PRODUCTION OF ANTISERUM TO MYOTOXIN A FROM PRAIRIE RATTLESNAKE (CROTALUS VIRIDIS VIRIDIS) VENOM AND LOCALIZATION OF MYOTOXIN A IN MOUSE SKELETAL MUSCLE

By

WILLIAM MICHAEL WOODS

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Oklahoma State University

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Thesis Approved:

Charlotte L. Ownby
Thesis Adviser

Duane T. Gamer

Theodore E. Staley

Norman N. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Rattlesnake bite is a serious medical problem in the world today. In 1968, the Department of the Navy recorded 700 to 1000 bites due to rattlesnakes and 20 of those resulted in death (Vick, 1971). At the University of Texas Medical Branch clinic alone there were 214 patients treated for rattlesnake bite from 1952 to 1977 (Huang et al., 1977). Deaths due to rattlesnake bite are substantially lower in the United States than the rest of the world due primarily to the widespread use of commercially available antivenin (antiserum). Although the use of this antivenin is highly effective in preventing death it may not be effective against the extensive local tissue damage that results in severe disfiguring or even loss of an extremity (Huang et al., 1977). This damage can occur even if the antivenin is injected immediately after the bite. Techniques such as surgical debridement, cryotherapy, and the use of various chemicals, designed to neutralize the effects of the toxin, have not been entirely successful (Ownby, 1975; Ownby and Tu, 1977), therefore another treatment is needed to prevent local tissue damage.

The venoms of most Crotalids produce hemorrhage and myonecrosis. Stringer et al. (1972) reported the Crotalus viridis viridis (Prairie rattlesnake) venom produces extensive hemorrhage and edema in muscle at the site of envenomation. When viewed with the light microscope, hemorrhage can be observed between fibers, and myonecrosis is found in focal

areas. Unaffected muscle fibers are interspersed among necrotic fibers. With the electron microscope, degeneration is first seen as dilation of the sarcoplasmic reticulum. Myeloid figures form in dilated sarcoplasmic reticulum, however, transverse tubules remain intact. Myofibrils break down, losing their striations and myofibrillar organization, until they become coagulated light and dark masses. Mitochondria appear to degenerate more slowly, not undergoing extreme degeneration until the fibrillar structure is lost (Stringer et al., 1972).

The isolation of a small, basic polypeptide from C. v. viridis venom by gel filtration and cation exchange chromatography has been reported (Ownby et al, 1976; Cameron and Tu, 1977). This polypeptide, named myotoxin a (molecular weight of 4100 daltons, isoelectric pH of 9.6), was found to induce local myonecrosis, indistinguishable from that induced by the crude venom (Ownby et al, 1976). When muscle tissue was observed with the light microscope, 72 hrs post-injection, extensive vacuolation was evident in most fibers. Electron microscopic studies (Ownby et al, 1976) showed that dilation of the sarcoplasmic reticulum and perinuclear space was evident with the transverse tubules retaining a normal configuration. Many of the cisternae of the sarcoplasmic reticulum contained myeloid figures. At 72 hrs after injection sarcomeres of some fibers had lost their structural integrity. Those fibers that remained intact has sarcomeres that were separated only by loosely arranged actin and myosin filaments. Only the Z lines were evident, the A and I bands had disappeared. Neither hemorrhage nor edema was found in muscle injected with myotoxin a.

Since myotoxin a-induced myonecrosis is essentially the same as that induced by crude C. v. viridis venom, and since commercial antivenin does not prevent this local damage, a method of treatment is needed that would

prevent the myonecrosis induced by rattlesnake bites. This study was concerned with the production of antibody against purified myotoxin a, subsequently called myotoxin, and the use of it in localization studies for the investigation of the binding site of the myotoxin. This information is useful in the discovery of the mode of action of myotoxin on skeletal muscle cells. Other studies will concern the use of the antiserum in neutralization of myonecrosis induced by pure myotoxin, crude venom and other Crotalid venoms in order to prevent disfiguration of extremities in actual snakebite cases.

CHAPTER II

MATERIALS AND METHODS

Isolation and Homogeneity of Myotoxin

Myotoxin was supplied by Dr. George V. Odel, Department of Biochemistry, Oklahoma State University. The isolation procedure, except for minor changes, was the same as described by Ownby et al. (1976) and later by Cameron and Tu (1977). Crude Prairie rattlesnake (C. v. viridis) venom in lyophilized form, purchased from Miami Serpentarium Laboratories, Miami, Florida, was first fractionated by gel-filtration on a Sephadex G-50 column (81 x 2 cm). A sample of crude venom (250 mg) was dissolved in 3 to 4 ml of a buffer composed of 0.05M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 9.0 at 21°C) and 0.1M KCl. The column was developed with this buffer at a flow rate of 30 ml per hr and 5 ml fractions were collected. All procedures were done at 4°C. Eluate absorbance at 280 nm was monitored with a Perkin-Elmer model Coleman 101 spectrophotometer. Salt was removed from each of these fractions by dialysis using benzoylated dialysis tubing (Sigma, St. Louis, Missouri) having an exclusion limit of 2000 daltons.

After desalting and lyophilization the fractions were tested for myotoxic activity by intramuscular injection into white mice. Each fraction was dissolved in physiologic saline (0.85% NaCl), PSS, to give a final concentration of approximately 50 μg per 0.1 ml. Seventy-two hours after injection of the solutions (0.1 ml per 20 gm mouse) into the lateral

aspect of the right thigh, the mice were killed by cervical dislocation, and a portion of muscle was removed from the medial aspect of the injected thigh. The muscle was fixed, processed and embedded in plastic (Epon 812) as previously described (Ownby et al., 1976). Thick (1 μm) sections were cut, stained with Mallory's Azure II Methylene Blue (Richardson et al., 1960) and examined with the light microscope for damaged fibers.

Fractions from the G-50 column which caused myonecrosis in mice were further fractionated by cation exchange chromatography on a C-25 (carboxymethyl Sephadex) column (56 x 1.5 cm). The C-25 column was equilibrated with the same buffer as used with the G-50 column. After developing the column with a stepwise KCl salt gradient (0.1M, 0.5M, 0.75M) in the 0.05M Tris buffer, 5 ml fractions were collected. Absorbance of the eluate was again monitored at 280 nm. Protein homogeneity was demonstrated by polyacrylamide disc gel electrophoresis in the β-alanine system as previously reported (Reisfield et al., 1962; Ownby et al., 1976) using 15% gels, pH 4.3, reversed electrodes.

