ISOLATION OF HEMORRHAGIC TOXIN B FROM *CROTALUS ATROX* VENOM: AFFINITY PURIFICATION OF ANTIBODIES AND THEIR USE IN NEUTRALIZATION OF HEMORRHAGE, DETECTION OF THE TOXIN IN OTHER SNAKE VENOMS, AND IMMUNOHISTOCHEMICAL LOCALIZATION OF HTB IN SKELETAL MUSCLE

By

MICHAEL SCOTT SMITH

Bachelor of Science
Guilford College
Greensboro, North Carolina
1979

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1984

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1987

ISOLATION OF HEMORRHAGIC TOXIN B FROM CROTALUS ATROX VENOM: AFFINITY PURIFICATION OF ANTIBODIES AND THEIR USE IN NEUTRALIZATION OF HEMORRHAGE, DETECTION OF THE TOXIN IN OTHER SNAKE VENOMS, AND IMMUNOHISTOCHEMICAL LOCALIZATION OF HTB IN SKELETAL MUSCLE

Thesis Approved:

Charlotte L. Overby
Thesis Adviser

Bruce A. Carlson

George V. Odell

Calvin H. Beamer, Jr.

Norman N. Durham
Dean of the Graduate College

PREFACE

I wish to extend my sincere gratitude to Dr. Charlotte L. Ownby for her continual support throughout my graduate program, acting both as advisor and friend. She was always willing to help in every possible way. I would also like to thank Dr. Bruce Lessley for his excellent assistance, and Drs. George Odell and Calvin Beames for serving as my committee members.

A word of thanks is also due to the Veterinary Medicine Research Advisory Council and Solvay America for deeming this project as one worth funding. I am grateful to the McAlester Scottish Rite Foundation and the OSU Foundation for the fellowships awarded to me. I would like to express my appreciation to my fellow graduate students and the staff of the department of Physiological Sciences for their many contributions and smiling faces, especially Dr. Mary Bober, Ed Johnson, and Terry Colberg

Finally, I want my family to know just how much their support meant to me throughout this program. Thanks are due to my brothers, Chip and Jeff, my sister, Debbie, my neat nephew, Logan, and my new dad, Don. I dedicate this and future accomplishments to my mother and my wife for their uncompromising faith and love. My need for them cannot be expressed.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

A254	Absorbance at 254 nm
A280	Absorbance at 280 nm
A340	Absorbance at 340 nm
A405	Absorbance at 405 nm
A578	Absorbance at 578 nm
BAEE	Benzyol-L-Arginine Ethyl Ester
BSA	Bovine Serum Albumin
^{o}C	Degree Centigrade
CaCl2	Calcium Chloride
cm	Centimeter
DW	Distilled Water
ELISA	Enzyme-Linked Immunosorbent Assay
FITC	Fluoroscein Isothiocyanate
gm	Gram
HCl	Hydrochloric Acid
hr	Hour
HTb	Hemorrhagic Toxin b
I.B.	Incubation Buffer
IgG	Immunoglobulin G
i.m.	Intramuscular
i.v.	Intravenous
i.p.	Intraperitoneal

kg	Killogram
LD_{50}	Lethal Dose 50%
M	Molarity (moles/liter)
mA	Milliamperes
mg	Milligram
$MgCl_2$	Magnesium Chloride
MHD	Minimum Hemorrhagic Dose
min	Minute
ml	Milliliter
mM	Millimolar
mm	Millimeter
mU/ml	Milliunits per Milliliter
NaCl	Sodium Chloride
Na_2CO_3	Sodium Carbonate
$NaHCO_3$	Sodium Bicarbonate
NaN_3	Sodium Azide
NaOH	Sodium Hydroxide
ng	Nanogram
$(NH_4)_2SO_4$	Ammonium Sulfate
nm	Nanometer
pI	Isoelectric Point
PBS	Phosphate Buffered Saline
PSS	Physiological Saline Solution
r	Sample Correlation Coefficient
rpm	Revolutions per Minute

rs	Crude Rabbit Antiserum
s.c.	Subcutaneous
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
TNBS	Trinitrobenzene Sulfonic Acid
Tween 20	Polyoxyethylene Sorbitan Monolaurate

CHAPTER I

INTRODUCTION

The world population of rattlesnakes consists of thirty-one species and seventy subspecies (Klauber, 1972), with six new subspecies awaiting recognition (Glenn and Straight, 1982). Of these, fifteen species and tweny-three subspecies reside in the United States (Glenn and Straight, 1982). Rattlesnake venom poisoning represents a serious medical problem, both in the United States and throughout the world, though the significance of these problems vary. Lethal effects of venoms, obviously the most serious of sequelae, are not a major concern, statistically, in the United States. Parrish (1980) recorded only fifteen deaths out of 6,680 total rattlesnake bites, based on a survey of hospitals for the year 1959. This represents an approximate 0.2% fatality rate. Deaths due to poisonous snake bites are estimated to exceed 40,000 per year, however, throughout the world (Swaroop and Grab, 1956). There are two primary reasons for the markedly fewer deaths in the United States: 1) the exceedingly large number of medical facilities, which can usually be reached relatively quickly, and 2) the widespread use of Wyeth's polyvalent (Crotalidae) antivenin, an antiserum shown to be effective

in preventing lethal effects of many different snake venoms (Minton, 1954; Ownby et al., 1983; Russell et al., 1973).

The major medical concerns in the United States are the local effects that often accompany rattlesnake venom poisoning. These effects include hemorrhage, myonecrosis, and edema, often leading to serious debilitation of the affected appendage. Local reactions to venom poisoning are due to a variety of toxins present in the venoms. Polyvalent antivenin has been shown to be effective against local hemorrhage (Ownby et al., 1984; Smith and Ownby, 1985). However, this antivenom offers little protection to myonecrosis induced by some venoms (Ownby et al., 1983b), although Ownby and Colberg (1986) reported the neutralization of a significant dose of Crotalus atrox venom in mice.

Current research includes studies on toxins present in venoms. These efforts include, among others, isolation and biochemical characterization of toxins, as well as studies on mechanisms of action and pathogenesis of the various sequelae. Because of the complexity of rattlesnake venoms, such research is no small task. The results of these multiple studies will hopefully aid in the development of more effective treatments, perhaps an antivenom capable of preventing or reducing not only lethal effects, but local reactions as well.

As previously stated, hemorrhage is a major result of rattlesnake venom poisoning and has received a great deal

of attention in the literature. Current views on mechanisms of hemorrhage and characteristics of many hemorrhagic toxins thus far isolated are presented in the following chapter. One of these toxins, hemorrhagic toxin b (HTb), was originally isolated by Bjarnason and Tu (1978) from the venom of the western diamondback rattlesnake (C. atrox). It is characterized as a metalloprotease, requiring zinc for activity, representing approximately 3.7% of the total venom protein. It is capable of inducing not only hemorrhage, but myonecrosis as well (Ownby et al., 1978). The following paper describes the isolation of HTb. Also presented are procedures employed in the production and affinity purification of antibodies to this toxin, and the ability of these antibodies to neutralize hemorrhage induced by HTb. Venom samples from several species of snakes have been screened, using the enzyme-linked immunosorbent assay, for the presence of HTb, or cross-reacting proteins, utilizing the affinity-purified antibodies. Finally, results of the immunohistochemical localization of this toxin in skeletal muscle are presented.

CHAPTER II

REVIEW OF LITERATURE

In recent years, efforts of researchers have been directed at the isolation and characterization of snake venom components, largely due to the complexity of these venoms and the difficulty in studying the biological effects of the venoms as a result of their complex nature. Snake venoms are known to contain a wide variety of enzymes, mostly hydrolases, that include phospholipases, proteases, arginine esterases, phosphodiesterases, amino acid oxidases, deoxyribo- and ribonucleotidases, coagulant and anticoagulant factors (Iwanaga and Suzuki, 1979), and collagenases (Hong, 1982). Karlsson (1979) also points out the presence of nonenzymatic toxic proteins in venoms, such as the cardiotoxins and neurotoxins. Members of the Elapidae and Hydrophiidae families contain no known arginine esterase activities in their venoms, while venoms from elapids have little endopeptidase activity (Iwanaga and Suzuki, 1979). However, crotalid and viperid venoms contain extensive proteolytic and arginine esterolytic activities (Deutch and Diniz, 1955; Hamberg and Rocha E. Silva, 1957; Devi et al., 1959). Much of the clotting and bradykinin releasing activities, resulting in the

hemostatic alterations typical of these venoms, have been shown to be due to the esterase activities of these venoms (Hamberg and Rocha E. Silva, 1957; Devi et al., 1959). The complexity of snake venoms becomes very obvious when one considers not only the above mentioned enzymatic constituents but also the pharmacological and biological effects of venoms.

The effects of snake venoms on mammals have been extensively studied (Mebs, 1978), and there are many reports on the pharmacological and biological actions of venoms and their compnonets. These activities include: (1) hemorrhage, due to the presence of hemorrhagic toxins; (2) myonecrosis, due to myotoxins, and as a secondary effect to ischemia induced by hemorrhage; (3) hemostatic alterations, due to coagulant and anticoagulant enzymes; (4) effects due to neurotoxins; (5) cardiovascular compromise due to fluid loss during hemostatic alterations and hemorrhage as well as a result of the direct action of cardiotoxins; and (6) cytotoxic effects. Members of the Elapidae and Hydrophiidae families have venoms that result primarily in neurotoxic, myotoxic and cardiotoxic effects. Members of the Crotalidae and Viperidae families contain venoms that induce primarily hemorrhagic and myotoxic effects and hemostatic dysfunction. Exceptions to this general classification exist, including the two species Crotalus durissus terrificus and Crotalus scutulatus scutulatus, which are known to induce serious neurotoxic

effects due to the presence in the venoms of crotoxin and mojave toxin, respectively.

Some Biological Activities of Snake Venoms

Hemorrhage: Hemorrhage is the most pronounced effect observed with rattlesnake venom poisoning and is an activity common to all viperid and crotalid snakes. Exceptions are C. s. scutulatus (Homma and Tu, 1971; Glenn et al., 1983) and Vipera russelli siamensis (Homma and Tu, 1971). There are two mechanisms that allow loss of blood from the vascular system as a result of snake venom poisoning (Ownby, 1982): (1) through either damaged endothelial cells, hemorrhage per rhexis, and (2) through widened intercellular junctions, hemorrhage per diapedesis. Crude C. atrox venom (Ownby et al., 1974), as well as three hemorrhagic toxins isolated from this venom, hemorrhagic toxins a, b, and e (Ownby et al., 1978), induce hemorrhage per rhexis. In an electron microscopy study, Ownby et al. (1978) described the pathogenesis of hemorrhage induced by these three toxins. Hemorrhagic toxins a and e both induced hemorrhage rapidly while hemorrhagic toxin b (HTb) demonstrated a latency period prior to the onset of hemorrhage. All three appeared to act directly on the endothelial cell, resulting in hemorrhage per rhexis. The endothelium after exposure to these toxins was described as having gaps adjacent to intact intercellular junctions,

with areas of the cell becoming very thin prior to rupture. The basal lamina was disrupted in all cases, and platelet aggregations were also typical (Ownby, 1982). The same type of necrosis was observed with the crude venom, although some myonecrosis was also present. In a recent study on the hemorrhagic proteinase HP-IV, isolated from the venom of C. horridus horridus (Civello et al., 1983a), Ownby and Geren (1987) showed that this toxin induced hemorrhage per rhexis. An intramuscular injection of the toxin caused hemorrhage within five minutes, resulting in gaps in the endothelial cells, many of which were severely swollen, especially at later time periods following injection of the toxin. Intact intercellular junctions were observed adjacent to damaged areas of the cells. Some regions of the basal lamina remained intact while other areas were disrupted. This toxin caused the release of proteins from the glomerular basement membrane (Civello et al., 1983b), an activity that may aid the hemorrhagic activity of the toxin. The toxin also induced hemolysis at all time periods, caused platelet aggregations, seen as thrombi in vessels, and resulted in fibrin deposition, an activity not observed with the hemorrhagic proteases isolated from C. atrox venom (Ownby et al., 1978).

Hemorrhage per diapedesis is caused by the venom of Trimersurus flavoviridis. Ohsaka et al. (1975) showed in an electron microscopic study that erythrocytes leave the vascular compartment through widened intercellular

junctions following treatment with this venom. The endothelial cells remained intact. This type of hemorrhage was also demonstrated using the purified hemorrhagic component HR1, isolated from T. flavoviridis, using cinematographic and electron microscopic analyses (Tsychiya et al.,1974). According to Ohsaka et al. (1973), the movement of erythrocytes through widened intercellular junctions was aided by the fact that the hemorrhagic principles of the crude venom all disrupted the basement membrane (Ohsaka et al., 1973), compromising the structural integrity of the vessel wall. Also, the hemorrhagic principles, including HR1, HR2a, and HR2b, induce the release of vasoactive mediators, such as histamine and serotonin, from some organs and tissues, which may facilitate the widening of the junctions (Ishida et al., 1975).

Table I lists some of the hemorrhagic components isolated from snake venoms. Hemorrhagic toxins isolated from snake venoms are heterogeneous, having different isoelectric points and molecular weights. Kurecki and Kress (1985) reported the isolation of a single protein from the venom of C. adamanteus responsible for all hemorrhagic activity of that venom. This toxin, proteinase H, has a molecular weight of 85,700 daltons and an isoelectric point of 6.1. C. atrox venom, however, has at least seven hemorrhagic components, some with basic and some with acidic isolelectric points, with molecular

TABLE I

SOME PROPERTIES OF HEMORRHAGIC TOXINS
ISOLATED FROM SNAKE VENOMS

Toxin	Species	Molecular Weight	Isoelectric Point	Proteolytic Activity	Reference
HTa	Crotalus atrox	68,000	acidic	+	Bjarnason and Tu (1978)
HTb	Crotalus atrox	24,000	basic	+	Bjarnason and Tu (1978)
HTc	Crotalus atrox	24,000	acidic	+	Bjarnason and Tu (1978)
HTd	Crotalus atrox	24,000	acidic	+	Bjarnason and Tu (1978)
HTe	Crotalus atrox	25,700	5.6	+	Bjarnason and Tu (1978)
HTf	Crotalus atrox	64,000	7.7	+	Nikai et al. (1984)
HTg	Crotalus atrox	60,000	6.8	+	Nikai et al. (1985)
Protein H	Crotalus adamanteus	85,000	6.1	+	Kurecki and Kress (1985)

TABLE I (Continued)

Toxin	Species	Molecular Weight	Isoelectric Point	Proteolytic Activity	Reference
HP-IV	Crotalus horridus horridus	57,000	5.1	(-)casein (+)hide powder	Civello et al. (1983)
Viriditoxin	Crotalus viridis viridis	115,000	4.8	+	Fabiano and Tu (1981)
HF2	Bothrops jararaca	50,000	n.d.	+	Mandelbaum et al. (1976)
NHFa	Bothrops neuwiedi	46,000	4.2	+	Mandelbaum et al. (1984)
NHFb	Bothrops neuwiedi	58,000	4.2	+	Mandelbaum et al. (1984)
AC1	Agkistrodon acutus	24,500	4.7	+	Nikai et al. (1977)
AC4	Agkistrodon acutus	33,000	4.4	+	Sugihara et al. (1980)
AC5	Agkistrodon acutus	24,000	6.7	+	Mori et al. (1984)
AaHI	Agkistrodon acutus	22,000	4.6	+	Xu et al. (1981)

TABLE I (Continued)

Toxin	Species	Molecular Weight	Isoelectric Point	Proteolytic Activity	Reference
AaHII	Agkistrodon acutus	22,000	5.3	+	Xu et al. (1981)
AaHIII	Agkistrodon acutus	22,000	>9.0	+	Xu et al (1981)
Proteinase B	Agkistrodon halys blomhoffii	95,000	4.18	+	Oshima et al. (1972)
HR 1	Trimeresurus flavorviridis	104,000	4.3	(-)casein (+)glomerular basement membrane	Omori-Satoh and Ohsaka (1970) Ohsaka et al. (1973)
HR 2a	Trimeresurus flavoviridis	n.d.	basic	(-)casein (+)glomerular basement membrane	Takahashi and Ohsaka (1970) Ohsaka et al. (1973)
HR 2b	Trimeresurus flavoviridis	n.d.	basic	(-)casein (+)glomerular basement membrane	Takahashi and Ohsaka (1970) Ohsaka et al. (1973)
Mucrotoxin A	Trimeresurus flavoviridis	94,000	4.3	+	Sugihara et al. (1983)

TABLE I (Continued)

Toxin	Species	Molecular Weight	Isoelectric Point	Proteolytic Activity	Reference
HR 1	Trimeresurus gramineus	23,500	acidic	+	Ouyang and Huang (1979)
HR 2	Trimeresurus gramineus	81,500	acidic	+	Huang et al. (1984)
Hemorrhagin	Vipera palestinae	44,000	acidic	+	Grotto et al. (1967)
HR 1	Vipera palestinae	60,000	basic	+	Ovadia (1978)
HR 2	Vipera palestinae	60,000	acidic	+	Ovadia (1978)
HR 3	Vipera palestinae	60,000	acidic	n.d.	Ovadia (1978)

n.d. = not determined

(-) = absence of proteolytic activity; (+) = presence of proteolytic activity

weights ranging from 68,000 daltons to 24,000 daltons (Bjarnason and Tu, 1978; Nikai et al., 1984; Nikai et al., 1985).

Thus far, all hemorrhagic toxins isolated from crotalid venoms have proteolytic activity on a common substrate, such as casein or hide powder azure, with the exception of the three principles, HR1, HR2a, and HR2b, isolated from T. flavoviridis venom. These principles do, however, cause the release of peptides and carbohydrates from the isolated glomerular basement membrane preparation (Ohsaka et al., 1973). The hemorrhagic protease, HP-IV, from C. h. horridus venom, showed no activity on casein but did hydrolyze hide powder azure (Civello et al., 1983a). It has been pointed out that venom proteases often have very specific substrate requirements, and the lack of protease activity on one substrate is not exclusive to activity on another substrate (Bjarnason and Tu, 1978; Civello et al., 1983b). With time, it may become evident that all hemorrhagic toxins are proteolytic on one type of substrate or another (Bjarnason and Tu, 1978; Huang and Perez, 1980). There is some question as to the relationship between proteolytic activity and hemorrhagic activity. Bjarnason and Tu (1978) found that there was an inverse relationship between the hemorrhagic activity and proteolytic activity of hemorrhagic toxin a. Fox et al. (1986) proposed that the major step leading to snake-venom induced hemorrhage "appears to be the disruption of the

pericellular collagenous basement membrane and other connective tissue collagens by proteolytic toxins...," though Civello et al. (1983b) detected no collagenous activity of HP-IV, a potent hemorrhagin isolated from the venom of C. h. horridus (Civello et al., 1983a). The toxin did, however, cause the release of proteins from the glomerular basement membrane preparation (Civello et al., 1983b). The differences in the mechanisms of action leading to hemorrhage, i.e., hemorrhage per rhexis or diapedesis, however, indicates the need for more work before a clear classification of the actions of hemorrhagic toxins can be made.

Myonecrosis: Myonecrosis is a complicated sequelae to snake venom poisoning. It results from the direct action of toxins on muscle (Ownby, 1982) and may result from the indirect effect of ischemia due to hemorrhagic components in the venoms (Gleason et al., 1983). There are several reports of myonecrosis following poisonous snakebites, and all four families of snakes are known to have venoms capable of damaging muscle (Reid, 1964; Tu, 1977; Campbell, 1979; Efrati, 1979; Reid, 1979). In a report by Homma and Tu (1971), of 37 crotalid, viperid and elapid venoms tested, all induced the necrosis of muscle except one elapid, Ophiophagus hannah. Also in this report, Homma and Tu (1971) proposed the classification of skeletal muscle necrosis based on light microscopic observations into the following categories: (1) myolytic necrosis - typical of

elapid venoms, is characterized by the presence of dense, clumped myofilaments; (2) coagulative necrosis - typical of viperid and crotalid venoms, demonstrates a hyaline, amorphous appearance of the cell; and (3) mixed necrosis - typical of crotalid venoms, is characterized by the presence of cells undergoing both myolytic and coagulative necrosis. However, in all cases, no "completely pure type of muscle necrosis" was present. The fact that all samples were taken at 24 hours following injection may indicate that the types of necrosis described may represent a sequential series of steps or stages leading to necrosis and cellular death (Ownby, personal communication). The resolution of this question requires a study involving the sampling of tissue at various times following injection to discern a pattern of necrosis, if such a pattern of sequential stages does exist. There have been several myotoxic components isolated from snake venoms. Table II presents some of these toxins.

Cardiotoxic Myotoxins: The cardiotoxins are small polypeptides that are frequently referred to as membrane active proteins (Chang, 1979). Experimental evidence indicates that these toxins bind the cell membrane, possibly through electrostatic interactions, and then insert a hydrophobic portion of it's molecule into the membrane, disrupting the membranal integrity (Klibansky et al., 1968; Dufourcq et al., 1982). These toxins are also directly hemolytic (Mebs, 1978). There have been several

TABLE II

MYOTOXIC COMPONENTS ISOLATED
FROM SNAKE VENOMS

Toxin	Species	Molecular Weight	pI	Enzymatic/Toxic Activities	Reference
		SMALL BASIC MYOTOXINS			
Myotoxin a	Crotalus viridis viridis	4,400	9.6	Hemolytic	Cameron and Tu (1977) Ownby et al. (1976)
Crotamine	Crotalus durissus terrificus	4,900	>10.3	None	Goncalves (1956)
Peptide C	Crotalus viridis helleri	4,990	>10	Vasculo-toxic	Maeda et al. (1978) Russell et al. (1978)
Myotoxin I	Crotalus viridis concolor	4,500	>10.6	None	Engle et al. (1983)
Myotoxin II	Crotalus viridis concolor	n.d.	>10.6	None	Engle et al. (1983)
		HEMORRHAGIC MYOTOXINS			
HTb	Crotalus atrox	24,000	basic	Protease Hemorrhagic	Bjarnason and Tu (1978)

TABLE II (Continued)

Toxin	Species	Molecular Weight	pI	Enzymatic/Toxic Activities	Reference
Viriditoxin	Crotalus viridis viridis	115,000	4.8	Protease Hemorrhagic	Gleason et al. (1983)
Mucrotoxin A	Trimersurus flavoviridis	94,000	4.3	Protease Hemorrhagic	Sugihara et al. (1983)
		PHOSPHOLIPASE MYOTOXINS			
Myotoxin	Agkistrodon bilineatus	15,070	acidic	Phospholipase	Mebs and Samejima (1986)
Myotoxin	Agkistrodon mokeson	14,570	acidic	Phospholipase	Mebs and Samejima (1986)
Myotoxin	Agkistrodon piscivorus	13,720	acidic	Phospholipase	Mebs and Samejima (1986)
Myotoxin	Bothrops asper	10,700	basic	Phospholipase Hemolytic	Gutierrez et al. (1984a)
Myotoxin	Bothrops nummifer	16,000	basic	None	Gutierrez et al. (1986)
Crotoxin	Crotalus durissus terrificus	24,550	4.7	Phospholipase Neurotoxic	Breithaupt et al. (1974)

TABLE II (Continued)

Toxin	Species	Molecular Weight	pI	Enzymatic/Toxic Activities	Reference
Mojave toxin	Crotalus scutulatus	22,700	acidic	Phospholipase Neurotoxic	Cate and Bieber (1978)
Myotoxin	Trimersurus flavoviridis	13,914	acidic	Phospholipase	Mebs and Samejima (1986)
Notexin	Notechis scutatus	13,500	acidic	Phospholipase Neurotoxic	Karlsson et al. (1972)
Taipoxin	Oxyuranus scutellatus	46,700	5.0	Phospholipase Neurotoxic	Fohlman et al. (1976)
Mulgotoxin A	Pseudechis australis	13,710	9.8	Neurotoxic	Leonardi et al. (1979)
Myotoxin VIIIA	Pseudechis australis	13,400	basic	Phospholipase Neurotoxic	Mebs and Samejima (1980)
Myotoxin IB	Pseudechis porphyriacus	13,400	basic	Phospholipase Neurotoxic	Mebs and Samejima (1980)
Myotoxin IV	Pseudechis colletti	14,100	basic	Phospholipase Neurotoxic	Mebs and Samejima (1980)
Myotoxin VI:5B	Enhydrina schistosa	13,500	basic	Phospholipase	Fohlman and Eaker (1977)

n.d.=not determined
none=no activity detected

studies on muscle degeneration following experimental envenomation with crude venoms and purified toxins. Stringer et al. (1971) studied the local effects following intramuscular injection of Naja naja kaouthia venom, a venom known to contain a cardiotoxin (Sarkar, 1947) and capable of inducing local necrosis in humans (Reid, 1964). At the light microscopic level, the venom induced the myolytic type of necrosis. Ultrastructurally, the mitochondria were described as being dramatically altered, containing dense material and clearly altered cristae. The sarcomeres became disrupted and the sarcoplasmic reticulum was fragmented and vacuolated. The degeneration appeared to be due to the cardiotoxin present in this venom (Stringer et al., 1971), potentiated by phospholipases (Lai et al., 1972).

Small Basic Myotoxins: Necrosis of skeletal muscle is also due to the presence of small molecular weight toxins, represented by myotoxin a, isolated from C. viridis viridis venom (Ownby et al., 1976; Cameron and Tu, 1977). This family of toxins have low molecular weights, around 4000 daltons, are highly basic, and have been isolated from the venoms of C. d. terrificus (Gonclaves, 1956; Cameron and Tu, 1978; Beltran et al., 1985), C. v. concolor (Engle et al., 1983), and C. v. helleri (Russell et al., 1978). The pharmacological action of these toxins indicates a possible interaction with the sodium-potassium ATPases found in the sarcoplasmic reticulum (Ownby et al., 1976;

Chang and Tseng, 1978; Hong and Chang, 1985), though recent studies showed an interaction of myotoxin a with the calcium ATPase of the sarcoplasmic reticulum (Liddle and Tu, 1985). Stringer et al. (1972) published the first report describing the ultrastructural changes in muscle resulting from rattlesnake venom. C. v. viridis venom, known to contain myotoxin a (Ownby et al., 1976), was the venom studied. An intramuscular injection of a one-fourth LD_{50} was the selected dose. Initial changes included dilatation of the normally flattened sarcoplasmic reticulum, followed by disintegration of the vacuolated sarcoplasmic reticulum into small vesicles. The myofilaments became disrupted with the disappearance of the H and M bands, followed by disruption of the Z line. The basal lamina and the sarcolemma remained unaffected until late in the degenerative stages. The effect of purified myotoxin a was also studied at the ultrastructural level (Ownby et al., 1976). Vacuolation of the muscle cells was first observed at six hours following the intramuscular injection of a 1.5 mg/kg dose in mice. By 72 hours, complete vacuolation and loss of normal striations were observed. The vacuolation observed at the light microscopic level was attributed to dilatation of the sarcoplasmic reticulum and widening of the perinuclear space. By 48 hours, the sarcoplasmic reticulum vacuoles had broken down into many smaller vacuoles, though the T-tubules appeared normal. Mitochondria demonstrated some

swelling and the myofibrillar structure was becoming compromised. By 72 hours, normal sarcomeric structure was dissolved. The basal lamina and sarcolemma of the necrotic cells, however, remained intact.

Phospholipase Myotoxins: A third group of toxins present in many venoms capable of inducing myonecrosis are the phospholipases. It has been proposed that these toxins may cause necrosis, as well as neurotoxicity, by altering calcium transport across nerve and muscle plasma membranes, the sarcoplasmic reticulum, and into mitochondria (Ng and Howard, 1980). These toxins are represented by notexin, from Notechis scutatus venom (Karlsson et al., 1972; Harris and MacDonnell, 1981), taipoxin, from Oxyuranus scutellatus venom (Fohlman et al, 1976), and crotoxin, from C. d. terrificus venom (Slotta and Fraenkel-Conrat, 1939). Although these toxins all have phospholipase activity and induce myonecrosis, there are distinct pharmacological and biochemical differences between them. Notexin is a single polypeptide neurotoxin capable of inhibiting the release of the presynaptic neurotransmitter acetylcholine (Harris et al., 1975). Taipoxin is a complex protein consisting of three subunits, also capable of inhibiting the release of acetylcholine (Fohlman et al., 1976; Harris and Maltin, 1982). Crotoxin is a protein having two subunits, one of which is a basic phospholipase, capable of causing myonecrosis (Gopalakrishnakone and Hawgood, 1984; Kouyoumdjian et al., 1986). The other subunit, which is

nontoxic by itself and acidic, is capable of increasing the toxicity of the basic subunit when complexed with it, apparently by preventing the non-specific attachment to receptor sites (Habermann and Breithaup, 1978; Bon et al., 1982). Crotoxin is a potent neurotoxin which also damages the neuromuscular junction (Gopalakrishnakone et al., 1979; Gopalakrishnakone et al., 1980; Gopalakrishnakone and Hawgood, 1984). Other venoms from which myotoxic phospholipases have been isolated include C. scutulatus (Bieber et al., 1975; Cate and Bieber, 1978), Pseudechis australis (Leonardi et al., 1979), P. porphyriacus (Mebs and Samejima, 1980), Bothrops asper (Alagon et al., 1980; Gutierrez et al., 1984a), T. flavoviridis (Mebs and Samejima, 1986), and Agkistrodon piscivorus (Welches et al, 1985; Mebs and Samejima, 1986).

The pathogenesis of myonecrosis induced by the venoms containing phospholipases has been studied using the electron microscope. Harris and Maltin (1982) described the myonecrosis following injection of Oxyuranus scutellatus venom, known to contain taipoxin. The plasma membrane was disrupted by one hour, and myofibrils were hypercontracted, giving rise to the myolytic necrosis described by Homma and Tu (1971). The basal lamina appeared unaffected. Within three hours, phagocytic cells had invaded the area and were often located within necrotic muscle cells. The toxin notexin, from Notechis scutatus, presents a histological picture very similar to that

described above. Because notexin is capable of hydrolyzing synthetic and membrane phospholipids (Harris and MacDonell, 1981), the mode of action leading to necrosis is most likely due to the destruction of the muscle cell plasma membrane. Gutierrez et al. (1984b) have reported the pathogenesis of myonecrosis induced by B. asper venom. This venom, which contains a myotoxic phospholipase (Gutierrez et al., 1984b), irreversibly damaged muscle cells within 30 minutes. Like Oxyuranus scutellatus venom, the crude venom and the myotoxic phospholipase destroyed the plasma membrane but in the absence of basal lamina damage. An influx of calcium following venom injection (Gutierrez et al., 1984b) presented further evidence that the venom acted on the muscle cell membrane leading to myonecrosis. The muscle cells were described as being of the myolytic type at early time periods, but by three hours, the myofibrils were more loosely packed, giving rise to a hyaline appearance. With both the toxin and crude venom, the mitochondria were severely affected. Many were swollen with vesiculated cristae, while others had flocculent densities. The sarcoplasmic reticulum and T-tubules were also affected, resulting in small vesicles dispersed throughout the cytoplasm.

Hemorrhagic Myotoxins: Another group of toxins found in snake venoms that are described as being directly myotoxic are the hemorrhagic myotoxins. Only three such toxins have been reported: HTb from C. atrox venom

(Bjarnason and Tu, 1978), viriditoxin (Fabiano and Tu, 1981) and viriditoxin-variant (Gleason et al., 1983), isolated from C. v. viridis venom, and mucrotoxin A, from T. flavoviridis venom (Sugihara et al., 1983). HTb has been previously described. Viriditoxin and viriditoxin-variant are described as high molecular weight (115,000 daltons) acidic proteins consisting of two subunits. The two toxins are very similar, but differences in amino acid compositions and stoichiometric relationships between the two subunits are reported (Gleason et al., 1983). Mucrotoxin A is an acidic protein with a molecular weight of 94,000 daltons. Myonecrosis three hours post-injection has been reported for both HTb (Ownby et al., 1976) and mucrotoxin A (Kishida et al., 1985). In the case of viriditoxin, myonecrosis was reported, but only after 24 hours post-injection (Fabiano and Tu, 1981). An important question concerning these toxins involves the relationship between ischemia and myonecrosis and whether these toxins cause myonecrosis directly or if the necrosis results from the compromised blood flow with the onset of ischemic conditions, since these toxins are all hemorrhagic. Ischemic conditions have been shown to cause necrosis and death of myocardial cells (Jennings and Reimer, 1981). Also, ischemia alters the membrane structure in liver cells which react much like the myocardial cell sarcolemma that has been exposed to ischemic conditions (Farber et al., 1981). Ischemic conditions result in the accelerated

degradation of phospholipids from the cellular plasma membrane and the endoplasmic or sarcoplasmic reticulum, disrupting the integrity of the membrane with the influx of calcium, leading eventually to cell death (Farber et al., 1981). In myocardial tissue exposed to severe ischemia, the mitochondria have been described as being swollen, having distorted cristal arrangements and containing dense matrix granules (Jennings and Reimer, 1981). Similar descriptions of skeletal muscle injected with hemorrhagic toxins (Ownby et al., 1978) and crude snake venom (Stringer et al, 1972) have been reported. Ownby et al. (1978) reported the presence of intact capillaries adjacent to necrotic muscle cells three hours following injection of HTb, indicating a possible direct myolytic action of the toxin. In contrast, Gleason et al. (1983) reported for viriditoxin that the necrosis of muscle was most likely due to indirect ischemic conditions since necrosis was observed only after the destruction of blood vessels and the apparent onset of such conditions.

