times that apparently used by Fabiano and Tu (1981). The main difference between the II$\underline{m}$ and II$\underline{wo}$ fractionations was the resolution of the 77 kdal protease prior to the II$\underline{m}$3 or II$\underline{wo}$3 peak. In the II$\underline{m}$ fractionation this protein eluted as a shoulder on the II$\underline{m}$3 peak, and as a seperate peak, II$\underline{wo}$3A, in the II$\underline{wo}$ fractionation.

However, Fabiano and Tu (1981) obtained two salt eluted peaks (B3 and B4) using the conditions they described, with viriditoxin present in the second eluted peak (B4). Their results can be most simply explained by assuming that their salt gradient, instead of being linear, started out very shallow and then became linear and very much steeper than what has been used successfully in this study. With such a gradient, the 77 kdal protease, which elutes prior or just prior to the VT$\underline{wo}$ or VT$\underline{m}$-containing peak in our hands, would have all eluted from the column in a peak before II$\underline{m}$3, and then the II$\underline{m}$3 and II$\underline{m}$4 major proteins would have come off the column together. Hyperbolic gradients of this sort can easily result when care is not taken to leave as little buffer as possible on top of the ion-exchange resin. The linearity of their salt gradient is questionable since they did not report having determined the conductivity of the fractions in their salt gradient. The gradients used in this study were determined to be linear and were a third to a tenth as steep as what they had reported. All other conditions of ion exchange chromatography were essentially the same as theirs.

The identification of their first peak as our 77 kdal protease is further substantiated by the activities they attributed to it. They report that it is a peak of myotoxic activity with a proteolytic activity against hide powder azure very much greater than that of

viriditoxin. They did not check it for hemorrhagic activity. These characteristics in fact aptly describe the 77 kdal protease, which we have additionally identified in the case of the western Oklahoma venom to cause hemorrhage as well as myonecrosis when injected in mice. The later steepness of their gradient is demonstrated by our data and their data with Tris-glycinate disc PAGE tube gels of these pools. Their system and our system of Tris-glycinate disc PAGE tube gels were nearly identical. Their peak B4 contains two major proteins of electrophoretic mobilities similar to what would have been observed for the II$\underline{m}$3 and II$\underline{m}$4 major proteins seen in Figure 6 if they had been mixed prior to electrophoresis. Their solution to the purification of viriditoxin away from the II$\underline{m}$4 proteins was an additional sizing step on a Sephadex G150-40 column.

Fabiano and Tu (1981) did not report the separation of a third peak of hemorrhagic-myotoxic activity in their DEAE fractionation step, or in their later step. This means that while they separated away the 77 kdal protease by ion exchange chromatography and the II$\underline{m}$4 major protein by gel filtration, they did not separate away a II$\underline{m}$4 minor protein, which we have found to be responsible for the II$\underline{m}$4 peak's hemorrhagic and myotoxic activity in this study.[13] No SDS-PAGE analysis of this or any other fraction was shown. Thus, our suggestion is that "viriditoxin" as first isolated was not pure and that the first electrophoretically homogeneous viriditoxin-like protein was produced by this isolation procedure and reported in this thesis. Of course it is possible that the Miami Serpentarium crude venom used by

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[13]They reported their gel filtration peak, corresponding to the II$\underline{m}$4 major protein, as having no hemorrhagic or myotoxic activity.

Fabiano and Tu (1981) differed in composition from the Miami Serpentarium crude venom used in this study.

Discussion

The biochemical characterization of venom components was pioneered by Slotta (1938). The objectives are to separate the many toxic activities of snake venom and to thereby achieve a better understanding of the mechanism of injury and of its prevention. In this study, the biochemical characterization of one snake venom toxin was undertaken, correlated with observation of its toxicity.

Hemorrhagic and Myotoxic Fractions and Isolates in Two Venoms From Crotalus viridis viridis

The initial goal of this project was to reproduce the isolation of viriditoxin, the hemorrhagic-myotoxin from Crotalus viridis viridis venom, and to examine the nature of its pathological effects. Somewhat surprisingly, several fractions in various stages of purification were found to be both hemorrhagic and myotoxic.

The first step in the purification of viriditoxin was gel filtration (Fabiano and Tu, 1981). This step was first employed by Cameron and Tu (1977) to resolve myotoxin a activity from the high molecular weight hemorrhagic and myotoxic activities in Crotalus viridis viridis venom. Pools Im through IVm, containing proteins ranging from greater than 265 to ~39 kdal in molecular weight (by K_{av} calculation; see Table II) all produced hemorrhage and myonecrosis 24 hours after injection into mouse skeletal muscle. Similar results were obtained with gel fractionation of crude C. viridis viridis

(western Oklahoma) venom. The previous study (Fabiano and Tu, 1981) also found hemorrhagic and myotoxic activities in the fractions corresponding to IIm through IVm in this study. The inability to resolve hemorrhage from myonecrosis in the high molecular weight fractions implied that several myotoxic hemorrhagins might be present, for four major reasons. First, the molecular weight range involved would seem to exclude the presence of members of the other crotalid myotoxin classes: phospholipases A_2 and small basic myotoxic polypeptides.[14] Second, SDS-PAGE analysis of fractions in the Im, IIIm, and IVm pool regions showed that VTm was present in too low of a concentration in these other fractions to account for their activity.[15] Third, the peak of myotoxicity as assayed by histology correlates well with the peak of EDTA-sensitive hemorrhage as assayed by Kondo's method, since Figures 3 and 15 are directly comparable. Fourth, as discussed in the results, proteolytic activity, which is EDTA-sensitive, does not elute smoothly with the hemorrhagic activity, which is also EDTA-sensitive, but rather elutes as three clusters of peaks of activity against various artificial substrates.

The suspicion that there might be multiple myotoxic hemorrhagins was confirmed by the DEAE fractionation of pools IIm and IIwo. When

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[14]The molecular weight of crotalid phospholipases as reviewed by Tu (1977) ranges from 31.9 to 9.4 kdal. While a myotoxic phospholipase has not been isolated from C. viridis viridis venom, weak phospholipase activity was detected in pool Vm in this work, and it seems likely that a phospholipase, if present, would be found in this fraction. The C. viridis viridis small basic myotoxic polypeptide, myotoxin a, was found in pool VIm, consistent with previous reports (Cameron and Tu, 1977).

[15]Indeed, this study as well as the previous study (Fabiano and Tu, 1981) showed that a greater hemorrhagic activity was present in the fractions not contained in the IIm pool.

IIwo was subjected to ion-exchange chromatography, three peaks of potent myotoxic-hemorrhagic activity eluted in the salt gradient. Pools IIwo3A, IIwo3 and IIwo4 were all indistinguishable by histological assay, all causing severe hemorrhage and muscle damage by 24 hours after injection. IIwo3A had a fairly high EDTA-sensitive proteolytic activity against hide powder azure and was composed mainly of the 77 kdal protease, as shown by SDS-PAGE analysis. The 77 kdal protease, identified and shown to be hemorrhagic and myotoxic in the western Oklahoma venom, was nearly identical in size, proteolytic properties, and Bio-Gel A DEAE elution characteristics to the 77 kdal protease present in the leading edge of the IIm3 peak. IIwo3 and the center of the IIm peak were shown by SDS-PAGE analysis to be comprised mainly of VTwo and VTm, respectively. IIm4 amd IIwo4 were both hemorrhagic, proteolytic and myotoxic, although here the picture is less clear, as these pools were shown to be complex by SDS-PAGE analysis, and the elution of hemorrhage and proteolysis was complex, as can be seen in Figure 17. The major hemorrhagic activity in IIm4 was prior to the major peak of eluted protein. Since protein absorbance at 280 nm depends almost entirely on the absorbance of tryptophan and tyrosine residues, the data could be interpreted to mean that a myotoxic-hemorrhagic protein essentially lacking these amino acids was eluting at this position, and/or that the myotoxic-hemorrhagin was extremely potent and present in very low quantity. Thus, at least two, and probably three, separate myotoxic hemorrhagins can be resolved from Crotalus viridis viridis venom.[16]

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[16]An additional weak hemorrhagin, IIm2, which did not bind DEAE, was only seen in early fractionations. Its precise nature is unknown.