Production of Antiserum

The immunogen was prepared by first dissolving 1.5 mg of the desalted, lyophilized myotoxin in 2 ml of physiologic saline. The final dose injected was 0.25 mg per kg for each of two rabbits. One male New Zealand white rabbit, and one male black satin rabbit, 4-5 months of age weighing about 3 kg, were first bled for pre-immune serum, and then each was injected with the immunizing antigen according to the following procedure. For the first injection, the myotoxin solution was emulsified with an equal volume of Freund's complete adjuvant, and 2 ml of this emulsion was injected intramuscularly between the shoulder blades. One

week later a booster injection was given, which consisted of 2 ml of this emulsion of equal amounts of myotoxin solution and Freund's complete adjuvant. Bleedings were made at weekly intervals for three weeks then the procedure was repeated. Blood was collected by centrifugation and stored in sterile bottles at -20°C until used or used immediately.

Separation and Purification of Antibodies

Separation and purification of antibodies was by the procedure described by Axelsen et al. (1973) with some minor changes. Antiserum from three bleedings of one rabbit was mixed with ammonium sulfate (25 gm per 100 ml of serum) and left for 21 hrs at room temperature. Antibodies were precipitated by centrifugation at 4,000 x g for 15 min (I.E.C. refrigerated centrifuge). The supernate was discarded and the precipitate washed by resuspending with a solution of 25 gm $(NH_4)_2SO_4$ per 100 ml of distilled water and centrifuging at 4,000 x g for 15 min. The washing steps were repeated until the supernate was clean, at which time the precipitate was transferred to a dialysis bag by redissolving in a small amount of water. The antibody-containing precipitate was dialyzed at 4°C against distilled water for two changes of 12 hrs each; against 0.05M sodium acetate, 0.021M hydrogen acetate, pH 5.0, for 24 hrs; against distilled water for two changes of 12 hrs each; and against 0.025M acetate buffer, pH 4.9, for 24 hours. A precipitate of lipoproteins and immunoglobulin M (IgM) formed during dialysis, was removed by centrifugation for 20 min at 20,000 x g in a Sorvall RC-2B refrigerated centrifuge. The supernate was saved, placed on an agarose column equilibrated with 0.025M acetate buffer and eluted with the same buffer. The eluate containing immunoglobulin G (IgG) and immunoglobulin A (IgA) was collected in 3 ml

fractions and monitored at 280 nm for determination of the antibody containing fractions. Pre-immune serum was treated in the same way for IgG separation.

Detection of Antibodies

The Ouchterlony agar-gel double diffusion technique (Clausen, 1969) was used to detect precipitating antibodies. Microdiffusion plates were prepared by pouring 7 ml of a 1.0% agarose solution in 0.85% NaCl into small Petri dishes. The agar was cut with a template to give one center well and six outer wells, 0.5 cm between wells. Each well was filled twice with approximately 15 µl of solution, either antigen or antiserum. The wells were not allowed to dry between the two fillings. Precipitin bands formed where antigens reacted with antibodies in the agar. Plates were incubated at room temperature in a moist chamber for 24 hrs after which no additional bands appeared. They were then photographed for a permanent record.

Conjugation of Antibodies with Fluorescein

Conjugation of globulins with fluorescein was by a procedure described by Dr. Konrad C. Hsu, Professor of Microbiology, Department of Microbiology, College of Physicians and Surgeons, Columbia University (Andres et al., 1973).

First, the protein concentration of antimyotoxin a IgG and preimmune IgG was determined by the Lowry method (Lowry et al, 1951). A solution was made to contain 20 mg of globulin per ml in a mixture containing 1 ml of 0.5M carbonate-bicarbonate buffer (pH 9.5) and 4 ml of physiological saline (0.85% NaCl). Fluorescein isothiocyanate (FITC) power (0.05 mg

per ml of protein in the reaction mixture) was added, and the mixture was stirred with a magnetic stirrer overnight at 4°C. This was followed by dialysis against phosphate buffered saline, PBS (0.05M sodium phosphate, 0.85% NaCl, pH 7.4) for 5-7 days, changing PBS twice daily, until the dialyzing fluid showed no fluorescence in ultraviolet light. Following dialysis, the conjugated globulin was passed through a Millipore filter to insure sterility, and stored at 4°C. The absorbance was determined at 495 nm (E'_{495}) and 280 nm (E'_{280}) for calculation of the molecular fluorescein/protein ratio (mol. F/P). The mol. F/P was calculated according to the equation given by The et al. (1970) who assumed a molecular weight of 160,000 for IgG and of 390 for FITC in its derivation.

$$\text{Mol. F/P} = \frac{2.87\ E'_{495}}{E'_{280} - 0.35\ E'_{495}}$$

Localization of Myotoxin

Injection Procedures

A mouse was injected with 0.1 ml of myotoxin (5.0 µg/gm mouse weight) into the medial aspect of the right thigh. Another mouse was injected with an equivalent volume of physiologic saline (PSS). Two hours post-injection the mice were killed by cervical dislocation, and the medial portion of the right thigh was exised. The muscle was either fixed in 1% paraformaldehyde and washed overnight in PBS (0.1 M sodium phosphate) or placed directly into 0.7M phosphate buffer. It was then rinsed in 0.7M phosphate buffer (pH 7.4) which included 5% sucrose as a cryoprotectant and frozen in liquid nitrogen. Cryostat sections (4 µm) were placed on

coverslips coated with 1% gelatin then air dried and stored at -25°C in the cryostat until used. Serial sections were taken so adjacent sections could be used in localization studies. This made possible direct comparison of specific muscle cells between experimental and controls. Some sections of muscle injected with PSS were subsequently incubated in myotoxin (0.1 mg/ml) then washed four times at 15 min each with PBS (0.01 M sodium phosphate). These sections composed the tissue for the in vitro treatment with myotoxin.

Direct Fluorescein Labeling

Sections used in this procedure consisted of: 1) myotoxin-injected muscle (in vivo), 2) PSS-injected muscle (control), and 3) PSS-injected muscle incubated in myotoxin (in vitro). Antibody preparations were all absorbed with homogenized muscle for 12 hrs to reduce nonspecific fluorescence and then diluted 1:20 with PBS prior to use. Sections were all incubated one hour in either preimmune IgG conjugated with FITC absorbed with muscle; antimyotoxin IgG conjugated with FITC absorbed with muscle; anitmyotoxin IgG conjugated with FITC absorbed with muscle and myotoxin. After this incubation sections were washed four times for 15 min each with PBS and coverslipped using 90% glycerol. Sections were viewed with a Zeiss fluorescent photomicroscope and recorded on Kodak Tri-X Pan film.