Activities and Composition of Crotalus atrox Venom

C. atrox venom has been shown to induce a wide variety of biological effects, including hemorrhage, myonecrosis, and hemostatic alterations. The complexity of C. atrox venom is typical of most crotalid venoms, containing many toxins with a variety of activities. Table III lists some

of the activities that have been identified for crude C. atrox venom. Table IV is a list of the components that have been isolated from C. atrox venom to date. All of the components have some type of toxic or enzymatic activity, excepting nerve growth factor. The primary technique used to isolate the toxins was column chromatograpy, including anion and cation exchange chromatography, gel filtration chromatography, and hydrophobic interaction chromatography.

Hemorrhage: All rattlesnake venoms thus far studied induce hemorrhage, with the exception of C. s. scutulatus venom. This activity of C. atrox venom is due to the presence of seven hemorrhagic toxins isolated from the venom, hemorrhagic toxins a, b, c, d, e, f, and g (Bjarnason and Tu, 1978; Nikai et al., 1984; Nikai et al., 1985). These toxins ranged in molecular weights from 24,000 daltons to 68,000 daltons. Five had acidic isoelectric points, HTf was almost neutral (pI 7.7), while HTb was very basic. All of the toxins are metalloproteases; both proteolytic and hemorrhagic activities were inhibited by treatment with ethylenediaminetetraacetic acid (EDTA). The proteolytic specificities for hemorrhagic toxins a, b, c, and d have been determined on oxidized insulin B chain. Hemorrhagic toxins b, c, and d cleaved the substrate at five identical sites (Hagihara et al., 1985; Fox et al., 1986). Toxins c and d may be isozymes since both are acidic and have similar molecular weights (Bjarnason and Tu, 1978). HTb

TABLE III

SOME ACTIVITIES OF *CROTALUS ATROX* VENOM

Activity	Selected Reference
Proteolytic	Wagner and Prescott (1966)
Esterolytic	Simpson and Taylor (1973)
Collagenolytic	Simpson and Rider (1971)
Phosphodiesterolytic	Richards et al. (1965)
Phosphomonoesterolytic	Richards et al. (1965)
Hemorrhagic	Ownby et al. (1974)
Myotoxic	Huang and Perez (1982)
Phospholipolytic	Wu and Tinker (1969)
Oxidation of L-amino acids	Kocholaty et al. (1971)
Hypotension and Cardiotoxic	Posner et al. (1981)
Fibrinogenolytic	Pandya et al. (1983)
Fibrinolytic	Bajwa et al. (1981)
Fibrinogen clotting	Pfleiderer and Sumyk (1961)
Anticoagulant	Pandya and Budzynski (1984)
Procoagulant	Pfleiderer and Sumyk (1961)
Hemolytic and Cytotoxic	Tu (1977)
Platelet aggregation	Ownby et al. (1974)

TABLE IV

TOXINS ISOLATED FROM THE VENOM OF *CROTALUS ATROX*

Toxin	Activities	Molecular Weight	pI	Characteristics/ Enzymatic Activity	Reference
HTa	Hemorrhage	68,000	acidic	Metalloprotease	Bjarnason and Tu (1978)
HTb	Hemorrhage	24,000	basic	Metalloprotease	Bjarnason and Tu (1978)
HTc	Hemorrhage	24,000	acidic	Metalloprotease	Bjarnason and Tu (1978)
HTd	Hemorrhage	24,000	acidic	Metalloprotease	Bjarnason and Tu (1978)
HTe	Hemorrhage	25,700	5.6	Metalloprotease	Bjarnason and Tu (1978)
HTf	Hemorrhage	64,000	7.7	Metalloprotease	Nikai et al. (1984)
HTg	Hemorrhage	60,000	6.8	Metalloprotease	Nikai et al. (1985)
Hypotensin	Hypotensive	n.d.	n.d.	Nonenzymatic	Bonilla (1976)
MDP	Myocardial depressant	10,000	n.d.	Nonenzymatic	Bonilla and Rammel (1976)

TABLE IV (Continued)

Toxin	Activities	Molecular Weight	pI	Characteristics/ Enzymatic Activity	Reference
PR I	Anti-coagulant	20,000	basic	Fibrinolytic Metalloprotease	Pandya and Budzynski (1984)
PR II	Anti-coagulant	31,000	acidic	Fibrinolytic Metalloprotease	Pandya and Budzynski (1984)
PR III	Anti-coagulant	24,000	acidic	Fibrinolytic Metalloprotease	Pandya and Budzynski (1984)
PR IV	Anti-coagulant	46,000	acidic	Fibrinolytic Metalloprotease	Pandya and Budzynski (1984)
Alpha-proteinase	Protease	n.d.	n.d.	Inhibited by EDTA Clots fibrinogen	Pfleiderer and Sumyk (1961)
Beta-proteinase	Protease	n.d.	n.d.	Inhibited by EDTA	Pfleiderer and Sumyk (1961)
Gamma-proteinase	Protease	n.d.	n.d.	Inhibited by EDTA	Pfleiderer and Sumyk (1961)
Enzyme	Phospho-lipase	29,500	acidic	Dimer	Hachimori et al. (1971)
Q1	Anti-complement	19,200	n.d.	Acts directly on complement	Man and Minta (1977)

TABLE IV (Continued)

Toxin	Activities	Molecular Weight	pI	Characteristics/ Enzymatic Activity	Reference
Q2	Anti-complement	14,500	n.d.	Acts directly on complement	Man and Minta (1977)
Q3	Anti-complement	12,600	n.d.	Acts directly on complement	Man and Minta (1977)
Q4	Anti-complement	55,000	n.d.	Acts directly on complement	Man and Minta (1977)
EI	Esterase	27,500	4.7	Releases bradykinin	Bjarnason et al. (1983)
EII	Esterase	29,200	4.3	Releases bradykinin	Bjarnason et al. (1983)
Fibrino-genase	Esterase Protease	31,000	4.6	Fibrinolytic	Nikai et al. (1983)
Enzyme	Collagenase	58,000	5.1	PZ-peptidase	Hong (1981)
NGF	Nerve growth	40,000	n.d.	nonenzymatic	Angeletti (1968)

n.d. = not determined

also hydrolyzed fibrinogen, although it was different from thrombin and did not produce a fibrin clot (Komori et al., 1985). Hemorrhagic toxin a also cleaved oxidized insulin B chain, sharing four common cleavage sites with HTb (Fox et al., 1986).

Ownby et al. described the pathogenesis of hemorrhage induced by the crude venom (1974) and by the three toxins HTa, HTb, and HTe (1978). In all cases, the hemorrhage was per rhexis, meaning the erythrocytes escaped the vascular compartment through gaps in the endothelial cell wall, not through widened intercellular junctions. The pathogenesis of hemorrhage induced by the venom and these three toxins has been previously described.

Perhaps associated with hemorrhage is the presence of a collagenase in the venom, which has been characterized as having a molecular weight of 58,000 and an isoelectric point of 5.1. The toxin also hydrolyzed the PZ-peptidase substrate (Hong, 1982). Disruption of the basal lamina may aid in the induction of hemorrhage (Ohsaka et al., 1973; Fox et al., 1986). The collagenolytic activity of the venom due to this component may be responsible for the disruption of the basal lamina described by Ownby et al. (1974), resulting in the weakening of the vessel wall, aiding the extravasation of erythrocytes.

Myonecrosis: C. atrox venom (Homma and Tu, 1971; Ownby et al., 1974), as well as HTb (Ownby et al.,1978), cause myonecrosis. Thus far, HTb is the only directly

acting myotoxin isolated from this venom. Many phospholipases isolated from snake venoms are known to induce myonecrosis (Karlsson et al., 1972; Gutierrez et al., 1984a; Mebs and Samejima, 1986). Only one phospholipase has been isolated from C. atrox venom, a protein with a molecular weight of 29,500 daltons with an acidic isoelectric point (Wu and Tinker, 1969; Hachimori et al., 1971). However, there is no report in the literature that this toxin causes myonecrosis. One feature of myonecrosis induced by a phospholipase from B. asper venom was the presence of "delta lesions," wedged shape areas seen at the light microscopic level in skeletal muscle, resulting from damage to the plasma membrane of the cell (Gutierrez et al., 1984b). Myonecrosis induced by crude C. atrox venom demonstrated lesions that appeared similar to these delta lesions (Ownby, personal communication), though it is unknown what toxin or toxins caused the lesions. One possibility is the presence of a cofactor or associated protein required by the phospholipase for activity and subsequently myonecrosis.

Hemostatic Alterations: By far, the largest number and types of toxins and components isolated from C. atrox venom are those that have some effect on normal hemostasis. These effects include the release of vasoactive compounds such as bradykinin, anti-coagulant and fibrinolytic activities, procoagulant activities, the induction of increases in capillary permeability, platelet aggregation,

and cardiotoxic and hypotensive effects.

Posner et al. (1981) demonstrated that C. atrox has cardiotoxic activity, showing that the venom could reduce the action potential amplitude, action potential duration, and the resting membrane potential of feline papillary muscle and Purkinje fibers. The maximum developed tension of papillary muscle was also significantly reduced. All of these factors would result in a potent depression of cardiac output, contractile force, and heart rate, resulting in a severe systemic arterial pressure hypotension, dramatically altering normal hemostasis. Bonilla and Rammel (1976) isolated from the venom a protein called myocardial depressor protein. It is characterized as an acidic protein containing no known proteolytic, esterolytic, or phospholipolytic activities, capable of severely depressing arterial pressure and cardiac output. Also in this venom is a very small molecular weight peptide (20 amino acids), known as hypotensin, which induces an immediate decrease in systemic arterial pressure upon injection (Bonilla, 1976).

Pandya and Budzynski (1984) isolated four metalloproteases, PR I, PR II, PR III, and PR IV, that were anticoagulant, capable of degrading fibrinogen, though no fibrin clot was formed, indicating activities different from thrombin. The molecular weights ranged from 20,000 to 46,000 daltons, and all had acidic isoelectric points, except PR I, which was basic. Nikai et al. (1983) reported

the isolation of fibrinogenase, a toxin with a molecular weight of 31,000 daltons and an isoelectric point of 4.6. It had both proteolytic and esterolytic activities, caused an increase in capillary permeability, degraded fibrinogen, and released bradykinin from kininogen. This toxin is probably PR II, isolated by Pandya and Budzynski (1984), which also has a molecular weight of 31,000 daltons and an acidic isoelectric point. Two other kallikrein-like proteins that release bradykinin from kininogen have been isolated from C. atrox venom (Bjarnason et al., 1983). These toxins, EI and EII, had respective molecular weights of 27,000 and 29,200 daltons. Their reported isoelectric points were 4.7 and 4.3, respectively. Both proteins had arginine esterase activity. Bradykinin is a potent vasodilator and causes an increase in vascular permeability, both of which would induce hypotension. Bradykinin is also the most probable source of pain associated with rattlesnake venom poisoning (Ownby, 1982).

Looking at the fibrinolytic and fibrinogen clotting enzymes in C. atrox, Bajwa et al. (1981) found ony two fibrinolytic enzymes. Their molecular weights were 60,000 and 21,500 daltons. No fibrin clot forming activity was found. Pfleiderer and Sumyk (1961), however, reported the presence of a fibrinogen clotting metalloprotease, alpha-protease, which was inhibited by EDTA. The differences in these reports may be due to variations in individual snake venoms. Pfleiderer and Sumyk (1961) also reported the

isolation of two other metalloproteases, beta-protease and gamma-protease, although these had no effect on fibrinogen. C. atrox venom also induced platelet aggregation and thrombi formation (Ownby et al., 1974), although no purified toxin responsible for this activity has been reported.

Other Isolated Components: Man and Minta (1977) reported the isolation of four toxins, Q1, Q2, Q3, and Q4, that act directly on complement factors. Although no enzymatic activities were determined, the toxic effects were inhibited by EDTA, indicating the need of metal ions for activity. The effects of the toxins appeared to be due to the direct action on complement factors, inhibiting the activation of the complement sequence.

Angeletti (1968) reported the isolation of nerve growth factor from C. atrox venom, having an approximate molecular weight of 40,000 daltons. No enzymatic activity was determined for the factor.

Lethal Toxicities: There are many papers that report the LD_{50} values for C. atrox venom, tested using intravenous, intraperitoneal, and subcutaneous injections. These values range from 1.0 mg/kg (i.v.) to 28.0 mg/kg (s.c.). Table V lists some of the reported LD_{50} values for C. atrox venom, determined using the above three routes of injection in mice.

TABLE V

REPORTED LETHAL TOXICITIES
OF *CROTALUS ATROX* VENOM

LD_{50} mg/kg			Reference
Route of Injection			
i.v.	i.p.	s.c.	
2.66	4.26	-	Kochalaty et al. (1971)
4.20	3.71	-	Emery and Russell (1963)
-	8.42	19.25	Minton (1956)
-	-	28.0	Minton (1975)
-	5.5 *	-	Macht (1937)
-	-	16.11	Schoettler (1951)
3.56	3.7	-	Freiderich and Tu (1971)
-	4.8	-	Glenn et al. (1972)
1.0	-	-	Theakston and Reid (1978)
-	4.5	-	Glenn and Straight (1978)
-	8.0	-	Perez et al. (1978)

* Minimum lethal dose

Treatments of Snake Venom Poisoning

An important reason for studying snake venoms is to develop better methods of treating snake bites in the clinical case. There are several methods used to treat snake venom poisoning, each of which has apparent attributes as well as problems. The major problem facing the treating physician, however, is the fact that no two snake bite cases present the same problem. There are many factors which influence an individual's response to a snake bite, all of which must be considered by the attending physician. These factors include the quantity of venom injected, the health of the individual, the depth and location of the bite, and the time between the bite and the treatment (Minton, 1954). Each of these factors must be considered by the physician when choosing a particular treatment.

Pressure-Immobilization: One treatment advocated by some researchers is the pressure-immobilization technique, which involves wrapping the bitten limb tightly with a bandage and immobilization with a splint (Sutherland and Coulter, 1981; Glenn and Straight, 1985). Sutherland and Coulter (1981) measured a delay in the movement of the venom in experimentally treated monkeys using radioimmune assays. One problem with this technique, however, would be the potential of concentrating within a small area those toxins present in the venom capable of inducing serious local effects, such as hemorrhage, edema and myonecrosis.

Ligature-Cryotherapy: Similar to the pressure-immobilization technique is the ligature-cryotherapy method, involving the placement of a ligature around the affected limb and immersion into a cold environment (Stahnke et al., 1957; Stahnke and McBride, 1966). Stahnke (1981) proposed that since most enzymes present in the venoms are active at temperature between 37 and 45 degrees centigrade, the cryotherapy technique would prevent or reduce the activity of these enzymes. Karlsson (1979), however, presented the point that many toxins present in snake venoms capable of inducing various effects are not enzymes. Myotoxin a, for example, is a nonenzymatic component capable of inducing serious myonecrosis (Ownby et al., 1976; Ownby, 1982). Often, the combination of the toxins and the tissue-damaging effects of the cold environment proves to cause more damage to the affected limb than the toxins in the venom alone left untreated (Arnold, 1982).

Surgical Intervention: Surgical intervention is often the treatment selected, and includes incision-suction and debridement and fasciotomy. There is some concern, however, in the amount of venom injected in a given bite. Sutherland and Coulter (1981) estimate that approximately 26% of the snake bites in the United States result in no venom deposition, cases in which surgical intervention is certainly contraindicated. Yet there are physicians that propose the use of surgery in all cases of snake bite

(Glass, 1976). Incision-suction, where an incision is made at the site of venom injection and direct suction is applied, is used in hopes of removing as much of the venom as possible. The effectiveness of this treatment requires administration very soon if not immediately after poisoning (McCollough and Gennaro, 1970; Russell et al., 1973; Efrati, 1979). The second surgical method includes debridement of the wound and fasciotomy (Glass, 1976; Efrati, 1979), and involves the surgical removal of the tissue at the site of injection, based on the idea that most of the venom remains in the local area. Huang et al. (1974), treating 54 patients, claimed that by mechanically removing the tissue containing the injected venom, most of the venom can conceivably be eliminated, thereby reducing the magnitude of the systemic intoxication and local effects. Garfin et al (1984), however, in an experiment using subcutaneous injections of C. v. helleri venom in dogs, demonstrated that fasciotomy did not reduce the necrosis induced by the venom. In addition, muscle cells have been shown to regenerate following necrosis induced by taipoxin (Oxyuranus scutellatus), a myotoxin isolated from B. asper, and crude B. asper venom (Maltin et al., 1983; Gutierrez et al., 1984c). Surgical removal of muscle tissue during fasciotomy could possibly result in the removal of satellite cells responsible for regeneration.

Steroids: Steroids have been used in conjunction with other treatments, based on their ability to reduce systemic

reactions (Stahnke and McBride, 1966; Glass, 1976). Reid et al. (1963), however, showed no improvement in response to A. rhodostoma envenomation in a clinical study using prednisone. Schaeffer et al. (1979) also reported that corticosteroid analogs offered no benefical effects against C. v. viridis venom shock. However, corticosteroids, antihistimines, and fluid infusion may prove helpful in treating systemic effects such as shock, anaphylaxis, and serum sickness (Schottler, 1954; Allam et al., 1956; McCollough and Gennaro, 1970; Schaeffer et al., 1978).

Antivenoms: The use of antivenoms specific for individual snake venoms, or polyvalent antivenoms produced against a group of snake venoms, is by far the most utilized treatment for snake venom poisoning. Polyvalent and monovalent antivenoms are commercially available in 22 countries and are produced against the venoms of 54 different species (Minton, 1967). The use of antivenoms is based on the presence of specific, neutralizing antibodies directed against specific toxic components in the venom. In the United States, the most widely used polyvalent antivenom is Wyeth's Polyvalent (Crotalidae) Antivenom (Wyeth's Laboratories, Marietta, Pennsylvania), subsequently referred to as polyvalent antivenin, produced from horses inoculated with venoms from four snake species: B. atrox, C. atrox, C. adamanteus, and C. d. terrificus. These venoms were chosen because of the paraspecific protection against other venoms offered by the antivenom

due to common antigens in venoms (Criley, 1956; Minton, 1976). Paraspecific protection by antivenoms for venoms not utilized in the antivenom production has been reported (Minton, 1967). Baxter and Gallichio (1974) demonstrated the ability of antivenom produced against Notechis scutatus to neutralize lethal effects of multiple sea snake species. Polyvalent antivenoms do not offer protection against all venoms from genera utilized in the inoculation, however. The Instituto Clodomiro Picado in Costa Rica produces a polyvalent antivenom capable of offering protection against members of the genus Micrurus, with the exception of M. mipartitus, which is unaffected by the antiserum and requires an antivenom directed specifically against it (Bolanos et al., 1975). The use of a specific antiserum, however, requires that the snake inflicting the bite be identified.

There have been several reports concerning the production of monovalent and polyvalent antisera, and the abilities of antisera to neutralize local and lethal effects induced by crude venoms and their toxic components. Ownby et al. (1979) successfully produced an antiserum in rabbits against purified myotoxin a, isolated from C. v. viridis venom (Ownby et al., 1976). In a comprehensive study published as a series of reports, Ownby et al. tested the ability of the monovalent antiserum to myotoxin a to neutralize local and lethal effects of myotoxin a, in vitro and in vivo, and crude C. v. viridis venom, in vitro (Ownby

et al., 1983a; Ownby et al., 1984). Also tested was the ability of a mixture of antiserum to myotoxin a and Wyeth's polyvalent antivenin to neutralize these same effects (Ownby et al., 1985; Ownby et al., 1986). The results showed that antimyotoxin a serum significantly neutralized a 0.75 mg/kg dose of myotoxin a and a 1.5 mg/kg dose of crude venom, in vitro, as measured by the vacuolation index, which is a measure of the myotoxin a activity of the venom (Ownby, 1982). Neutralization of the higher dose of crude venom is explained by the fact that myotoxin a is only a fraction, 18% (Cameron and Tu, 1977), of the total crude venom protein. Polyvalent antivenin did not neutralize any of the mytoxin a activity of the crude venom tested. In vivo, antimyotoxin a serum significantly neutralized a 0.75 mg/kg dose of myotoxin a if injected prior to toxin injection, and neutralization of a 0.38 mg/kg dose if injected immediately afterwards. No protection was offered if antiserum injection was delayed fifteen minutes. A 1:1 mixture of antimyotoxin a serum and polyvalent antivenin significantly neutralized myonecrosis at a dose of 12.0 mg/kg crude venom, in vitro. The same mixture was able to neutralize a 1.5 mg/kg dose of crude venom if injected intravenously up to thirty minutes following in intramuscular injection of venom. These results demonstrate that toxin-specific antisera may prove useful in diminishing local effects resulting from snake venom poisoning, especially if used in conjunction with a

polyvalent antiserum. Smith and Ownby (1985) showed that polyvalent antivenin significantly neutralized myonecrosis induced by a 6.0 mg/kg dose of C. h. horridus venom, in vitro. Ownby and Colberg (1986) reported the ability of polyvalent antivenin to neutralize myonecrosis induced by a 15.0 mg/kg dose of C. atrox venom.

Polyvalent antivenin is much more effective in neutralizing hemorrhage and lethality induced by crude crotalid venoms. Ownby et al. (1984) showed that this antivenin could significantly neutralize hemorrhagic activity of a 24.0 mg/kg dose of C. v. viridis venom and that of a 12.0 mg/kg dose of C. atrox venom, while Smith and Ownby (1985) reported neutralization of a 12.0 mg/kg dose of C. h. horridus venom. Ownby et al. (1983) reported neutralization of six times the LD_{50} of C. v. viridis venom, though Criley (1956) reported neutralization of only 2.21 times the LD_{50} for this venom. Criley, however, used an i.v. injection route while Ownby et al. injected the venom i.m. It is interesting that Minton (1954) reported no significant neutralization of lethal effects by polyvalent antivenin induced by either C. atrox or C. adamanteus venom, despite the fact that both venoms are used in the production of the antiserum.

The ability of polyvalent antivenin to neutralize lethal effects is dependent on the time between venom deposition and antiserum administration. Russell et al. (1973) showed with C. v. helleri venom that administration

of antivenin immediately after venom injection resulted in better protection against lethality than the delayed injection of antivenin. Minton (1954) reported that 50% of mice receiving antivenin fifteen minutes prior to an intraperitoneal injection of C. h. horridus venom survived, while only 35% survived if antivenin was delayed for fifteen minutes post-injection.

Cross-Reactivity of Antigens: As previously mentioned, the use of polyvalent antivenoms is based on the paraspecific or cross-neutralization reactions offering protection against venoms other than those used in the inoculation (Criley, 1956; Minton, 1976). Rosenfeld and Kelen (1966) hypothesized that many snake species share common antigens in their venoms, especially within the same genus, and that cross-neutralization depended on the ability of antibodies to recognize common toxic antigens.

Minton (1967) surveyed the paraspecific protection offered by elapid and sea snake antivenoms. The results showed that antivenoms raised against the Iran cobra (Naja naja oxiana), the tiger snake (Notechis scutatus), and the death adder (Acanthophis antarticus) were effective in neutralizing lethal effects of eight species of the genus Naja as well as members of the genera Enhydrina, Bungarus, and Micrurus. Even those antivenoms that failed to protect the animal against particular venoms prolonged survival time. Rosenfeld and Kelen (1966) looked at neutralization of coagulant activity in various genera of snakes, using

primarily antivenoms raised against Bothrops species. Their results showed extensive cross-neutralization by many monovalent antivenoms of venoms within the same genus, as well as neutralization of some species of a different genus. The polyvalent antivenom produced against venom from snakes of the Bothrops genus significantly neutralized the coagulant activity of all Bothrops species studied, in addition to offering some protection against venoms from the genus Crotalus. The most striking example of common antigens, or at least common antigenic sites, involved the use of a monoclonal antibody produced against a specific coelenterate lethal toxin. Russo et al. (1983) reported the detection of homologous antigenic sites on toxic proteins from C. d. terrificus, the hornet, Vespa orientalis, and the sea wasp, Chironex fleckeri. They also reported the use of an anti-Portugese man-of-war toxin antibody for the isolation of a toxin from C. d. terrificus venom.

The use of monoclonal and monovalent antibodies against isolated toxin preparations for studying cross-reactivity with other snake venoms and toxins has been reported. Rael et al. (1986) produced a monoclonal antibody to Mojave toxin, isolated from the venom of C. s. scutulatus, and used this antibody to detect common antigens with immunoblots of electrofocused crude venoms. Cross-reacting proteins were recognized in the venoms of C. basilicus, C. v. concolor, C. d. terrificus, and C. d.

durissus. Five bands, with isoelectric points ranging from 5.1 to 6.1, were recognized in the venom of C. s. scutulatus. Kaiser et al (1986) demonstrated that antiserum raised against crotoxin, a potent neuro- and myotoxin in C. d. terrificus venom, successfully neutralized lethal activity of Mojave toxin (C. s. scutulatus) and concolor toxin (C. v. concolor), both of which are also neurotoxins. These results indicate the possible utility of antisera raised against these neurotoxins in treatment of venom poisoning by snakes containing homologous neurotoxins.

A primary objective of venom research is to develop better methods of treatment. The use of antisera mixtures, such as polyvalent antivenin and antimyotoxin a serum (Ownby et al., 1985; Ownby et al., 1986), and the inclusion of toxins or venoms known to cross-react with other venoms (Minton, 1967; Baxter and Gallichio, 1974; Rael et al., 1986; Kaiser et al., 1986), may prove useful in the clinical snake bite treatment, hopefully reducing both lethal and local effects of poisoning. Other methods are also being pursued in attempts to improve antivenom effectiveness. Cross-linking the low molecular weight toxic fraction of the Malayan cobra, Naja naja sputatrix, with a neurotoxin isolated from the venom of the common sea snake, Enhydrina schistosa (Karlsson et al., 1972), with 1% glutaraldehyde prior to immunization in rabbits, Tan (1983) reported the production of an antivenom four to eight times

more potent against lethal effects than the commercially used antiserum. Another method being utilized in attempts to improve antisera is affinity purification of specific antibodies and toxins (Lee and Chao, 1978; Lomonte et al., 1985; Russell et al., 1985).

Affinity Chromatography

Affinity chromatography involves the separation of molecules based on biospecific interactions between the material being purified and the chromatographic column (Chaiken, 1983). Although this method is considered by some to include hydrophobic affinity, metal-ion affinity, as well as other interactions, most uses of affinity chromatography are based on biospecific interactions (Katchalski-Katzir, 1983). Column preparation requires the attachment of one member of the interacting pair to an insoluble matrix, such as sepharose, which is used to pack the column. The material containing the other member of the interacting pair is applied to the column. Any compound having an affinity for the solid phase matrix will bind, primarily through hydrophobic forces, electrostatic interactions, hydrogen bonding, and/or Van der Waals forces (Geld, 1973; Katchalski-Katzir, 1983), while all other materials pass through as the unbound material. The attached compound is then eluted by changing either the pH or ionic strength of the elution medium (Kennedy and Barnes, 1981; Sullivan and Russell, 1982; Bureau and

Daussant, 1983). This technique is relatively simple and allows the isolation of the material in a single step (Egarov, 1975). The purity of the isolate depends entirely on the purity of the material which is bound to the solid phase matrix. The types of interactions that have been exploited with this technique include antigen-antibody recognition (Lee and Chao, 1978; Sullivan and Russell, 1982; Clegg and Smith, 1983), enzyme-inhibitor interactions (Cuatrecasas and Anfinsen, 1971; Schultz et al., 1983), hormone-carrier protein associations (Abercrombie et al., 1983), and hydrophobic interactions (Boedeker and Cheney, 1983; Werber et al., 1983).

The major use of affinity chromatography in snake venom research has been in the purification of antibodies directed at either crude venoms or purified toxins. The purpose for this is two-fold. First, one of the serious drawbacks to polyvalent antivenoms, usually produced in horses against crude venoms, is the serum reactions many patients exhibit towards the foreign equine proteins in the antivenom, giving rise to anaphylactic and serum sickness reactions (McCollough and Gennaro, 1970; Sullivan and Russell, 1983). This problem is often compounded by the inability of the physician to determine the amount of antivenom required to neutralize the clinical symptoms of envenomation, leading to the administration of large amounts of antiserum (Russell et al., 1985). Second, the concentration of antibodies specific for a given toxin, one

that may be important in lethal or local effects, are often in very low concentrations in the polyvalent antivenom. Ownby et al. (1983b) demonstrated the very low titer of antibodies against myotoxin a, a potent myotoxin from C. v. viridis venom, with the enzyme-linked immunosorbent assay. The use of affinity chromatography allows not only the removal of excess serum proteins, most of which offer no protection against snake venom poisoning yet are very integral in serum reactions, but also allows for an increase in total concentration of the venom- or toxin-specific antibodies, permitting the administration of more antiserum with a diminished likelihood of a serum reaction by the patient.

Lee and Chao (1978) coupled Bungarus multicinctus venom to a cyanogen-bromide activated Sepharose 4b matrix. The crude horse antivenom specific for this venom was able to neutralize 0.76 LD_{50} per milligram protein, injected subcutaneously in mice. The reconstituted lyophilyzed product following affinity chromatography neutralized 22.5 LD_{50} per milligram protein, representing a 29.6-fold increase in neutralization capacity, clearly demonstrating an improvement in protection against the lethal effects of this venom.

Cobrotoxin, isolated from the venom of Naja atra (Yang, 1965), is the main toxic component of this venom. Isolation of antibodies from the polyvalent antivenom produced against this toxin by gel filtration (Chang and

Yang, 1969) produced an antiserum that showed a 17.5-fold increase in neutralization capacity over the crude antiserum. However, affinity chromatography allowed the purification of an antibody fraction demonstrating a 23-fold increase in neutralization capacity. Lomonte et al. (1985) purified antibodies against a myotoxin from B. asper venom (Gutierrez et al., 1984a) using this technique. The results showed the ability of the affinity-purified antibody to neutralize most of the myotoxic effects of the crude B. asper venom. The significance of these findings are two-fold. First, the increased neutralization capacity of affinity-purified antibodies is indicated. Second, the importance of individual venom components, in this case a myotoxin, in local reactions is clear. Ownby et al. (1985; 1986) reported that a mixture of polyvalent (Crotalidae) antivenin and antimyotoxin a serum provided greater protection against myonecrosis induced by the crude venom than the polyvalent antivenin offered alone. The use of affinity-purified antibodies, in conjunction with other affinity-purified antibodies or polyvalent antivenoms, may prove successful in the clinical treatment of both lethal and local effects of snake venom poisoning.

Affinity purification of antibodies from antisera does not always result in an increase in neutralization. Bober (1987) purified antibodies using affinity chromatography to myotoxin a from an antiserum produced in rabbits against this toxin (Ownby et al., 1979). The affinity-purified

antibody was non-precipitating (as determined with immunodiffusion), nor did the antibody neutralize myonecrosis induced by the toxin at the amounts tested. The affinity-purified antibody produced by Yang et al. (1977) against cobrotoxin was also non-precipitating, yet demonstrated a 23-fold increase in neutralization capacity of the lethal effects over the crude antiserum. The inability of the purified antibody produced by Bober (1987) to neutralize myonecrosis may be due to the inability of the antibody to recognize the antigenic site or sites associated with the induction of necrosis or to the relatively low amounts of antibody tested.

The use of purified toxins and affinity-purified antibodies have other uses in snake venom research. The detection of particular toxins in different species of snakes has been discussed. By determining the prevalence of a toxin in a group of snakes, and depending on the importance of the toxin in the local and lethal effects of venom poisoning, more effective antisera may be produced by selecting venoms containing the particular toxin. The use of mixtures of antisera in the treatment of snake venom poisoning has also been indicated. Another use of purified toxins and antibodies involves the localization of the toxin in the tissue with immunohistochemical techniques, perhaps offering some insight into the mechanism of action of the toxin.