Since this work was reported (Gleason et al., 1983), two more myotoxic-hemorrhagic toxins, HTf and HTg, have been isolated from Crotalus atrox venom (Nikai et al., 1984, 1985a). Additionally, nine other hemorrhagic toxins which also have myotoxic acitivity have been reported (Kishida et al., 1985; Nikai et al., 1985b; Yagihashi et al., 1986; Mori et al., 1987; Nikai et al., 1987) HTb, isolated from Crotalus atrox venom by Bjarnason and Tu (1978), was the first myotoxic-hemorrhagin to be discovered (Ownby et al., 1978). Fabiano and Tu (1981) reported that two peaks of myotoxic activity were eluted during the ion-exchange step of the viriditoxin purification. One of the peaks was highly proteolytic and the other not very proteolytic. No assay of hemorrhagic activity in these peaks was reported. That they chose to purify the myotoxic fraction containing low proteolytic activity suggests that they had originally intended to isolate a new high molecular weight class of myotoxin from C. viridis viridis venom, and presumably found later that they had purified a myotoxic hemorrhagin. Thus, one thing this study has accomplished is to establish firmly that C. viridis viridis venom (from two sources) does contain more than one myotoxic-hemorrhagin.

Intraspecial Variation

Individual variations (Glenn and Straight, 1977; Willemse, 1978; Faure and Bon, 1987) and geographical variations (Ohsaka, 1979; Lee, 1972; Glenn and Straight, 1978; Moran and Geren, 1979a; Sadahiro and Omori-Satoh, 1980; Gutiérrez et al., 1980, Minton and Weinstein, 1986) in venom composition have been observed in the venoms of several species. While morphological characteristics of snakes may be

unchanged within a given species, qualitative and quantitative differences in their venom components may still exist. Indeed, electrophoretic differences in venom composition were observed between the two venoms of Crotalus viridis viridis used in this study as seen in Figure 21. Differences were also observed in the fractionation of some activities during purification and in the isolates, VTm and VTwo.

There are two probable explanations for the divergence of venom components between geographically distinct populations. First, the venom components might diverge by random, silent mutation. If a hemorrhagic component comprises a only small part of the total hemorrhagic activity of a crude venom, the selective pressures on this minor hemorrhagic component should be reduced. Clearly VTm is a minor hemorrhagic component, and pressure to maintain its sequence may have been low. Second, different toxicities might be required in different geographical areas, due to regional variations in the prey on which the snakes feed or the enemies against which they must defend themselves. Immunity of prey to venom components has been demonstrated and, in particular, sera from prairie voles and woodrats contain components capable of partially neutralizing hemorrhage (DeWit, 1982; Perez et al., 1979). Rodents are not affected by the hemorrhagin in the venom of Ophiphagus hannah (Elapidae), but rabbits and hares are sensitive to it (Weissenberg et al., 1987). While the exact role of toxic venom components in killing prey is not clear, it is clear that hemorrhagic components are an important part of the snake's arsenal of toxins.

Both of these factors may apply to Crotalus viridis viridis venom and its viriditoxin-like components. The collections of prairie

rattlesnakes extracted to obtain Crotalus viridis viridis venoms used in this study are undeniably different. The wide range of this rattlesnake as seen in Figure 1 suggests that the collective gene pool is quite large, and that diverse habitats, with differing species of prey and natural enemies, are available.

When purified VTwo and VTm were analysed side by side by SDS-PAGE electrophoresis, two points of difference were noted. First, the subunits of VTwo were larger by approximately 2 kdal. Second, the VTwo smaller polypeptide bound considerably less dye than the VTm smaller polypeptide. This may indicate a difference between VTwo and VTm in amino acid compositions and/or glycolsylation states of perhaps the lower molecular weight polypeptide. Alternatively, but less likely, this may indicate a difference in the abundance of a putative high molecular weight polypeptide homodimer and a mixed heterodimer.[17] The amino acid composition of the VTwo isolate differed from that of viriditoxin (Fabiano and Tu, 1981), but both toxins were similar in their high acidic residue content. Observations such as these could be explained equally well by either of the above explanations. However, the minimum hemorrhagic doses of the VTwo and VTm isolates were not significantly different. This indicates that the differences between the two isolates do not affect their function and argues against the homodimer theory unless only the high molecular weight subunit is ever active. The differences observed in the VTwo and VTm isolates constitute an intraspecial variation for this toxin.

Other examples of intraspecial variation of specific venom

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[17]SDS-PAGE profiles across gel filtration columns do not support the existance of any homodimer.

components have been reported. The pooled venom of Crotalus durrissus terrificus contains at least 15 different isoforms of crotoxin, many of which are thought to be due to variations in amino acid content (Faure and Bon, 1987). A geographic variant of Crotalus durissus terrificus contains none of the myotoxin-a like component, crotamine, whereas another population does contain this toxin (Lee, 1972). Mojave toxin of C. scutulatus scutulatus venom shows similar geographical variation (Glenn and Straight, 1978). Similarly, Trimeresurus flavoviridis venom collected from the Okinawa Islands contains none of the HR-1B fraction found in the venom collected from the Anami Oshima Islands (Sadahiro and Omori-Satoh, 1980). However, both were found to contain the closely related HR-1A fraction. Since HR-1A seems to be very similar to the viriditoxin-like isolates in that it is a dimeric hemorrhagin with subunits of ~60 kdal molecular weight (albeit a concentration dependent dimer), these differences in the venoms of two populations of Trimeresurus flavoviridis venom consitute a close intraspecial variation parallel to the variations in the venoms of the two populations of Crotalus viridis viridis studied here.

Comparison of the Two Isolates with Other Known Hemorrhagins

Of the viriditoxin-like toxins isolated thus far, the apparent molecular weight was precisely determined by gel filtration only for the VTm isolate. The apparent molecular weights of the other preparations can be inferred from their positions in the elution profile. Fabiano and Tu (1981) stated that viriditoxin's molecular

weight was consistent with that of a dimer by gel filtration, but offered no data. The apparent molecular weight of VTm was 129 kdal and the molecular weight of VTwo was estimated to be 132 kdal by K_{av} calculation.

The only other hemorrhagin which is known to exist as a dimer is HR-1A from Trimeresurus flavoviridis venom (Omori-Satoh and Sadahiro, 1979). HR-1A exhibits a so-called concentration-dependent polymerization. The molecular weight of the HR-1 fraction, from which HR-1A and HR-1B can be purified, was previously determined by analytical sedimentation to be 104 kdal (Ohsaka et al., 1971b).[1e] In order to assure the reader that this "polymerization" was in fact a dimerization of HR-1A, consider the following. When an 0.8% solution of HR-1A was sedimented, an $s_{20,w}$ of 5.4S was obtained. The $s_{20,w}$ value for the always monomeric HR-1B, obtained under conditions identical to those used for HR1A, was 3.5S. At low concentrations of HR-1A, the $s_{20,w}$ value decreased, indicating that dissociation was taking place. As additional evidence of dimerization, consider the gel filtration data. Sephadex G-200 Superfine gel filtration of HR-1A at high (6.4%) and low (0.4%) concentrations showed a shift of 6-7 mls in the elution position of the peak. The void volume of their column was 67 ml and elution volume of HR-1A chromatographed at the high concentration was 84 ml, which gives a K_{av} consisitent with a dimer of 120 to 130 kdal. The authors discussed the HR-1A "polymerization" as if they meant dimerization, but apparently were reluctant to state this unequivocally since certain controls were missing from their

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[1e]When a 0.7% solution of HR-1 was sedimented, an $s_{20,w}$ of 5.8S was obtained.

experiments. The lability of the dimer form of viriditoxin-like isolates has not been rigorously determined, but non-denaturing separation of the subunits might be possible, since SDS-PAGE analysis without prior reduction of disulfide bridges with β-mercaptoethanol or heat denaturation resolved the two subunits of VTm. If the viriditoxin-like isolates did dissociate into monomers at low concentrations, this might explain the observation of two precipitin lines in Ouchterlony assays using antisera made to the homogeneous isolate (Ownby, personal communication). Self-proteolysis of viriditoxin or the formation of a denatured aggregrate might also explain this observation.

The observation that there seem to be two molecular weight classes of hemorrhagic polypeptides has been discussed in Chapter II. The viriditoxin-like myotoxic hemorrhagins in Crotalus viridis viridis venom(s) (Fabiano and Tu, 1981; Gleason et al., 1983) probably consist of dimers of hemorrhagins belonging to the 50-100 kdal class. It apears that the 77 kdal protease identified in this study may also be a member of the 50-100 kdal class. Our previous attribution of hemorrhagic and myotoxic activity to a group of low molecular weight polypeptides from IIwo4 (Gleason et al., 1983) has been shown to be doubtful by the use of shallower gradients in the ion-exchange purification of the hemorrhagic activity of IIm4, as can be seen in Figure 17. The IIm4 activity seems to be attributable to an unknown component in the leading edge of this fraction. This evidence, together with the estimated lower molecular weight limit of the last sizing pool to have hemorrhagic activity, seems to make less likely the existence of any 20-30 kdal class hemorrhagins in Crotalus viridis

viridis, similar to those that have been observed in Crotalus atrox, Trimeresurus flavoviridis, and other venoms.[19] Only the venoms of Crotalus atrox and Trimeresurus flavoviridis are known to contain hemorrhagins belonging to both molecular weight classes. The venom of Crotalus horridus horridus is only known to contain a member of the 50-95 kdal class (Civello et al., 1983a) and in this regard seems similar to the venom of Crotalus viridis viridis.