Indirect Fluorescein Labeling

Cryostat sections were obtained as described for the direct labeling technique and washed four times at 15 min each with PBS. They were then incubated in a moist chamber for one hour at room temperature in one of the following: preimmune IgG, antimyotoxin IgG (10 mg/ml) both absorbed

with muscle or antimyotoxin IgG absorbed with muscle and myotoxin. All sera were diluted 1:10 with PBS (0.01 M sodium phosphate) prior to use. Following incubation sections were washed four times at 15 min each with PBS and incubated one hour at room temperature in a 1:20 dilution of goat anti-rabbit IgG fluorescein conjugated (Grand Island Biological Company, Grand Island, New York). Sections were washed four times at 15 min each with PBS (0.01 M sodium phosphate), coverslipped using 90% glycerol, viewed and recorded as described above.

Indirect Ferritin Labeling for Electron Microscopy

Mice were injected and tissue was excised as previously described. Tissue was either placed immediately into 0.7M phosphate buffer plus 5% sucrose (pH 7.4) and frozen in liquid nitrogen, or the tissue was first fixed for one hour in 1% paraformaldehyde then washed overnight in PBS, (0.1 M sodium phosphate), rinsed in 0.7M phosphate buffer plus 5% sucrose and frozen in liquid nitrogen. Cryostat sections (21 μm) on coverslips were air-dried and stored until use at -25°C. Agarose (2% at 40-45°C) was poured over the sections which were mounted on coverslips, and cooled. The agarose was cut into blocks containing the tissue on one surface, and placed into vials containing PBS (0.01 M sodium phosphate). Some blocks containing PSS-injected sections, were incubated in 0.1 mg/ml myotoxin for one hour and washed four times at 15 min each in PBS (0.01 M sodium phosphate). Blocks containing PSS-injected muscle incubated in myotoxin were incubated one hour in antimyotoxin IgG (10 mg/ml) absorbed with muscle or preimmune IgG absorbed with muscle both diluted 1:10 with PBS (0.1 M sodium phosphate). Following this incubation blocks were washed four times at 15 min each with 0.05M phosphate buffer, pH 7.2, then

incubated one hour in sheep anti-rabbit IgG, ferritin conjugated, (Cappel Laboratories, Downington, Pennsylvania) diluted 1:20 with 0.05M phosphate buffer, pH 7.2. Following incubation blocks were washed four times at 15 min each with 0.05 M phosphate buffer, pH 7.2, then rinsed with cacodylate buffered wash (0.1M sodium cacodylate and 6% sucrose at pH 7.4). The tissue was fixed one hour in 2% glutaraldehyde, washed four times at 15 min each with cacodylate buffered wash, postfixed one hour in 1% osmium tetroxide, dehydrated in a graded acetone series, and embedded in Epon 812 in flat molds. The muscle blocks were sectioned with an LKB ultratome I and viewed unstained with a Philips EM 200 electron microscope. Some sections were double stained with uranyl acetate and lead citrate for electron microscopic examination to compare morphology of fixed versus unfixed tissue.

CHAPTER III

RESULTS

Isolation of Myotoxin

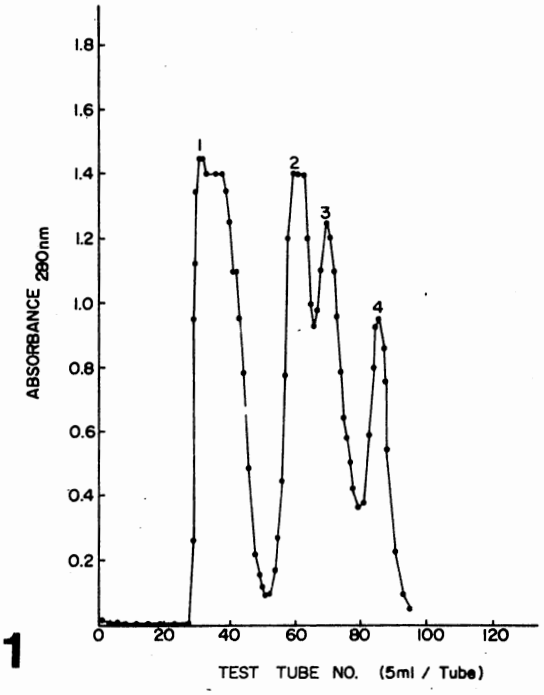
Four fractions were obtained from fractionation of crude venom on the Sephadex G-50 column, as is shown by the elution profile in Figure 1. Each fraction was tested for myotoxic activity by injection into mice; only fractions 2 and 3 caused myonecrosis. Further fractionation of fractions 2 and 3 by cation exchange chromatography on a CM Sephadex (C-25) column resulted in the elution profile shown in Figure 2. Out of five fractions obtained only fractions 1 and 3 caused muscle necrosis in mice. Fraction 3 corresponded to myotoxin a (Ownby et al., 1976; Cameron and Tu, 1977) and was used in all these experiments. Protein homogeneity was demonstrated by ployacrylamide disc gel electrophoresis in the β-alanine system and the purified C-25 fraction 3 is shown in Figure 3. A portion of this fraction was assayed and an example of the myonecrosis induced is shown in Figure 4.

Antiserum to Myotoxin

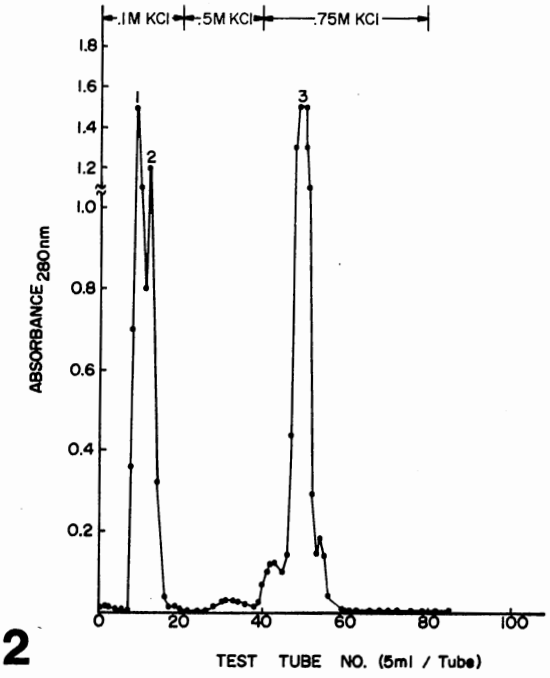
When antiserum from the first bleeding was tested in gel-diffusion plates against dilutions of myotoxin (0.4 mg/ml and diluted 1:2, 1:4, 1:8, 1:16 down to 1:512) or as shown in Figure 5a when myotoxin IgG [110 mg/ml (Lowry et al., 1951) and diluted 1:2, 1:4, 1:8, and 1:16], one precipitin line was formed against all solutions down to a dilution of 1:16.