Immunohistochemistry

The location of proteins within tissues can be determined with the techniques of immunohistochemistry and immunocytochemistry, techniques which are predicated on the specificity of antigen-antibody interactions (Griffith, 1982). The term immunohistochemistry is used for visualization at the light microscopic level, while immunocytochemistry is used for the electron microscopic level, though the terms are often used interchangeably. The basic steps of these techniques are as follows: (1) the tissue containing the protein to be localized is either chemically fixed with a cross-linking molecule, such as glutaraldehyde or formaldehyde, or rapid-frozen in an appropriate solvent, such as isopentane or acetone; (2) the tissue is embedded in a resin (unless frozen) and sectioned; (3) a specific monovalent, monoclonal or affinity-purified antibody, referred to as the primary antibody, produced against the protein to be localized is applied to the tissue; (4) a secondary antibody conjugated to a specific label, such as fluorescein, produced against the primary antibody is applied to the tissue; (5) the section is treated so that the label is visable, for example, with ultraviolet light if fluorescein is the label. The major difference between the two techniques, besides the method of viewing the tissue, occurs at the labelling process. For immunohistochemistry, the label must be visible with the light microscope, while, for the

electron microscope and immunocytochemistry, the label must be electron dense and capable of withstanding the energy of the electron beam. Also, there are multiple variations on these techniques relative to the labels used. For example, the label may be the result of an enzymatic reaction which forms an insoluble precipitate at the site of the antigen. This is the basis of the peroxidase-antiperoxidase technique (Mohanty, 1982). The use of protein A conjugated to colloidal gold has also been used at both the light and electron microscope levels (Holgate et al., 1983). This techique is based on the fact that protein A binds the Fc portion of the IgG primary antibody, eliminating the secondary antibody. Fluorescein and rhodamine are used as labels due to their easy viewing following excitation (Williamson et al., 1980; Dongen et al., 1985). Immunohistochemistry has been used to localize proteases in murine skeletal muscle. Stauber et al. (1985) used flourescent labeled antibodies in the localization of cathepsin proteases in frozen thick sections of muscle.

There are only two reports in the literature concerning the localization of snake venoms or their components in mammalian tissues. Schiff et al. (1984) described the localization of C. atrox venom in pulmonary tissue of mice following "in vivo envenomation," an intraperitoneal injection of a 10 mg/kg dose. The tissue was processed one hour following the injection. Horseradish peroxidase (HRP) was conjugated to Wyeth's

polyvalent (Crotalidae) antivenin and used as the antigen-specific antiserum. No secondary antibody was required. The results of this study were as follows: (1) the reaction product was found on the alveolar surface of type I and II cells, in cytosomes of type II cells, and in membrane bound vesicles of type I cells; (2) macrophages in the area contained large quantities of the reaction product; and (3) no reaction product was found on or in endothelial cells, or in the surrounding connective tissues spaces. Although no reaction product was seen in or around endothelial cells, the authors proposed that the venom components were carried across the blood-air barrier where they came to lie at the air/surfactant interface. This conclusion was based on the presence of membrane-bound vesicles, although these were seen only in type I cells, and only rarely in these cases. The authors offered no evidence to explain how the venom crossed the endothelial cell or the connective tissue septum. The authors also proposed that there was affinity for the phophatidylcholine-rich surfactant by the phospholipase in the venom, explaining the location of the reaction product and the association with the type II cells.

Tu and Morita (1983) reported the localization of myotoxin a, isolated from the venom of C. v. viridis, in vitro, using myotoxin a conjugated directly to HRP. No immunoglobulins were utilized. Human biopsy samples were frozen and sectioned at 10-20 microns in thickness. The

myotoxin a-HRP conjugate was applied directly to the tissue. After washing and treatment with the peroxidase substrate, the sections were embedded in plastic and prepared for the electron microscope. The results demonstrated binding of the myotoxin a-HRP conjugate only to the sarcoplasmic reticulum and occasionally in the cisternae of this organelle. No reaction product was observed at the sarcolemma or in association with other cellular organelles. Because myotoxin a causes severe vacuolation of the sarcoplasmic reticulum (Ownby et al., 1976), Morita and Tu concluded that myotoxin a induces myonecrosis through a possible interaction with the calcium regulatory function associated with the sarcoplasmic reticulum.

CHAPTER III

ISOLATION OF HEMORRHAGIC TOXIN B FROM *CROTALUS ATROX* VENOM

Introduction

Hemorrhagic toxin b (HTb) is only one of many hemorrhagic toxins found in *Crotalus atrox* venom. In 1978, Bjarnason and Tu reported the isolation of five hemorrhagic components from *C. atrox* venom. Since that time, two additonal hemorrhagic toxins have been isolated (Nikai et al., 1984; Nikai et al., 1985). All seven hemorrhagic toxins from *C. atrox* are described as metalloproteases with zinc being the necessary metal ion. Removal of the zinc ion with metal chelators, such as EDTA and ortho-phenanthroline, destroys both the proteolytic activities and hemorrhagic activities of all the toxins (Ownby et al., 1975; Bjarnason and Tu, 1978).

The pathogenesis of hemorrhage induced by snake venoms and their components can be of two types, per rhexis or per diapedesis (Ohsaka et al., 1971; Ownby et al., 1974). In hemorrhage per diapedesis, such as is seen with the hemorrhagic principle HR-1 from the venom of *Trimeresurus flavoviridis*, erythrocytes are described as exiting the vascular compartment through gaps in intercellular

junctions in the absence of endothelial cell lysis (Ohsaka et al., 1971; Tsuchiya et al., 1974). Since the hemorrhagic components from T. flavoviridis are able to cause the release of proteins and carbohydrates from the isolated glomerular basement membrane preparation, the hemorrhage that results from the action of the hemorrhagic principles is presumed to be due to the lysis of the underlying basement membrane of endothelial cells, resulting in a weakening of the intercellular junctions and escape of erythrocytes (Ohsaka et al., 1973). In contrast, hemorrhage per rhexis is described as the escape of erythrocytes through damaged endothelial cells rather than through compromised intercellular junctions. This type of hemorrhage is found with C. atrox venom (Ownby et al., 1974) and with the purified hemorrhagic toxins a, b and e from this venom (Ownby et al., 1978). Hemorrhagic toxin b, however, demonstrates an unusual hemorrhagic pattern. Unlike many other toxins, including the other toxins from C. atrox, hemorrhage does not ensue until after a latent period of at least 20 min after initial injection of the toxin (Smith et al., unpublished results).

HTb has also been described as a myotoxin, causing myonecrosis in addition to hemorrhage (Ownby et al., 1978). It is unclear, however, whether the toxin is directly myotoxic, causing necrosis due to a direct lytic action of the toxin on the muscle cells, or if the myotoxicity is of an indirect origin, resulting from the ischemic conditions

induced by the hemorrhage. Ownby et al. (1978) described the presence of intact capillaries next to necrotic muscle cells using electron microscopy techniques, indicating a possible direct myotoxic effect of the toxin.

Further study of the pathogenesis of hemorrhage and myonecrosis caused by HTb requires a purified protein preparation. The present chapter describes the isolation of HTb and some biochemical properties of both the toxin and fractions obtained from C. atrox venom.

Materials and Methods

Venom

Lyophilized C. atrox venom was purchased from Dr. John Perez. The venom was collected from adult specimens captured around Sweetwater, Texas at the Big Spring Rattlesnake Roundup in 1983 and was pooled, lyophilized, and stored at -20^{o}C until use. Before beginning the purification, 2.0 gm of venom was dissolved in 50 ml of 0.01 M Tris-Cl buffer, pH 8.6 and centrifuged at 40,060 g in a Sorvall SM-24 rotor (Dupont Co., Newtown, CN) for one hour to remove particulate matter. The supernatant was desalted in an Amicon Model 400 ultrafiltration cell (Amicon Corp., Danvers, MA) equipped with a YM-10 membrane (exclusion limit - 10,000 daltons) and was subsequently concentrated to 5.0 ml in an Amicon Model 50 ultrafiltration cell equipped with a YM-10 membrane.

Isolation of Hemorrhagic Toxin B

Figure 1 is a flow diagram of the steps used in the isolation of HTb. The selection of fractions to be further chromatographed was based on the presence of hemorrhagic and proteolytic activity. Protein concentration was monitored by the absorbance at 280 nm. The concentrated, desalted venom sample was applied to a 2.6 X 27 cm column of DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.01 M Tris-Cl buffer, pH 8.6. Elution with the equilibration buffer was continued until the unbound material had eluted, at which time a 2000 ml linear gradient of 0.0 M to 0.25 M NaCl in 0.01 M Tris-Cl, pH 8.6, was started. A flow rate of 30 ml/hr was maintained by a peristaltic pump. Fractions of 8.2 ml were collected until elution of the unbound material, after which fractions of 19.0 ml were collected. The unbound material was collected, pooled and concentrated to 5.0 ml in an Amicon ultrafiltration cell over a YM-10 membrane. The concentrated fractions from the ion-exchange were applied to a 2.6 X 85 cm column of Ultrogel AcA 54 (LKB Corp., Rockville, MD) equilibrated in 0.01 M TAPS-Cl buffer, 0.15 M NaCl, pH 8.6. A flow rate of 30 ml/hr was maintained by a peristaltic pump and fractions of 6.0 ml were collected. The second major absorbance peak was collected and pooled. Buffer exchange into 0.01 M TAPS-Cl, pH 8.6, was performed by dialfiltration in an Amicon Model 400 ultrafiltration cell equipped with a YM-10 membrane, followed by

Figure 1. Isolation procedure used in the purification of hemorrhagic toxin b.

ISOLATION PROCEDURE

CRUDE VENOM SOLUTION

▼

DESALTING AND CENTRIFUGATION

▼ (supernatant)

ANION EXCHANGE ON DEAE-SEPHACEL
(0.01 M Tris-Cl, pH 8.6, 0-0.25 M NaCl)

▼ (fraction A1)

GEL FILTRATION ON LKB ULTROGEL ACA 54
(0.01 M Na-MOPS, pH 8.6, 0.15 M NaCl)

▼ (fraction B2)

CATION EXCHANGE ON CM-FRACTOGEL
(0.01 M Na-MOPS, pH 8.6, 0-0.15 M NaCl)

▼ (fraction C6)

HYDROPHOBIC INTERACTION ON PHENYL-SEPHAROSE CL-4B
(0.01 M Tris-Cl, pH 7.2, 1.5-0 M Ammonium Sulfate,
0-50% Ethylene Glycol)

▼

PURIFIED HTb

concentration to approximately 5.0 ml. The concentrated fractions from the gel filtration separation were applied to a 1.6 X 33 cm column of Fractogel TSK CM-650S cation exchanger (EM Sciences, Gibbstown, NJ) equlibrated with 0.01 M TAPS-Cl, pH 8.6. The column was eluted with a 750 ml linear gradient of 0.0 M to 0.15 M NaCl in 0.01 M TAPS-Cl immediately after sample loading. The flow rate was 30 ml/hr and 6.0 ml were collected. The sixth major absorbance peak was collected and pooled. A buffer exchange into 0.01 M Tris-Cl buffer, 1.5 M ammonium sulfate, pH 7.2, was performed using an Amicon Model 400 ultrafiltration cell equipped with a YM-10 membrane . The sample was concentrated to 5.0 ml and applied to a 1.6 X 27 cm column of Phenyl-Sepharose CL-4B (Pharmacia Fine Chemicals) hydrophobic interaction column equilibrated with 0.01 M Tris-Cl, pH 8.6. The column was eluted with a 600 ml linear gradient immediately after sample application. The composition of the starting buffer for the gradient was 1.5 M ammonium sulfate, 0.01 M Tris-Cl, pH 7.2 and the limit buffer was 0.01 M Tris-Cl, pH 7.2, containing 50% ethylene glycol. The flow rate was 25 ml/hr and 6.0 ml fractions were collected. One broad peak eluted and collected, pooled, concentrated into 0.01 M Na-MOPS buffer, 0.01 M NaCl, pH 7.2, and stored at 20^{o}C until use.

The concentration of HTb was determined by dividing the absorbance at 280 nm by a molar extinction coefficient with a value of 1.2756. This value was calculated using

the amino acid composition reported by Bjarnason and Tu (1978) which included four tryptophan and seven tyrosine residues.

Electrophoresis

The purified HTb was analyzed using SDS-polyacrylamide slab gel electrophoresis (SDS PAGE) according to the method of Laemmli (1970) under conditions of complete denaturation and reduction. The resolving gel was 10% total acrylamide with 2.7% bisacrylamide. The stacking gel was 4% total acrylamide with 2.7% bisacrylamide. The following molecular weight standards, purchased from Sigma Chemicals, were used: myosin (205,000 Da), beta-galactosidase (116,000 Da), phosphorylase b (97,000 Da), bovine serum albumin (66,000 Da), egg albumin (45,000 Da), glyceraldehyde (36,000 Da), carbonic anhydrase (29,000 Da), trypsinogen (24,000 Da), trypsin inhibitor (20,100 Da), and alpha-lactalbumin (14,200 Da). A sample of the purified HTb containing 0.10 mg of protein was combined with an equal volume of sample buffer, 0.125 M Tris-Cl, 4% SDS, 2% sucrose, and 10% 2-mercaptoethanol, pH 6.8, and heated at 100^{o}C for 3.0 min. The sample was electrophoresed at 35 mA for approximately 2 hr in a LKB (Rockville, MD) electrophoresis unit cooled to 15^{o}C. The gel was fixed in 3.46% sulphosalicylic acid containing 11.5% trichloracetic acid for several hours. Following fixation, the gel was washed for 20 min in destaining solution consisting of 25%

ethanol and 4% acetic acid. The gel was then stained overnight in 1% Coomassie blue R-250. Destaining was carried out until the gel became clear.

Enzyme Assays

Protease activity was measured according to the method of Lin et al. (1969) using N,N-dimethylcasein as the substrate. The following reagents were necessary for the assay: (1) 0.1% N,N-dimethylcasein (Sigma Chemical Co.); (2) 0.1% trinitrobenzene sulfonic acid (TNBS, Sigma Chemical Co.); (3) 4.0% sodium bicarbonate; (4) 10% SDS; and (5) 1.0 N HCl. A 0.02 mg sample from every third fraction of all columns was incubated with 1.0 ml of the dimethylcasein in 0.005 M Na-MOPS at 38^{o}C for 30 min at a pH of 7.2. The enzymatic reaction was stopped by immersing the solution in a boiling water bath for 1.0 min. Added to the sample solution was 1.0 ml TNBS and 1.0 ml sodium bicarbonate. This solution was incubated for 30 min at 50^{o}C in the dark. The reaction was stopped by the addition of 1.0 ml SDS and 0.5 ml HCl. The absorbance at 340 nm was read. The number of free amino terminals was determined by dividing the A340nm by the extinction coefficient 1.3×10^{4} moles^{-1} cm. One unit is defined as one mole of peptide bonds cleaved during the thirty minute incubation period. The protease activity of the fractions following hydrophobic interaction chromatography was not determined since the ammonium sulfate interferred with the TNBS

reaction. The protease activity of the pooled fraction after buffer exchange, however, was determined.

Arginine esterase activity was determined according to the method of Rick (1963) using benzoylarginine ethyl ester (BAEE, Sigma Chemical Co.) as the substrate. The substrate solution consisted of a 3.43% BAEE in 0.05 M Tris-Cl, pH 8.0, containing 0.222% $CaCl_2$. The procedure consisted of adding 0.1 ml of every third fraction from anion exchange chromatography to 1.0 ml of the substrate solution and monitoring the change in absorbance at 254 nm, recording the change every 30 sec for 4.0 min. The micromoles substrate hydrolyzed per minute was calculated by dividing the change in A254/min by 1.046. One unit is defined as one mole of BAEE hydrolyzed per minute.

Phospholipase activity was determined according to a modification on the method of Bon and Saliou (1983) as follows. The following solutions were used in the assay: (1) lecithin solution: 1.0 gm lecithin (Sigma Chemicals) was dissolved in 10 ml methanol and 3.0 Tween 20, filtered and brought to 20 ml with distilled water; (2) indicator solution: 6.0 mg cresol red (Sigma Chemicals) was dissolved in 1.0 ml methanol and brought to 100 ml with distilled water; (3) buffer substrate solution: 4.0 ml of the lecithin solution was mixed with 7.0 ml of indicator solution, 3.0 ml of 0.1 M glycylglycine solution, 1.0 ml 90 mM $CaCl_2$ solution and brought to 30 ml with distilled water. The pH was adjusted to 9.5 with 2.0 N NaOH. The

assay consisted of adding 0.01 ml of every third tube from anion exchange chromatography to 1.0 ml of the buffer substrate solution and reading the change in absorbance at 578 nm, recording the value at 30 sec intervals. The extinction coefficient used in the calculation of activity was determined by adding 0.03 ml of a 0.1 N HCl solution to 3.0 ml of the buffer substrate solution. The absorbance at 578 nm of the buffer substrate solution after addition of the acid was subtracted from the absorbance of the solution prior to acid addition. The difference was used in the calculation. The volume activity was determined by the following equation:

$$\text{Vol. act} = \frac{[(1.01) \times (\text{Change in A578/min})]}{(\text{extinct. coeff.}) \times (1.0) \times (0.01)}$$

The activity was recorded as units/ml sample. One unit is defined as a change of 2.42×10^{-3} absorbance units per minute.

Hemorrhage

The maintenance of hemorrhagic activity was monitored following each chromatographic step by injection of 0.05 ml of the pooled sample into the thigh of a mouse. After three hours, the mouse was killed and the skin removed to evaluate the presence or absence of hemorrhage.

Chromatofocusing

A Pharmacia 2.6 X 30 cm column of polybuffer exchanger 118 was equilibrated with triethanolamine buffer, pH 10.5

and eluted with polybuffer exchanger 118, pH 8-10.5 buffer. A sample of 25 mg of HTb dissolved in the triethanolamine buffer, pH 10.5, was applied to the column and a flow rate of 20 ml/hr was maintained with a peristaltic pump. Fraction sizes of 6.0 ml were collected and the absorbance at 280 nm recorded.

PH Optimum

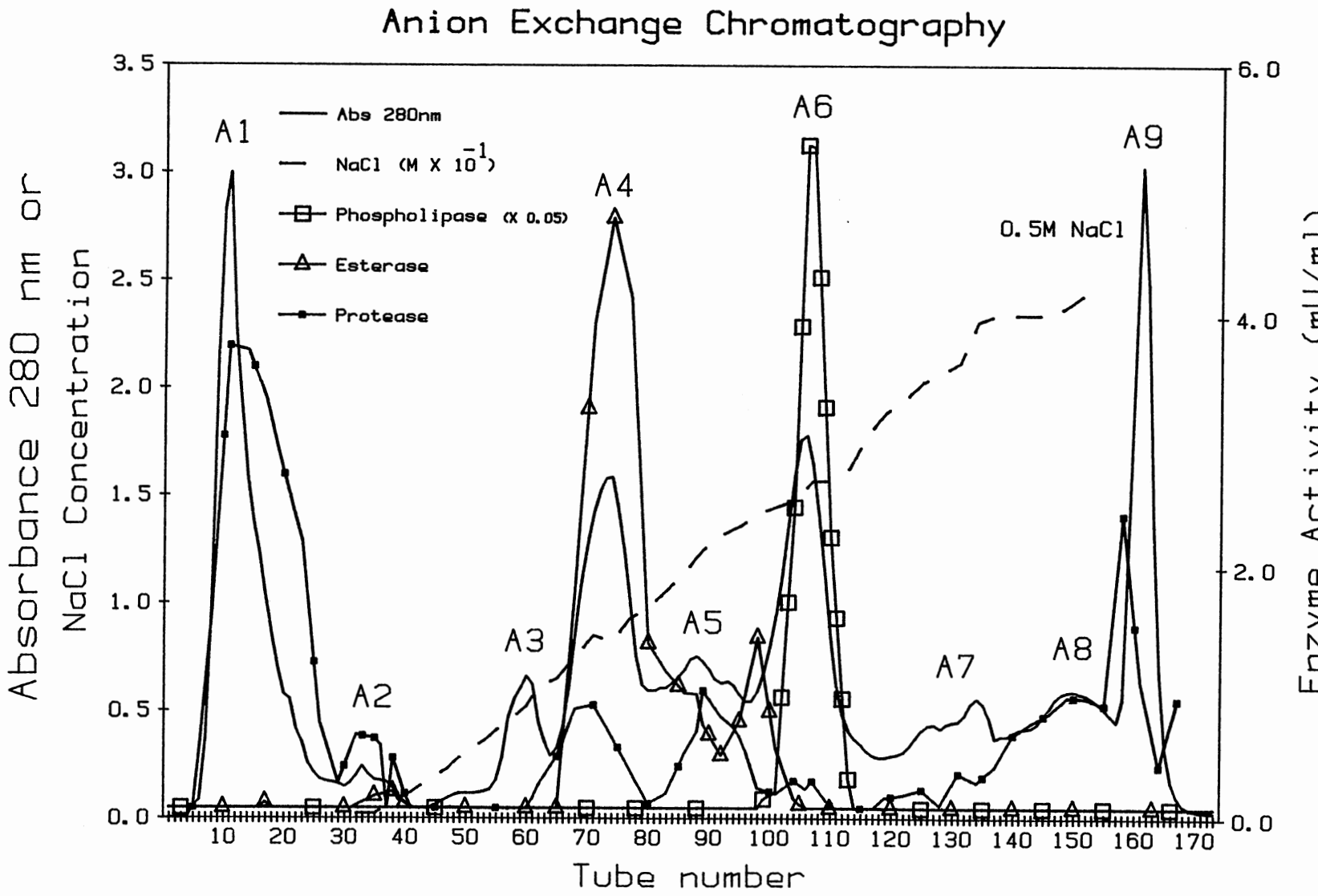
The pH optimum of the protease activity of the partially purified toxin (following cation exchange chromatography) was determined by performing the protease assay described previously, but preparing the dimethylcasein in 0.005 M Na-MOPS buffer titrated to the following pH values: 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0, 10.5, and 11.0.

Results

Isolation

Figure 2 is the elution profile obtained for crude C. atrox with anion exchange chromatography. Nine major fractions were obtained, and all contained protease activity with fraction A1 having the largest activity. Only fractions A2, A4, A5, and A6 had arginine esterase activity while only fraction A6 had phospholipase A_2 activity. Because HTb is a basic protein, only fraction A1, tubes 7 through 25, which also demonstrated hemorrhagic activity, was collected, pooled, and concentrated for gel

Figure 2. Fractionation of 2 gm crude C. atrox venom on DEAE-Sephacel. The column was eluted with equilibration buffer, 0.01 M Tris-Cl, pH 8.6, until elution of the unbound material, followed by a 2000 ml linear gradient of 0.0 M - 0.25 M NaCl at a flow rate of 30 ml/hr. Peak one, tubes 7 - 25, was pooled and concentrated for further purification. Fractions of 8.2 ml were collected during the elution of the unbound fraction, and increased to 19.0 for the remainder of the separation.Figure 2. Column size: 2.6 X 27 cm.


Anion Exchange Chromatography
Absorbance 280 nm or
NaCl Concentration
Enzyme Activity (mU/ml)
Tube number
Abs 280nm
NaCl (M X 10^-1)
Phospholipase (X 0.05)
Esterase
Protease
0.5M NaCl
A1
A2
A3
A4
A5
A6
A7
A8
A9
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6.0
4.0
2.0
0.0
10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160 170

filtration chromatography.

Figure 3 is the elution profile obtained from the separation of fraction A1 with gel filtration. Two major fractions were obtained with a very minor third fraction. Only fractions B1 and B2 had protease activity, and only fraction B2 was hemorrhagic. Esterase and phospholipase activities were not measured after anion exchange chromatography since these activities were not observed in the unbound fraction, A1. Fraction B2, tubes 53 through 64, was collected, pooled, and concentrated for application to cation exchange chromatography.

Figure 4 is the elution profile of the separation of fraction B2 on cation exchange chromatography. Six fractions were obtained with only fraction C5 and C6 having protease activity. Fraction C5 had the greatest protease activity, but was only slightly hemorrhagic. Fraction C6, tubes 91 through 100, however, was very hemorrhagic and was therefore collected, pooled, concentrated, and applied to the hydrophobic interaction column.

Figure 5 is the elution profile obtained with hydrophobic interaction chromatography. One broad peak, eluted which was collected, concentrated an exchanged into a 0.01 M Na-MOPS buffer containing 0.01 M NaCl, pH 7.2, and stored frozen until use. The pooled protein, tubes 31 through 62, contained protease activity measuring 1.57 mU/ml.

Figure 6A shows the homogeneity of the purified HTb

Figure 3. Gel filtration of fraction A1 from anion exchange chromatography on LKB AcA 54, equilibrated with 0.01 M Na-MOPS, 0.15 M NaCl, pH 8.6. A flow rate of 30 ml/hr was applied. Peak two, tubes 53 - 64, was pooled and concentrated for cation exchange chromatography. Fractions of 6.0 ml were collected. Column size: 2.6 X 85 cm.

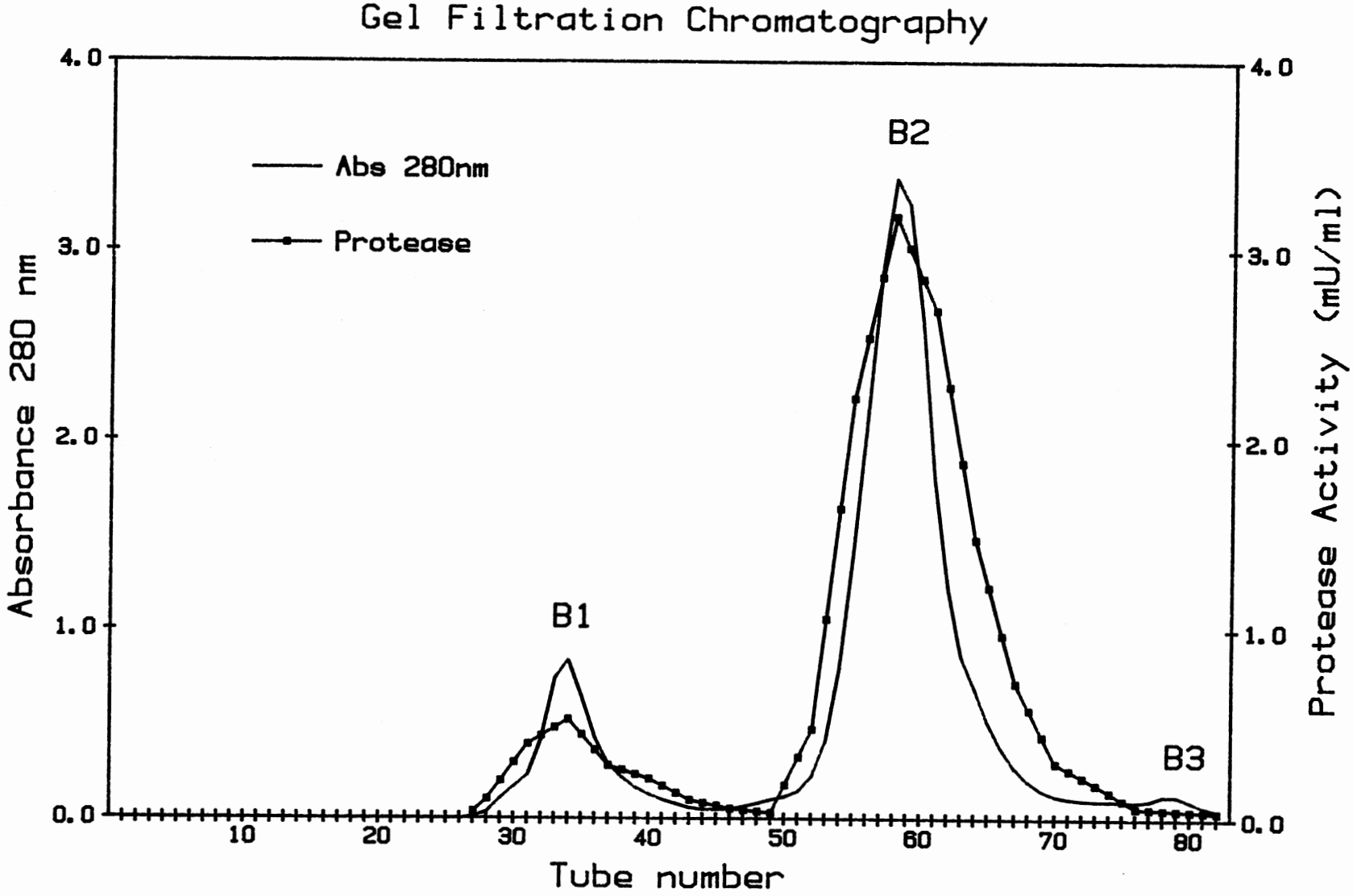

Gel Filtration Chromatography
Abs 280nm
Protease
B1
B2
B3
Absorbance 280 nm
Protease Activity (mU/ml)
Tube number
4.0
3.0
2.0
1.0
0.0
10
20
30
40
50
60
70
80

Figure 4. Fractionation of fraction B2 from gel filtration chromatography on Fractogel TSK 650S cation exchanger, equilibrated with 0.01 M TAPS-Cl, pH 8.6. A 750 ml linear gradient of 0.0 M - 0.15 M NaCl was applied at a flow rate of 30 ml/hr immediately after sample loading. Peak six, tubes 91 - 100, was pooled and concentrated for hydrophobic interaction chromatography. Fractions of 6.0 ml were collected. Column size: 1.6 X 33 cm.