Most of the isolated hemorrhagic toxins appear to be acidic proteins. The isoelectric points of VTm and VTwo were not determined; however, the pI for viriditoxin was found to be pH 4.8 (Fabiano and Tu, 1981). All of the hemorrhagic and myotoxic components of the IIm and IIwo pools appear to be somewhat acidic, in that they bind to DEAE. However, the myotoxic and hemorrhagic toxin HTb from Crotalus atrox venom is a basic 24 kdal class protein. It appears that myotoxic-hemorrhagins can belong to either polypeptide class and can be either acidic or basic proteins.

Crotalid venoms contain significant proteolytic activity compared to the venoms of other snake families (Tu, 1982). Proteolytic activity in Crotalus viridis viridis crude venom and fractions of it has been previously described (Friederich and Tu, 1971; Fabiano and Tu, 1981). In this study, at least three proteolytic activities were discerned in the crude venom of Crotalus viridis viridis (Miami). The VTm isolate was found to have significant but low activity against the N,N′-dimethylated substrates. Previous workers (Fabiano and Tu, 1981)

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[19]Personal communication (November, 1987) from Dr. Ownby; recent evidence indicates that there may be at least one low-molecular weight class heorrhagin in the Crotalus viridis viridis venom.

reported that viriditoxin had approximately 3% of the proteolytic activity against N,N′-dimethylated casein observed in hemorrhagins HT*a*-*d* from *Crotalus atrox* venom. Using their data and data from Table IV, it can be calculated that the VT*m* isolate had approximately 1% of the proteolytic activity against N,N′-dimethylated casein reported for HT*a*-*d*. This value is commensurate with theirs, considering the slight differences between their assay and this study's assay. The proteolytic nature of the 77 kdal protease was also elucidated. This protein was found to have a proteolytic activity against hide powder azure ~50 fold greater than that found for VT*m*.

Not all venom proteases are hemorrhagic, but it appears that all hemorrhagins are likely to be proteases (Tu, 1982). Hence, observations made in this work that VT*m*, VT*wo*, and the 77 kdal protease are hemorrhagic and proteolytic are consistent with the bulk of research available. The effect in this study of EDTA treatment of gel filtration fractions prior to hemmorrhagic assay and assay for caseinolytic activity once again indicates the involvement of metal cations with these activities. Inhibition of viriditoxin's hemorrhagic and proteolytic activities by metal chelators was not reported (Fabiano and Tu, 1981).

The pathogenic action of hemorrhage by hemorrhagins is very specific. Correspondingly, their proteolytic activity should be as specific. In fact, the ability of the hemorrhagin HP-IV from *Crotalus horridus horridus* venom to proteolyze was strongly affected by the conformation of the substrate used (Civello et al., 1983b). VT*m* also had a much higher proteolytic activity against some substrates than others.

The observation of multiple myotoxic and hemorrhagic associated activities in Crotalus viridis viridis venom fractions, and the dual activities of hemorrhage and myotoxicity observed in the viriditoxin-like isolates, as in twelve other hemorrhagins, necessitates an explanation of the commonalities of these two biological effects. It has been proposed, based on pathogenetic studies with VTwo, that the hemorrhagic activity is direct consequence of treatment with this toxin and that myonecrosis is indirectly caused by the hemorrhaged tissue (Gleason et al., 1983). Evidence and arguments for this proposed mechanism of action by VTwo and possibly other hemorrhagins will be presented in the next chapter.

CHAPTER IV

PATHOGENESIS OF HEMORRHAGE AND MYONECROSIS INDUCED BY VIRIDITOXIN-LIKE VTm AND VTwo IN MOUSE SKELETAL MUSCLE

Introduction

The pathogenesis of injury caused by rattlesnke venoms has been extensively reviewed by Ownby (1982). In addition, the pathogenesis of injury caused by two myotoxic-hemorrhagic toxins has been studied (Ownby et al., 1978; Gleason et al., 1983). When the pathogenic effects of HTb from Crotalus atrox venom on mouse skeletal muscle were studied by light and electron microscopy, it was observed that 3 hours after injection of the toxin into mouse muscle, significant damage to muscle cells had occurred (Ownby et al., 1978). The hemorrhagin viriditoxin has also been reported to have myotoxic activities (Fabiano and Tu, 1981). The investigation of pathogenesis caused by our preparations of VTm and VTwo is discussed below.

Materials and Methods

Many of the materials and methods used were described in the previous chapter. Only those unique to this chapter are described here.

Histology

The preparation of Crotalus viridis viridis venom fractions and purified VTwo used in these studies was as described previously in Chapter III. These were injected (i.m.) at a dose of 2.5 µg/gm into the dorsolateral aspect of the right hind thigh of 20-30 gm white mice, as previously described in Chapter III. After varying lengths of time, ranging from 15 minutes to 96 hr, as will be specified as experiments are described, mice were sacrified as before. The injected muscle was then immediately processed as previously described in Chapter III. Photomicrographs of tissue sections were taken using a Zeiss photomicroscope and Kodak Pan-X film.

Creatine Kinase Assay

Five minutes prior to sacrifice of mice injected (i.m.), a blood sample was withdrawn from the tail into heparinized capillary tubes. Plasma was then used to quantitate creatine kinase via the CK catalyzed ADP/phosphocreatine reaction as described by Lee et al. (1974).

Hemoglobin Method of Hemorrhagic Assay

Extravasated erythrocytes resulting from hemorrhage can be easily monitored in muscle tissue, due to its homogeneity, by determination of the hemoglobin content per gram of excised muscle (Ownby et al., 1984b). Twenty to 30 gm white mice were injected (i.m.) into the dorsolateral aspect of the right hind thigh at a dose of 2.5 µg/gm of venom fractions or purified toxins. After an appropriate

time mice were sacrified and the entire thigh musculature was excised in as reproducible manner as possible, weighed, and stored temporarily at 4 °C in a nalgene tube. Muscle was then homogenized for 30 sec in 3 mL of quartz-distilled water using a Brinkmann Polytron tissue homogenizer at a setting of five. Supernatants were obtained by centrifugation for 5 min at setting seven in an IEC clinical centrifuge. These supernatants were centrifuged at 18,500 RPM (41,000 x g) in a Beckmann JA-20 rotor for 30 min. An aliquot of 1.8 mL of supernatant was added to 5 mL of 2x Drabkin's solution, 0.2% (w/v) $NaCO_3$, 0.04% (w/v) potassium ferricyanide and 0.01% (w/v) KCN. The hemoglobin content of the supernatants was then determined at by measuring the absorbance at 540 nm on a Beckman Model DU-8 spectrophotometer along with methemoglobin standards as described by Ownby et al. (1984b). Myoglobin does not interfere with this assay. Values were calculated as mg% hemoglobin.