Figure 1. Elution Profile for Sephadex G-50 Gel-Filtration Column. Crude C. v. viridis venom (250 mg) was dissolved in elution buffer consisting of 0.05M Tris and 0.1M KCl (pH 9.0 at 21°C). Elution with the same buffer results in four fractions. Fractions 2 and 3 were found to cause myonecrosis and were placed on the cation exchange column.

Figure 2. Elution Profile for CM Sephadex (C-25) Cation Exchange Column. Fractions 2 and 3 were dissolved in elution buffer consisting of 0.05M Tris and 0.1M KCl (pH 9.0 at 21°C). After equilibration with this buffer, the column was eluted with a stepwise KCl gradient (0.1M, 0.5M, 0.75M) in 0.05M Tris buffer. Fractions 1 and caused myonecrosis. Only fraction 3 was used in this study.


ABSORBANCE 280nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
4
0
20
40
60
80
100
120
TEST TUBE NO. (5ml / Tube)

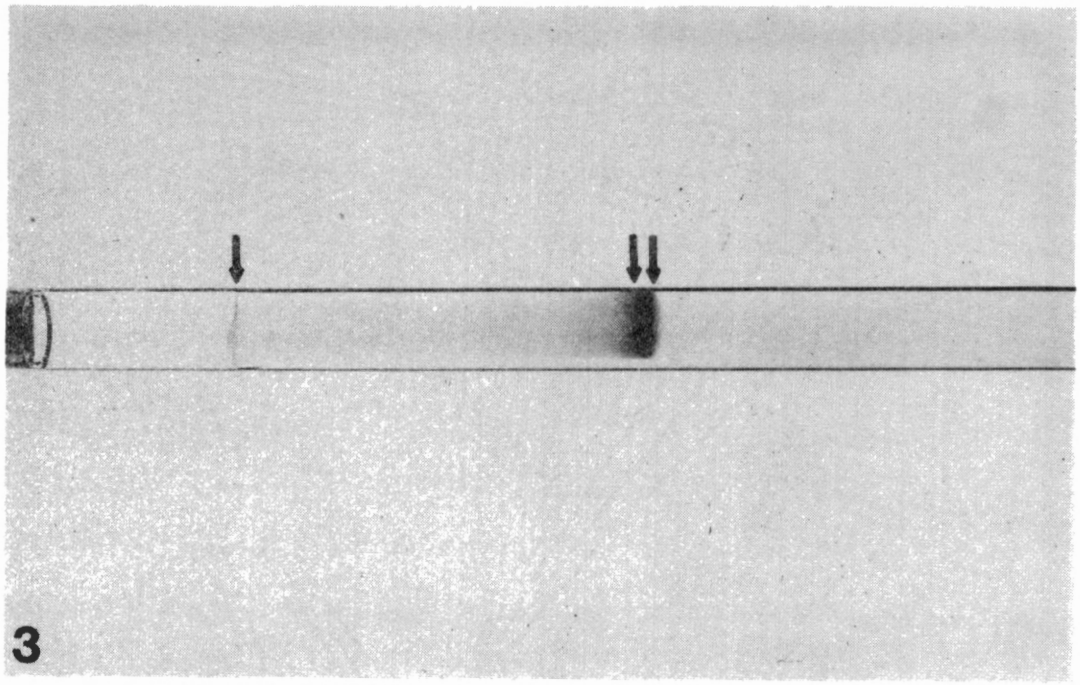
1


.1M KCl
.5M KCl
.75M KCl
ABSORBANCE 280nm
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
1
2
3
0
20
40
60
80
100
TEST TUBE NO. (5ml / Tube)

2

Figure 3. β-Alanine Disc Gel Electrophoresis. Electrophoretic homogeneity of myotoxin a is demonstrated by the presence of only one band in the electrophoresis gel. Approximately 100 µg of myotoxin a (fraction 3 from the CMS-25 column, Figure 2) was applied to 15% acrylamide gels which were run at pH 4.3 with electrodes reversed. Gels were stained with 0.05% Coomassie blue and 10% trichloroacetic acid, destained with 20% Cl_3AcOH and stored in 7.5% acetic acid. Anode is on left. Top of gel is at single arrow. Myotoxin a is between double arrows.

Figure 4. Photomicrograph of Mouse Skeletal Muscle Injected With Myotoxin a. Mice were injected intramuscularly with 2.0 µg/gm of myotoxin a (fraction 3 from CMS-25 column, Figure 2) dissolved in physiologic saline. Muscle was taken 24 hrs after the injection and routinely processed for plastic embedding. Thick (1 µm) sections were taken and stained with Mallory's Azure II Methylene Blue. Note extensive vacuolation of muscle cells which indicates damage to cell (V). Also, note adjacent normal appearing muscle cells (M). No vacuolation was observed in control muscle injected with physiologic saline and processed along with the experimental tissue.



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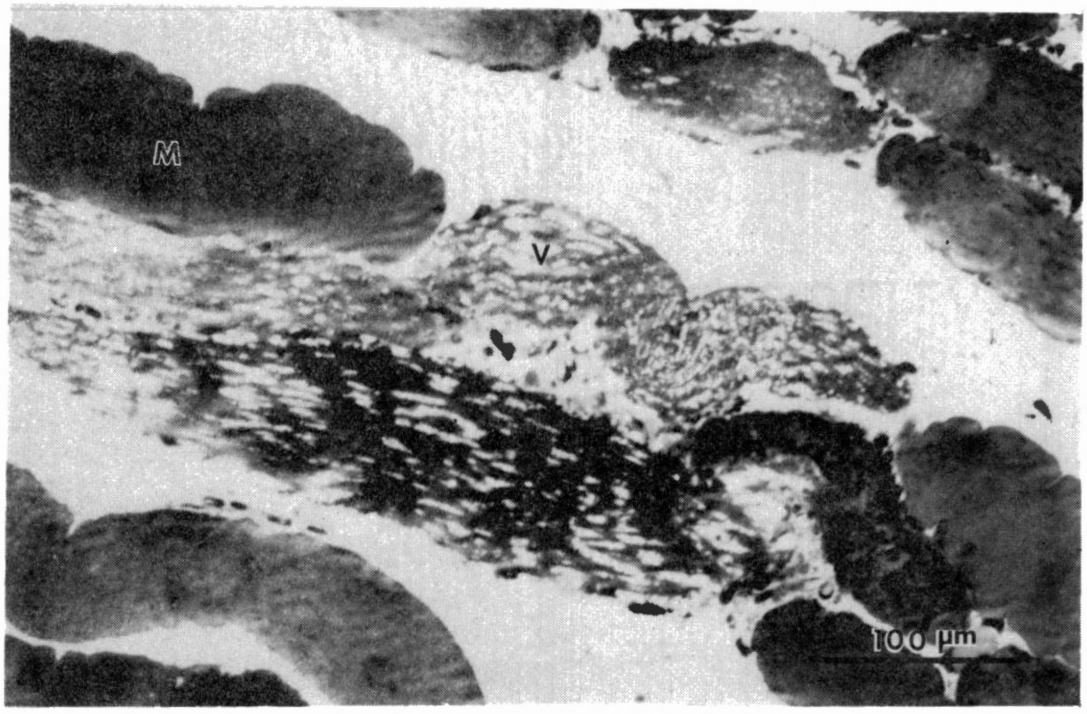