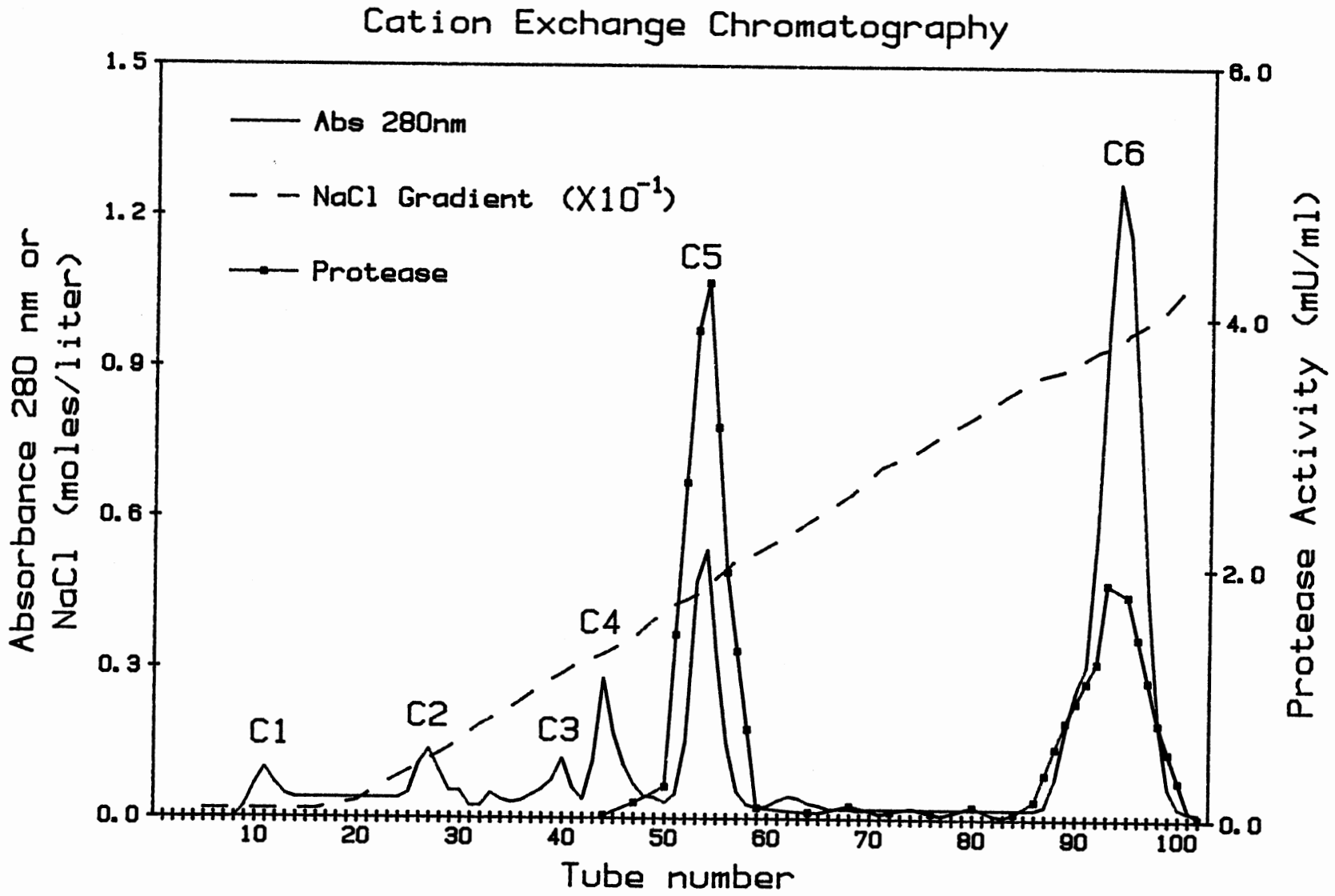
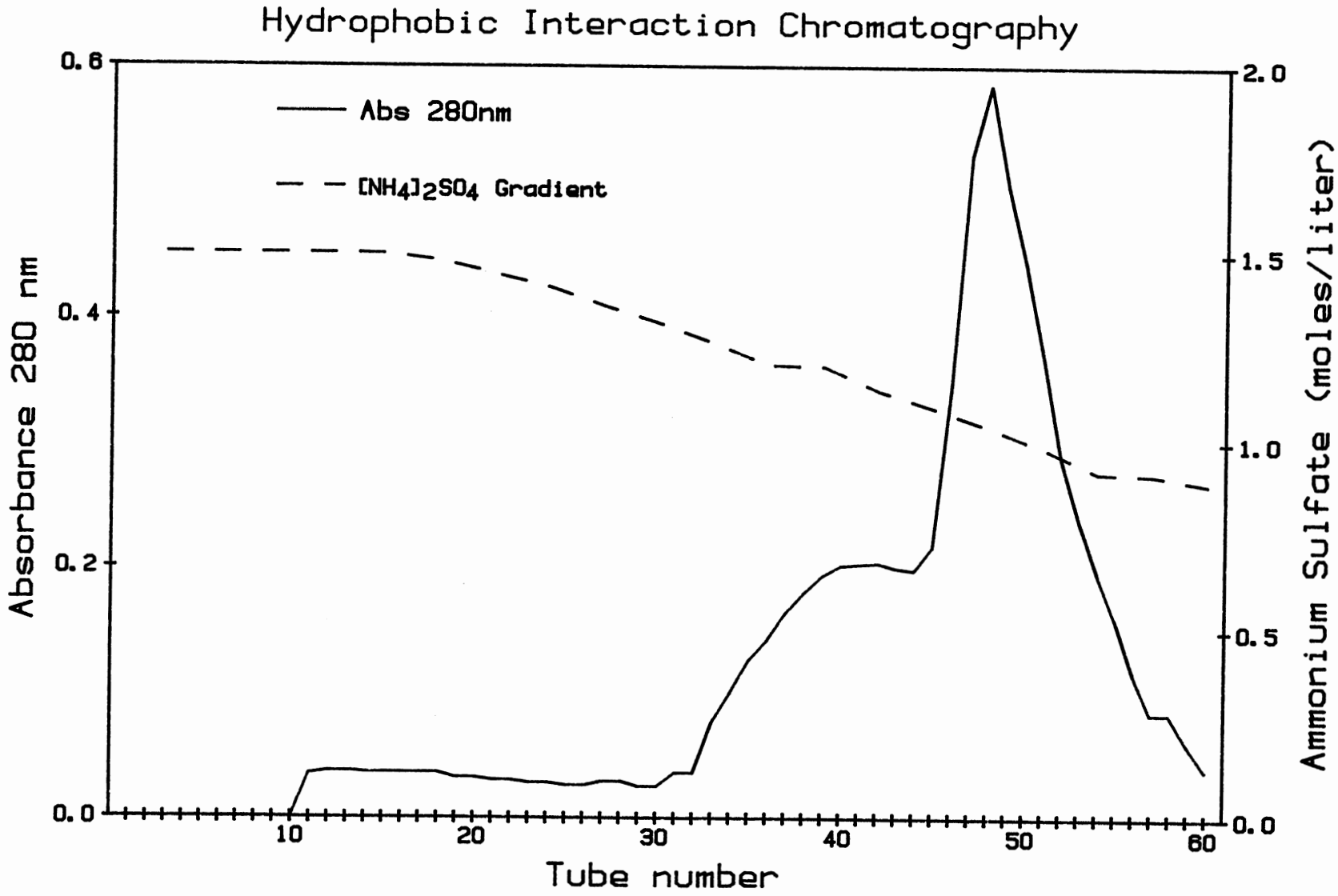

Cation Exchange Chromatography
Abs 280nm
NaCl Gradient (X10^{-1})
Protease
C1
C2
C3
C4
C5
C6
Absorbance 280 nm or NaCl (moles/liter)
Protease Activity (mU/ml)
Tube number
1.5
1.2
0.9
0.6
0.3
0.0
6.0
4.0
2.0
0.0
10
20
30
40
50
60
70
80
90
100

Figure 5. Fractionation of fraction C6 from cation exchange chromatography on Phenyl-Sepharose CL-4B, equilibrated with 0.01 M Tris-Cl, 1.5 M ammonium sulfate, pH 7.2. A 600 ml linear gradient of decreasing ammonium sulfate, 1.5 M - 0.0 M, concentration and increasing ethylene glycol, 0% - 50%, was applied at a flow rate of 25 ml/hr. A single broad peak was collected, tubes 31 - 62, subjected to a buffer exchange, and stored at -20^{o}C until use. Fractions of 6.0 ml were collected. Column size: 1.6 X 27 cm.


Hydrophobic Interaction Chromatography
Abs 280nm
[NH4]2SO4 Gradient
Absorbance 280 nm
Ammonium Sulfate (moles/liter)
Tube number
0.0
0.2
0.4
0.6
0.5
1.0
1.5
2.0
10
20
30
40
50
60

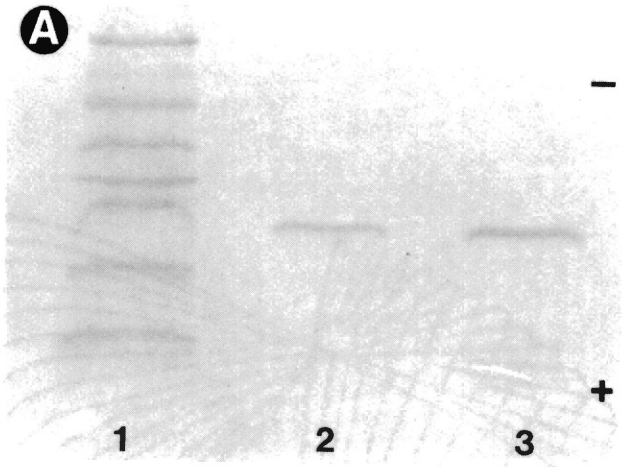
using a 10% SDS polyacrylamide gel electrophoresis. In lane 1 were the molecular weight standards, excluding myosin. In lane 2 was HTb at 0.005 mg/well, and in lane 3 was HTb at 0.01 mg/well. Only one band was visible, demonstrating a homogeneous protein. The HTb protein band migrated slightly farther than the trypsinogen standard, which has a molecular weight of 24,000 daltons, indicating that HTb had a molecular weight of approximately 23,000 daltons. Bjarnason and Tu (1978) reported a molecular weight of 24,000 daltons by SDS disc gel electrophoresis and 19,000 daltons by gel filtration. Figure 6B is a photograph of the SDS-polyacrylamide gel of the protein following the cation exchange chromatography which shows a second lower molecular weight contaminating band. The molecular weight standards were the same as those described above. Lanes 2 and 3 also contained 0.005 and 0.01 mg/well respectively.

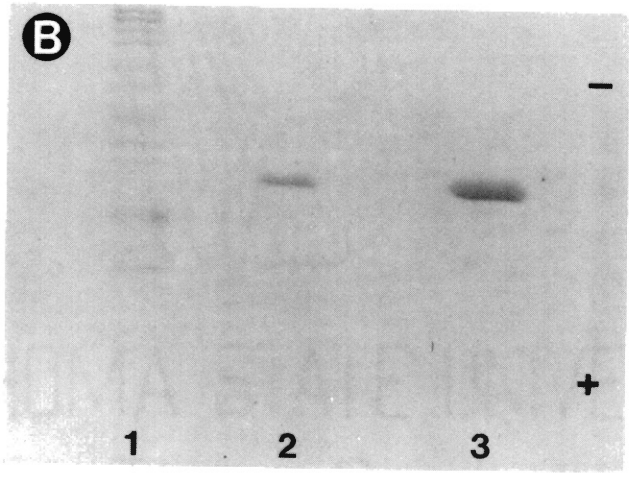
A total of 68.46 mg of purified toxin was recovered from the isolation procedure described above. This represents at least 3.42% of the crude venom. Bjarnason and Tu (1978) reported a value of 3.70%.

The hemorrhagic activity of the toxin was maintained throughout the isolation procedure until the final hydrophobic interaction chromatographic step. During this chromatographic procedure, the hemorrhagic activity of the venom was destroyed even though the toxin was still proteolytic. However, this step was necessary to produce a

Figure 6A. 10% SDS-polyacrylamide slab gel demonstrating the single, homogeneous band of HTb following hydrophobic interaction chromatography. Lane 1: molecular weight standards (beta-galactosidase, 116,000 Da; phosphorylase b, 97,000 Da; bovine serum albumin, 66,000 Da; egg albumin, 45,000 Da; glyceraldehyde, 36,000 Da; carbonic anhydrase, 29,000 Da; trypsinogen, 24,000 Da; trypsin inhibitor, 20,100 Da; and alpha-lactalbumin, 14,200 Da); lane 2: HTb at 0.005 mg/well; lane 3: HTb at 0.01 mg/well.

Figure 6B. 10% SDS-polyacrylamide slab gel demonstrating the lack of homogeneity of the protein following cation exchange chromatography. Lane 1: same molecular weight standards described for Figure 5; lane 2: 0.005 mg/well; lane 3: 0.01 mg/well.


A
−
+
1
2
3


B
−
+
1
2
3

purified toxin required in immunization for antibody production, since the fraction following cation exchange chromatography contained more than a single protein.

During the chromatofocusing procedure, if the isoelectric point of the toxin was less than 10.5 pH units, the protein would have bound to the column. However, the HTb did not bind at all, eluting with the unbound fraction. This demonstrates that the isoelectric point of HTb must be greater than 10.5.

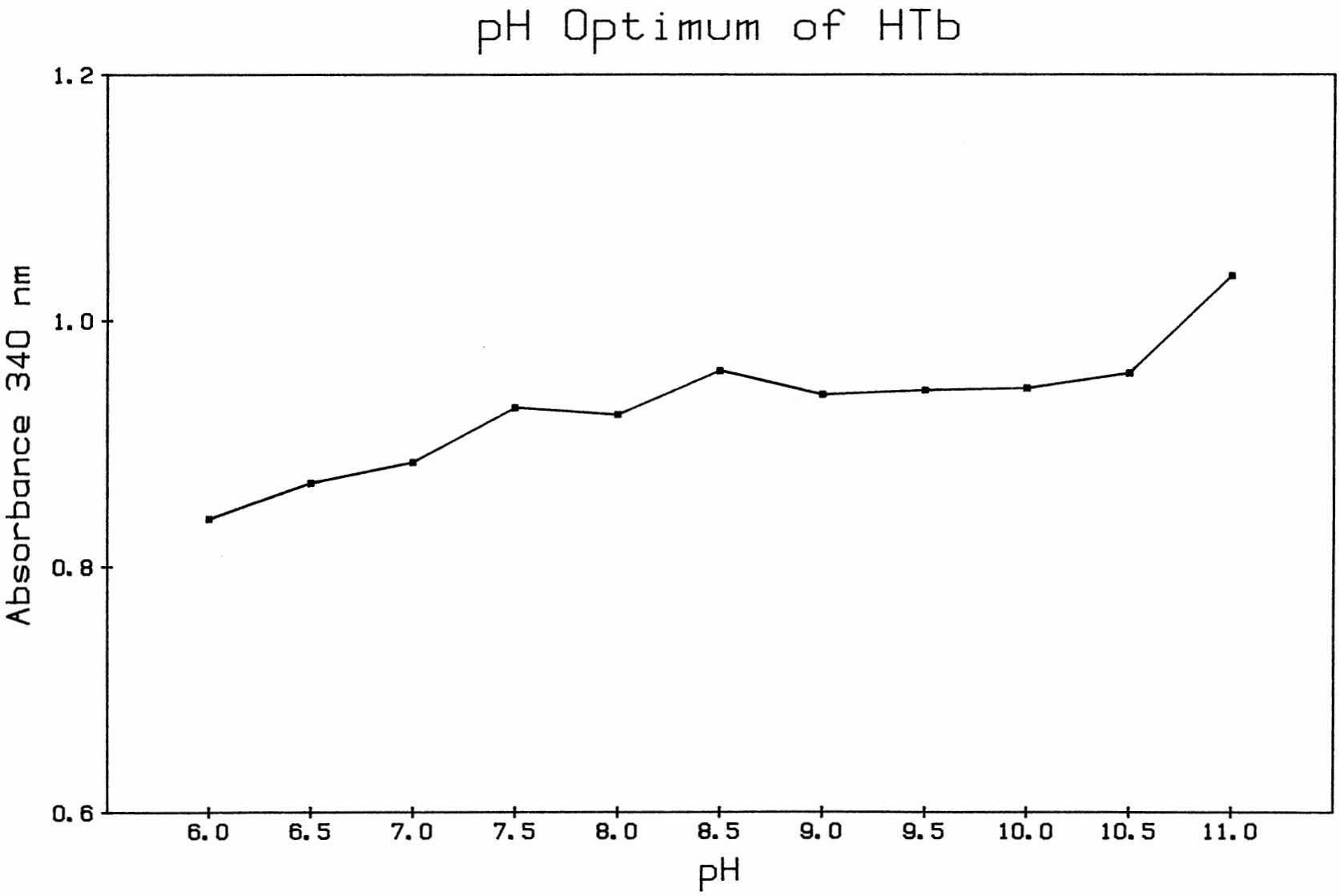
The results of the pH optimum experiment are shown in Figure 7. The values are graphed as the absorbance at 340 nm according to the procedure described for the protease assay in Materials and Methods. The protein had a very broad pH range, over which it maintained the ability to hydrolyze dimethylcasein.

Discussion

Hemorrhagic toxin b has been isolated to homogeneity as evidenced by SDS PAGE. It has a molecular weight of approximately 23,000 daltons and an isoelectric point greater than 10.5. It possesses proteolytic activity with dimethylcasein as a substrate, and is devoid of both esterase and phospholipase activity with BAEE and egg yolk lecithin as substrates, respectively. It represents approximately a minimum of 3.4% of the crude venom protein.

Bjarnason and Tu (1978) reported the isolation of HTb to homogeneity as measured by SDS disc gel electrophoresis

Figure 7. Determination of the pH optimum of the proteolytic activity of HTb on dimethylcasein. Htb used was fraction C6 following cation exchange chromatography. Values are plotted as absorbance at 340 nm according to the protease assay method of Lin et al. (1969) as described in Materials and Method. Twenty microliter samples of fraction C6 were incubated for thirty minutes with the substrate (0.1% dimethylcasein) in 0.01 M Na-MOPS buffer adjusted to pH values ranging from 6.0 to 11.0.


pH Optimum of HTb
Absorbance 340 nm
1.2
1.0
0.8
0.6
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
pH

using the following isolation procedure: fractionation on a Whatman DE 32 anion exchange column; fractionation on a Sephadex G-75 superfine; fractionation on a Whatman CM 32 cation exchange resin; a second fractionation on the cation exchange column. In the present study, an additional method of purification, hydrophobic interaction chromatography on phenyl sepharose CL-4B, was required to yield a homogeneous protein, since the isolate following cation exchange chromatography demonstrated contaminants (Figure 5b). A possible reason for the additional purification step requirement as presented here could be due to a difference in the venoms. Glenn and Straight (1978) reported variations in toxicity of the venoms from C. scutulatus scutulatus specimens collected from different geographic regions in the United States and Mexico. Minton and Weinstein (1986) also reported differences in proteolytic and lethal activities of venoms from C. atrox collected from various geographic areas. These reports demonstrate that venom compositions can vary depending on their origin, and may explain the difference in the isolation protocol used by Bjarnason and Tu (1978) and that reported here. Also, Bjarnason and Tu (1978) reported that HTb represented 3.7% of the crude venom protein, while the results presented here demonstrate that HTb is only 3.46% of the protein. This difference could be due to three possible reasons: (1) variations in the venoms; (2) the conditions, such as which tubes should be collected and

concentrated for a given elution peak or fraction, used in pooling the protein fractions following each chromatographic step; or (3) the the removal in the present study of contaminating proteins present in the "purified" HTb reported by Bjarnason and Tu (1978). The possibility of a combination of these factors should also be considered.

The inability of the toxin to bind the chromatofocusing column equilibrated with a buffer at a pH of 10.5 indicates the toxin has an isoelectric point greater than 10.5. This is considerably more basic than any other hemorrhagic toxin reported in the literature, although AaHIII, from the venom of Agkistrodon acutus, has a reported pI greater than 9.0. There are several myotoxins that have reported isoelectric points greater than 9.8. Some of these are peptide C, from the venom of C. viridis helleri (Maeda et al, 1978), myotoxins I and II, from the venom of C. v. concolor (Engle et al., 1983), and mulgotoxin A, from the venom of Pseudechis australis (Leonardi et al.. 1979). The basicity of HTb may be important in the mechanism of action leading to hemorrhage and/or myonecrosis. However, mucrotoxin A, from the venom of T. flavoviridis, is also a hemorrhagic myotoxin, but with a pI of 4.3, indicating that both basic and acidic toxins can induce hemorrhage and myonecrosis.

A question that has been raised by reports in the literature concerns the relationship between proteolytic

activity and hemorrhagic activity of hemorrhagic toxins. It is interesting that Civello et al. (1983a) make the statement "...very few of the hemorrhagins have been shown to contain proteolytic activity," because a review of the literature has shown that all of the hemorrhagic toxins thus far isolated have proteolytic activity on either casein or hide powder azure, with the notable exceptions of HR-1, HR-2a, and HR-2b, toxins isolated from the venom of T. flavoviridis (Takahashi and Ohsaka, 1970; Ohsaka et al., 1973). These venoms, however, do cause the release of proteins from the glomerular basement membrane preparation. Bjarnason and Tu (1978) did, however, demonstrate a lack of direct relationship between proteolytic activity and hemorrhagic activity of five of the hemorrhagic toxins present in the venom of C. atrox. HTa, for example, was the most hemorrhagic of the toxins, yet it had the least activity on casein. The presence or absence of proteolytic activity, or the degree of activity on a given substrate, depends to a large degree on the particular substrate used in the assay (Bjarnason and Tu, 1978). Because a protein lacks proteolytic activity on a given substrate does not necessarily mean the protein is not a protease. Given the right substrate, many toxins previously described as being non-proteolytic may very well be proteolytic. HTb, which has been described as a specific protease (Bjarnason and Tu, 1978), is able to hydrolyze insulin B chain and glucagon (Hagihara et al., 1985), casein (Bjarnason and Tu,

1978), and fibrinogen (Komori et al., 1985). Fox et al. (1983) propose that the disruption of the basal lamina by proteases in the venom would be an important mechanism in the hemorrhagic response. Obviously, the disruption of the basal lamina could be facilitated by the presence of a protease capable of hydrolyzing proteins that are a part of this structure. The hemorrhagic toxin HP IV, isolated from the venom of C. horridus horridus (Civello et al., 1983a), which has no collagenase activity (Civello et al., 1983b), is capable of disrupting the basal lamina (Ownby and Geren, in press), despite the fact that the major protein of the basal lamina is collagen. Treating the purified hemorrhagic toxins from C. atrox venom with EDTA, Bjarnason and Tu (1978) demonstrated the complete loss of hemorrhagic activity concomitant with loss of proteolytic activity. The same results were reported by Friederich and Tu (1971) using crude venoms from the genera Agkistrodon, Crotalus, Sisturus, Vipera, and Bothrops. These results clearly demonstrate at the least a relationship between the two activities. However, in the present study, it was noted that application of the partially purified HTb to the hydrophobic interaction column resulted in a loss of hemorrhagic activity when tested i.m. in mice, yet without loss of proteolytic activity on dimethlycasein. Application of the protein to this column, which was eluted with a decreasing linear gradient of 1.5 M ammonium sulfate and increasing ethylene glycol, was necessary to acheive

the purity required for immunization. Obviously, proteolytic activity can be retained even with the loss of hemorrhagic activity. This is not surprising since there are a number of proteolytic toxins, such as the proteases I, II, III, and IV, from the venom of C. atrox, that do not induce hemorrhage. The loss of hemorrhagic activity with elution from the hydrophobic interaction column could possibly be due to a conformational change resulting from the following: (1) interaction with the phenyl-sepharose matrix; (2) the high ionic strength of the ammonium sulfate which was required for the toxin-matrix binding interaction; or (3) the presence of the ethylene glycol, which was included to facilitate hydrophilicity of the toxin and a more efficient purification step. A combination of these effects could also be possible. Further work considering these factors could help elucidate the mechanism of action of HTb, especially related to it's proteolytic activity. It is possible that there are two regions on the molecule involved in the hemorrhagic response in vivo, one region responsible for the binding of the toxin and one region responsible for the proteolytic activity. Any alteration in the conformation of either of these proposed regions could result in the loss of the hemorrhagic response. Perhaps future research will one day clarify the relationship between the proteolytic activity of a toxin and it's presence or absence of hemorrhagic activity.

HTb is not vastly different from other hemorrhagic toxins thus far isolated from snake venoms, although it is the most basic toxin reported. Not all toxins isolated have had exact isoelectric points determined. It is possible that there are other hemorrhagic toxins with isoelectric points as basic as HTb, perhaps even more so. However, for those reported with pI values, HTb is the only one above 10 (based on the present study). Hemorrhagic toxins have been isolated from both crotalid and viperid species, with molecular weights ranging from 22,000 to 115,000 daltons. As previously stated, all have proteolytic activity on at least one substrate provided glomerular basement membrane is included as a substrate. A unique feature of HTb as compared to most other hemorrhagic toxins, however, is it's ability to induce myonecrosis. Two other hemorrhagic toxins, mucrotoxin A (Sugihara et al., 1983) and viriditoxin (Fabiano and Tu, 1981), from the venoms T. flavovirids and C. v. viridis, respectively, have also been reported to cause myonecrosis. Gleason et al. (1983), however, have concluded for viriditoxin that myonecrosis due to this venom results not from a direct myotoxic activity of the toxin, but from ischemic conditions created by the hemorrhage. There is still some question as to the direct myotoxic effects of the remaining two hemorrhagic-myotoxins.

The complexity of rattlesnake venoms makes the study of their biological actions very difficult. The use of

purified components will certainly be an advantage in describing the pathogenesis of the sequelae typical of these venoms. The pathogenesis of myonecrosis induced by myotoxins isolated from B. asper venom (Gutierrez et al., 1984) and C. v. viridis venom (Ownby et al., 1976) has been described. The ability of an antiserum to myotoxin a to neutralize the myotoxic effects of the toxin and the crude C. v. viridis venom has been described by Ownby et al. (1983). The purified HTb described in this chapter has been used to produce an antibody. The toxin and the antibody have been used to measure the presence of HTb or similar toxins in other venoms and to study one aspect of the pathogenesis of the myonecrosis induced by the toxin. In addition, the ability of the antibody to neutralize the hemorrhage induced by the purified toxin has been measured. The results are described in the following chapters.

CHAPTER IV

AFFINITY PURIFICATION OF ANTIBODIES TO HTB: ABILITY TO NEUTRALIZE HEMORRHAGE

Introduction

The inability of polyvalent antivenoms to neutralize local and lethal effects of snake venom poisoning (Minton, 1954; Ownby et al., 1983) has resulted in attempts to develop more effective antisera. Ownby et al. (1985; 1986) have shown more effective prevention of myonecrosis induced by Crotalus viridis viridis venom and myotoxin a with a mixture of an antiserum produced against myotoxin a and Wyeth's polyvalent (Crotalidae) antivenin, both in vitro and in vivo. Tan (1983) demonstrated an improvement in protection against lethality due to the Malayan cobra by conjugating the toxic low molecular weight components present in this venom to a neurotoxin isolated from the venom of Enhydrina schistosa. Another method utilized in hopes of producing better antisera is the affinity purification of antibodies, both to crude venoms and to purified toxins. Yang et al. (1977) showed an improvement in the neutralization of lethal effects induced by cobrotoxin using affinity-purified antibodies to this toxin. Lomonte et al. (1985) purified antibodies to a

myotoxin by this technique from the polyvalent antivenom used against the venom Bothrops asper that were more effective in neutralizing myotoxic effects caused by the crude venom. Lee and Chao (1978) demonstrated a 29.6-fold increase in neutralizing capacity of affinity-purified antibodies to Bungarus multicinctus as compared to the polyvalent antivenom produced against the crude venom. These results encourged the development of an affinity-purified antibody to purified hemorrhagic toxin b (HTb) in hopes of eventually improving the ability of commercial antisera to neutralize toxins and better treat snake venom poisoning. The production and affinity-purification of antibodies to HTb and their ability to neutralize hemorrhage in vitro is presented.

Materials and Methods

Hemorrhagic Toxin b

HTb was isolated as previously described (Chapter III). Briefly, 2.0 grams of desalted crude Crotalus atrox venom was purified by a four-step chromatographic procedure utilizing anion exchange, gel filtration, cation exchange, and hydrophobic interaction chromatography. The purified toxin thus obtained contained only a single protein band after analysis by SDS-PAGE, and was considered homogenous. The toxin was desalted, diafiltered into 0.01 M Na-MOPS, pH 7.2, and stored at -20^{o}C until needed.

Anti-HTb serum

One ml of Freund's complete adjuvant was mixed with one ml of a 0.1 mg/ml solution of purified HTb and injected i.m. between the shoulder blades of a New Zealand white female rabbit. Booster injections were of the same concentration of HTb, but were mixed with one ml of Freund's incomplete adjuvant. Booster injections were administered two weeks following the initial injection of toxin and subsequently at monthly intervals. Approximately two weeks following the second and third booster injections, the rabbit was bled from the marginal ear vein. Serum was collected and stored at -20^{o}C until needed.

Affinity Column Preparation

Approximately 27 mg of HTb was conjugated to eight ml of an Affi-Gel 10 agarose gel bead support (Bio-Rad Corp.) in 0.1 M Na-MOPS, ph 7.0, overnight at 4^{o}C. The gel was packed into a 1.5 X 12 cm glass chromatography column and equilibrated in phosphate buffered saline, pH 7.4 (Sigma Chemical Co.). The four eluants to be used, PBS, 0.05 M acetic acid, and distilled water were applied to the column and fractions were collected and measured for protein to ensure that HTb would remain bound to the column. Protein concentration of the fractions was measured with the protein determination method of Bradford (1976).

Affinity Purification of Antibodies

Prior to application to the column, the crude rabbit serum was centrifuged at 40,060 g, 4°C, in a Sorvall SM-24 centrifuge (Dupont Corp.) for 30 min. One ml of the supernatant was applied to the affinity column and eluted with PBS at a flow rate of 5.0 ml/hr until elution of the unbound fraction was complete. The column was then successively eluted with distilled water, 0.05 M acetic acid, and then re-equilibrated in PBS. Fractions of 2.2 ml were collected and the absorbance at 280 nm was read using a Beckman DU-8 spectrophotometer (Beckman Corp., Irvine, CA). The peak eluting with the acetic acid was subjected to a buffer exchange, into 0.01 M Na-MOPS buffer, pH 7.2, in an Amicon ultrafiltration cell and pooled, concentrated, and stored frozen until use.

Enzyme-Linked Immunosorbent Assay (ELISA)

The composition of reagents used for the ELISA were as follows: (a) coating buffer - 0.015 M sodium bicarbonate, 0.035 M sodium carbonate, 0.003 M sodium azide, 1% BSA; (b) washing buffer - 0.15 M Nacl, 0.05% Tween 20; (c) incubation buffer - 0.05% Tween 20, 0.003 M sodium azide, 1% BSA, dissolved in PBS; (d) diethanolamine buffer -0.1 M diethanolamine-Cl, 0.0011 M magnesium chloride, pH 9.8; (e) phosphatase substrate - p-nitrophenyl phosphate disodium, 40 mg/ml, dissolved in diethanolamine buffer. The

immunological activity of the fractions eluting from the column was monitored using the ELISA technique. Polystyrene microtiter plates (Flow Laboratories, McLean, VA) were coated with 0.2 ml of a 1.0 mg/ml solution of HTb, diluted in coating buffer, overnight at 4°C. The plates were washed 3 X 5 min with washing buffer followed by addition of 0.2 ml of each fraction from the affinity column for 2 hr at room temperature. The plates were washed 3 X 5 min with washing buffer and 0.2 ml of a 1/2000 dilution of goat anti-rabbit IgG conjugated to akaline phosphatase diluted in incubation buffer was added and allowed to incubate for 2 hr at room temperature. The plates were washed 3 X 5 min with washing buffer. For color development, 0.2 ml of phosphatase substrate was added and allowed to incubate for 30 min at room temperature. The absorbance at 405 nm was measured using an EIA ELISA manual reader (Biotek Instruments, Inc.).

Immunodiffusion

The Ouchterlony double-diffusion technique (Clausen, 1969) was used to detect precipitation reactions between HTb, crude venom and antibodies present in the crude serum, polyvalent antivenin, and the affinity-purified antibody solutions. Serial two-fold dilutions, from undiluted to 1/32, of a 1.0 mg/ml solution of HTb and the crude venom were tested against the same dilutions of concentrated polyvalent antivenin and rabbit antiserum to HTb. The

affinity antibodies were diluted as above, but the undiluted material was 0.76 mg/ml. The gels were of 1% agarose dissolved in PBS, pH 7.4. The gels were 10 X 15 cm and the distance between wells was 1.0 cm. Each well received 0.02 ml of the test solutions, and the gels were allowed to incubate in a humid chamber at room temperature for 24 hr. The gels were washed for 24 hr in PBS and stained with Crowle double stain (Crowle and Cline, 1977), dried, and photographed.

Western Blot Technique

The specificity of the affinity purified antibody was measured using the western blot immunoblotting technique according to the method of Towbin et al. (1979). HTb was electrophoresed on a 10% SDS polyacrylamide gel, with 0.005 mg protein/well, and electrophoretically transferred to a nitrocellulose membrane in a Hoeffer Scientific Instruments (San Francisco, CA) transfer unit cooled to 15^{o}C. The nitrocellulose membrane was washed two times at 5 min in 0.5 M NaCl containing 20 mM Tris-Cl buffer (TBS) and then coated for 2 hr in three concentrations of primary antibody. These concentrations were 0.01 mg/ml, 80 ng/ml, and 0.64 ng/ml. The membrane was washed twice in TBS, twice in TBS containing 0.05% Tween 20, all at 5 min, and twice in distilled water for a total of 2 min. Protein A-colloidal gold was applied to the membrane for 4 hr, followed by two washings in TBS for five minutes each,

thorough rinsing in distilled water, and equilibration in 0.2 M citrate buffer for 5 min. The bands made visible with the protein A-colloidal gold were enhanced with 0.11% silver lactate dissolved in 0.85% hydroquinone for 5 min, giving rise to distinct dark bands. Following enhancement, the membrane was fixed in fixing solution for 5 min. The staining procedure described above was performed using the Protein A Gold Immun-blot assay kit obtained from Bio Rad Laboratories (Richmond, CA), catalog number 170-6510.

Minimum Hemorrhagic Dose Determination

The minimum hemorrhagic dose (MHD) of HTb was determined with the method of Kondo (1960). Briefly, the method involved the intradermal injection of 0.05 ml of the test solution into the shaved backs of mice. Six hr post-injection, the mice were killed with ether and the skin removed, placed between two glass plates, and examined on the inside surface. The hemorrhagic spot was measured at the widest point. A second measurement at right angles to the first was also made. The average of these two measurements was recorded as the hemorrhagic spot size. Three doses of HTb, 0.1 mg/kg, 0.3 mg/kg, and 0.5 mg/kg, were tested in three groups of five mice each. A dose response curve was then used to determine the MHD, that dose producing a spot size of 10.0 mm.