Results

Intial Pathogenetic Studies

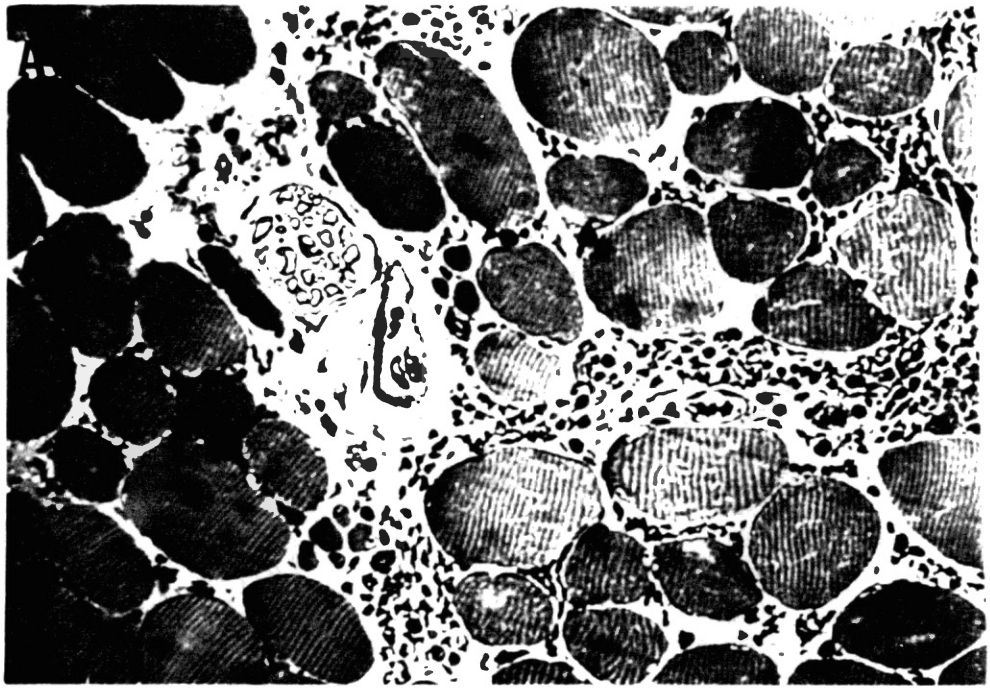
The isolation and proof of homogeneity of VTm and VTwo was described in Chapter III. In addition, it was demonstrated that VTm and VTwo cause both myonecrosis and hemorrhage 24 hr after injection (i.m.) into mouse skeletal muscle. Initial studies with the VTm isolate seen in Figure 5A of Chapter III revealed that if the muscle was observed 1 hr after injection, only hemorrhage was observed, as can be seen in Figure 22. However, by 24 hr in addition to the hemorrhage myotoxicity was observed, as can be seen in Figure 8 of

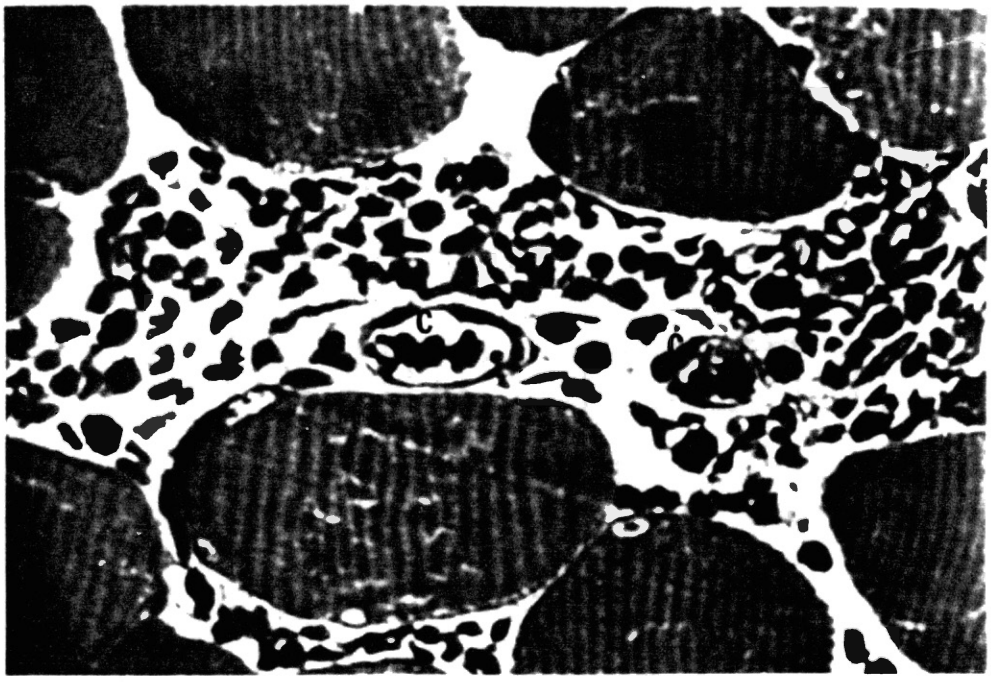
Figure 22. Light Photomicrographs of Hemorrhage in Mouse Skeletal Muscle One Hour After Injection with VTm.

Light micrographs of thick (1 μm) plastic sections stained with Mallory's trichrome.

A. Field of muscle showing extensive hemorrhage by 1 hr after injection (i.m.) with VTm. Muscle cells look normal. Magnification is x300.

B. Enlarged view of Plate A. Two capillaries (C) in the center, especially the one to the left, appear to be congested with erythrocytes. Magnification is x750.





Chapter III. This indicated that myotoxicity was occurring at some time after hemorrhage and led to more detailed studies with VTwo which have been previously reported (Gleason et al., 1983).

Outline of the Pathogenetic Study

The amount of VTwo injected into mouse skeletal muscle was 2.5 μg/gm of mouse weight. Muscle was taken from mice 15 min, 1 hr, 3 hr, 6 hr, 12 hr, 24 hr, 48 hr, 72 hr and 96 hr after intramuscular injection with VTwo and prepared and examined by light microscopy as described in the Materials and Methods of Chapter III. Plasma samples were taken from the injected mice and levels of creatine kinase subsequently determined. Total hemoglobin present in the injected mouse muscle was also measured. Histology, CK levels, and hemoglobin determinations were completed in the same mice.

General Aspects of the Pathogenesis of Hemorrhage and Myonecrosis in Histologically Examined Mouse Skeletal Muscle

As seen in Figure 23A, some hemorrhage was observed 15 min after the injection. As can be seen in Figure 23B, 6 hr after injection hemorrhage had become quite intense, yet no myonecrosis was observed. By 12 hr, striking evidence of necrosis, erythrocytes inside necrotic muscle cells, was visible as seen in Figure 24A. This anomaly is indicative of disruption of the sacrolemma, as described by others (Ownby et al., 1974, 1978). Dissolution and disorientation of myofibrils was prominent by 24 hr as seen in Figure 23B. By 72 hr macrophages were apparent in the cellular debris, and they became

Figure 23. Effect of VTwo After Injection Into Mouse Skeletal Muscle After 15 Minutes and Six Hours.

Light micrographs of thick (1μm) plastic sections stained with Mallory's trichrome.

A. 15 min after injection (i.m.) with VTwo. Note the presence of erythrocytes (E) in connective tissue, capillaries (C) still intact and normal muscle cells (NM). Magnification is x300.

B. Six hours after injection (i.m.) with VTwo. Note dense packing of erythrocytes (E) in connective tissue and normal muscle cells (NM); peripheral nerve (PN) is normal. Magnification is x250.