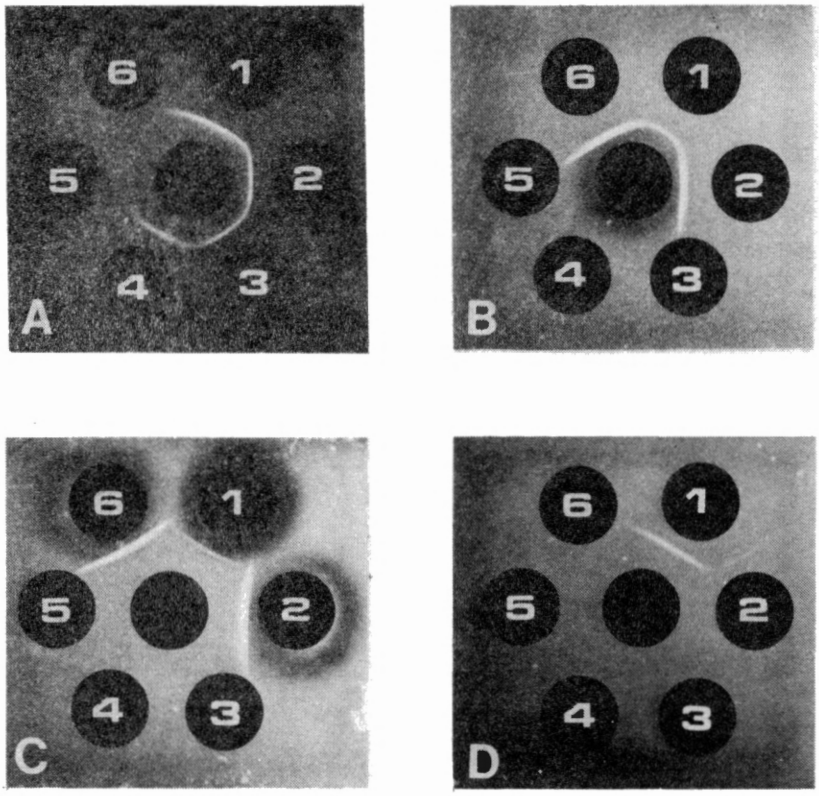
Figure 5. Ouchterlony Agar Gel-Diffusion Plates (1% Agarose in PSS)

a. Center well: myotoxin *a*, 0.1 mg/ml. Peripheral wells: (1) antimyotoxin *a* IgG, 110 mg/ml; (2) antimyotoxin *a* IgG diluted 1:2; (3) antimyotoxin *a* IgG diluted 1:4; (4) antimyotoxin *a* IgG diluted 1:8; (5) antimyotoxin *a* IgG diluted 1:16; (6) preimmune IgG.

b. Center well: antimyotoxin *a* IgG. Peripheral wells: (1) myotoxin *a*, 0.1 mg/ml; (2) and (6) crude *C. v. viridis* venom, 1 mg/ml; (3), (4), and (5) empty.

c. Center well: crude *C. v. viridis* venom, 1 mg/ml. Peripheral wells: (1) Wyeth's Polyvalent *Crotalidae* antivenin; (2) and (6) antimyotoxin *a* IgG; (3), (4), and (5) empty.

d. Center well: myotoxin *a*, 0.1 mg/ml. Peripheral wells: (1) antimyotoxin *a* IgG; (2) antimyotoxin *a* IgG absorbed with myotoxin *a*; (6) Preimmune IgG; (3), (4), and (5) empty.


6
1
5
2
4
3
A
6
1
5
2
4
3
B
6
1
5
2
4
3
C
6
1
5
2
4
3
D

One precipitin line formed between myotoxin a (fraction 3) from two different isolations, but no line formed against pre-immune serum. Both rabbits gave essentially the same results, except that one produced antiserum of a slightly higher titer. Neither pre-immune serum ever produced a precipitin line against myotoxin or crude venom. When anitmyotoxin IgG was run against myotoxin and crude C. v. viridis venom, precipitin lines formed which showed total identity (Figure 5b). No precipitin lines were formed between myotoxin and Wyeth's polyvalent Crotalidae antivenin, however, one line formed between crude C. v. viridis venom and antivenin. Figure 5c shows the lack of identity between the immunoprecipitin line between antimyotoxin serum and crude venom and the line between commercial antivenin and crude C. v. viridis venom. Figure 5d shows lack of a precipitin line between myotoxin and preimmune IgG, presence of a line between myotoxin and antimyotoxin IgG, and absence of line between myotoxin and antimyotoxin absorbed with myotoxin indicating the specificity of the antibodies for myotoxin. The precipitin line between antimyotoxin IgG and antimyotoxin IgG absorbed with myotoxin is probably due to excess myotoxin not bound for IgG in the absorbed antiserum.

Localization by Immunofluorescence

Tissue that had been fixed in 1% paraformaldehyde showed much non-specific binding of fluorescein in all controls. Some muscle fibers were brightly fluorescent, while other fibers were relatively low in fluorescence. However, morphology was good in fixed tissue. Muscle cells in longitudinal section showed distinct striations with the sarcolemma appearing to remain intact. In unfixed tissue non-specific fluorescein binding was greatly reduced, but morphology was not as good as in fixed

tissue. Muscle striations were less apparent, although the sarcolemma appeared to remain intact.

Sections of muscle incubated in myotoxin (in vitro) and reacted with anti-myotoxin IgG conjugated with FITC (mol. F/P ratio of 1.8) showed strong fluorescence along the sarcolemma of muscle cells as shown in Figures 6a and 7a. Controls showed decreased fluorescence along the sarcolemma (Figures 6b-c and 7b-c). Non-specific fluorescence using the direct immunofluorescent technique was minimal. Sections of muscle taken from mice injected with myotoxin (in vivo) showed very little fluorescence (Figure 8). Localization, by indirect immunofluorescence, showed much nonspecific binding of fluorescein in both fixed and unfixed tissue. Due to this intense background staining, it was impossible to determine localization of myotoxin.