Neutralization of Hemorrhage

The ability of the affinity-purified antibodies to HTb and Wyeth's polyvalent (Crotalidae) antivenin to neutralize hemorrhage in vitro was tested with Kondo's method. The toxin was concentrated to ten times one MHD, and 0.1 ml of this solution was added to 0.9 ml of the antisera, giving a 1/10 dilution of the toxin, or one MHD. The test solution was allowed to react overnight at $4^{\circ}C$. The solution was centrifuged for 2 min in an Brinkman 3200 microfuge to remove any precipitate. The following solutions were tested for their neutralization ability: 1.79, 8.26, and 16.20 mg/ml solutions of affinity-purified antibodies to HTb, reconstituted concentrated polyvalent antivenin, crude rabbit serum containing antibodies to HTb, preimmune rabbit serum, normal horse serum, and PBS. Controls included normal rabbit and horse serum and PBS.

Statistical Analysis

An analysis of variance was performed to determine significance of variation within treatments and the least significant difference test was used to determine significance of differences in treatment means. Significance was recorded at $p < 0.01$.

Results

Affinity Column and Antibody Purification

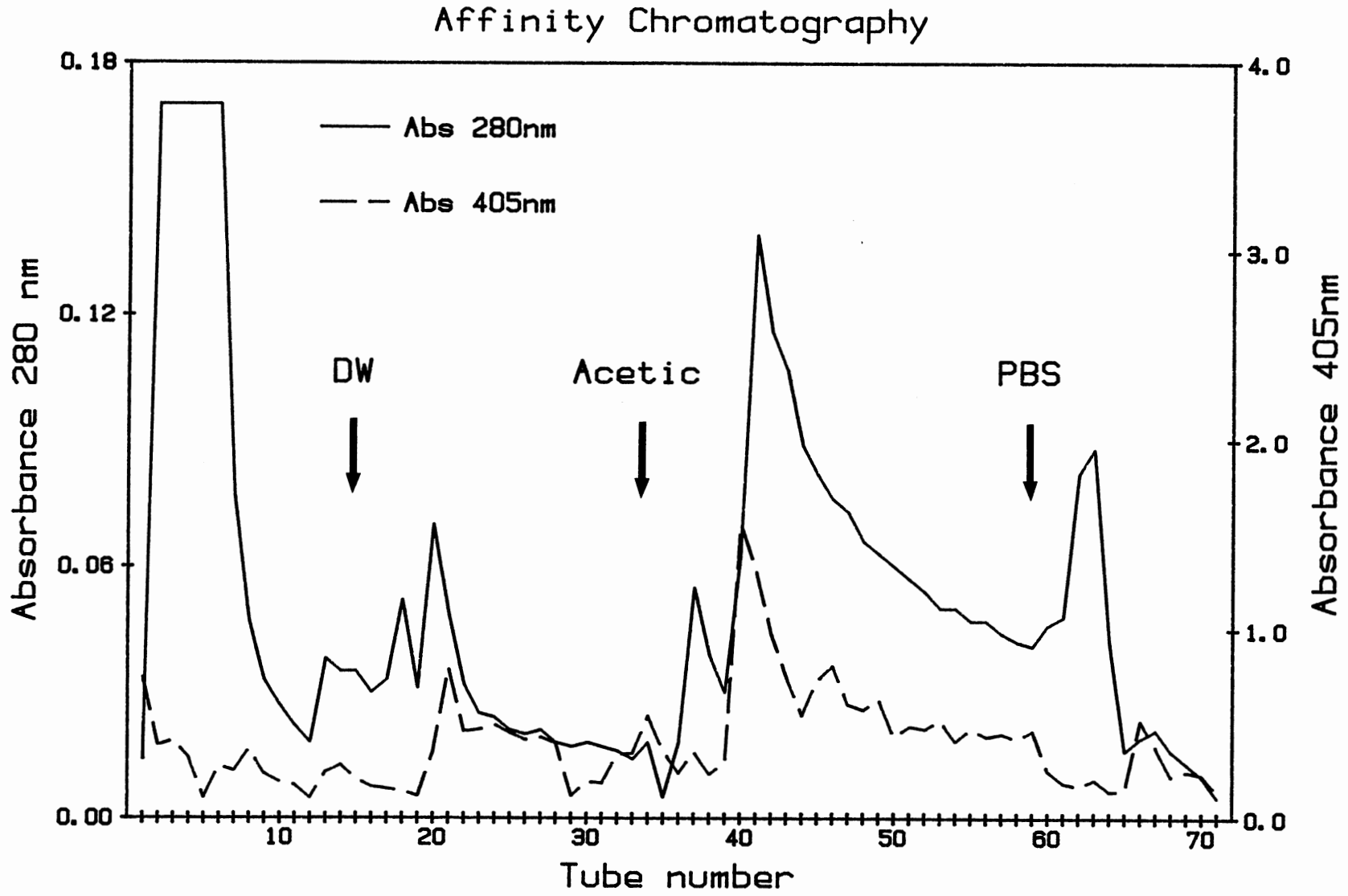
A high coupling efficiency for covalent attachment of purified HTb to the affinity matrix as evidenced by the absence of protein eluting from the column during the washing procedures was expected since the protein binding capacity of the column (35 mg/ml of gel) exceeded the amount of toxin added. Also, none of the elution conditions, including PBS, distilled water, or 0.05 M acetic acid, resulted in loss of the toxin from the column.

Figure 8 is a typical elution profile obtained with the Affi-Gel 10 affinity column. Also presented on this graph is the absorbance at 405 nm, the results of the ELISA of the column fractions. With the exception of the unbound fraction, the largest protein peak, eluted with the acetic acid in tubes 35 through 58. This fraction also contained the highest antibody concentration as measured with the ELISA and was therefore collected and used in the subsequent immunodiffusion and neutralization studies.

Minimum Hemorrhagic Dose

The MHD was determined to be 0.3 mg/kg based on three doses tested, five mice per dose. The mean values for each dose were 0.1 mg/kg = 2.80 mm; 0.3 mg/kg = 10.4 mm; 0.5 mg/kg = 19.4 mm. In most cases, the hemorrhagic spot was

Figure 8. Elution profile of the affinity-purification of antibodies to HTb from of 1.0 ml crude rabbit serum immunized against 0.1 mg HTb. ELISA results showing antibody activity. The left-hand scale represents protein determination by absorbance of each fraction at 280 nm; the right-hand scale is a measure of antibody content as determined with the ELISA at 405 nm. The affinity column was equilibrated in PBS and the sample was applied at a flow rate of 5.0 ml/hr. DW = distilled water; Acetic = 0.05 M acetic acid, pH 3.0; PBS = phosphate buffered saline, pH 7.4. Fraction size was 2.2 ml/tube. Column size: 1.0 X 12 cm.


Affinity Chromatography
Abs 280nm
Abs 405nm
DW
Acetic
PBS
Absorbance 280 nm
Absorbance 405nm
Tube number
0.18
0.12
0.06
0.00
4.0
3.0
2.0
1.0
0.0
10
20
30
40
50
60
70

clearly delineated and relatively round.

Immunodiffusion

Figure 9 shows the immunodiffusion results of each antisera against the crude C. atrox venom. Wyeth's polyvalent antivenin produced two precipitin bands against the crude venom. One band was at an approximate point of equivalence at a 1/8 dilution of the crude venom, while the other precipitin band was at an approximate point of equivalence at a 1/2 dilution of the crude venom (Figure 9 A and B). A single band was visible when the crude rabbit antiserum was tested against the venom, with an approximate equivalence point at a 1/4 dilution of the crude venom (Figure 9 C and D). A single band was also visible when the affinity antibody was tested against the crude venom, with the equivalence point occurring when both test solutions were undiluted (Figure 9 E and F).

Figure 10 shows the results of the antisera tested against the purified HTb. A single band was visible for all three antisera when tested against the purified HTb. The undiluted Wyeth's antivenin and the rabbit antiserum had equivalence points only at the most dilute concentration of HTb, a 1/32 dilution (Figure 10, A and B, C and D). The affinity-purified antibodies produced a band with a zone of equivalence when the test solutions were both undiluted (Figure 10 E and F).

Figure 9. Results of the immunodiffusion assay testing crude C. atrox venom versus polyvalent antivenin, crude rabbit antiserum, and the affinity-purified antibodies to HTb.
A = Wyeth's polyvalent antivenin;
C = crude rabbit antiserum;
E = affinity-purified antibodies;
B, D, F = C. atrox venom;
Dilutions of antisera and venom: (1) 1/32, (2) 1/16, (3) 1/8, (4) 1/4, (5) 1/2, (6-11) undiluted.

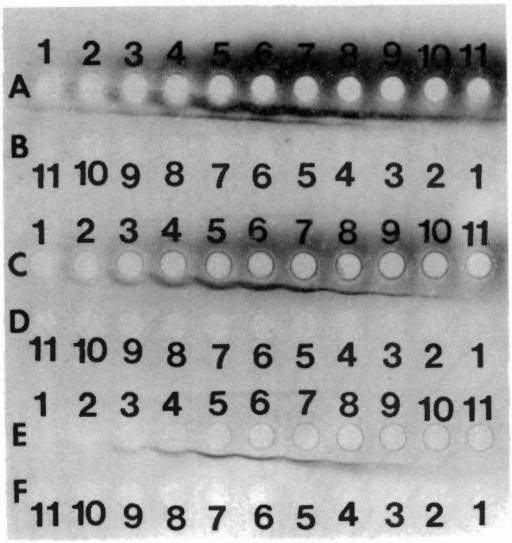
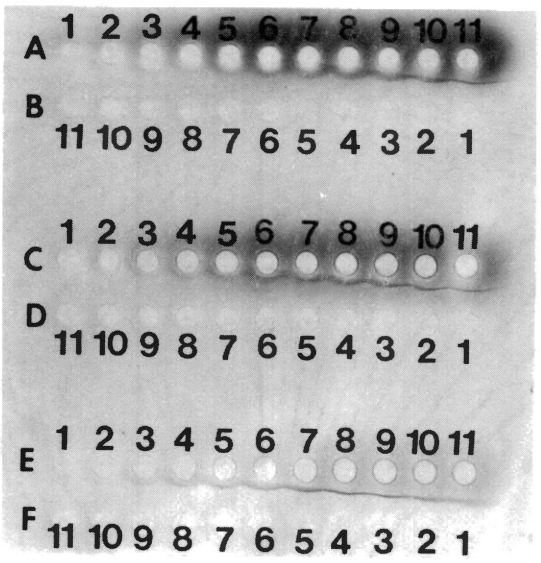

1 2 3 4 5 6 7 8 9 10 11
A
B
11 10 9 8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8 9 10 11
C
D
11 10 9 8 7 6 5 4 3 2 1
1 2 3 4 5 6 7 8 9 10 11
E
F
11 10 9 8 7 6 5 4 3 2 1

Figure 10. Results of the immunodiffusion assay testing purified HTb against polyvalent antivenin, crude rabbit antiserum, and affinity-purified antibodies against HTb.
A = Wyeth's polyvalent antivenin;
C = crude rabbit antiserum;
E = affinity-purified antibodies;
B, D, F = Purified HTb;
Dilutions of antisera and venom: (1) 1/32, (2) 1/16, (3) 1/8, (4) 1/4, (5) 1/2, (6-11) undiluted.


A 1 2 3 4 5 6 7 8 9 10 11
B 11 10 9 8 7 6 5 4 3 2 1
C 1 2 3 4 5 6 7 8 9 10 11
D 11 10 9 8 7 6 5 4 3 2 1
E 1 2 3 4 5 6 7 8 9 10 11
F 11 10 9 8 7 6 5 4 3 2 1

Western Blot

Figure 11 shows the results of the western blot. In lane one were the molecular weight standards. Lane 2 contained the HTb following cation exchange chromatography, which showed a very faint second band with an approximate molecular weight of 14,000 daltons. Lanes 3, 4, and 5 contained purified HTb with antibody concentrations of 0.64 ng/ml, 80 ng/ml, and 0.01 mg/ml, respectively. At all antibody concentrations, only one band was demonstrable, indicating the specificity of the antibody.

Neutralization of Hemorrhage

Partial neutralization of hemorrhage was acheived with the affinity-purified antibody solution at a concentration of 8.3 mg/ml, while 100% neutralization was observed when the concentration was 16.2 mg/ml. All other test solutions were ineffective in reducing or preventing hemorrhage induced by HTb. The results of the tests are presented in Figure 12. The control solutions, PBS, normal rabbit serum, and normal horse serum, produced no hemorrhage. Only the last two antisera solutions, consisting of the affinity antibodies, were significant in neutralizing capacity. Complete neutralization was at a molar ratio of approximately 15.6 moles antibody to one mole of toxin, based on molecular weights of 160,000 daltons and 24,000 daltons, respectively. The polyvalent antivenin caused a slight, though insignificant, increase in hemorrhage.

Figure 11. Western blot of HTb using affinity purified antibodies specific for HTb. The stain was silver enhanced according to BIO-RAD Protein A Gold Immun-Blot assay kit. Lane 1: molecular weight standards (myosin, 205,000 daltons; beta-galactosidase, 116,000; phosphorylase b, 97,000; bovine serum albumin, 66,000; egg albumin, 45,000; glyceraldehyde, 36,000; carbonic anhydrase, 29,000; trypsiongen, 24,000; trypsin inhibitor, 20,100; and alpha-lactalbumin, 14,200); lane 2: HTb following cation exchange chromatography (note second faint band at approximately 14,000 daltons); lanes 3, 4, and 5: purified HTb (following hydrophobic interaction chromatography) with antibody concentrations of 0.64 ng/ml, 80 ng/ml, and 0.01 mg/ml respectively.

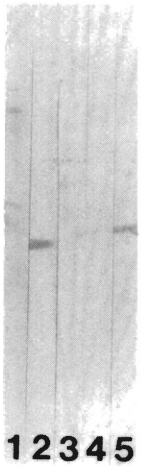
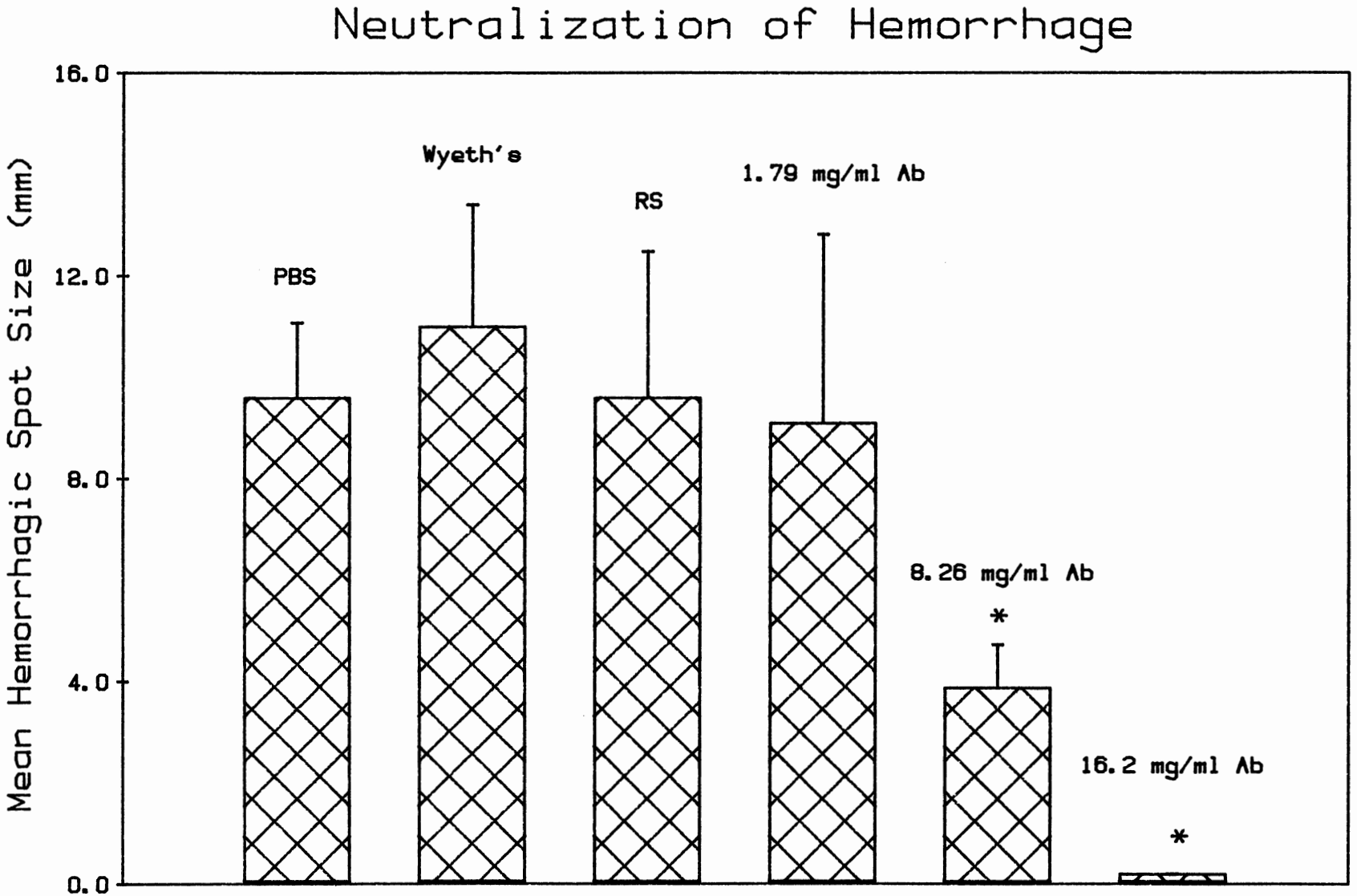

1 2 3 4 5

Figure 12. Results of the in vitro neutralization of hemorrhage induced by HTb, testing neutralization capacities of Wyeth's polyvalent antivenin, crude rabbit antiserum to HTb, and three concentrations of the affinity-purified antibodies to HTb - 1.79 mg/ml, 8.26 mg/ml, and 16.2 mg/ml. Bars are plotted as the mean hemorrhagic spot size + standard deviations. PBS = phosphate buffered saline, pH 7.4; Wyeth's = polyvalent (Crotalidae) antivenin; RS = crude rabbit serum immunized against HTb; Ab = affinity antibody at the specified concentration. * Significant at the $p < 0.01$ level compared to the PBS + HTb group.


Neutralization of Hemorrhage
Mean Hemorrhagic Spot Size (mm)
16.0
12.0
8.0
4.0
0.0
PBS
Wyeth's
RS
1.79 mg/ml Ab
8.26 mg/ml Ab
*
16.2 mg/ml Ab
*

Discussion

Antibodies specific for HTb have been isolated using affinity chromatography from serum obtained from rabbits immunized with HTb . These antibodies form one precipitin band when tested against crude C. atrox venom and purified HTb using immunodiffusion in agarose gels. The MHD dose for HTb reported here, 0.3 mg/kg, agrees with that previously reported by Bjarnason and Tu (1978), also 0.3 mg/kg.

Wyeth's polyvalent antivenin has been shown to be quite effective in neutralizing hemorrhagic effects of crude venoms. Ownby et al. (1984) reported complete neutralization of a 6.0 mg/kg dose of C. atrox venom and a 12.0 mg/kg dose of C. v. viridis. Smith and Ownby (1985) reported the ability of antivenin to neutralize a 12.0 mg/kg dose of C. horridus horridus venom. The results presented here demonstrate that polyvalent antivenin has little capacity to neutralize hemorrhage induced by HTb. This is most likely due to the low titer of antibodies present in the antiserum directed against HTb, despite the fact that the concentration of HTb in C. atrox venom is greater than the other hemorrhagic toxins found in this venom (Bjarnason and Tu, 1978) and that this venom is used in the immunization protocol for production of the antiserum. Minton (1954) reported the inability of polyvalent antivenin to neutralize lethal effects of C. atrox or C. adamanteus venom, both of which are used in the

production of the antivenin. In the present study, the polyvalent antivenin actually resulted in an increased, though insignificant, hemorrhagic response. Ownby et al. (1983) demonstrated an exacerbation of vacuolation of muscle cells induced by C. v. viridis venom when the venom was mixed with polyvalent antivenin. Similar results were observed with C. h. horridus venom (Smith and Ownby, 1985). Ownby and Colberg (1986) reported the ability of polyvalent antivenin to neutralize myonecrosis induced by a 15.0 mg/kg dose of C. atrox venom, which is significantly better than that reported for either C. v. viridis venom or C. h. horridus venom.

In contrast, the affinity-purified antibodies did neutralize the hemorrhagic activity of the toxin, but only at very high concentrations. Complete neutralization was accomplished when the antibody/toxin ratio was 104.6 mg antibody/1.0 mg toxin. Lomonte et al. (1985) used affinity-purified antibodies specific for a myotoxin isolated from the venom of B. asper. These antibodies were effective in neutralizing myonecrosis induced by the crude venom, but only at a ratio of 29 mg antibody/1.0 mg venom. This antiserum was not effective in neutralizing lethal effects of the crude venom. The poor ability of the affinity-purified anti-HTb immunoglobulins to reduce hemorrhage may be due to a low affinity for the toxin. However, elution of the antibodies from the affinity column required 0.05 M acetic acid at a pH of 3.0, suggesting that

the the affinity was relatively high. In addition, this was a precipitating antibody, though the ability of antibodies to precipitate with the antigen may be insignificant. Chang and Yang (1977) used an affinity-purified antibody fraction from serum immunized against cobrotoxin which showed a 23-fold increase in neutralizing capacity for lethal effects that proved not to be a precipitating antibody. Bober (1987) produced an affinity-purified antibody to myotoxin a that was nonprecipitating and incapable of neutralizing myonecrosis induced by the toxin.

Despite the need for high concentrations of the affinity antibody to neutralize hemorrhage, the fact that neutralization was possible is promising since polyvalent antivenin, the commercial antivenom used for treatment of rattlesnake venom poisoning in the United States, proved completely ineffective in reducing hemorrhagic activity of the toxin. Tan (1983) reported the production of a very effective antiserum against lethal effects of the Malayan cobra by chemically cross-linking the major toxins from this venom with a neurotoxin from a sea snake. Perhaps the immunogenicity of HTb, or other purified toxins important in venom poisoning sequelae, could be improved by such measures, including the conjugation of the toxins to larger or more strongly immunogenic carriers, thereby increasing the liklihood of the presence of antibodies in the antiserum used to treat snake bites.

CHAPTER V

DETECTION OF HEMORRHAGIC TOXIN B FROM *CROTALUS ATROX* VENOM IN OTHER SNAKE VENOMS

Introduction

Hemorrhagic toxin b (HTb) is a metalloprotease present in the venom of the western diamondback (*Crotalus atrox*) rattlesnake. It has an approximate molecular weight of 23,000 daltons, is highly basic with an isoelectric point above ten, and requires zinc for proteolytic and hemorrhagic activity (Bjarnason and Tu, 1978). It comprises at least 3.7% of the crude protein in the venom, and is one of seven hemorrhagic toxins found in *C. atrox* venom. HTb is also capable of inducing myonecrosis (Ownby et al., 1978).

Most rattlesnake venoms induce hemorrhage, a serious sequelae to venom poisoning. Of eleven rattlesnake venoms tested, Friederich and Tu (1971) found that only the Mojave rattlesnake (*C. scutulatus*) was nonhemorrhagic. Several hemorrhagic toxins have been isolated from snake venoms, including venoms of the following genera: *Crotalus*, *Agkistrodon*, *Trimersurus*, *Bothrops*, and *Vipera*. The predominance of hemorrhagic toxins in venoms indicates the

importance of this reaction medically. If a particular hemorrhagic toxin is widely distributed in a variety of snake venoms, then an antivenom to be used in treatment must contain antibodies to this toxin. The currently used antivenom in the United States, Wyeth's polyvalent (Crotalidae) antivenin, has been shown to be moderately effective in neutralizing hemorrhagic effects of rattlesnake venoms in vitro (Ownby et al., 1984a; Smith and Ownby, 1985). However, this antivenom was ineffective in preventing hemorrhage induced by HTb (Chapter IV). This result was surprising because this toxin comprises approximately 3.5% of the crude venom protein, the C. atrox venom from which it was isolated is used in the immunization protocol for production of the polyvalent antivenin, and that the polyvalent antivenin does contain antibodies to the toxin (Chapter IV) as measured with immunodiffusion. Consequently, little reduction in hemorrhage due to HTb, or similar toxins, would be expected clinically when polyvalent antivenin is used. It is therefore important to determine the presence of this potent hemorrhagin in other snake venoms.

Bober (1987), using the ELISA and an affinity purified antibody to myotoxin a, a myotoxin isolated from the venom of C. viridis viridis (Ownby et al., 1976), screened eighty-two venoms from six genera for the presence of myotoxin a. Previous reports have demonstrated the ineffectiveness of polyvalent antivenin to prevent myotoxic

effects of C. v. viridis venom (Ownby et al., 1983a), and the low titer of antibodies to this toxin in the polyvalent antivenin (Ownby et al., 1983b). Bober (1987) found detectable levels of myotoxin a in thirty-five of the venoms tested, all from members of the genus Crotalus.

The present study describes the use of ELISA in screening venom samples representing fifty-three species and subspecies of snakes for the presence of HTb, or cross-reacting proteins, using the affinity-purified antibody previously described. Also recorded are the geographical locations of the snakes used in the study, where available.

Materials and Methods

Hemorrhagic Toxin b

HTb was isolated as previously described (Chapter III). Briefly, 2.0 grams of desalted crude Crotalus atrox venom was purified by a four-step chromatographic procedure utilizing anion exchange, gel filtration, cation exchange, and hydrophobic interaction chromatography. The purified toxin thus obtained contained only a single protein band after analysis by SDS-PAGE, and was considered homogenous. The toxin was desalted, diafiltered into 0.01 M Na-MOPS, pH 7.2, and stored at -20^{o}C until needed.

Affinity Purification of Antibodies to HTb

Affinity purification of antibodies to HTb was

conducted as described previously in Chapter IV. Briefly, serum from rabbits immunized with HTb was applied to an affinity column consisting of 8.0 ml of Affi-Gel 10 affinity matrix conjugated with 27.0 mg of purified HTb. After elution of unbound serum components, HTB-specific antibodies were eluted with 0.05 M acetic acid, pH 3.0.

Antigen Detection ELISA

The composition of reagents used for the ELISA were as follows: (a) coating buffer - 0.015 M sodium bicarbonate, 0.035 M sodium carbonate, 0.003 M sodium azide, 1% BSA; (b) washing buffer - 0.15 M Nacl, 0.05% Tween 20; (c) incubation buffer - 0.05% Tween 20, 0.003 M sodium azide, 1% BSA, dissolved in PBS; (d) diethanolamine buffer - 0.1 M diethanolamine-Cl, 0.0011 M magnesium chloride, pH 9.8; (e) phosphatase substrate- p-nitrophenyl phosphate disodium, 40 mg/ml, dissolved in diethanolamine buffer. Microtiter plates (Flow Laboratories) were coated overnight at $4^{o}C$ with 0.2 ml of antibodies to HTb at a concentration of 0.001 mg/ml, diluted in coating buffer. The plates were washed 3 X 5 min with washing buffer, followed by the addition of 0.2 ml of known amounts of HTb, ranging in concentrations from 0.01 mg/ml to 1000 ng/ml, for 2 hr at $37^{o}C$. Blank wells, containing only incubation buffer, were also run with each plate. The plates were washed 3 X 5 min with washing buffer, followed by a 2 hr incubation with 0.2 ml affinity-purified antibodies at a concentration of 0.001

mg/ml at 37°C. After washing 3 X 5 min with washing buffer, 0.2 ml of secondary antibody, goat anti-rabbit IgG antibody (Sigma Chemical Co.), conjugated with alkaline phosphatase, diluted 1/2000 in incubation buffer, was added for 2 hr at 37°C. The plates were again rinsed for 3 X 5 min with washing buffer and 0.2 ml of the enzyme substrate, p-nitrophenyl phosphate disodium (Sigma Chemical Co.), was added and allowed to incubate at room temperature for 30 min. The enzyme/substrate reaction was stopped with the addition of 0.05 ml 3 N NaOH. Absorbance at 405 nm was determined with an EIA Manual ELISA spectrophotometric reader.

Assay of Venoms for HTb

Fifty-three venoms samples, representing six genera, were tested for the presence of HTb, or cross-reacting proteins, using the antigen-detection ELISA method. The venoms were generously supplied by Jim Glenn, Salt Lake City, Utah. They were obtained from snakes collected over wide geographic areas and consisted of both males and females. One venom sample was from a snake captured in Costa Rica, while the others were collected from various regions in Mexico and the following ten states: Florida, Georgia, North Carolina, New Mexico, Utah, Arizona, Montana, Colorado, Wyoming, and South Dakota. Each venom was tested initially at a concentration of 1.0 mg/ml. Those venoms producing values greater than 1.0 absorbance

units were diluted 1/100 (0.01 mg/ml) and tested. Again, any reading above 1.0 absorbance units were diluted 1/10 (1000 ng/ml) and tested. One venom was diluted to 100 ng/ml. All venoms were originally tested once on each of three plates, recorded as values at n=3. Many of these venoms were tested again, repeated five times on one plate, recorded as values at n=5. Also, serial two-fold dilutions of five venoms were measured to test the validity of the technique, insuring that a 50% dilution resulted in an approximate reduction by 50% of the absorbance value. A standard curve of HTb was run with each plate as a positive control.

Statistical Analyses

A student's t-test was used to determine significance between treatment means at the $p < 0.05$ level. Mean absorbance values for each venom were tested against mean absorbance values for the incubation buffer blanks. At n=3, the means of two blank wells/plate were averaged for the three plates. At n=5, each venom mean was tested against the mean of five blank wells from the single plate.

Results

Antigen Detection ELISA

Figure 13 is a diagram illustrating the steps of the ELISA antigen detection plate. Incubation control blanks, a measure of nonspecific binding, were very low, with

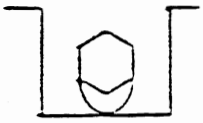
Figure 13. A flow chart illustrating the steps involved in the hemorrhagic toxin b detection plate.

ELISA FOR HEMORRHAGIC TOXIN b DETECTION

1. Anti-HTb Adsorbed to Plate

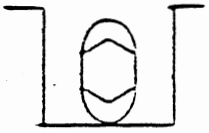
WASH

2. Solution Containing HTb Added



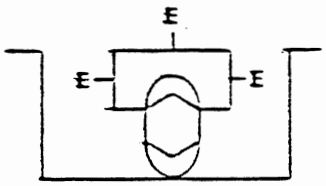
WASH

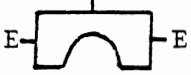
3. Solution Containing Anti-HTb Added



WASH

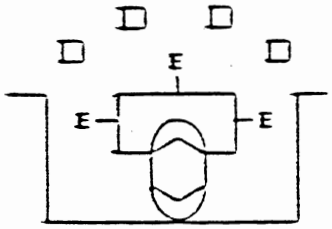
4. Alkaline Phosphatase Labelled Antibody to Rabbit IgG Added





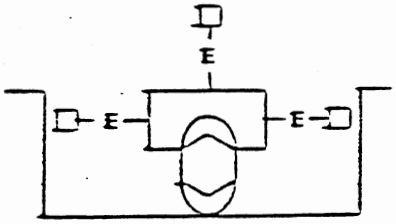
WASH

5. Enzyme Substrate Added



INCUBATE

6. Read Absorbance of Reaction Product at 405 nm



values ranging between 0.084 and 0.191 absorbance units. Table VI illustrates the values obtained with a standard curve, measuring dilutions of HTb. The values are recorded as the mean (n = 4) of absorbance units at 405 nm ± the standard error. The blanks were subtracted from the absorbance readings.

Assay of Venoms for HTb

Results of the ELISA for the detection of HTb in the venom samples are presented in Table VII. Four of the five genera evaluated contained venoms testing positive for HTb. Only the genus Naja, with the single venom from N. n. kaouthia (Thailand cobra), tested negative. Of the remaining fifty-two venom samples, forty (75.5%) tested positive for HTb. Twenty-three of the venoms had values above 1.0 and were tested at 0.001 mg/ml. All tested positive at this dilution. Of these, ten venoms had values above 1.0 and were tested at a concentration of 1000 ng/ml. Again, all tested positive. One venom, obtained from C. mitchelli pyrrhus, tested positive at a concentration of 100 ng/ml. Table VIII represents the data obtained in testing the dilutions of five venoms, C. lepidus klauberi, C. m. pyrrhus, C. exsul, C. v. abyssus, and A. contortrix mokeson, demonstrating high correlation between concentrations of venom and the absorbance at 405 nm.