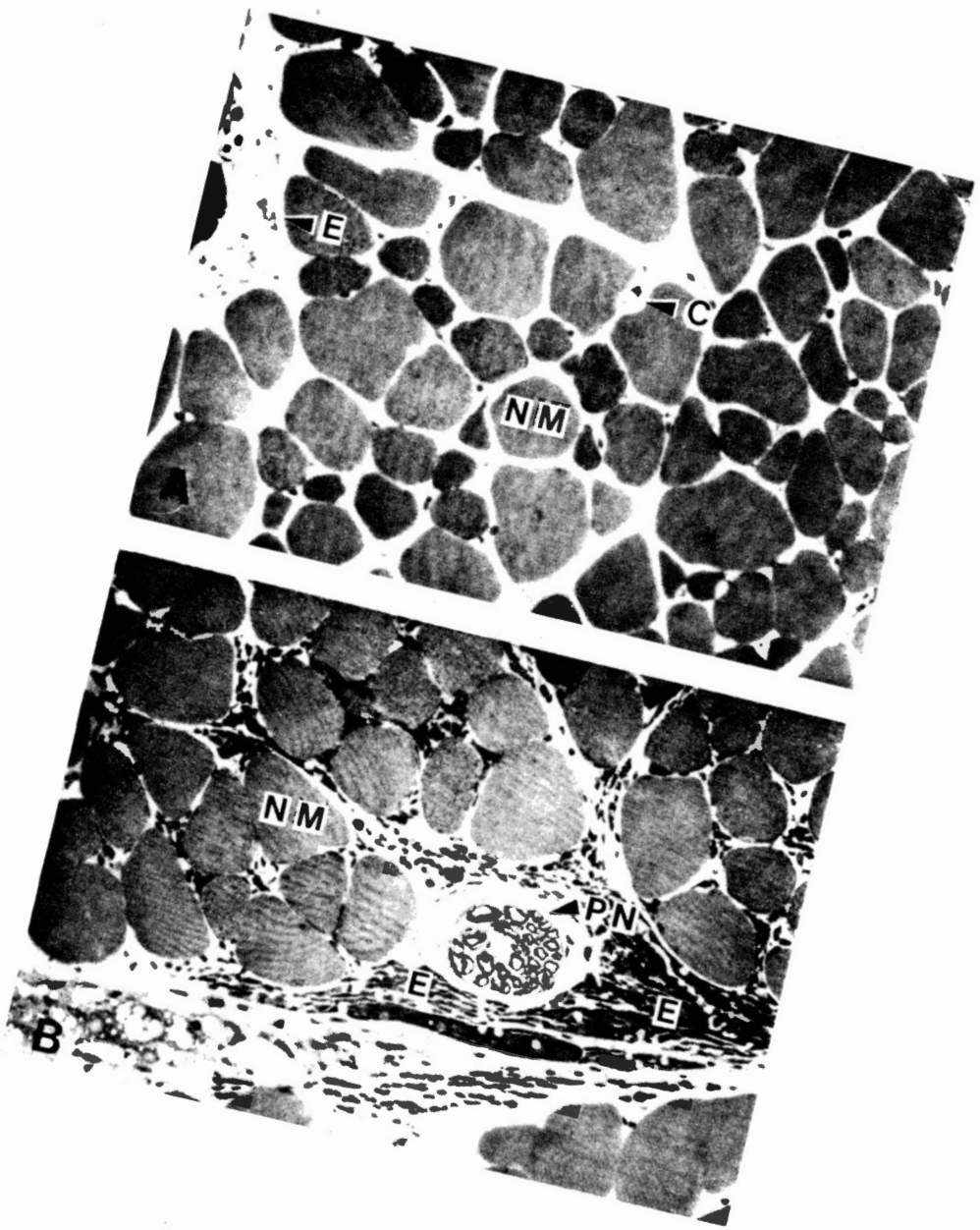
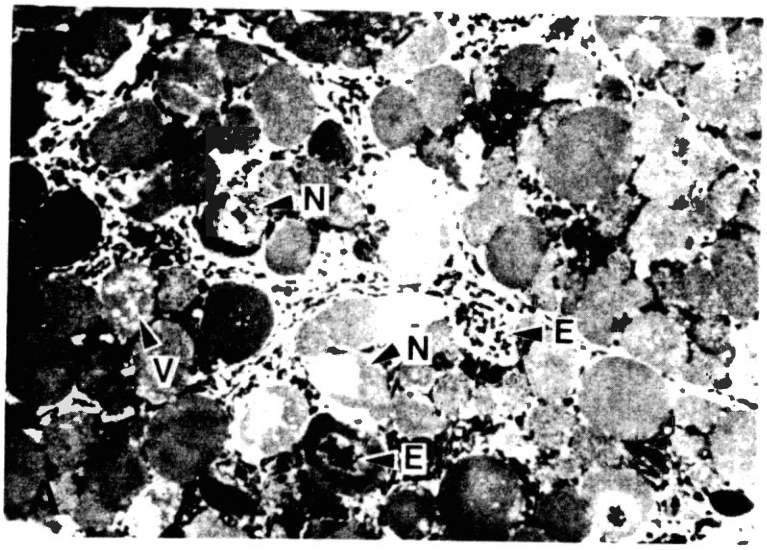

E
C
NM
NM
PN
E
E
B

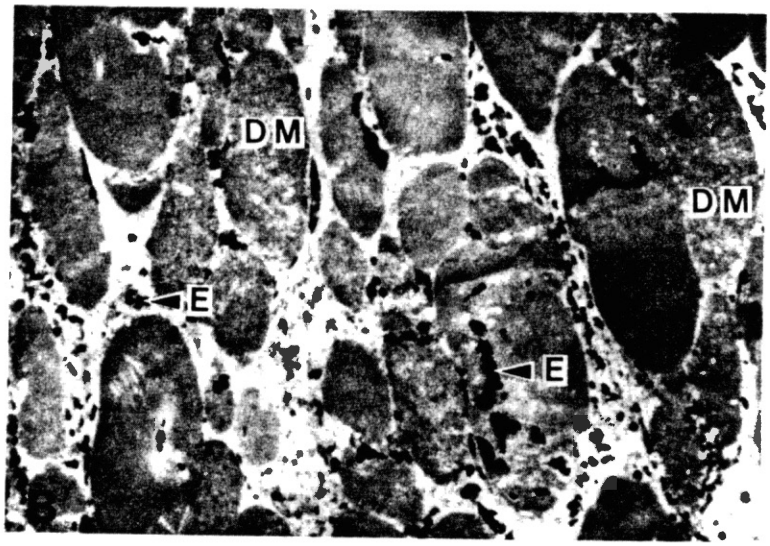
Figure 24. Effect of VTwo 12 Hours and 24 Hours After Injection Into Mouse Skeletal Muscle.

Light micrographs of thick (1μm) plastic sections stained with Mallory's trichrome.

A. Twelve hours after injection (i.m.) with VTwo. Note vacuolation (V), necrotic muscle cells (N) and erythrocytes (E) in connective tissue and within muscle cells. Magnification is x200.

B. Twenty-four hours after injection (i.m.) with VTwo. Note dispersed myofibrils (DM) and erythrocytes (E) in connective tissue and within muscle cells. Magnification is x250.


N
V
N
E
E


DM
DM
E
E

abundant by 96 hr as seen in Figure 25. At 96 hr a field of cellular debris devoid of capillaries lies adjacent to a field of normal muscle cells in which intact capillaries are present.

Histology of the Development of Hemorrhage

The progression of hemorrhage can be observed in detail by closer examination of the early histological time points. In Figure 23A, a small number of extravasated erythrocytes was visible 15 min after injection, but many intact blood vessels were still apparent. In Figure 22, from the initial study with VTm, it can be seen that after 1 hr a capillary has become plugged and that hemorrhage is quite visible. Such an observation was not made with VTwo-treated muscle; however, only a limited number of tissue sections were viewed. At 1 hr after injection with VTwo, the connective tissue had become filled with erythrocytes and no intact capillaries were visible (data not shown). The connective tissue was packed with aggregrated erythrocytes by 6 h as can be seen in Figure 23B. The presence of erythrocytes in the connective tissue was readily visible at all time points examined.

Biochemical Assessment of the Pathogenesis

Determination of the hemoglobin content of muscle tissue and of the release of creatine kinase (CK) into the plasma after injection (i.m.) of VTwo into mouse skeletal muscle provided an independent assessment of the histological data by biochemical means. The data are shown in Figure 26. Replicates of each injection were made for statistical validity. Muscle hemoglobin levels rose as an almost

Figure 25. Effect of VTwo After Injection Into Mouse Skeletal Muscle After 96 Hours.

Light micrograph of thick (1μm) plastic sections stained with Mallory's trichrome, 96 hr after injection (i.m.) with VTwo. Note highly necrotic muscle cells (N), and cellular debris being cleared by macrophages (M). Normal muscle cells (NM) have intact capillaries (C) nearby. Note the presence of erythrocytes (E) throughout the connective tissue. Magnification is x300.