Localization by Immunoferritin

Poor morphology was noted in localization of myotoxin by indirect ferritin labeling, tissue that was immediately frozen and not fixed. The sarcolemma was discontinuous, detached, and laminated. There were membrane whorls present indicating a degeneration of cellular organelles. Also, the sarcoplasmic reticulum was swollen (Figure 9a).

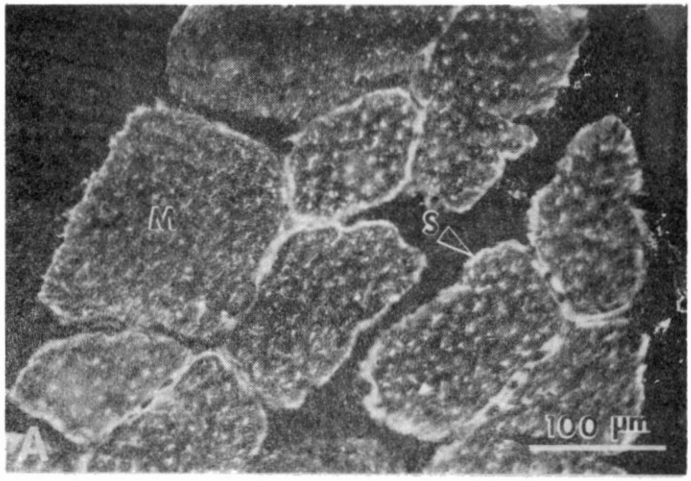
In fixed tissue, morphology was quite good. The sarcolemma appeared intact, and mitochondria were normal with intact cristae. The sarcoplasmic reticulum retained a normal appearance in PSS-injected muscle and a swollen appearance in mytoxin-injected muscle. Transverse tubules were intact and myofibrils appeared normal with striations prominent. Ferritin was located along the sarcolemma of both experimental (Figure 10a) but also of control (Figure 10b) tissue, indicating a high degree of

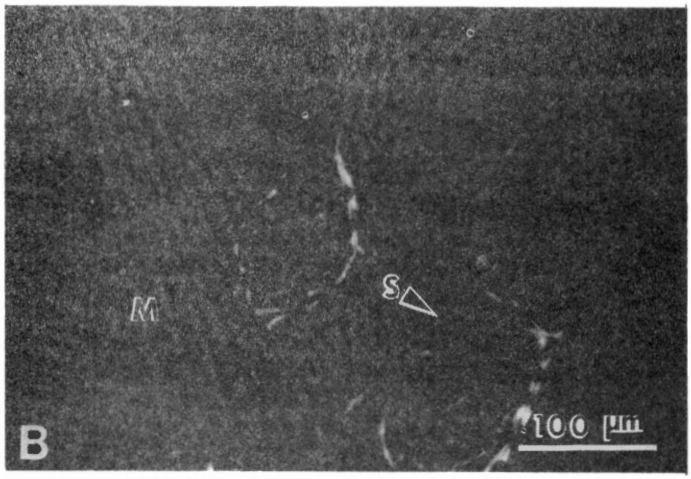
Figure 6. *In Vitro* Localization by Immunofluorescence: Cross Sections of Muscle

a. Muscle cells (M) incubated with myotoxin *a*, 0.1 mg/ml, and in antimyotoxin *a* IgG conjugated with fluorescein and absorbed with muscle. Myotoxin is evidenced by bright fluorescence along the sarcolemma (S) and within the muscle cells (M).

b. Muscle cells (M) incubated in myotoxin *a*, 0.1 mg/ml, and in antimyotoxin *a* IgG conjugated with fluorescein, absorbed with myotoxin *a* as a negative control and absorbed with muscle. Note the decreased fluorescence of the sarcolemma (S) and of muscle cells (M), as compared with Figure 6a.

c. Muscle cells (M) incubated in myotoxin *a*, 0.1 mg/ml, and in preimmune IgG conjugated with fluorescein and absorbed with muscle. Note the decreased fluorescence of the sarcolemma (S) and of muscle cells (M), as compared with Figure 6a.


M
S
100 µm
A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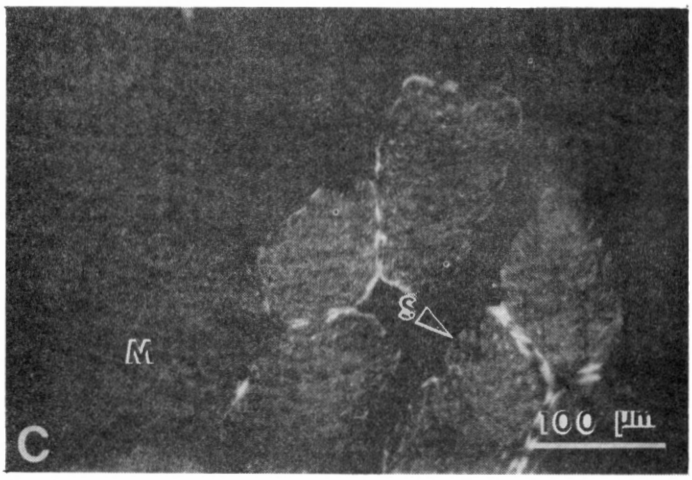
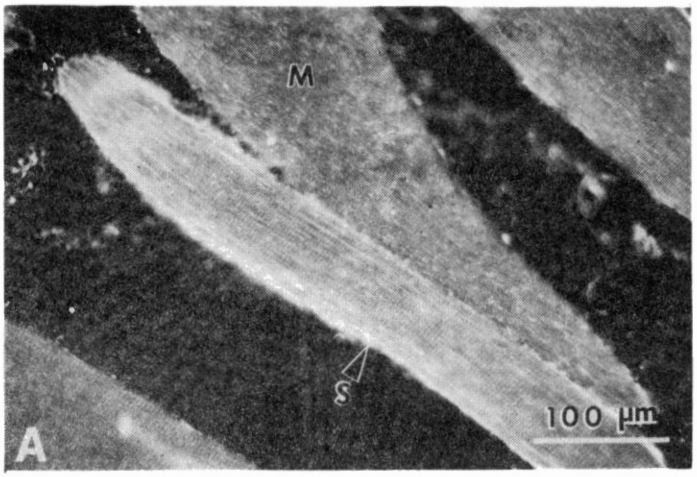