There was marked variation in the presence of HTb in venoms from the same genus. For example, two species from

TABLE VI

DETECTION OF HTb USING ELISA

HTb (microgm/ml)	Absorbance (405 nm)
10.0	0.980 ± 0.17
8.0	0.754 ± 0.06
5.0	0.457 ± 0.05
3.0	0.181 ± 0.03
1.0	0.077 ± 0.02
I.B.	0.138 ± 0.02
	(r=0.99)

HTb was assayed using one well/plate over four plates. The values are expressed as mean ± standard error. (n = 4)

I.B. = incubation buffer blank with 1% bovine serum albumin. The background values have been subtracted from each absorbance reading.

r = sample correlation coefficient

TABLE VII

DETECTION OF HEMORRHAGIC TOXIN B IN SNAKE VENOMS USING ELISA

Venom	1.0 mg/ml	0.01 mg/ml	0.001 mg/ml
A. bilineatus bilineatus Miami Serpentarium	0 (3)		
A. contortrix contortrix Biotoxins, Inc.	*0.274 ± 0.020 (3)		
A. contortrix mokeson Miami Serpentarium	*1.590 ± 0.046 (3)	*0.527 ± 0.052 (3)	
A. pictigaster pictigaster Miami Serpentarium	*1.062 ± 0.066 (5)	*0.157 ± 0.012 (5)	
B. asper Costa Rica	*0.044 ± 0.005 (5)		
C. adamanteus Florida	*0.310 ± 0.069 (3)		
C. atrox Texas	*0.300 ± 0.029 (3)		
C. basiliscus basiliscus unknown origin	* > 2.0 (3)	*0.469 ± 0.046 (3)	

TABLE VII (Continued

Venom	1.0 mg/ml	0.01 mg/ml	0.001 mg/ml
C. catalinensis Baja, Mexico	0.012 ± 0.006 (3)		
C. cerastes cerastes Utah	*0.195 ± 0.023 (3)		
C. cerastes cerastes Miami serpentarium	*1.336 ± 0.191 (3)	*0.146 ± 0.010 (5)	
C. durissus culminatus Houston Zoo	*0.224 ± 0.029 (5)		
C. durissus durissus unknown origin	* > 2.0 (3)	*1.530 ± 0.139 (3)	*0.111 ± 0.007 (5)
C. durissus totonacus unknown origin	*0.096 ± 0.007 (5)		
C. enyo enyo Baja, Mexico	0 (3)		
C. enyo enyo Baja, Mexico	0.016 ± 0.008 (3)		
C. exsul unknown origin	* > 2.0 (3)	*0.512 ± 0.075 (3)	
C. exsul unknown origin	*0.224 ± 0.035 (3)		

TABLE VII (Continued)

Venom	1.0 mg/ml	0.01 mg/ml	0.001 mg/ml
C. horridus atricaudatus Valdosta, Georgia	0 (3)		
C. horridus atricaudatus North Florida	0.003 ± 0.002 (5)		
C. horridus atricaudatus unknown origin	0 (5)		
C. horridus horridus North Carolina	*0.053 ± 0.003 (5)		
C. lepidus klauberi Zacatacus, Mexico	* > 2.0 (3)	*0.977 ± 0.064 (3)	*0.198 ± 0.009 (5)
C. lepidus klauberi Zacatacus, Mexico	* > 2.0 (3)	*1.195 ± 0.191 (3)	*0.136 ± 0.003 (5)
C. lepidus klauberi Durango, Mexico	*0.031 ± 0.003 (5)		
C. lepidus klauberi New Mexico	*0.061 ± 0.005 (5)		
C. mitchelli mitchelli Baja, Mexico	0.019 ± 0.005 (5)		
C. mitchelli pyrrhus Bahia de los Angeles, Mexico	* > 2.0 (3)	*1.655 ± 0.248(3)	*0.171 ± 0.004 (5)

TABLE VII (Continued)

Venom	1.0 mg/ml	0.001 mg/ml	0.0001 mg/ml
C. mitchelli pyrrhus Baja, Mexico	* > 2.0 (3)	*1.095 ± 0.133 (3)	*0.051 ± 0.002 (5)
C. mitchelli pyrrhus Bahia de los Angeles, Mexico	* > 2.0 (3)	*0.449 ± 0.052 (3)	
C. mitchelli pyrrhus Virgin River, Arizona	* > 2.0 (3)	* > 2.0 (3)	* > 2.0 (3)
C. mitchelli pyrrhus Utah	* > 2.0 (3)	*1.357 ± 0.196 (3)	*0.098 ± 0.003 (5)
C. mitchelli pyrrhus Utah	* > 2.0 (3)	*0.593 ± 0.017 (3)	
C. mitchelli pyrrhus Utah	* > 2.0 (3)	*0.383 ± 0.016 (5)	
C. mitchelli stephensi unknown origin	* > 2.0 (3)	*0.326 ± 0.012 (3)	
C. molassus molassus Miami Serpentarium	* > 2.0 (3)	* > 2.0 (3)	*0.336 ± 0.012 (3)
C. molassus nigrescens Durango, Mexico	* > 2.0 (3)	*0.655 ± 0.144 (3)	
C. ruber ruber Miami Serpentarium	* > 2.0 (3)	*0.335 ± 0.012 (3)	

TABLE VII (Continued)

Venom	1.0 mg/ml	0.001 mg/ml	0.0001 mg/ml
C. scutulatus scutulatus Miami Serpentarium	0 (3)		
C. vergrandis Venezuela	*0.516 ± 0.098 (3)		
C. viridis abyssus unknown origin	* > 2.0 (3)	*0.308 ± 0.007 (5)	
C. viridis cereberus unknown origin	* > 2.0 (3)	*0.399 ± 0.028 (5)	
C. viridis concolor Sage Creek Den, Wyoming	0.004 ± 0.006 (3)		
C. viridis helleri unknown origin	* > 2.0 (3)	*1.251 ± 0.046 (3)	*0.116 ± 0.006 (5)
C. viridis lutosus Magna, Utah	* > 2.0 (3)	*1.191 ± 0.191 (3)	*0.049 ± 0.008 (5)
C. viridis oreganus Miami Serpentarium	*0.040 ± 0.006 (5)		
C. viridis viridis Slater, Colorado	0.012 ± 0.002 (5)		
C. viridis viridis Colorado	*0.048 ± 0.005 (5)		

TABLE VII (Continued)

Venom	1.0 mg/ml	0.01 mg/ml	0.001 mg/ml
C. viridis viridis Colorado	*0.028 ± 0.004 (5)		
C. viridis viridis Eddy County, New Mexico	0.017 ± 0.005 (5)		
C. viridis viridis Martin, South Dakota	*0.156 ± 0.020 (3)		
Naja naja kaouthia Miami Serpentarium	0 (3)		
Trimeresurus flavoviridis Miami Serpentarium	*0.471 ± 0.036 (5)		

* significant at $p < 0.05$

A.= *Agkistrodon*; C.= *Crotalus*; B.= *Bothrops*

Results presented as absorbance value with blank value subtracted; number in parentheses indicates number of wells tested

TABLE VIII

TEST OF VENOM DILUTIONS WITH ELISA

Antigen (microgm/ml)	C. klauberi	C. pyrrhus	C. exsul	C. abyssus	A. mokeson
			ABSORBANCE 405 nm		
40	-	-	0.60 ± .03	0.56 ± .04	-
20	-	1.11 ± .28	0.39 ± .04	0.29 ± .04	0.42 ± .04
10	1.49 ± .13	0.70 ± .20	0.30 ± .03	0.21 ± .03	0.35 ± .02
5	0.83 ± .13	0.33 ± .08	0.17 ± .14	0.10 ± .03	0.23 ± .04
2	0.38 ± .08	0.22 ± .04	0.11 ± .03	-	0.11 ± .01
1	0.11 ± .04	0.12 ± .04	-	-	-
0.625	0.09 ± .02	-	-	-	-
I.B.	0.10 ± .003	0.10 ± .003	0.01 ± .003	0.01 ± .003	0.01 ± .003
	(r=0.99)	(r=0.99)	(r=0.97)	(r=0.99)	(r=0.92)

Antigen = Venom sample. The venom samples were assayed as single wells from three plates. Expressed as the mean absorbance (405 nm) ± standard error.
I.B. = incubation buffer blank with 1% bovine serum albumin. The background values have been subtracted from each absorbance reading; r = sample correlation coefficient.
(-) not assayed at this concentration; C.= Crotalus; A.= Agkistrodon.

the genus Agkistrodon had very high amounts of the toxin in their venoms, while two other species had very low amounts. This same type of variation was also observed within the same species. In two venom samples from C. cerastes cerastes, one had very large amounts while the other had quite small amounts of HTb. Similar variations were observed within the following species: C. exsul, C. l. klauberi, and C. viridis subspecies. Other venom samples, such as those from C. m. pyrrhus, consistently had very high amounts of the toxin, while others, such as C. horridus atricaudatus, consistently had very low amounts of HTb. C. atrox venom, despite being the homologous venom from which the toxin was isolated, showed with the ELISA to have relatively small amounts of the toxin.

The fifty-three venom samples tested showed a tendency towards geographic variation, with those from northern regions having less amounts of the toxin and those from more southern regions having higher amounts. This variation was only a tendency, however, since there were exceptions.

Discussion

The evaluation of venoms for common antigens, especially medically important components such as hemorrhagins, could be very useful for the development of snake bite treatments. For an antiserum to be effective against a given venom poisoning reaction, it must have

antibodies to those toxins responible for the reaction, especially if these toxins are ubiquitous among snake venoms. The enzyme-linked immunosorbent assay is an effective tool for the determination of common antigenic components in snake venoms. An affinity- purified antibody to HTb was produced and used to test fifty-three venom samples representing five genera for the presence or absence of this toxin.

The large number of cross-reacting venoms indicates the potential importance of HTb in snake bites, expecially in those venoms containing relatively high amounts. Although polyvalent antivenin has been shown to be effective against hemorrhage induced by rattlesnakes (Ownby et al., 1984b; Smith and Ownby, 1985), neutralization studies reported in this paper demonstrate the ineffectiveness of polyvalent antivenin in reducing or preventing hemorrhage caused by HTb. This antiserum was unable to neutralize even a single minimum hemorrhagic dose. Since HTb is found in such a wide variety of snake species, an antiserum developed for the treatment of snake venom poisoning should be effective against HTb.

The homologous crude venom, C. atrox, showed relatively small amounts of HTb using the ELISA, despite the fact that the toxin represents 3.46% of the venom protein. These results could be due to an inhibition in the antigen-antibody reaction due to the high concentration of antibodies to HTb. Similar results were observed by

Ownby et al. (1983b) when the ELISA was used to measure antibody titers in antimyotoxin a serum, antiserum to C. v. viridis venom, and Wyeth's polyvalent antivenin. It would also seem unlikely that such a large number of venoms would contain a toxin identical to HTb, and in some cases, in such large amounts. A more plausible explanation would be the presence of proteins with common antigenic sites capable of cross-reacting with the anti-HTb antibodies, proteins which may or may not have hemorrhagic activity.

The results obtained in this study showed a tendency towards geographical variations in the presence of HTb in the venoms, although there were exceptions. Generally, venoms from snakes indigenous to southern regions of North and Central America had relatively high amounts of the toxin, while venoms from northern species tended to have lower amounts. For example, all of the venoms from the species C. m. pyrrhus, typically found in the Baja region of Mexico and in southern New Mexico, Utah and California, had very high amounts of HTb. C. d. durissus, indigenous to Central America, also had high amounts of the toxin in the venom. However, the two other C. durissus subspecies, C. d. totonacus and C. d. culminatus had little toxin in their venoms. None of the species tested from the eastern United States, C. h. horridus, C. adamanteus, and C. h. atricaudatus, had high amounts of HTb. Bober (1987) showed with the ELISA a geographical variation in the distribution of myotoxin a, from C. v. viridis venom, with a greater

distribution of the toxin in the north and southwest regions of the United States, Mexico and South America.

Bonilla and Horner (1969) made the statement, "...venoms represent body fluids whose compositions are characteristic for a given species," based on an electrophoretic analysis of Crotalus and Agkistrodon species. However, there are other reports describing the presence of cross-reacting components in venoms of differing species. Kaiser et al. (1986) reported the cross-reactivity and neutralization by rabbit antisera produced against crotoxin, a potent neurotoxin present in the venom of C. d. terrificus, of Mojave toxin, from the venom of C. s. scutulatus, and concolor toxin, from the venom of C. v. concolor. Glenn and Straight (1985) tested for the presence of mojave toxin in the venom of six crotalid species using immunodiffusion and found a cross-reactive protein in the venom of C. mitchelli mitchelli. Rosenfeld and Kelen (1966) demonstrated cross-reacting antigens in the venom of multiple species from the two genera Bothrops and Crotalus. The use of monoclonal antibodies to Mojave toxin have shown the presence of antigenically similar components in the venoms of C. basiliscus, C. d. durissus, C. d. terrificus, C. h. horridus, and C. v. concolor (Rael et al., 1986). Tests for HTb were positive for three of these same venoms, clearly indicating that some venom components are not necessarily characteristic for a given species.

The determination of common components in snake venoms has also been used to study relationships to geographic distribution. Glenn and Straight (1978) reported the variation in toxicity of the venom from C. s. scutulatus depending on the geographical origin. There were distinct differences in lethality induced by the venom and the ability of polyvalent antivenin to neutralize lethal effects between venom specimens from southern California and those from north central and northwest Arizona. Such variations are very important in the consideration of medical treatments. Minton and Weinstein (1986) reported differences in proteolytic activity and lethality of C. atrox venom relative to geographic origin. There was no apparent relationship between hemorrhagic activity and geographical distribution. The results presented in this study indicate a possible geographical distribution of HTb.

Pooling of venoms for study often "hides" variations in snake venoms (Glenn and Straight, 1982), a characteristic that should be considered important, especially for medical reasons. Clearly, snake venoms do contain common antigenic components, characteristics that may be exploited in developing better treatments. Common components may also be useful in mapping ontogenic relationships between species and genera.

CHAPTER VI

IMMUNOHISTOCHEMICAL LOCALIZATION OF HEMORRHAGIC TOXIN B IN SKELETAL MUSCLE

Introduction

Hemorrhagic toxin b is capable of causing not only hemorrhage, but myonecrosis as well (Ownby et al., 1978). It represents approximately 3.7% of the crude venom protein, has a molecular weight around 24,000 daltons, and is highly basic (Bjarnason and Tu, 1978). It is the only reported directly-acting myotoxin isolated from Crotalus atrox venom (Ownby et al., 1978). The pathogenesis of hemorrhage induced by HTb has been reported (Ownby et al., 1978), and has previously been described in Chapter II.

Myonecrosis is caused by a variety of toxins, including toxins that act directly on the muscle, and toxins which apparently act indirectly through ischemia. These latter toxins include viriditoxin, from C. viridis viridis venom (Fabiano and Tu, 1981; Gleason et al., 1983), which was originally reported as a "tissue-damaging hemorrhagin" (Fabiano and Tu, 1981). Hemorrhagic toxin b has been reported to cause myonecrosis, though it was unclear whether the action was direct or due indirectly to ischemia (Ownby et al., 1978). The presence of intact

capillaries next to damaged muscle cells indicated a possible direct action of the toxin. Necrosis in muscle due to ischemia does not ensue until about six hours after the onset of the condition (Ownby and Colberg, publication submitted), and unlike the other hemorrhagic toxins in C. atrox venom, HTb causes myonecrosis by three hours in skeletal muscle (Ownby et al., 1978), presenting further evidence for a possible direct action of the toxin on muscle. There is only one other reported hemorrhagic myotoxin, mucrotoxin A from the venom of Trimeresurus mucrosquamatous, capable of inducing myonecrosis by three hours (Sugihara et al., 1983).

There are only two reports in the literature describing the localization of snake venoms or their components in mammalian tissues. Schiff et al. (1984) reported the immunocytochemical localization of C. atrox venom in the lungs of mice using Wyeth's polyvalent (Crotalidae) antivenin conjugated to horse-radish peroxidase. Tu and Morita (1983) described the localization of myotoxin a, from C. v. viridis venom, on the sarcoplasmic reticulum at the electron microscopic level, avoiding the use of immunoglobulins by conjugating myotoxin a with HRP directly.

Because HTb causes hemorrhage only after a latency period, and since there is still some question as to the mechanism of induction of myonecrosis by the toxin, it would be useful to know whether the toxin binds to the

capillary wall and muscle cell, and if so, at what time period after the injection of the toxin. Information concerning the binding of the toxin to the tissue could then be used to direct studies in the ultrastructural localization of the toxin.

Material and Methods

Hemorrhagic Toxin b and Antibody Purification

HTb was isolated as previously described (Chapter III). Briefly, 2.0 grams of desalted crude Crotalus atrox venom was purified by a four-step chromatographic procedure utilizing anion exchange, gel filtration, cation exchange, and hydrophobic interaction chromatography. The purified toxin thus obtained contained only a single protein band after analysis by SDS-PAGE, and was considered homogenous. The toxin was desalted, diafiltered into 0.01 M Na-MOPS, pH 7.2, and stored at -20°C until needed.

Antibodies to HTb, subsequently referred to as primary antibodies, were purified using affinity chromatography as previously described (Chapter IV). Briefly, serum from rabbits immunized with HTb was applied to an affinity column consisting of 8.0 ml of Affi-Gel 10 (Bio-Rad Corp.) affinity matrix containing approximately 3.5 mg of covalently coupled HTb per ml. The affinity-purified antibodies were eluted with 0.05 M acetic acid, pH 3.0, and stored frozen in PBS until needed.

Goat anti-rabbit antibodies labelled with fluorescein isothiocyanate, subsequently referred to as secondary antibodies, were purchased from Sigma Chemical Company. These were affinity-purified whole immunoglobulin molecules.

Mice

All experiments were performed on female white CD-1 mice purchased from Charles River (Rockville, MD). The weights ranged from 25 to 30 gm.

Immunohistochemistry: Tissue Preparation and Sectioning

Four groups of five mice were injected intramuscularly with the toxin following cation exchange chromatography. Each mouse was injected in the dorsolateral aspect of the right thigh with 8.4 mg/kg of HTb in a volume of 0.05 ml. The five mice from each group were killed by cervical dislocation at 5 min, 30 min, 3 hr and 24 hr after toxin injection and samples of tissue were removed for processing. A fifth group of five mice received a physiological saline injection as a contol and the tissue from these mice was processed at 5 min following injection. The tissue was cut into approximately 1 X 1 mm blocks and placed in 1% freshly prepared paraformaldehyde in 0.27 M cacodylate buffer, pH 7.4, for 1 hr. The tissue was washed 3 X 10 min with 0.27 M cacodylate buffer, washed for 30 min

in 0.02 M glycine, 0.15 M sodium chloride, 0.01 M sodium phosphate, pH 7.4, to block unreacted formaldehyde groups, and then placed in 1% gelatin for 30 min. The tissue blocks were mounted on cork bases with OCT compound (Lab-Tek Products, Naperville, IL) and rapid frozen in acetone cooled to -60°C with dry ice. Sections of approximately eight micron thickness cut using a SLEE cryostat (SLEE Corp., London, England) and collected on glass slides coated with poly-L-lysine (Sigma Chemical Co.).

Immunohistochemistry: Staining

The sections were allowed to warm to room temperature, at which time they were rehydrated for 10 min in PBS and subsequently treated with 0.02 M glycine in PBS for 10 min. The sections were washed 2 X 1 min with PBS and placed in a 1/20 dilution of normal goat serum for 1 hr. After washing 4 X 5 min in PBS, 0.2 ml of a 0.05 mg/ml solution of primary antibodies was applied to the sections and allowed to react overnight at 4°C in a humid chamber. The sections were washed 4 X 5 min with PBS and 0.1 ml of a 1/500 dilution of secondary antibodies conjugated with FITC was applied to the sections and allowed to react for 2 hr in a humid chamber. The sections were washed 4 X 5 min in PBS and placed under coverslips in a 50% glycerol (in PBS) solution. The antibody solutions were always centrifuged for 2 min in an Brinkmann Microfuge to remove any aggregated material. The sections were examined

immediately after staining with a Carl Zeiss incident fluorescence microscope with the following filters: (1) excitation filter, BP 485/20; (2) beam splitter filter, FT 510; and (3) barrier filter, LP 520. Photographs were taken using Kodak ektachrome P800/1600 film exposed during photography for two minutes.

Three control staining solutions were also incorporated on serial sections of the tissue: (a) a PBS control, which included treatment of the section with PBS in place of primary and secondary antibody solutions; (b) a secondary antibody control, which included treatment of the section with PBS in place of primary antibody; and (c) a preabsorbed antibody control, which included treatment of the section with the preabsorbed antibody solution in place of the primary antibody. The preabsorbed antibody was prepared by mixing 45 mg HTb per mg primary antibody. The mixture was allowed to react overnight at 4^{o}C, followed by centrifugation in a Brinkmann microfuge.

Histology

A serial section of the frozen tissue was always stained with Multiple Stain for frozen sections (Polysciences, Inc, Warrington, PA). A section of the muscle used for the immunohistochemical localization was also prepared for plastic embedding. The tissue was fixed for 2 hr in 1% glutaraldehyde, followed by washing and fixation with 0.5% osmium tetroxide for 1 hr. The tissue

was subjected to dehydration in acetone and placed in Poly Bed 812 (Polysciences, Inc.) plastic resin. One micron thick sections were cut using a Sorvall MT-5000 ultramicrotome (Dupont Corp.) and stained with Mallory's trichrome stain. Photographs were taken with a Nikon (Garden City, NY) Labophot microscope equipped with a UFX II photomicrographic attachment.

Results

Preliminary Studies

Several preliminary studies were performed in order to optimize the conditions for the experiments. (1) Several dilutions of primary and secondary antibody solutions were tested to determine the lowest possible concentrations of antibodies that could be used so as to reduce the chances of nonspecific reactions; (2) fixation in 1% glutaraldehyde, 0.5% glutaraldehyde with 1% paraformaldehyde, and 1% paraformaldehyde was evaluated to determine which fixative could be used without destroying antigenicity of the toxin; and (3) the tissue was also sectioned with and without pretreatment with 1% gelatin to determine if the treatment stabilized the tissue during sectioning. The preliminary studies showed that the primary antibody solution was optimal at 0.05 mg/ml while the secondary antibody solution, purchased from Sigma Chemical Co., was optimal at a 1/500 dilution, which was approximately 0.002 mg/ml. Treatment of the sections with

either of the fixatives containing glutaraldehyde resulted in no fluorescence of the tissues receiving treatment with primary and secondary antibodies. Only fixation with 1% paraformaldehyde resulted in fluorescence. The treatment with gelatin stabilized the tissue during sectioning. This was unnecessary at the 5 and 30 min time periods, but was helpful at the 3 and 24 hour time periods. At these time periods, the extensive edema resulted in a marked loss of integrity of the tissue.

Figure 14 shows the results for the control on specific toxin binding, i.e., tissue from mice receiving no toxin. The PBS (Figure 14A) and secondary antibody (Figure 14B) control sections had some autofluorescence. It was predominantly the smaller muscle cells in the tissue that autofluoresced, usually around the periphery of the cell. When viewed with the microscope, this fluorescence was noticeably more yellow than the fluorescence of the FITC, which was bright green. In the preabsorbed antibody control sections (Figure 14C), the entire muscle cell fluoresced, although at a low intensity. Figure 14D shows that there was no specific fluorescence after treatment of the section with primary and secondary antibodies, which was expected since no toxin was introduced into the tissue. This excludes the possibility of non-specific binding of the primary or secondary antibody to the tissue. The normal morphology of the tissue is seen in Figures 14E and 14F.

Figure 14. Light micrographs of sections of muscle from control mice which were not injected with HTb.
(A) PBS control - no primary or secondary antibody;
(B) Secondary antibody control - no primary antibody;
(C) Preabsorbed antibody control;
(D) Section treated with both primary and secondary antibodies;
(E) Untreated frozen section stained with Multiple Stain;
(F) Plastic embedded tissue section.
M = normal muscle cell.

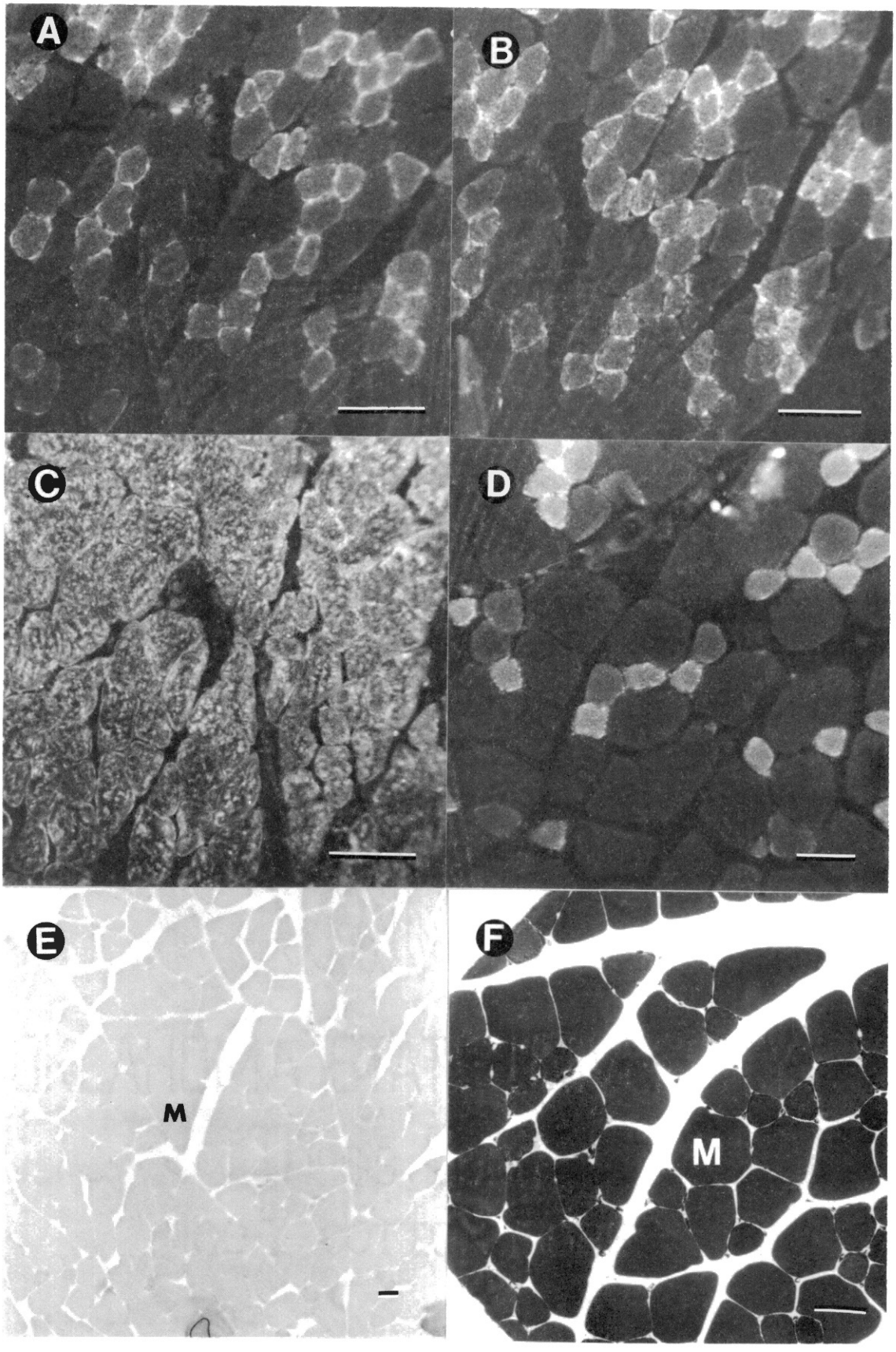

A
B
C
D
E
M
F
M

Figure 15 shows the results of the localization of HTb in muscle tissue removed 5 min after injection of the toxin. The PBS (Figure 15A) and secondary antibody (Figure 15B) control sections had some endogenous fluorescence. The preabsorbed antibody control section (Figure 15C) had cells that also fluoresced in their entirety. Figure 15D shows a section treated with both primary and secondary antibodies. There was a bright, specific fluorescence around the periphery of the muscle cells. However, only 10 to 20% of the cells within the section demonstrated fluorescence at the 5 min period. Binding of the toxin to capillary walls was not detected since the fluorescence around the muscle cell was so intense. There was no edema to allow separation of the muscle cells and thus visualization of the capillaries. No fluorescence was seen in the connective tissue spaces where binding to capillaries would occur. Figure 15E is the section of muscle stained with Multiple Stain, and Figure 15F is the plastic embedded tissue, allowing visualization of the muscle section morphology. None of the cells appeared damaged and no hemorrhage was present.

Figure 16 shows the results of the localization of HTb at 30 min following toxin injection. The control sections, Figures 16A-C, had the same basic pattern described above. However, there was fluorescence over a larger portion of the section treated with primary and secondary antibodies (Figure 16D). Again, the fluorescence was very intense at

Figure 15. Light micrographs of sections of murine muscle taken 5 min following injection of HTb.
(A) PBS control - no primary or secondary antibody;
(B) Secondary antibody control - no primary antibody;
(C) Preabsorbed antibody control;
(D) Section treated with both primary and secondary antibodies;
(E) Untreated frozen section stained with Multiple Stain;
(F) Plastic embedded tissue section. M = normal muscle cell. Note fluroescence at arrow.