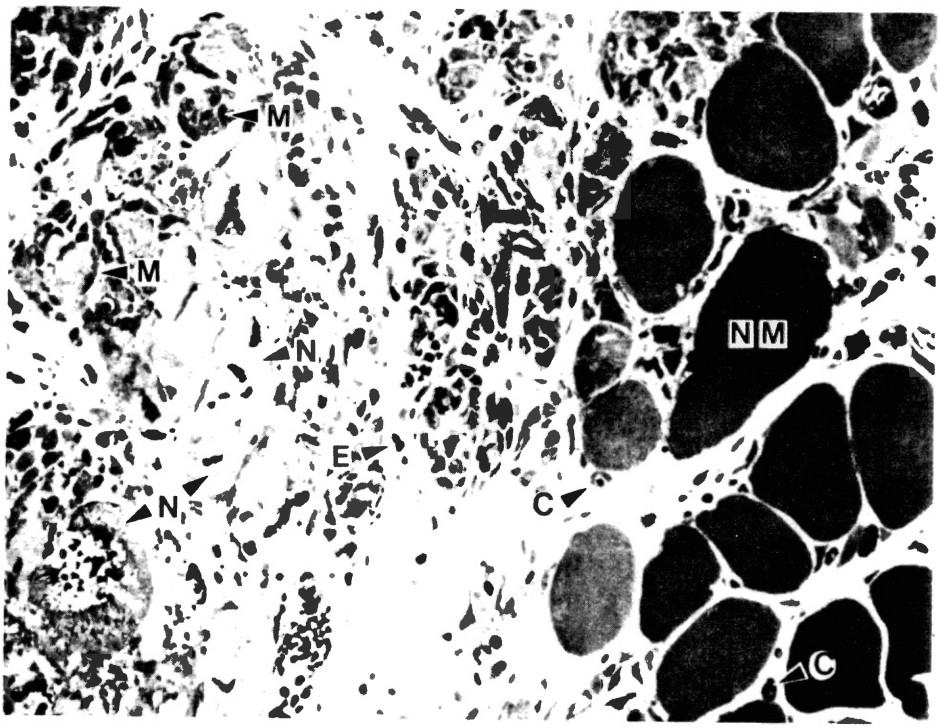
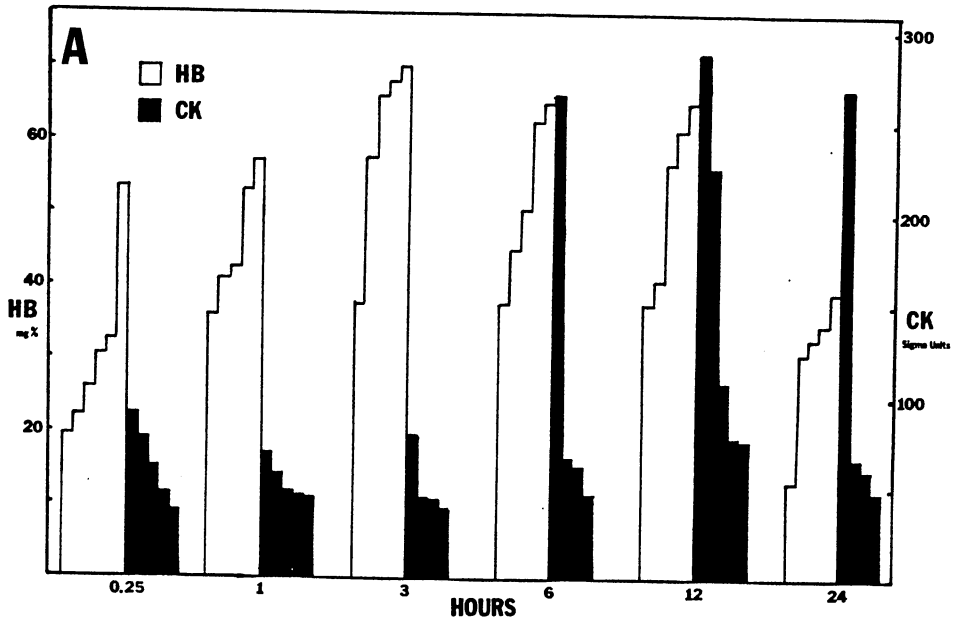

M
M
N
N
E
C
NM
C

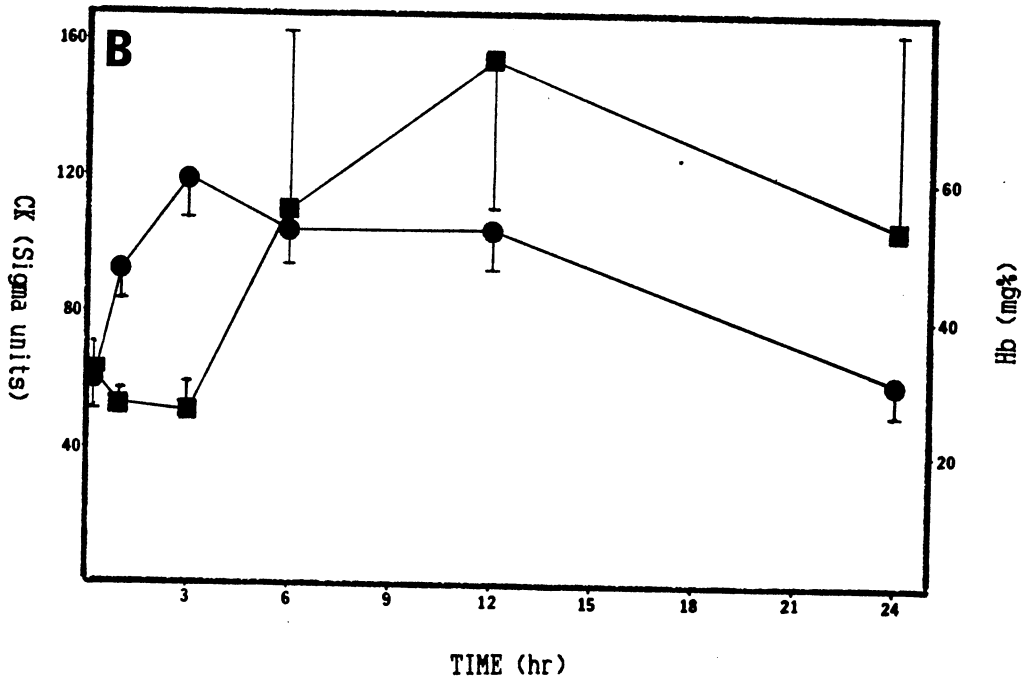
Figure 26. Time Course of Hemglobin Content and CK Level After VTwo Injection.

To obtain control values for the muscle hemoglobin content, four mice were injected with PSS and sacrificed after 15 min. The mean muscle hemoglobin content was 7.04 mg%, with a square error of the mean of ± 2.97 mg%. To obtain control values for the plasma CK level, two mice were injected (i.m.) with PSS and sacrificed after 24 hr. The mean plasma CK level was 38 Sigma units, with a square error of the mean of ± 6.0 Sigma units.

A. Levels of plasma CK (solid bars) and muscle hemoglobin (open bars) in muscle tissue obtained from individual mice injected (i.m.) with 2.5 μg/g of VTwo are presented in the histogram. Tissue and blood samples were taken for the assays as described in the Materials and Methods. Four to six mice (25-30 gm each) were used for each time interval.

B. Levels of plasma CK (■) and muscle hemoglobin (●), from the same experiment, presented graphically. Points represent mean values, and error bars represent one-half the square error of the mean.


A
HB
CK
60
40
20
HB
300
200
100
CK
0.25
1
3
6
12
24
HOURS


B
160
120
80
40
CK (Sigma units)
60
40
20
Hb (mg%)
3
6
9
12
15
18
21
24
TIME (hr)

immediate consequence of VTwo injection, becoming maximal at 3 hr. After 3 hr the muscle hemoglobin content slowly decreased, remaining quite high up to 12 hr after injection. Muscle hemoglobin content then decreased more rapidly to a medial level at least five-fold greater than controls injected with PSS. This medial level of hemoglobin content was maintained over the 24 to 96 hr time points. This correlates well with the histological observations of hemorrhage. Additionally, gross examination of dissected mice revealed that the injected muscle tissue was most edematous after 3-6 hr.

On the other hand, CK values did not sharply rise until 6 hr after injection, were maximal at 12 hr, and had begun to decline at 24 hr. CK values then slowly increased through 96 hr. The variance about the mean of the CK values at the 6 hr, 12 hr and 24 hr time points required additional analysis. While in some mice the CK levels were highly elevated above controls, the CK levels in other mice were only slightly elevated. Nevertheless, comparison of all of the 15 min, 1 hr, and 3 hr data (n=14) to all of the 6 hr, 12 hr, and 24 hr data (n=13) revealed they were significantly different by Student's t test ($p<0.02$). Additionally, the mice in which CK levels were only slightly elevated were reasonably higher in the 12 hr data (n=3) compared to the 15 min, 1 hr and 3 hr data ($p<0.2$). Additional data from this experiment are presented in Appendix C[1].

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[1]Results of CK determination in this experiment were comparable to results obtained in a prior experiment as can also be seen in Appendix C.