M
S
100 µm
C

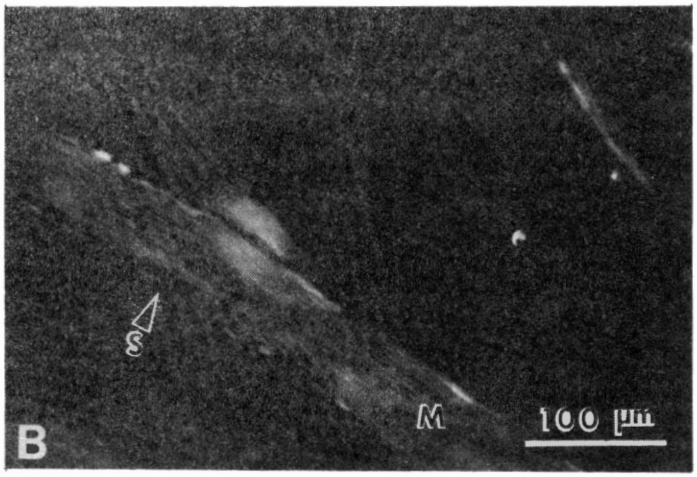
Figure 7. *In Vitro* Localization by Immunofluorescence: Longitudinal Sections of Muscle

a. Muscle cells (M) incubated in myotoxin *a*, 0.1 mg/ml, and in antimyotoxin *a* IgG conjugated with fluorescein and absorbed with muscle. Myotoxin is evidenced by bright fluorescence along the sarcolemma (S) and muscle cells (M).

b. Muscle cells (M) incubated in myotoxin *a*, 0.1 mg/ml, and in antimyotoxin *a* IgG conjugated with fluorescein, absorbed with myotoxin *a* as a negative control and absorbed with muscle. Note the decreased fluorescence of sarcolemma (S) and of muscle cells (M), as compared with Figure 7a.

c. Muscle cells (M) incubated in myotoxin *a*, 0.1 mg/ml, and in preimmune IgG conjugated with fluorescein and absorbed with muscle. Note the decreased fluorescence of sarcolemma (S) and muscle cells (M), as compared with Figure 7a.


M
S
100 µm
A


S
M
100 µm
B

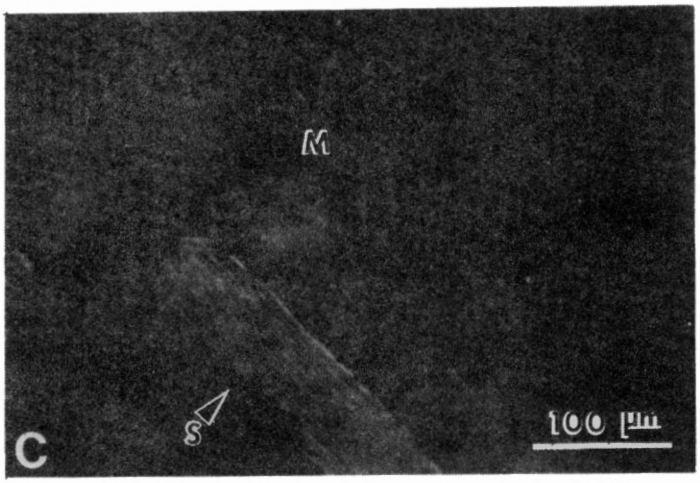
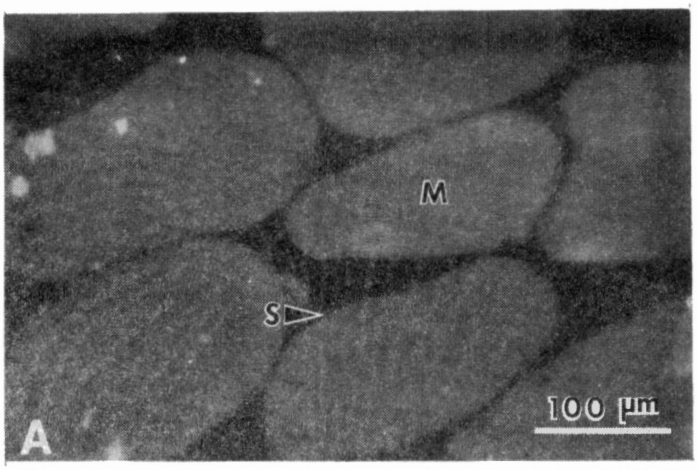

M
S
100 µm
C

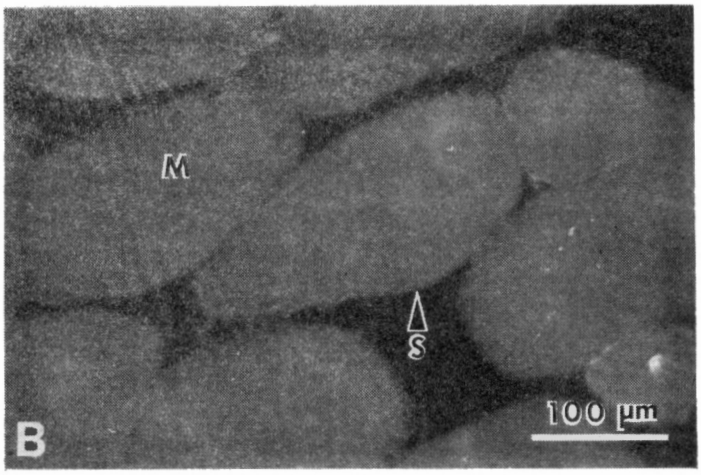
Figure 8. *In Vivo* Localization by Immunofluorescence: Cross Sections of Muscle

a. Muscle cells (M) of mouse muscle injected with myotoxin and incubated in antimyotoxin *a* IgG conjugated with fluorescein and absorbed with muscle. No fluorescence is evident along the sarcolemma (S) or within muscle cells (M).

b. Muscle cells (M) of mouse muscle injected with myotoxin *a* and incubated in antimyotoxin *a* IgG conjugated with fluorescein, absorbed with myotoxin *a*, and absorbed with muscle. Note absence of fluorescence along the sarcolemma (S) and within muscle cells (M).

c. Muscle cells (M) of mouse muscle injected with myotoxin *a* and incubated in preimmune IgG conjugated with fluorescein and absorbed with muscle. Note absence of fluorescence along the sarcolemma (S) and within muscle cells (M).