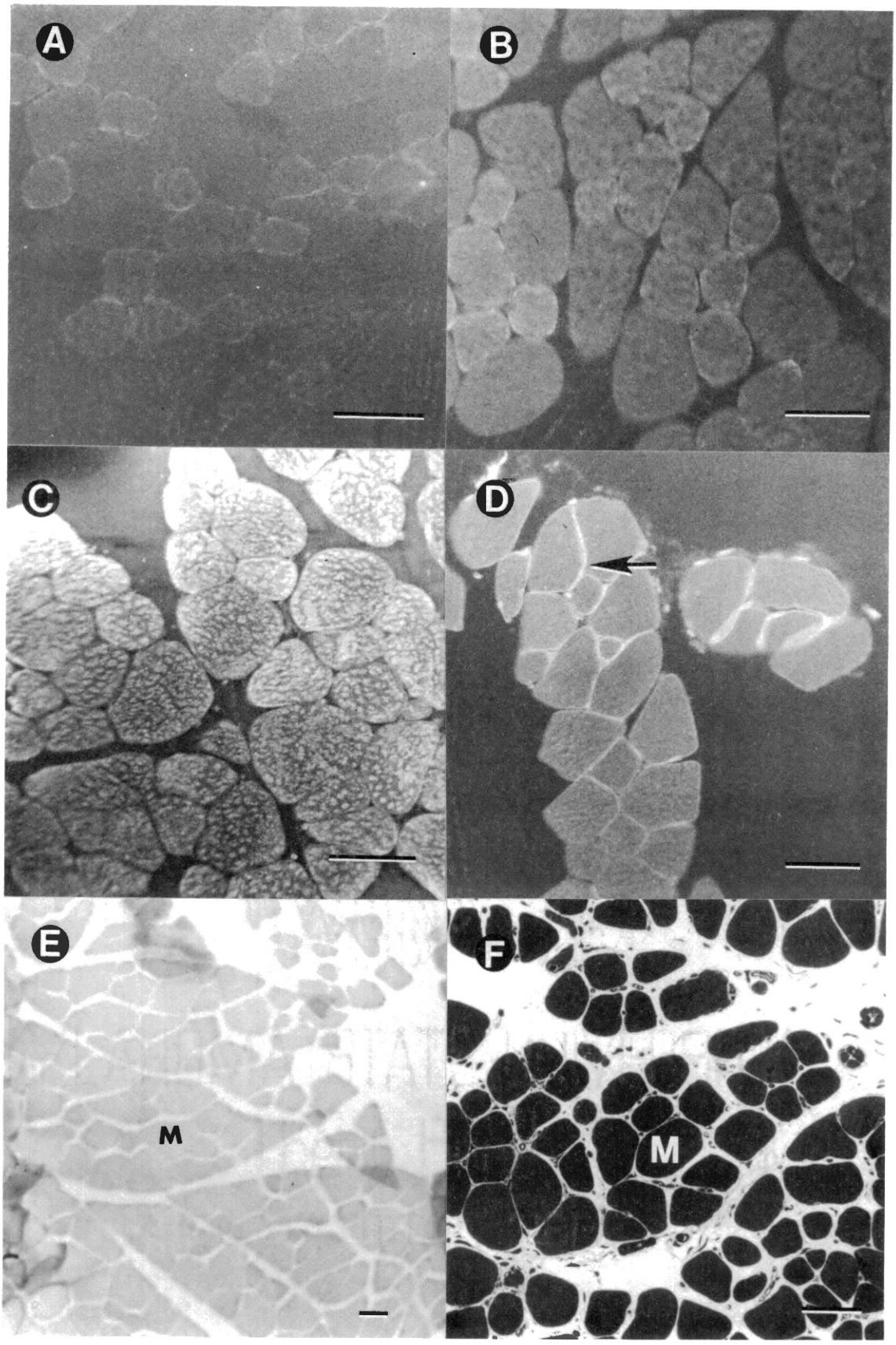
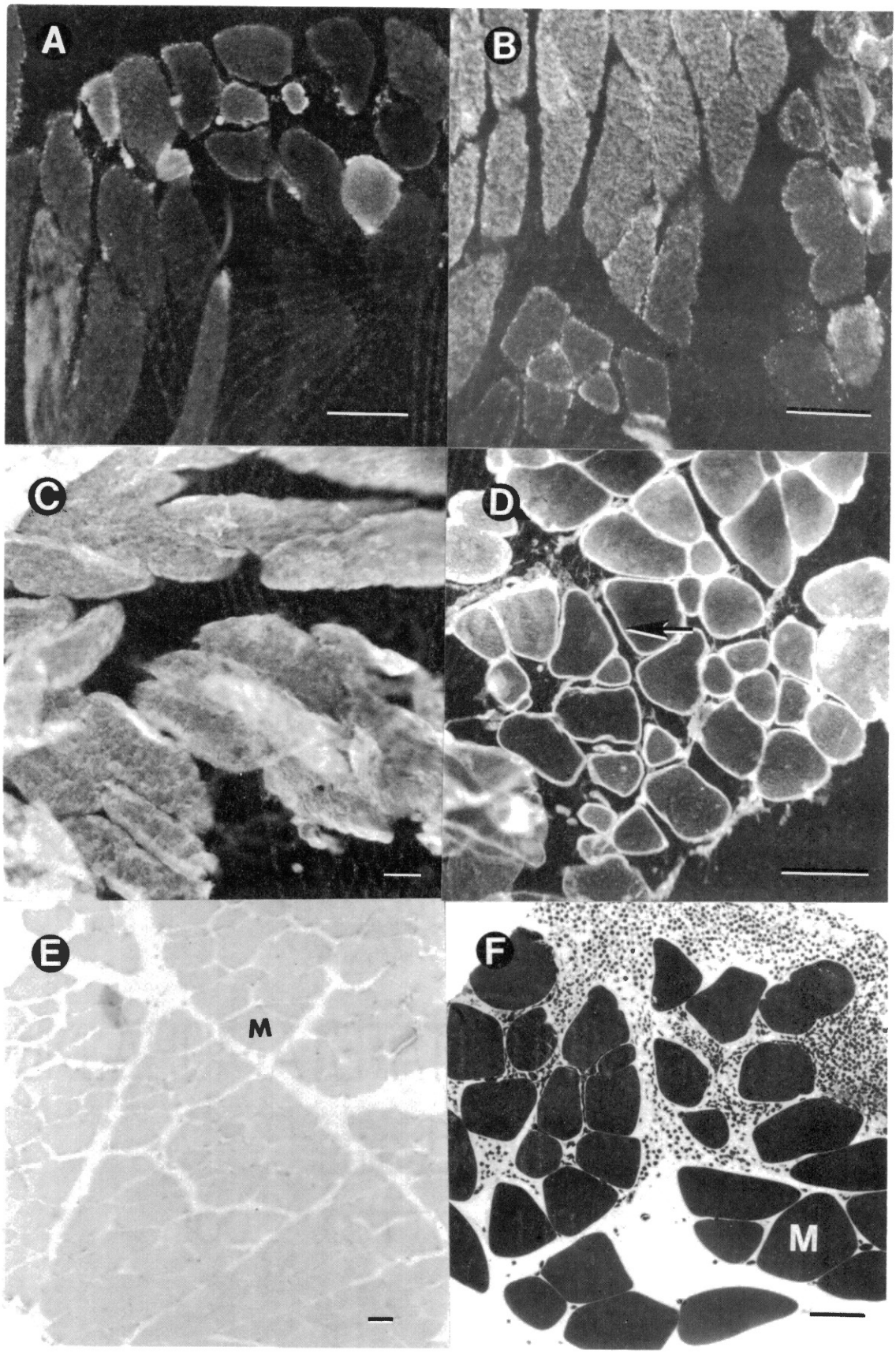

A
B
C
D
E
M
F
M

Figure 16. Light micrographs of sections of murine muscle taken 30 min following injection of HTb.
(A) PBS control - no primary or secondary antibody;
(B) Secondary antibody control - no primary antibody;
(C) Preabsorbed antibody control;
(D) Section treated with both primary and secondary antibodies;
(E) Untreated frozen section stained with Multiple Stain;
(F) Plastic embedded tissue section.
M = normal muscle cell. Note fluorescence at arrow.


A
B
C
D
E
M
F
M

the muscle cell periphery, and determination of toxin binding to the capillaries in this region was not possible. Approximately 70% of the cells observed showed binding of the toxin. The areas that did have fluorescence appeared to be of homogeneous intensity, as with 5 min post-injection, though slightly more intense than at five minutes. Figure 16E is the untreated frozen section of muscle stained with Multiple Satin and Figure 16F is the plastic embedded tissue, again demonstrating relatively normal morphology and no hemorrhage.

Figure 17 shows the results of localization of HTb at 3 hr after toxin injection. The control sections, Figures 17A-C, were similar to those described above for 5 and 30 min. However, treatment with secondary antibody control solution (Figure 17B) resulted in the specific green fluorescence of erythrocytes, making it impossible to visualize any reaction of the toxin with capillaries. By 3 hr following injection of the toxin, severe hemorrhage and some myonecrosis was observed, and virtually all of the cells observed had fluorescence at the muscle cell periphery (Figure 17D). The section receiving the Multiple Stain (Figure 17E), and the plastic embedded tissue section (Figure 17F), showed that hemorrhage and myonecrosis had developed by this time period. The tissue was edematous, making sectioning difficult.

Figure 18 shows the results of the localization at 24 hr after toxin injection. The PBS and secondary antibody

Figure 17. Light micrographs of sections of murine muscle taken 3 hr following injection of HTb.
(A) PBS control - no primary or secondary antibody;
(B) Secondary antibody control - no primary antibody;
(C) Preabsorbed antibody control;
(D) Section treated with both primary and secondary antibodies;
(E) Untreated frozen section stained with Multiple Stain;
(F) Plastic embedded tissue section.
M = normal muscle cell; N = necrotic muscle cell. Note fluorescence at arrow.

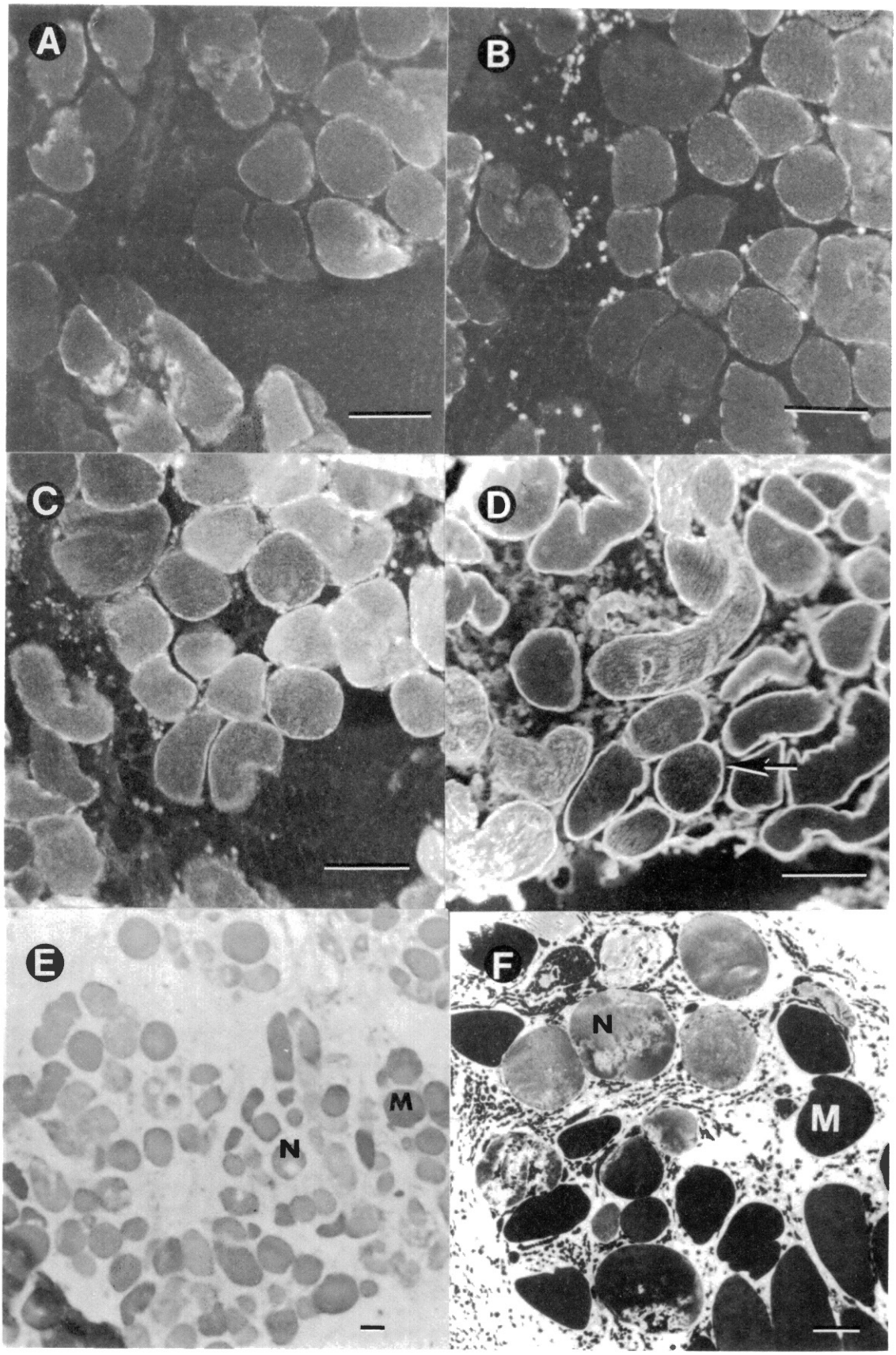
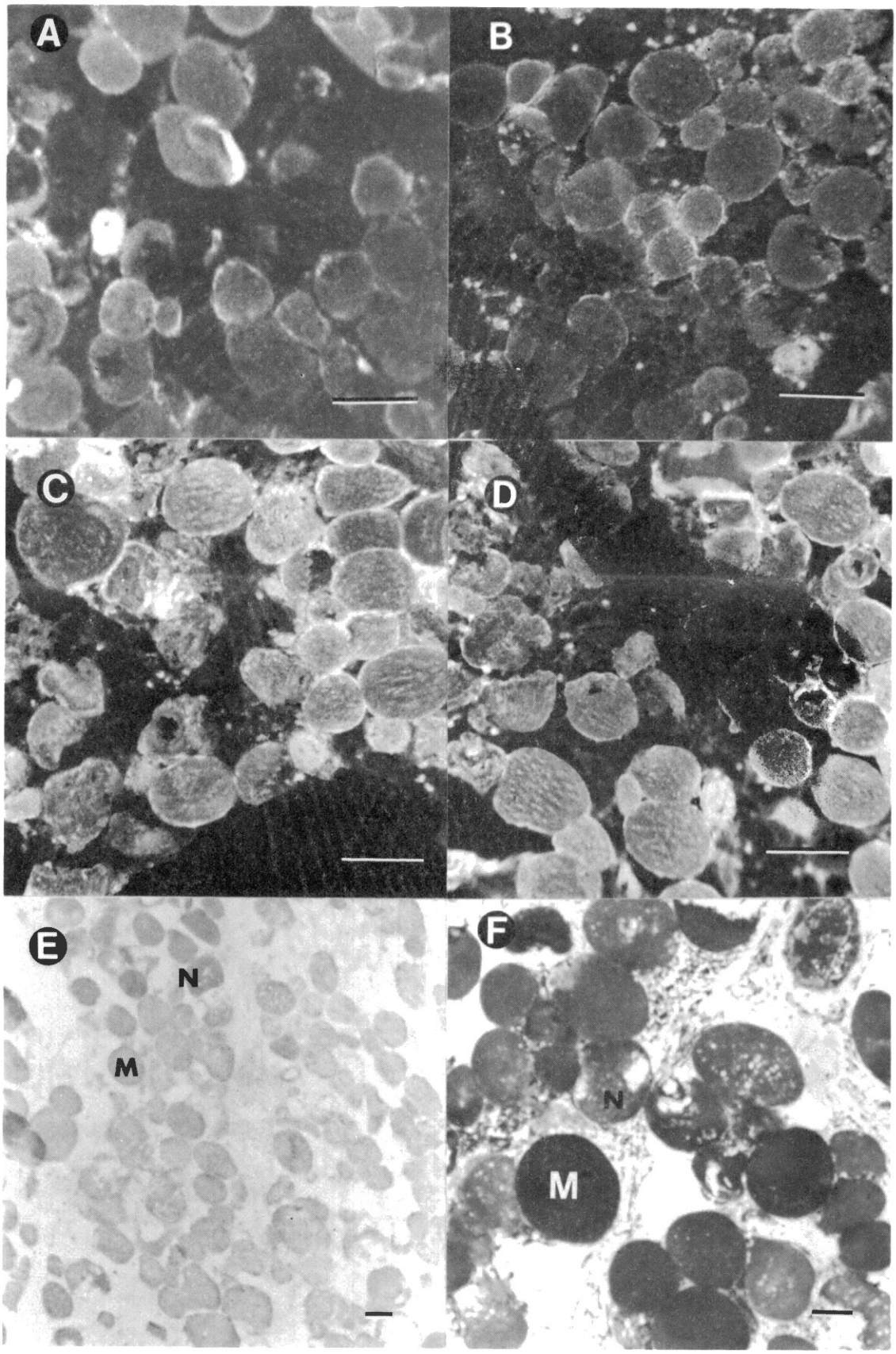

A
B
C
D
E
M
N
F
N
M

Figure 18. Light micrographs of sections of murine muscle taken 24 hr following injection of HTb.
(A) PBS control - no primary or secondary antibody;
(B) Secondary antibody control - no primary antibody;
(C) Preabsorbed antibody control;
(D) Section treated with both primary and secondary antibodies;
(E) Untreated frozen section stained with Multiple Stain;
(F) Plastic embedded tissue section.
M = normal muscle cell; N = necrotic muscle cell.


A
B
C
D
E
N
M
F
N
M

control sections, Figures 18A and 18B, showed an increase in the autofluorescence of the tissue. The preabsorbed antibody control section was similar to that observed with the three hour time period. By this time period, severe myonecrosis and hemorrhage was observed (Figures 18E and 18F). The necrotic cells included those with clumped myofilaments, delta lesions, and cells with a hyaline appearance. In some cases, only remnants of the cell membrane and basal lamina were visible. It was difficult to determine if the toxin was still associated with the muscle cell periphery based on the fluorescence because of the severity of the necrosis. There was some fluorescence associated with the muscle cells (Figure 18D), but much of the fluorescence was yellow, i.e., autofluorescence. Some of the cells fluoresced entirely, similar to that observed with the preabsorbed antibody controls, although the state of the muscle precluded any definitive conclusions concerning this. Also at this time period, the erythrocytes fluoresced following treatment with the secondary antibody control solution. Figures 18E and 18F are the frozen and plastic embedded tissue sections, clearly demonstrating the degree and extent of myonecrosis by this time period.

Discussion

The results presented here demonstrate that HTb clearly binds to the muscle cell, offering further evidence

that this toxin is indeed a direct-acting myotoxin as well as a hemorrhagic toxin. Fabiano and Tu (1981) reported the isolation of a tissue-damaging toxin, viriditoxin, from the venom of C. v. viridis, capable of inducing hemorrhage and myonecrosis. However, Gleason et al. (1983), showed that the myotoxic effect of this toxin was most likely due to myonecrosis resulting from ischemia, since necrosis was not seen until after the onset of ischemic conditions. HTb is different from viriditoxin in that myonecrosis is observed three hours after toxin injection (Ownby et al., 1978), indicating a possible direct action of the toxin on the muscle. The results presented here on the localization of the toxin support this hypothesis.

The binding of the toxin appeared to be an all-or-none type of binding, since the degree of fluorescence appeared to be relatively the same for all time periods for which it was observed, with differences only in the number of cells showing binding. The fluorescence observed at five minutes was only slightly less intense than at other time periods. Apparently a major limiting factor to the distribution of the toxin throughout the tissue is diffusion, though additional studies would be required to allow a conclusive statement. It was not possible to determine if the toxin was retained by the muscle cells at 24 hr after injection due to the severity of myonecrosis. There was fluorescence at the periphery of some cells, but the majority of it was yellow, which is indicative of autofluorescence. Some of

the cells did have green fluorescence due to FITC in their interior, which indicates a possible internalization of the toxin. However, the degree of necrosis and the state of the muscle cells at this time period prevented any definite conclusions concerning this.

The fluorescent antibody technique did not allow for determination of toxin binding to the capillary wall since the secondary antibody reacted, perhaps nonspecifically, with erythrocytes at the 3 and 24 hr time periods following toxin injection. This was compounded by the degree of fluorescence at the periphery of the muscle cells, although no binding of the toxin to vessels in the interstitial spaces was observed at 5 or 30 min.

The preabsorbed antibody control sections had cells that fluoresced entirely despite the use of excessive amounts of toxin. It is possible that the antibodies did not bind the tissue-binding region of the toxin, which would allow nonspecific binding of the toxin/antibody complexes to the tissue, explaining the fluorescence throughout the cell. Unpublished results (Chapter IV) demonstrated the poor ability of the affinity-purified antibodies to neutralize hemorrhage, neutralization occurring only when the antibodies were in extreme excess. These results possibly offer some support for the idea that the antibodies bind a region or regions of the toxin different from the tissue-binding region of HTb. However, further studies must be done on the mechanism of action of

the toxin for the induction of hemorrhage and myonecrosis before acceptance of this hypothesis is possible, since the active site of the toxin may be different for the two different effects.

Ownby et al. (1978) described the pathogenesis of hemorrhage induced by HTb. The endothelial cells were described as becoming very thin prior to rupture, even adjacent to intact intercellular junctions, resulting in hemorrhage per rhexis. Fox et al. (1986) proposed that disruption of the collagenous material around the capillary aids in hemorrhage. HTb has been shown to disrupt the basal lamina (Ownby et al., 1978), perhaps due to its proteolytic capabilities, supporting the view of Fox et al. (1986). HTb is capable of degrading casein (Bjarnason and Tu, 1978), fibrinogen (Komori et al., 1985), and insulin B chain (Hagihara et al., 1985), though there is no report in the literature of it's activity on collagen. It is of interest to know if the toxin does bind the capillary wall, and if so, whether it binds the basal lamina, the endothelial cell, or both. Demonstration of this would require an electron microscopic study. However, preliminary studies for the work presented here showed that fixation with glutaraldehyde apparently destroyed the antigenicity of the toxin. As cross-linking fixatives such as glutaraldehyde are usually required for adequate preservation of cell ultrastructure, this approach is not currently feasible. Efforts to develop a fixation method

that would not destroy the antigenicity of the toxin would be required before such an ultrastructural study could be undertaken.

Tu and Morita (1983) reported the localization of myotoxin a to the sarcoplasmic reticulum using horse-radish peroxidase conjugated directly to the toxin. Such a technique would not be affected by the fixation method used. However, one could not be sure that the HRP label did not affect the "normal" distribution of the toxin in the tissue, creating some questions as to the results of such a localization study.

CHAPTER VII

SUMMARY AND CONCLUSIONS

Hemorrhagic toxin b has been isolated by anion exchange, molecular sieve, cation exchange, and hydrophobic interaction chromatography from the venom of Crotalus atrox. Homogeneity was demonstrated with SDS polyacrylamide gel electrophoresis. The toxin has a highly basic isoelectric point, above 10.5, and proteolytic activity with dimethylcasein as the substrate, demonstrating a broad pH optimum. It is a single polypeptide with a molecular weight of approximately 23,000 daltons. It demonstrates no phospholipase or arginine esterase activities, and represents approximately 3.5% of the crude venom protein. The toxin has a minimum hemorrhagic dose of 0.3 mg/kg in mice, as measured with the method of Kondo (1960), to be compared with a minimum hemorrhagic dose of 0.2 mg/kg for crude C. atrox venom.

HTb is a peculiar toxin as compared to other hemorrhagic toxins. It is the most highly basic hemorrhagic toxin reported, although many of the toxins have not had specific isoelectric points determined. This toxin also induces myonecrosis directly, evident by the presence of necrotic muscle cells by three hours after

toxin injection, as opposed to necrosis due to ischemia, which requires at least six hours (C. L. Ownby, personal communication). The only other direct acting hemorrhagic myotoxin reported in the literature is mucrotoxin A, from T. mucrosquamatous venom, which is a high molecular weight toxin with an acidic isoelectric point. HTb also differs from the other hemorrhagic toxins isolated from C. atrox venom in that hemorrhage does not appear until at least twenty minutes following toxin injection. Histologically, muscle treated with HTb appears completely normal until after this latent time period, after which hemorrhage increases gradually to a maximum.

Antibodies to HTb have been affinity-purified from the serum of rabbits immunized with the toxin using an affinity column containing HTb coupled to an Affi-Gel 10 matrix. Partial neutralization of hemorrhage was observed when the antibody/toxin weight ratio was 58.58 mg/1.0 mg. Complete neutralization required a ratio of 104.6 mg/1.0 mg.

The enzyme-linked immunosorbent assay was used to detect the presence of HTb in the venom of fifty-three species and subspecies of snakes. The venoms were collected from snakes captured in Mexico, Costa Rica, and ten different states of the United States. In some cases, the origin of the venom was unknown. Of the five genera tested, Crotalus, Agkistrodon, Bothrops, and Trimeresurus had venoms that tested positive. The venom from Naja kaouthia, the only representative of this genus, tested

negative. Forty of the fifty-three venom samples tested positive, which represents over 75% of those tested. Ten of these venoms had detectable levels of HTb at a concentration 0.001 mg/ml, the highest dilution measured.

The results of the assay for the presence of HTb illustrated a tendency towards a geographic variation relative to HTb content. None of the species evaluated from the eastern United States had appreciable amounts of the toxin in their venoms, while there seemed to be a north-south variation with venoms from species typically found in the southern regions of the United States, Mexico and Central America having the greatest amounts of HTb. There also was marked variation in the venom content of HTb within specific genera as well as within species and subspecies.

Because of the question concerning the direct myotoxic action of HTb, the toxin was localized in skeletal muscle at the light microscopic level using immunohistochemical techniques. The toxin was shown to bind the muscle cell within five minutes following injection of the toxin. The only apparent limitation to the distribution of the toxin and it's subsequent binding to the muscle cells was diffusion, although the results presented here cannot offer definitive conclusions concerning this. Although no attempts were made to quantitate the number of cells demonstrating binding of the toxin, visual inspection showed that approximately 10-20% of the cells had bound the

toxin by five minutes. By thirty minutes, approximately 60-70% of the cells demonstrated binding, and by three hours post-injection, all of the cells observed showed binding of the toxin. It was not possible to determine the state of toxin binding at twenty-four hours following injection of the toxin due to the advanced state of necrosis.

It was impossible to determine when the toxin bound the capillaries, if such binding occurred at all, for several reasons. The degree of fluorescence around the periphery of the muscle cell was too intense to determine if there was any fluorescence at the capillaries in the region between the cells. Also, at the early time periods, five and thirty minutes, the lack of severe hemorrhage and edema did not allow for separation of the muscle cells, compounding the problem of observing the capillaries in these regions. However, there was no fluorescence around capillaries in the interstital spaces at five and thirty minutes after toxin injection, indicating that no binding had occurred by this time period. At three and twenty-four hours post-injection, the secondary antibodies nonspecifically bound the extravasated erythrocytes, precluding any determination of capillary binding. The presence of intact capillaries next to necrotic muscle cells and the onset of necrosis by three hours following injection of the toxin (Ownby et al., 1978), coupled with the information presented here showing that the toxin does

bind muscle cells within five minutes following injection, strongly indicates that HTb does have direct myotoxic activity.

There are many reasons for studying snake venoms, but two of the most important should be (1) to develop better methods of treating clinical cases, and (2) to better understand the characteristics of the toxins in venoms so that we may better understand the pathogenesis of their toxic activities. The development of better treatments is completely dependent on the understanding of pathogenesis and mechanisms of actions of toxic components. It is this dependency upon which the work presented here was based. By isolating and studying a single toxin, some insight may be offered concerning the effects of the whole venom. Hopefully, the results presented in this paper will aid in a more complete understanding of C. atrox venom poisoning, in addition to exposing some questions to be further studied. These questions might include an ultrastructural localization of the toxin to determine what morphological structure or structures are affected by the toxin.

Because HTb induces both myonecrosis and hemorrhage, and represents 3.5% of the crude venom, it would seem that this toxin would play a decisive role in the local tissue damage following C. atrox envenomation. Although Wyeth's polyvalent (Crotalidae) antivenin, the antiserum most commonly used for rattlesnake venom poisoning, was shown to be effective against hemorrhage induced by C. atrox venom

(Ownby et al., 1984b), the results presented here demonstrated no neutralization (in vitro) of even one minimum hemorrhagic dose of HTb. Because so many other crotalid venoms have a toxin that cross-reacts with antibodies produced against HTb, many of which may have tissue-damaging effects similar to HTb, an antiserum should have, at the least, some neutralizing capacity for such a toxin. It is hoped that the results presented in this paper may be used to develop treatments that will prove effective against toxins like HTb and, ultimately, against the crude venoms.

LITERATURE CITED

Abercrombie, D., Tamaoki, H., Angal, S. and Chaiken, I. (1983) Affinity chromatography studies of cooperative interactions in neurophysin-neuropeptide complexes. In: Affinity Chromatography and Biological Recognition, pp. 113-118 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Allam, M., Weiner, D. and Lukens, F. (1956) Comparison of cortisone and antivenin in the treatment of crotaline envenomation. In: Venoms, pp. 393-397 (Buckley, E. and Porges, N., Eds.). Washington, D.C., American Association for the Advancement of Science.

Angeletti, R. (1968) Studies on the nerve growth factor (NGF) from snake venom: gel filtration patterns of crude venoms. J. Chromatog. 36: 535.

Arnold, R. (1982) Treatment of rattlesnake bites. In: Rattlesnake Venoms, Their Actions and Treatment, pp. 315-338 (Tu, A., Ed.). New York: Marcel Dekker, Inc.

Bajwa, S., Markland, F. and Russell, F. (1981) Fibrinolytic and fibrinogen clotting enzymes present in the venoms of western diamondback rattlesnake, Crotalus atrox, eastern diamondback rattlesnake, Crotalus adamanteus, and southern pacific rattlesnake, Crotalus viridis helleri. Toxicon 19: 53.

Baxter, E. and Gallichio, H. (1974) Cross-neutralization by tiger snake (Notechis scutatus scutatus) antivenene and sea snake (Enhydrina schistosa) antivenene against several sea snake venoms. Toxicon 12: 273.

Beltran, J., Mascarenhas, Y., Craievich, A. and Laure, C. (1985) SAXS study of structure and conformational changes of crotamine. Biophys. J. 47: 33.

Bieber, A., Tu, T. and Tu, A. (1975) Studies of an acidic cardiotoxin isolated from the venom of Mojave rattlesnake (Crotalus scutulatus scutulatus). Biochem. Biophys. Acta. 400: 178.

Bjarnason, J., Barish, A., Direnzo, G., Campbell, R. and Fox, J. (1983) Kallikrein-like enzymes from Crotalus atrox venom. J. Biol. Chem. 258: 12: 566.

Bjarnason, J. and Tu, A. (1978) Hemorrhagic toxins from western diamondback rattlesnake (Crotalus atrox) venom: isolation and characterization of five toxins and the role of zinc in hemorrhagic toxin e. Biochemistry 17(16): 3395.

Bober, M. (1987) The use of an affinity purified antibody to develop the antigen and antibody ELISA, to measure the blood disappearance of antimyotoxin and to screen venoms for myotoxin a. Ph.D Dissertation, Oklahoma State University, Stillwater, Oklahoma.

Boedeker, E. and Cheney, C. (1983) Hydrophobic bacterial surface properties are determined by pili which mediate attachment to intestinal brush border membranes. In: Affinity Chromatography and Biological Recognition, pp. 501-502 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Bolanos, R., Cerdas, L. and Taylor, R. (1975) The production and characteristics of a coral snake (Micrurus mipartitus hertwigi) antivenin. Toxicon 13: 139.

Bon, C. (1982) Synergism of the two subunits of crotoxin. Toxicon 20(1): 105.

Bon, C. and Saliou, B. (1983) Ceruleotoxin: identification in the venom of Bungarus fasciatus, molecular properties and importance of phospholipase A_2 activity for neurotoxicity. Toxicon 21(5): 681.

Bonilla, C. (1976) Hypotensin: a hypotensive peptide isolated from Crotalus atrox venom: purification, amino acid composition and terminal amino acid residues. FEBS letters 68: 297.

Bonilla, C. and Horner, N. (1969) Comparative electrophoresis of Crotalus and Agkistrodon venoms from North American snakes. Toxicon 7: 327.

Bonilla, C. and Rammel, O. (1976) Comparative biochemistry and pharmacology of salivary gland secretions III. Chromatographic isolation of a myocardial depressor protein (MDP) from the venom of Crotalus atrox. J. Chromatog. 124: 303.

Breithaupt, H., Rubsamen, K. and Habermann, E. (1974) Biochemistry and pharmacology of the crotoxin complex. Eur. J. Biochem. 49: 333.

Bureau, D. and Daussant, J. (1983) Desorption following immunoaffinity chromatography; generalization of a gentle procedure for desorbing antigen. J. Immunol. Meth. 57: 205.

Cameron, D. and Tu, A. (1977) Characterization of myotoxin a from the venom of prairie rattlesnake (Crotalus viridis viridis). Biochemistry 16: 2546.

Cameron, D. and Tu, A. (1978) Chemical and functional homology of myotoxin a from prairie rattlesnake venom and crotamine from South American rattlesnake venom. Biochem. Biophys. Acta. 532: 147.

Campbell, C. (1979) Symptomatology, pathology and treatment of the bites of Elapid snakes. In: Snake Venoms, pp. 898-921 (Lee, C., Ed.). Berlin: Springer-Verlag.

Cate, R. and Bieber, A. (1978) Purification and characterization of mojave (Crotalus scutulatus scutulatus) toxin and its subunits. Arch. Biochem. Biophys. 189: 397.

Chaiken, I. (1983) Molecular interactions, affinity methods, and the developing synergism between affinity chromatography and biological recognition. In: Affinity Chromatography and Biological Recognition, pp. 3-6 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Chang, C. and Tseng, K. (1978) Effect of crotamine, a toxin from South American rattlesnake venom, on the sodium channel of murine skeletal muscle. Br. J. Pharmacol. 63: 551.

Childs, G. (1982) Use of immunocytochemical techniques in cellular endocrinology. In: Electron Microscopy in Biology, pp. 107-180 (Griffith, G., Ed.). New York: John Wiley and Sons, Inc.

Civello, D., Duong, H. and Geren, C. (1983a) Isolation and characterization of a hemorrhagic proteinase from timber rattlesnake venom. Biochemistry 22: 749.

Civello, D., Moran, J. and Geren, C. (1983b) Substrate specificity of a hemorrhagic proteinase from timber rattlesnake venom. Biochemistry 22: 755.

Clausen J. (1969) Immunological techniques for the identification and estimation of macomolecules. In: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 2(3) (Work, T. ans Work, E., Eds.). Amsterdam: North Holland.

Clegg, J. and Smith, M. (1983) Affinity chromatography with mouse monoclonal IgM. In: Affinity Chromatography and Biological Recognition, pp. 395-396 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Criley, B. (1956) Development of a multivalent antivenin for the family Crotalidae. In: Venoms, pp. 373-380 (Buckley, E. and Porges, N., Eds.). Washington, D.C., American Association for the Advancement of Science.

Crowle, A. and Cline, L. (1977) An improved stain for immunodiffusion tests. J. Immunol. Meth. 17: 379.

Cuatrecasas, P. and Anfinsen, C. (1971) Affinity chromatography. In: Methods in Enzymology: Enzyme Purification and Related Techniques, pp. 345-378 (Jakoby, W., Ed.). New York: Academic Press.