Discussion

The severity of hemorrhage and myonecrosis caused by rattlesnake venom toxins underscores the necessity of understanding the pathogenetic mechanisms of these activities and their relationships. While many myotoxins do not cause hemorrhage, it can be argued that hemorrhagic toxins in general can also have a myotoxic effect. The hemorrhagic and myotoxic effects of HTb from Crotalus atrox venom (Ownby et al., 1978) and viriditoxin from Crotalus viridis viridis venom (Fabiano and Tu, 1981) have been previously described. In Chapter III of this study, pools of varying degrees of purity have been found to possess associated hemorrhagic and myotoxic activities, and it was suggested that at least two myotoxic hemorrhagins were present in the venoms. Indeed, the purified viriditoxin-like isolates, VTm and VTwo, were definitely shown to be myotoxic hemorrhagins. The pathogenic effects of VTwo were characterized as reported in this chapter and previously, (Gleason et al., 1983). The pathogenic effects of HTb from C. atrox venom have been previously reported (Ownby et al., 1978), as observed at the light microscopic level and ultrastructural level. More recently, two more myotoxic hemorrhagins from this venom, HTf and HTg, have been purified and characterized (Nikai et al., 1984, 1985a). The pathogenic effects of HTa and HTe, two other hemorrhagic toxins from the same venom for which no myotoxic activity was discovered, have also been reported (Ownby et al., 1978). Two previously purified hemorrhagic principles from Trimeresusrus flavoviridis venom, HR-2a and HR-2b (Takahashi and Ohsaka, 1970a), have been reinvestigated and found to cause myonecrosis in skeletal

muscle 3 hr after injection (i.m.) (Nikai et al., 1987). Three hemorrhagic factors from the venom of Trimeresurus mucrosquamatus have been found to cause necrosis in skeletal muscle (Nikai et al., 1985b; Kishida et al., 1985). One of these toxins, mucrotoxin, causes myonecrosis 3 hr after injection (i.m.) in mouse skeletal muscle, and myonecrosis 2 hr after injection (i.v.) in mouse cardiac muscle. Hemorrhagic toxin Ac_3-proteinase from the venom of Agkistrodon acutus has been shown to cause myonecrosis in mouse skeletal muscle 1 hr after injection (i.m.) (Yagihashi et al., 1986). Three hemorrhagic toxins from Crotalus ruber ruber venom were found to have caused myonecrosis in mouse skeletal muscle 6 hr after injection (i.m.) (Mori et al., 1987). A comparison of the pathogenic effects caused by HTb and VTwo after injection into mouse skeletal muscle follows.

In pathogenetic studies on VTwo, slight hemorrhage was visible 15 minutes after injection, as well as intact capillaries. By 1 hr, hemorrhage was much more severe. The connective tissue had become filled with erythrocytes, and only one intact capillary was found in all the fields examined. In an initial experiment with VTm, 1 hr after injection two intact capillaries were found to be occluded by blood cells. When mouse skeletal muscle was observed 3 hr after injection of HTb, the endothelial cell plasma membranes were found to be disrupted (Ownby et al., 1978). Free erythrocytes were seen in the connective tissue, and the intact vessels that remained were seen to be packed with erythrocytes and platelets. The same features were seen in mouse skeletal muscle treated with HTa and HTe. Ultrastructurally, the Crotalus atrox toxins have been shown to cause hemorrhage per rhexis. While such proof is lacking for VTwo, the

rapid and nearly total destruction of the muscle's microcirculatory system by VTwo and the similarity in the histological appearance of hemorrhage caused by VTwo or C. atrox toxins tends to indicate such a mechanism.

When the effect of HTb was examined histologically 3 hr after injection of the toxin into mouse muscle, necrosis was prominent and erythrocytes were found within the necrotic muscle cells (Ownby et al., 1978). Ultrastructurally, the necrotic muscle cells were observed to have lost the integrity of the sarcolemma, as shown by the presence of erythrocytes within necrotic cells and free mitochondria in the connective tissue. In comparison, 12 hr after injection of VTwo into skeletal muscle, extravasated erythrocytes were seen within visibly necrotic muscle cells. While ultrastructural proof was not available, the disruption of the integrity of the sarcolemma is certainly implied by the presence of erythrocytes inside the necrotic muscle cells in this study and by analogy to the experiments with HTb (Ownby et al., 1978). No time course was performed on the pathogenesis of injury by HTb. Myonecrosis in this study was first visible at the 12 hr time point, becoming maximal at 24 hr after injection. Unfortunately, the pathogenetic studies on HTa and HTe were done in such a way as to preclude observation of myonecrosis occuring by any but the fastest mechanism, since only 5 min and 2 min, respectively, were allowed before sacrifice of the animal (Ownby et al., 1978). In contrast, the effects of HTf and HTg were observed 24 hr after injection (Nikai et al., 1984, 1985a) and necrosis of skeletal muscle cells was readily apparent. The slow increase in CK levels after 24 hours and clearing of cellular debris by macrophages by 72 and 96 hours observed in this

study is similar to that observed by Gutiérrez and coworkers (1986) in regenerating myonecrotic skeletal muscle damaged by Bothrops asper venom.

The development of myonecrosis after early onset of severe hemorrhage induced by VTwo implies a sequential causal correlation between the toxin's dual activities as opposed to a simple nonspecifity towards endothelium and muscle cells as previously proposed (Fabiano and Tu, 1981; Tu, 1982; Tu, 1983). This would seem to indicate an indirect rather than a direct myonecrotic mechanism, although a temporally late but direct effect can not be ruled out. One possible indirect mechanism of myonecrosis that can plausibly be induced by hemorrhage is ischemia[2].

The necrotic mechanism of ischemia has been discussed in detail in the Literature Review. Ischemic myonecrosis has been demonstrated to cause many of the same histological and ultrastructural features as hemorrhagic myonecrosis. Ischemic myonecrosis is slow, taking an hour to begin to develop in myocardial tissue after ischemia is complete. It involves dissolution of the myofibrils, disruption of the sarcolemma, dilation of the sarcoplasmic reticulum, extreme swelling of the mitochondria, accumulation of glycogen, and clotting in adjoining capillary beds. All of these effects have been noted as prominent features in hemorrhagic myonecrosis (Ownby et al., 1978; Gleason et al., 1983). Indeed when a mouse hind leg was experimentally ligated to produce ischemia, the resultant myonecrosis

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[2]Another possible indirect mechanism could be a non-muscle cell component "activated" by hemorrhage or by the hemorrhagin, acting on the muscle cells.

was difficult to distinguish from that caused by VT<u>wo</u> (Gutiérrez, unpublished data).

Certain stochastic features of the pathogenetic data support the ischemia hypothesis as well. If myonecrosis were caused by severe ischemia, it would make sense that more extensively hemorrhaged, very myonecrotic tissues would be found alongside less extensively hemorrhaged, healthier tissue. Examples of such differences observed within a field of tissue and between duplicate mice follow.

In Figure 25, a field of normal skeletal muscle containing intact capillaries as well as many extravasated erythrocytes can be seen adjacent to a field of highly necrotic muscle cells and cellular debris in which no capillaries are apparent. Examination of this photomicrograph raises an interesting question: why is there normal muscle tissue present when hemorrhage around this tissue and the adjacent necrotic tissue appears to have been so severe? The simplest answer is that the skeletal muscle did not become necrotic simply because its supply of oxygen and nutrients was not cut off. The intact capillaries of this muscle bundle, indicated in Figure 25, were probably capable of fully supplying its needs. Extravasated erythrocytes found in the healthy muscle bundle were probably from the damaged tissue and had infiltrated this area. In other words, this muscle group was not subject to the same ischemic conditions as the adjacent muscle group. This strongly implicates discontinued blood flow as a cause of myocyte death in the necrotic tissue.

Myonecrosis was unevenly distributed from mouse to mouse. Some mice displayed a severe rise in blood CK levels while others retained a near-normal but nevertheless elevated level. The simplest

explanation for this phenomenon is that hemorrhage was more extensive in some mice than in others, and that the extent of hemorrhage would have a dramatic effect on the development of myonecrosis. Moderate hemorrhage could be present without causing ischemic conditions in the muscle tissue, while somewhat more severe hemorrhage would create extensive regions of ischemic, necrotic muscle tissue. Differences in the extent of hemorrhage in different mice could be due to the individual nature of the mouse musculatures injected in combination with slight variations in the position of the injection and/or the injection's infusion into the tissue.

A relevant point about ischemia is that the extent of calcium loading in hepatic cells is not sufficient to cause necrosis of ischemic hepatocytes, unless sufficient Ca^{++} is supplied by reperfusion (Farber et al., 1981). This is in contrast to muscle cells, which have large intracellular stores of Ca^{++}. Nikai et al. (1984) investigated the effect of the myotoxic hemorrhagin HTf on non-muscle tissues, and observed hemorrhage in these tissues 24 hr after injection (i.v.), but no necrosis. Similar results have been obtained with hemorrhagic factors HF_a and HF_b from Trimeresurus mucrosquamatus venom (Nikai et al., 1985b). With both toxins, hemorrhage was observed in the stomach 24 hr after injection (i.v.). When the lungs and liver were investigated after injection (i.v.) of HF_b, hemorrhage was found. However, no necrosis was found in any of these tissues. In another reported example, hemorrhage but no necrosis was observed in the kidneys, stomach and lungs 2 hr after injection (i.v.) of mucrotoxin from Trimeresurus mucrosquamatus venom, while both hemorrhage and necrosis were observed in cardiac muscle with the same

treatment (Kishida et al., 1985). In light of the studies presented in the Literature Review, the probable reason why no necrosis occurred in these tissues after treatment with known myotoxic-hemorrhagins is that the flow of plasma was cut off and uptake of sufficient Ca^{++} to cause coagulative necrosis could not occur. Thus, the evidence in this case would not contradict an ischemic process at work in all tissues examined, but rather tends to support such a hypothesis.