Deutsch, H. and Diniz, C. (1955) Some proteolytic activities of snake venoms. J. Biol. Chem. 216: 17.

Dongen, J., Hooijkaas, H., Comans-Bitter, W., Benne, K., Os, T. and de Jong, J. (1985) Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J. Immunol. Meth. 80: 1.

Dufourcq, J., Faucon, J. Bernard, E. and Pezolet, M. (1982) Structure-function relationships for cardiotoxin interacting with phospholipids. Toxicon 20: 165.

Efrati, P. (1979) Symptomology, pathology and treatment of the bites of viperid snakes. In: Snake Venoms, pp. 956-977 (Lee, C., Ed.). Berlin: Springer-Verlag.

Egarov, T., Svenson, A. and Rydien, L. (1975) A rapid and specific method for isolation of thiol-containing peptides form large proteins by thiol-disulfide exchange on a solid support. Proc. Natl. Acad. Sci. USA 72: 3029.

Emery, J. and Russell, F. (1963) Lethal and hemorrhgaic properties of some North American snake venoms. In: Venomous and Poisonous Animals and Noxious Plants of the Pacific Region, (Keegan, H. and Macfarlane, W., Eds.). New York: The Macmillan Co.

Engl, C., Becker, R., Bailey, T. and Bieber, A. (1983) Characterization of two myotoxic proteins from the venom of Crotalus viridis concolor. Toxin Reviews 2(2): 267.

Fabiano, R. and Tu, A. (1981) Purification and biochemical study of viriditoxin, tissue damaging toxin, from prairie rattlesnake venom. Biochemistry 20: 21.

Farber, J., Chien, K. and Mittnacht, S. (1981) The pathogenesis of irreversible cell injury in ischemia. Am. J. Path. 102: 271.

Fohlman, J., Eaker, D., Karlsson, E. and Thesleff, S. (1976) Taipoxin, an extremely potent presynaptic neurotoxin from the venom of the Australian snake taipan (Oxyuranus scutellatus scutellatus). Isolation, characterization, quarternary structure and pharmacological properties. Eur. J. Biochem. 68: 457.

Fohlman, J. and Eaker, D. (1977) Isolation and characterization of a lethal myotoxic phospholipase A from the venom of the common sea snake Enhydrina schistosa causing myoglobinuria in mice. Toxicon 15: 385.

Fox, J., Campbell, R., Beggerly, L. and Bjarnason, J. (1986) Substrate specificities and inhibition of two hemorrhagic zinc proteases Ht-c and Ht-d from Crotalus atrox venom. Eur. J. Biochem. 156: 65.

Friederich, C. and Tu, A. (1971) Role of metals in snake venoms for hemorrhagic, esterase and proteolytic activities. Biochem. Pharmacol. 20: 1549.

Garfin, S., Castiloma, R., Mubarak, S., Hargens, A., Russell, R. and Akeson, W. (1984) Rattlesnake bites ans surgical decompression results using a laboratory model. Toxicon 22(2): 177.

Gelb, W. (1973) Affinity chromatography for separation of biological material. American Lab. Oct.: 61.

Glass, T. (1976) Early debridement in pit viper bites. JAMA 235(23): 2513.

Gleason, M., Odell, G. and Ownby, C. (1983) Isolation and biological activity of viriditoxin and a viriditoxin-variant from Crotalus viridis viridis venom. Toxin Rev. 2(2): 235.

Glenn, J. and Straight, R. (1978) Mojave rattlesnake Crotalus scutulatus scutulatus: variation in toxicity with geographical origin. Toxicon 16: 81.

Glenn, J. and Straight, R. (1982) The rattlesnakes and their venom yield and lethal toxicity. In: Rattlesnake Venoms, Their Actions and Treatment, pp. 3-120 (Tu, A., Ed.). New York: Marcel Dekker, Inc.

Glenn, J. and Straight, R. (1985) Venom properties of the rattlesnakes (Crotalus) inhabiting the Baja California region of Mexico. Toxicon 23(5): 769.

Glenn, J., Straight, R. and Snyder, D. (1972) Yield of venom obtained from Crotalus atrox by electrical stimulation. Toxicon 10: 575.

Glenn, J., Straight, R., Wolfe, M. and Hady, D. (1983) Geographical variation in Crotalus scutulatus scutulatus (Mojave rattlesnake) venom proteins. Toxicon 21: 119.

Goncalves, J. (1956) Purification and properties of crotamine. In: Venoms, pp. 261-274 (Buckley, E. and Porges, N., Eds.). American Association for the Advancement of Science: Washington, D.C.

Gopalakrishnakone, P. and Hawgood, G. (1984) Morphological changes induced by crotoxin in murine nerve and neuromuscular junction. Toxicon 22(5): 791.

Grotto, L., Moroz, C., de Vries, A. and Goldblum, N. (1967) Isolation of Vipera palestinae hemorrhagin and distinction between its hemorrhagic and proteolytic activities. Biochem. Biophys. Acta. 133: 356.

Gutierrez, J., Ownby, C. and Odell, G. (1984a) Isolation of a myotoxin from Bothrops asper venom: partial characterization and action on skeletal muscle. Toxicon 22(1): 115.

Gutierrez, J., Ownby, C. and Odell, G. (1984b) Pathogenesis of myonecrosis induced by crude venom and a myotoxin of Bothrops asper. Exp. Mol. Path. 40: 367.

Gutierrez, J., Ownby, C. and Odell, G. (1984c) Skeletal muscle regeneration after myonecrosis induced by crude venom and a myotoxin from the snake Bothrops asper (Fer-de-lance). Toxicon 22(5): 719.

Gutierrez, J., Lomonte, B. and Cerdas, L. (1986) Isolation and partial characterization of a myotoxin from the venom of the snake Bothrops nummifer. Toxicon 24(9): 885.

Hachimori, Y., Wells, M. and Hanahan, D. (1971) Observations on the Phospholipase A_2 of Crotalus atrox. Molecular weight and other properties. Biochemistry 10(22): 4084.

Hagihara, S. Komori, Y. and Tu, T. (1985) Proteolytic specificity of hemorrhagic toxin b from Crotalus atrox (western diamondback rattlesnake) venom. Comp. Biochem. Physiol. 82: 21.

Holgate, C., Jackson, P., Cowen, P. and Bird, C. (1983) Immunogold-silver staining: new method of immunostaining with enhanced sensitivity. J. Histochem. Cytochem. 31(7): 938.

Homma, M. and Tu, A. (1971) Morphology of local tissue damage in experimental snake envenomation. Br. J. Exp. Path. 52: 538.

Hong, B. (1982) Isolation and identification of a collagenolytic enzyme from the venom of the western diamondback rattlesnake (Crotalus atrox). Toxicon 20(3): 535.

Hong, S. and Chang, C. (1985) Electrophysiological studies of myotoxin a, isolated from prairie rattlesnake (Crotalus viridis viridis) venom, on murine skeletal muscle. Toxicon 23(6): 927.

Huang, S. and Perez, J. (1980) Comparative study on hemorrhagic and proteolytic activities of snake venoms. Toxicon 18: 421.

Huang, S. and Perex, J. (1982) A comparative electron microscopic study of myonecrosis induced by Crotalus atrox (western diamondback rattlesnake) in gray woodrats and mice. Toxicon 20: 443.

Huang, T., Lynch, J., Larson, D. and Lewis, S. (1974) The use of excisional therapy in the management of snakebite. Ann. Surg. 179: 598.

Huang, T., Chang, J. and Ouyang, C. (1984) Characterization of hemorrhagic principles from Trimersurus gramineus snake venom. Toxicon 22(1): 45.

Ishida, Y., Yamashita, S., Ohsaka, A., Takahashi, T. and Omori-Satoh, T. (1976) Pharmacological studies of the hemorrhagic principles isolated from the venom of Trimersurus flavoviridis, a crotalid. In: Animal, Plant and Microbial Toxins, pp. 263-272 (Okshka, A., Hayashi, K., and Sawai, Y., Eds.). New York: Plenum, Inc.

Iwanaga, S. and Suzuki, T. (1979) Enzymes in snake venoms. In: Snake Venoms, pp. 61-158 (Lee, C., Ed.). New York: Springer-Verlag.

Jennings, R. and Reimer, K. (1981) Lethal myocardial ischemic injury. Am. J. Path. 102: 241.

Kaiser, I., Middlebrook, J., Crumrine, M. and Stevenson, W. (1986) Cross-reactivity and neutralization by rabbit antisera raised against crotoxin, its subunits and two related toxins. Toxicon 24(7): 669.

Karlsson, E., Eaker, D., Tryklund, L. and Kadin, S. (1972) Chromatographic separation of Enhydrina schistosa (common sea snake) venom and the characterization of two principle neurotoxins. Biochemistry 11: 4628.

Karlsson, E. (1979) Chemistry of protein toxins in snake venoms. In: Snake Venoms, pp. 159-212 (Lee, C., Ed.). New York: Springer-Verlag.

Katchalski-Katzir, E. (1983) Some general considerations on the recognition by and of proteins. In: Affinity Chromatography and Biological Recognition, pp. 7-28 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Kishida, M., Nikai, T., Mori, N., Kohmura, S. and Sugihara, H. (1985) Characterization of mucrotoxin A from the venom of Trimersurus mucrosquamatous (the Chinese habu snake). Toxicon 23(4): 637.

Klibansky, C., London, Y., Frenkel, A., and de Vries, A. (1968) Enhancing action of synthetic and natural basic polypeptides on erythrocyte ghost phospholipid hydrolysis by phospholipase A. Biochem. Biophys. Acta. 150: 15.

Kocholaty, W., Ledford, E., Daly, J. and Billings, T. (1971) Toxicity and some enzymatic properties and activities in the venoms of crotalidae, elapidae and viperidae. Toxicon 9: 131.

Komori, Y., Hagihara, S. and Tu, A. (1985) Specificity of hemorrhagic proteinase from Crotalus atrox (western diamondback rattlesnake) venom. Biochemica et Biophysica Acta 829: 127.

Kondo, H., Kondo, S. Ikezawa, H., Murata, R. and Ohsaka, A. (1960) Studies on the quantitative method for determination of hemorrhagic activity of Habu snake venom. Jap. J. Med. Sci. Biol. 13: 43.

Kouyoumdjian, J., Harris, J. and Johnson, M. (1986) Muscle necrosis caused by the sub-units of crotoxin. Toxicon 24(6): 575.

Kurecki, T. and Kress, L. (1985) Purification and partial characterization of the hemorrhagic factor from the venom of Crotalus adamanteus (eastern diamondback rattlesnake). Toxicon 23(4): 657.

Lomonte, B., Gutierrez, J. and Mata, E. (1985) Isolation from a polyvalent antivenom of antibodies to a myotoxin in Bothrops asper snake venom. Toxicon 23(5): 807.

Lee, H. and Chao, Y. (1978) Purification of Bungarus multicinctus antivenin by affinity chromatography. Chinese J. Microbiol. 11: 122.

Leonardi, T., Howben, M. and Spence, I. (1979) A lethal myotoxin isolated from the venom of the Australian king brown snake (Pseudechis australis). Toxicon 17: 549.

Liddle, W. and Tu, A. (1985) Interaction of myotoxin a with artificial membranes: raman spectroscopic investigation. Biochemistry 24: 7635.

Macht, D. (1937) Comparative toxicity of sixteen specimens of Crotalus venom. Proc. Soc. Exp. Biol. Med. 36(4): 499.

Maeda, N., Tamiya, N., Pattabhiraman, T. and Russell, F. (1978) Some chemical properties of the venom of the rattlesnake, Crotalus viridis helleri. Toxicon 16: 431.

Maltin, C., Harris, J. and Cullen, M. (1983) Regeneration of mammalian skeletal muscle following the injection of the snake venom toxin, taipoxin. Cell. Tissue Res. 232: 565.

Man, D. and Minta, J. (1977) Purification, characterization and analysis of the mechanism of action of four anti-complementary factors in Crotalus atrox venom. Immunochemistry 14: 521.

Mandelbaum, R., Ruchl, A. and Asakura, M. (1976) Some physical and biochemical characteristics of HF2, one of the hemorrhagic factors in the venom of Bothrops jararaca. Toxicon 13: 109.

Mandelbaum, F., Assakura, M. and Reichl, A. (1984) Characterization of two hemorrhagic factors isolated from the venom of Bothrops neuwiedi (Jararaca pintada). Toxicon 22(2): 193.

McCollough, N. and Gennaro, J. (1970) Treatment of venomous snakebite in the United States. Clinical Toxicology 3(3): 482.

Mebs, D. (1978) Pharmacology of reptilian venoms. In: Biology of the Reptilia, pp. 437-560 (Gans, C., Ed.). London: Academic Press.

Mebs, D., Ehrenfield, M. and Samejima, V. (1983) Local necrotizing effect of snake venoms on skin and muscle: relationship to serum creatine kinase. Toxicon 21(3): 393.

Mebs, D. and Samejima, V. (1980) Purification, from Australian elapid venoms, and properties of phospholipases A which cause myoglobinuria in mice. Toxicon 18: 443.

Mebs, D. and Samejima, V. (1986) Isolation and characterization of myotoxic phospholipases A_2 from crotalid venoms. Toxicon 24(2): 161.

Minton, S. (1954) Polyvalent antivenin in the treatment of experimental snake venom poisoning. Am. J. Trop. Med. Hyg. 3: 1077.

Minton, S. (1956) Some properties of North American pit viper venoms and their correlation with phylogeny. In: Venoms, pp. 145-151 (Buckley, E. and Porges, N., Eds.). Washington, D.C.: American Association for the Advancement of Science.

Minton, S. (1967) Paraspecific protection by elapid and sea snake antivenins. Toxicon 5: 47.

Minton, S. (1975) A note on the venom of an aged rattlesnake. Toxicon 13: 73.

Minton, S. (1976) Neutralization of old world viper venoms by American pit viper antivenin. Toxicon 14: 146.

Minton, S. and Weinstein, S. (1986) Geographic and ontogenic variation in venom of the western diamondback rattlesnake (Crotalus atrox). Toxicon 24(1): 71.

Mori, N., Nikai, T. and Sugihara, H. (1984) Purification of a proteinase (AC5-Proteinase) and characterization of hemorrhagic toxins from the venom of the hundred pace snake (Agkistrodon acutus). Toxicon 22(3): 451.

Ng., R. and Howard, B. (1980) Mitochondria and sarcoplasmic reticulum as model targets for neurotoxic and myotoxic phospholipases A_2. Biochemistry 77(3): 1346.

Nikai, T., Kito, R., Mori, N., Sugihara, H. and Tu, A. (1983) Isolation and characterization of fibrinogenase from western diamondback rattlesnake and its comparison to the thrombin-like enzyme, crotalase. Comp. Biochem. Physiol. 76(4): 679.

Nikai, T., Mori, N., Kishida, M., Sugihara, H. and Tu, A. (1984) Isolation and biochemical characterization of hemorrhagic toxin f from the venom of Crotalus atrox (western diamondback rattlesnake). Arch. Biochem. Biophys. 231(2): 309.

Nikai, T., Mori, N., Kishida, M., Tsuboi, M. and Sugihara, H. (1985) Isolation and characterization of hemorrhagic toxin g from the venom of Crotalus atrox (western diamondback rattlesnake). Am. J. Trop. Med. Hyg. 34(6): 1163.

Ohsaka, A., Ohashi, M., Tsuchiya, M., Kamisaki, Y. and Fugishiro, Y. (1971) Action of Trimersurus flavoviridis venom on the microcirculatory system of the rat; dynamic aspects as revealed by cinephotomicrographic recording. Japan J. Med. Sci. Biol. 24: 34.

Ohsaka, A., Just, M. and Habermann, E. (1973) Action of snake venom hemorrhagic principles on isolated glomerular basement membrane. Biochem. Biophys. Acta. 323: 415.

Ohsaka, A., Suzuki, K. and Ohashi, M. (1975) The spurting of erythrocytes through junctions of the vascular endothelium treated with snake venom. Microvascular Research 10: 208.

Omori-Satoh, T. and Ohsaka, A. (1970) Purification and some properties of hemorrhagic principle 1 in the venom of Trimersurus flavoviridis. Biochem. Biophys. Acta. 207: 432.

Omori-Satoh, T. and Sadahiro, S. (1979) Resolution of the major hemorrhagic component of Trimersurus flavoviridis venom into two parts. Biochem. Biophys. Acta. 580: 392.

Oshima, G., Omori-Satoh, T., Iwanaga, S. and Suzuki, T. (1972) Studies on snake venom hemorrhagic factor I (HR I) in the venom of Agkistrodon halys blomhoffii. J. Biochem. 72: 1483.

Ovadia, M. (1978) Isolation and characterization of three hemorhagic factors from the venom of Vipera palestinae. Toxicon 16: 479.

Ownby, C., Kainer, R. and Tu, A. (1974) Pathogenesis of hemorrhage induced by rattlesnake venom: an electron microscopic study. Am. J. Path. 76(2): 401.

Ownby, C., Tu, A., and Kainer, R. (1975) Effect of diethylenetriaminepentacetic acid and procaine on hemorrhage induced by rattlesnake venom. J. Clin. Pharm. 15(5): 419.

Ownby, C., Cameron, D. and Tu, A. (1976) Isolation of myotoxic component from rattlesnake (Crotalus viridis viridis) venom: electron microscopic analysis of muscle damage. Am. J. Path. 85: 149.

Ownby, C., Bjarnason, J. and Tu, A. (1978) Hemorrhagic toxins from rattlesnake (Crotalus atrox) venom: pathogenesis of hemorrhage induced by three purified toxins. Am. J. Path. 93: 201.

Ownby, C., Wood, W. and Odell, G. (1979) Anitserum to myotoxin from prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 17: 373.

Ownby, C. (1982) Pathology of rattlesnake envenomation. In: Rattlesnake Venoms, Their Actions and Treatment, pp. 163-209 (Tu, A., Ed.). New York: Marcel Dekker.

Ownby, C., Gutierrez, J., Colberg, T. and Odell, G. (1982) Quantitation of myonecrosis induced by myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 20(5): 877.

Ownby, C., Odell, G., Woods, W. and Colberg, T. (1983a) Ability of antiserum to myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom to neutralize local myotoxicity and lethal effects of myotoxin a and homologous crude venom. Toxicon 21(1): 35.

Ownby, C., Odell, G. and Theakston, R. (1983b) Detection of antibodies to myotoxin a and prairie rattlesnake (Crotalus viridis viridis) venom in three antisera using enzyme-linked immunosorbent assay and immunodiffusion. Toxicon 21(6): 849.

Ownby, C., Colberg, T., Claypool, L. and Odell, G. (1984a) In vivo test of the ability of antiserum to myotoxin a from prairie rattlesnake (Crotalus viridis viridis) venom to neutralize local myonecrosis induced by myotoxin a and homologous crude venom. Toxicon 22(1): 99.

Ownby, C., Colberg, T. and Odell, G. (1984b) A new method for quantitating hemorrhage induced by rattlesnake venoms: ability of polyvalent antivenom to neutralize hemorrhagic activity. Toxicon 22(2): 227.

Ownby, C., Colberg, T. and Odell, G. (1985) Ability of a mixture of antimyotoxin a serum and polyvalent (Crotalidae) antivenin to neutralize myonecrosis, hemorrhage and lethality induced by prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 23(2): 317.

Ownby, C. and Colberg, T. (1986) Ability of polyvalent (Crotalidae) antivenin to neutralize local myonecrosis induced by Crotalus atrox venom. Toxicon 24(2): 201.

Ownby, C., Colberg, T. and Odell, G. (1986) In vivo ability of antimyotoxin a serum plus polyvalent (Crotalidae) antivenin to neutralize prairie rattlesnake (Crotalus viridis viridis) venom. Toxicon 24(2): 197.

Ownby, C. and Geren, C. (1987) Pathogenesis of hemorrhage induced by hemorrhagic porteinase IV from timber rattlesnake (Crotalus horridus horridus) venom. Toxicon, In press.

Pandya, B., Rubin, R., Olexa, S. and Budzynski, A. (1983) Unique degradation of human fibrinogen by proteases from western diamondback rattlesnake (Crotalus atrox) venom. Toxicon 21(4): 515.

Pandya, B. and Budzynski, A. (1984) Anticoagulant proteases from western diamondback rattlesnake (Crotalus atrox) venom. Biochemistry 23: 460.

Parrish, H. (1980) Poisonous Snakebites in the United States. New York: Vantage Press.

Perez, J., Haws, W., Garcia, V. and Jennings, F. (1978) Resistance of warm-blooded animals to snake venoms. Toxicon 16: 375.

Pfleiderer, G. and Sumyk, G. (1961) Investigation of snake venom enzymes: separation of rattlesnake venom proteinases by cellulose ion-exchange chromatography. Biochem. Biophys. Acta. 51: 482.

Pirkle, H., Markland, F., Throdor, I., Baumgartner, R., Bajwa, S. and Kirakossian, H. (1981) The primary structure of crotalase, a thrombin-like venom enzyme, exhibits closer homology to kallikrein than to other serine proteases. Bioch. Biophys. Res. Comm. 99(2): 715.

Posner, P., Macintosh, B. and Gerencser, G. (1981) Effects of western diamondback rattlesnake (Crotalus atrox) venom on heart muscle. Toxicon 19(2): 330.

Rael, E., Salo, R. and Zepeda, H. (1986) Monoclonal antibodies to mojave toxin and use for isolation of cross-reacting proteins in Crotalus venoms. Toxicon 24(7): 661.

Reid, H., Thran, P. and Martin, W. (1963) Specific antivenene and prednisone in viper-bite poisoning: controlled trial. British Medical Journal 2: 1378.

Reid, H. (1964) Cobra-bites. Br. Med. J. 2: 540.

Reid, H. (1979) Symptomatology, pathology, and treatment of the bites of sea snakes. In: Snake Venoms, pp. 922-955 (Lee, C., Ed.). Berlin: Springer-Verlag.

Richards, G, du Vair, G. and Laskowski, M. (1965) Comparison of the levels of phosphodiesterase, endonuclase and monophosphatase in several snake venoms. Biochemistry 4(3): 501.

Rick, W. (1963) Trypsin. In: Methods of Enzymatic Analysis, pp. 807-818 (Bergmeyer, H., Ed.). New York: Academic Press.

Rosenfeld, G. and Kelen, E. (1966) Cross neutralization of the coagulent activity of some snake venoms by antivenins. Toxicon 4: 7.

Russell, F., Ruzic, N. and Gonzales, H. (1973) Effectiveness of antivenin (Crotalidae) polyvalent following injection of Crotalus venom. Toxicon 11: 461.

Russell, F., Pattabhiraman, T., Schaeffer, R., Tamiya, N. and Maeda, N. (1978) Some properties of the venom of Crotalus viridis helleri. Toxicon 16: 425.

Russell, F., Sullivan, J., Egen, N., Jetter, W., Markland, F., Winger, W. and Bar-Or, D. (1985) Preparation of a new antivenin by affinity chromatography. Am. Soc. Trop. Med. Hyg. 34(1): 141.

Russo, A., Cobbs, C., Carlton, J. and Burnett, J. (1983) Detection of common antigenic sites in lethal proteins of non-related animal venoms. Toxicon 21(3): 433.

Sarkar, N. (1947) Existence of a cardiotoxic principle in cobra venom. Ann. Biochem. Exp. Med. 8: 11.

Schaeffer, R., Carlson, R., Puri, V., Callahan, G., Russell, F. and Weil, M. (1978) The effects of colloidal and crystalline fluids on rattlesnake venom shock in the rat. J. Pharm. Exp. Ther. 206: 687.

Schaeffer, R., Carlson, R. and Weil, M. (1979) The effects of antivenin and corticosteroid analogs on rattlesnake venom schock in the rat. J. Pharm. Exp. Ther. 211(2): 409.

Schiff, R., Cassai, N. and Gennaro, J. (1984) Ultrastructural localization in the mouse lung of venom from the western diamondback (Crotalus atrox) rattlesnake. Toxicon 22(6): 947.

Schoettler, W. (1951) Toxicity of the principal snake venoms of Brazil. Am. J. Trop. Med. Hyg. 31: 489.

Schoettler, W. (1954) Antihistamine, ACTH, cortisone, hydrocortisone and anesthetics in snake bite. Am. J. Trop. Med. Hyg. 3: 1083.

Schultz, R., Patel, A. and Ahsan, A. (1983) Covalent transition-state affinity chromatography of trypsin-like proteases. In: Affinity Chromatography and Biological Recognition, pp. 475-476 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Simpson, J. and Rider, L. (1971) Collagenolytic activity from the venom of the rattlesnake Crotalus atrox. Proc. Soc. Exp. Biol. Med. 137: 893.

Simpson, J. and Taylor, A. (1973) Elastolytic activity from the venom of the rattlesnake Crotalus atrox. Proc. Soc. Exp. Biol. Med. 144: 380.

Smith, M. and Ownby, C. (1985) Ability of polyvalent (Crotalidae) antivenin to neutralize myonecrosis, hemorrhage and lethality induced by the timber rattlesnake (Crotalus horridus horridus) venom. Toxicon 23(3): 409.

Stahnke, H., Allen, F., Horan, R. and Tenery, J. (1957) The treatment of snakebite. Am. J. Trop. Med. Hyg. 6: 323.

Stahnke, H. and McBride, A. (1966) Snake bite and cryotherapy. J. Occup. Med. 8(2): 72.

Straight, R. and Glenn, J. (1985) Effects of pressure-immobilization on the sysstemic and local actions of venoms in a mouse tail model. Toxicon 23(1): 40.

Stringer, J., Kainer, R. and Tu, A. (1971) Ultrastructural studies of myonecrosis induced by cobra venom in mice. Toxicol. Appl. Pharmacol. 18: 442.

Stringer, J., Kainer, R. and Tu, A. (1972) Myonecrosis induced by rattlesnake venom: an electron microscopic study. Am. J. Path. 67: 127.

Sugihara, H., Nikai, T., Kinoshito, C. and Tanaka, T. (1980) Enzymochemical studies on snake venoms. VII. Purification of hemorrhagic principle AC4-proteinase in the venom of Agkistrodon acutus. Yakugaku Zasshi 100: 855.

Sugihara, H., Mourira, M. and Nikai, T. (1983) Purification and properties of a lethal, hemorrhagic protein, mucrotoxin A, from the venom of the chinese habu snake (Trimersurus mucrosquamatous). Toxicon 21: 274.

Sullivan, J. and Russell, R. (1982) Isolation and purification of antibodies to rattlesnake venom by affinity chromatography. Proc. West. Pharmacol. Soc. 25: 185.

Sullivan, J. and Russell, F. (1983) Isolation, quantitation and subclassing of IgG antibody to Crotalidae venom by affinity chromatography and protein electrophoresis. Toxicon Suppl. 3: 429.

Sutherland, S. and Coulter, A. (1981) Early management of bites by the eastern diamondback rattlesnake (Crotalus adamanteus) studies in monkeys (Macaca fasicularis). Am. J. Trop. Med. Hyg. 30(20): 497.

Takahashi, T. and Oksaka, A. (1970) Purification and some properties of two hemorrhagic principles (HR2a and HR2b) in the venom of Trimersurus flavoviridis; complete separation of the principles from proteolytic activity. Biochem. Biophys. Acta. 207: 65.

Tan, N. (1983) Improvement of Malayan cobra (Naja naja sputatrix) antivenin. Toxicon 21(1): 75.

Theakston, R. and Reid, H. (1978) Changes in the biological properties of venom from Crotalus atrox with aging. Period. Biol. 80: 123.

Tin, Y. Means, G. and Feeney, R. (1969) The action of proteolytic enzymes on N,N-dimethyl protein. J. Biol. Chem. 244(4): 789.

Towbin, H., Stalhelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA 76(9): 4350.

Tsuchiya, M., Ohshio, C., Ohashio, M., Ohsaka, A., Suzuki, K., and Fujishiro, Y. (1974) Cinematographic and electron microscopic analyses of the hemorrhage induced by the main hemorrhagic principle, HR 1, isolated from the venom of Trimersurus flavoviridis. In: Platelets, Thrombosis, Inhibitors, pp. 439-446 (Didisheim, P., Shimamoto, T., and Yamazaki, H., Eds.). Stuttgart: F. K. Schattauer.

Tu, A. (1977) Nonneurotoxic basic proteins (cardiotoxins, cytotoxins and others). In: Venoms: Chemistry and Molecular Biology, pp. 301-320 (Tu, A., Ed.). New York: John Wiley and Sons, Inc.

Tu, A. (1977) Hemolysis. In: Venoms: Chemistry and Molecular Biology, pp. 321-328 (Tu, A., Ed.). New York: John Wiley and Sons, Inc.

Tu, A. and Morita, M. (1983) Attachment of rattlesnake venom myotoxin a to sarcoplasmic reticulum: peroxidase conjugated method. Br. J. Exp. Path. 64: 633.

Tu, A., Nikai, T. and Baker, J. (1981) Proteolytic specificity of hemorrhgaic toxin a isolated from western diamondback rattlesnake (Crotalus atrox) venom. Biochemistry 20: 7004.

Vick, J., Blanchard, R. and Perry, J. (1963) Effects of epsilon amino caproic acid on pulmonary vascular changes produced by snake venom. Proc. Exp. Biol. (New York) 113: 841.

Wagner, F. and Prescott, J. (1966) A comparative study of proteolytic activities in the venoms of some North American snakes. Comp. Biochem. Physiol. 17: 191.

Welches, W., Felsher, D, Landshuby, W. and Maraganore, J. (1985) A rapid method for the purification of monomeric and/or dimeric phospholipases A2 in crotalid snake venoms. Toxicon 23(5): 747.

Werber, M., Daphna, D., Goldman, J. Joseph, D., Radnay, J. Rozenszajn, L. and Manor, D. (1983) Preliminary purification of two human blood cell hormones by hydrophobic interaction chromatography. In: Affinity Chromatography and Biological Recognition, pp. 495-498 (Chaiken, I., Wilchek, M. and Parikh, I., Eds.). Orlando: Academic Press.

Williamson, J., Tilton, R., Kilo, C. and Yu, S. (1980) Immunofluorescent imaging of capillaries and pericytes in human skeletal muscle and retina. Microvascular Research 20: 233.

Wu, T. and Tinker, D. (1969) Phospholipase A_2 from Crotalus atrox venom. Purification and some properties. Biochemistry 8: 1558.

Xu, X., Wang, C., Lin, J. and Lu, Z. (1981) Purification and characterization of hemorrhatgic components from Agkistrodon acutus (hundred pace rattlesnake) venom. Toxicon 19: 633.

Yang, C. (1965) Crystallization and properties of cobrotoxin from Formosan cobra venom. J. Biol. Chem. 240: 1616.

Yang, C. and Chang, C. (1969) Immunochemical studies on cobrotoxin. J. Immunol. 102: 1437.

Yang, C., Lin, M. and Chang, C. (1977) Purification of anticobratoxin antibody by affinity chromatography. Toxicon 15: 51.

Yoshimura, N., Murachi, T., Heath, R., Kay, J., Jasani, B. and Newman, G. (1986) Immunogold electron-microscopic localisation of calpain I in skeletal muscle of rats. Cell Tissue Res. 244: 265.

VITA

Michael Scott Smith

Candidate for the Degree of

Doctor of Philosophy

Thesis: ISOLATION OF HEMORRHAGIC TOXIN B FROM CROTALUS ATROX VENOM: AFFINITY PURIFICATION OF ANTIBODIES AND THEIR USE IN NEUTRALIZATION OF HEMORRHAGE, DETECTION OF THE TOXIN IN OTHER SNAKE VENOMS, AND IMMUNOHISTOCHEMICAL LOCALIZATION OF HTB IN SKELETAL MUSCLE

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Charlotte, North Carolina, August 11, 1957, the son of Christine M. Stahl. Married to Ruth Ann on June 7, 1986.

Education: Received Bachelor of Science degree from Guilford College in May, 1979; received North Carolina secondary teaching certificate from the University of North Carolina at Charlotte in May, 1981; received Master of Science degree at Oklahoma State University in July, 1984; completed requirements for the Doctor of Philosophy degree at Oklahoma State University in July, 1987.

Professional Experience: High School Teacher, Forest Hills High School, Marshville, North Carolina, August, 1981 to June, 1982; Graduate Teaching Assistant, Department of Physiological Sciences, Oklahoma State University, August, 1982 to July, 1987; Recipient of Lewis Corey Distinguished Graduate Fellowship, 1986, and McAlester Scottish Rite Fellowship, 1986; Member, Sigma Xi Research Society, International Society on Toxinology.