Until these studies nothing was known about the time course of pathogenesis of injury by myotoxic-hemorrhagins. An excellent study since has much more completely detailed the pathogenesis of hemorrhage (Ownby and Geren, 1987). It has been speculated that these hemorrhagic toxins which have more than one biological activity have a decreased membrane specificity (Tu, 1983), thereby deleteriously interacting with both endothelial and sarcolemmal membranes. The evidence presented argues against this suggestion and supports the hypothesis that the myonecrosis observed is a consequence of ischemia caused indirectly by the hemorrhagic toxin.

CHAPTER V

SUMMARY AND CONCLUSIONS

The biochemistry of snake venoms is studied in order to investigate the nature of the toxic actions of snake venoms through the characterization of their components. The hope of such studies is to improve the treatment for and prevent injury from snakebite through the clarification of the mechanism of action of these toxins. In this study, the isolation and biochemical and pathogenetic characterization of a tissue-damaging toxin as isolated from two venoms of the prairie rattlesnake, Crotalus viridis viridis, was undertaken.

It was found that at least two myotoxic hemorrhagins were present in the venoms of Crotalus viridis viridis. The further characterization of VTm and VTwo, the components similar to the previously described viriditoxin (Fabiano and Tu, 1981) was undertaken. These proteins were found to vary slightly in molecular weight and in amino acid composition between the two venoms studied. VTm and VTwo were found to cause both hemorrhage and myonecrosis in mouse skeletal muscle. VTm was found to be weakly proteolytic against a variety of substrates, with a marked preference for some substrates over others, and was further found to be completely inhibited by metal chelation in both its proteolytic and hemorrhagic activities. Some evidence was found that this study may contain the first isolation of a completely pure viriditoxin-like component from C. viridis viridis

venom.

Another myotoxic hemorrhagin was also discovered in both venoms of Crotalus viridis viridis and partially characterized. It was found to vary slightly in its ion-exchange behavior between the two venoms. This toxin was found to be highly proteolytic against a variety of substrates, and was estimated to have a major polypeptide molecular weight of 77 kdal by SDS-PAGE.

In the study of the time course of pathogenesis of hemorrhage and myonecrosis in mouse skeletal muscle induced by VTwo, it was found that hemorrhage began immediately after injection and then became intense several hours before myonecrosis was seen. Muscle damage, as well as occuring late, was found to be non-uniform.

The above observations as well as various pieces of circumstantial evidence from this study and others point to ischemia as the mechanism of myonecrosis induced by hemorrhagic toxins such as VTm and VTwo. This could be substantiated by ultrastructural observation of calcium accumulation in the sarcoplasmic reticulum and mitochondria of damaged muscle tissue and by biochemical observation of thromboxane A_2 breakdown products and decreased membrane arachadonic acid content in the damaged tissue. The ischemia hypothesis could be further verified by prevention of myonecrosis in the hemorrhaged tissue through the use of calcium antagonists, thromboxane A_2 antagonists, and forced oxygenation with low-calcium reperfusion. If ischemia is indeed the mechanism of hemorrhagin-induced myonecrosis, there is a greater hope of treating the severe local effects of these toxins, as calcium antagonists (Nayler, 1981; Henry et al., 1977) thromboxane A_2 antagonists and synthase inhibitors (Lefer, 1985),

low-calcium reperfusion (Nayler, 1981; Shine, 1981), and mild hypothermia (Nayler, 1981) have been shown to reduce or prevent permanent damage to ischemic myocardium.

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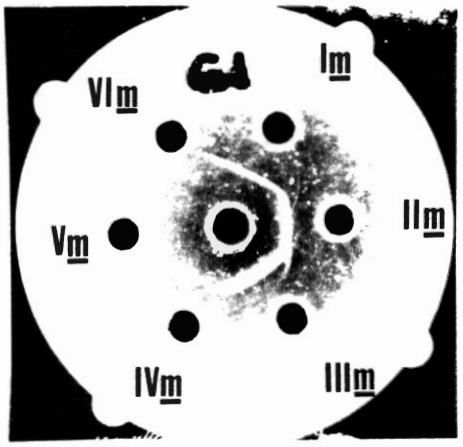
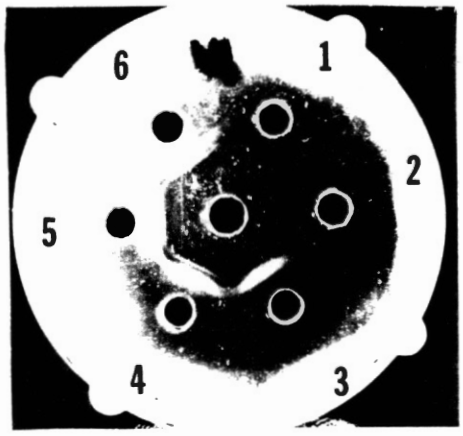
APPENDIXES

APPENDIX A

OUCHTERLONY AGAROSE GEL-DIFFUSION PLATE ANALYSIS

Rabbit antiserum to VTm (Figure 5A) was placed in the center well of the plates below. Plate A outer wells are gel filtration pools of crude venom as indicated. Plate B outer wells 1 and 2 are preimmune serum; wells 3 through 6 are VTm.

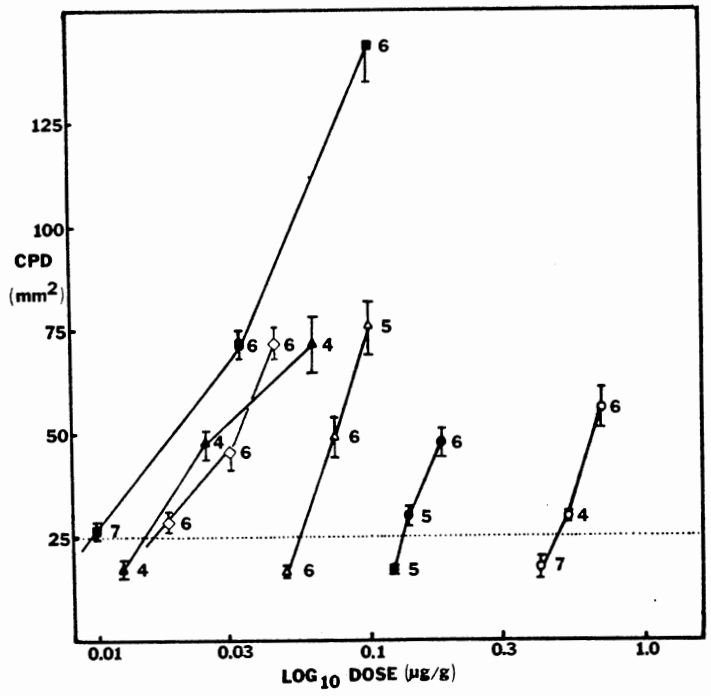




APPENDIX B

MINIMUM HEMORRHAGIC DOSE DETERMINATIONS

Graph shows the determination of MHD for crude venom, pools, and isolates. Numbers to the right of individual points are the number of mice used to determine the CPD at that dose. Error bars represent the square error of the mean. Where curves intersect the dotted line corresponds to the MHD. Curves shown are as follows: EDTA-treated crude venom (○); crude venom (●); pool II<u>m</u>, Figure 15 (△); VT<u>m</u>, Figure 19 (◇); VT<u>wo</u> (▲); Pool III<u>m</u>, Figure 15, pooled as in Figure 3 (■).



APPENDIX C

VALUES OF CREATINE KINASE AND MILLIGRAM PERCENT HEMOGLOBIN IN TIME COURSE OF PATHOGENESIS EXPERIMENTS

Additional Data from Experiment shown in Figure 26.

Time After Injection of VTm (hr)	Hb (mg%)	CK (Sigma units)
48	20.0,23.7	84.2,79.8
72	27.5,24.8	115.4,51.4
96	22.6,17.2	82.2,94.2

Previous Time Course Following Development of CK Levels

Time After Injection	CK (Sigma units)
0.25	116
1	112
3	88,152
6	68,120
12	248,196
24	280,72
48	44,32
72	44,72
96	80,52

VITA

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Doctor of Philosophy

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