M
S
100 µm
A


M
S
100 µm
B

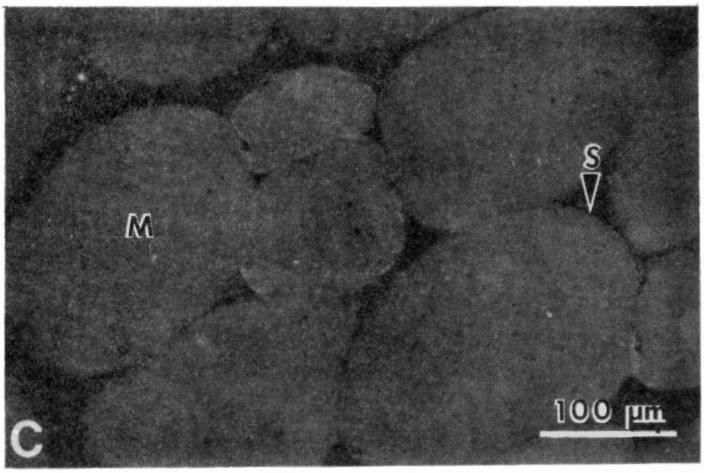
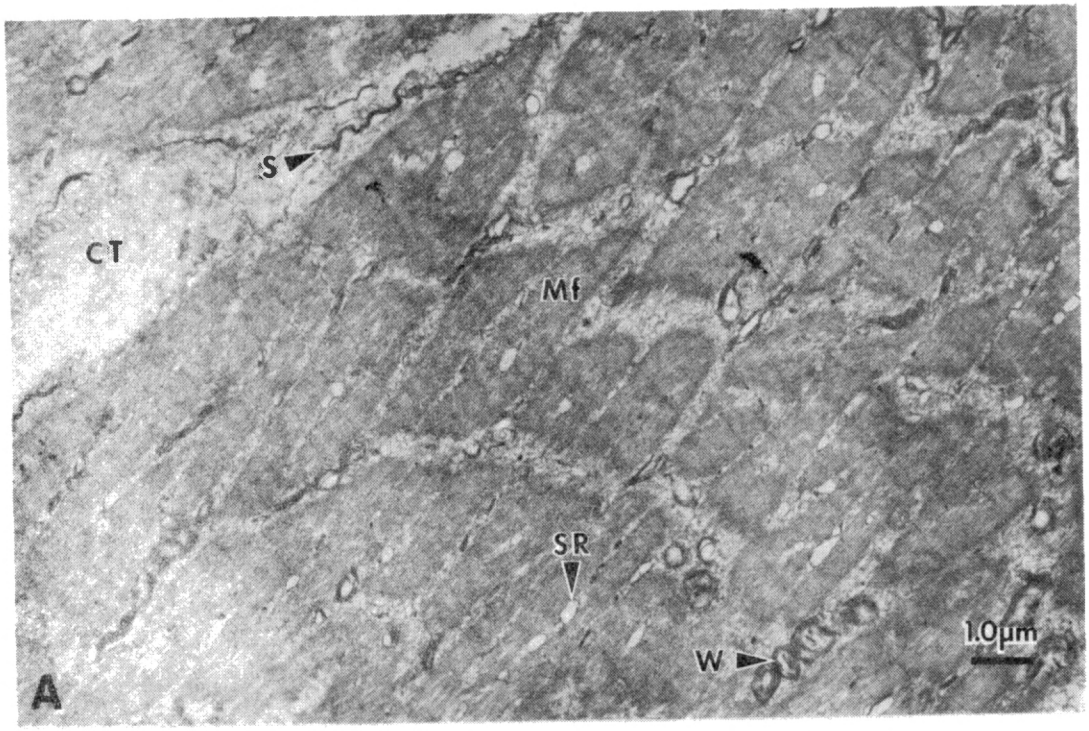

S
M
100 µm
C

Figure 9. Electron Micrographs of Unfixed and Fixed Mouse Skeletal Muscle

a. Section of unfixed muscle processed for electron microscopy and double stained with uranyl acetate and lead citrate. Note the ruptured sarcolemma (S) and the presence of membrane whorls (W). The sarcoplasmic reticulum (SR) is swollen. Connective tissue (CT). Myofibrils (Mf).

b. Section of muscle fixed in 1% paraformaldehyde, processed for electron microscopy and double stained with uranyl acetate and lead citrate. Muscle showing a more normal appearance of mitochondria (Mi), sarcoplasmic reticulum (SR), sarcolemma (S), and myofibrils (Mf). There was some damage from ice crystal formation (I) during processing. Connective tissue (CT).


S
CT
Mf
SR
W
1.0μm
A

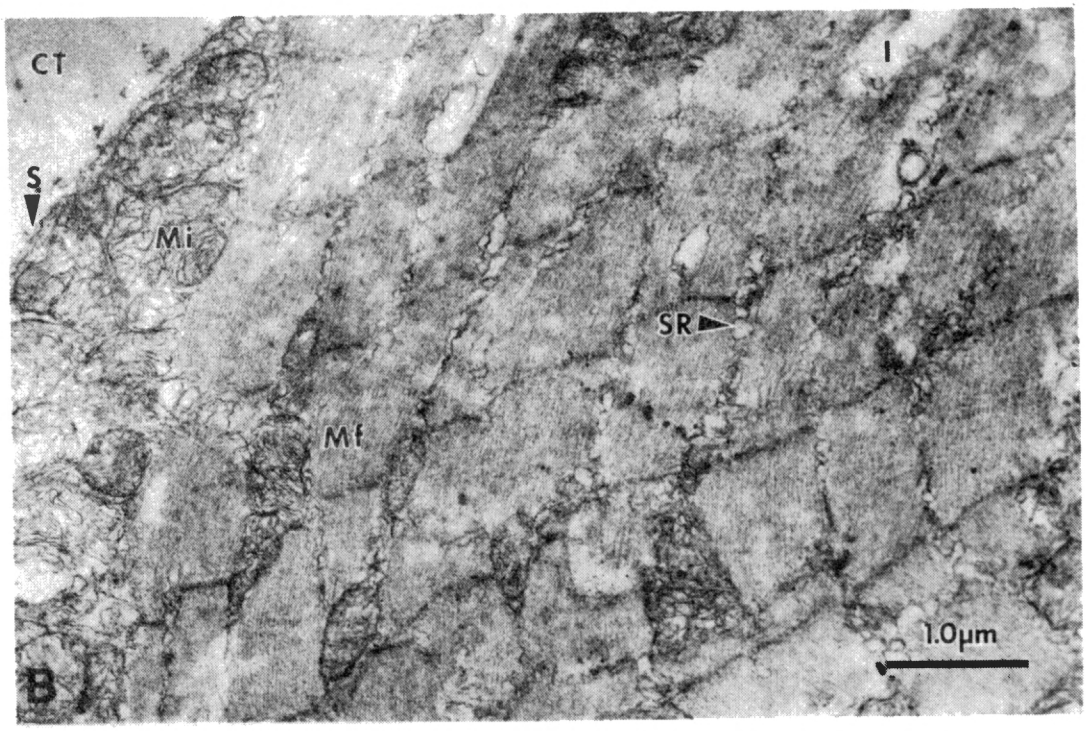
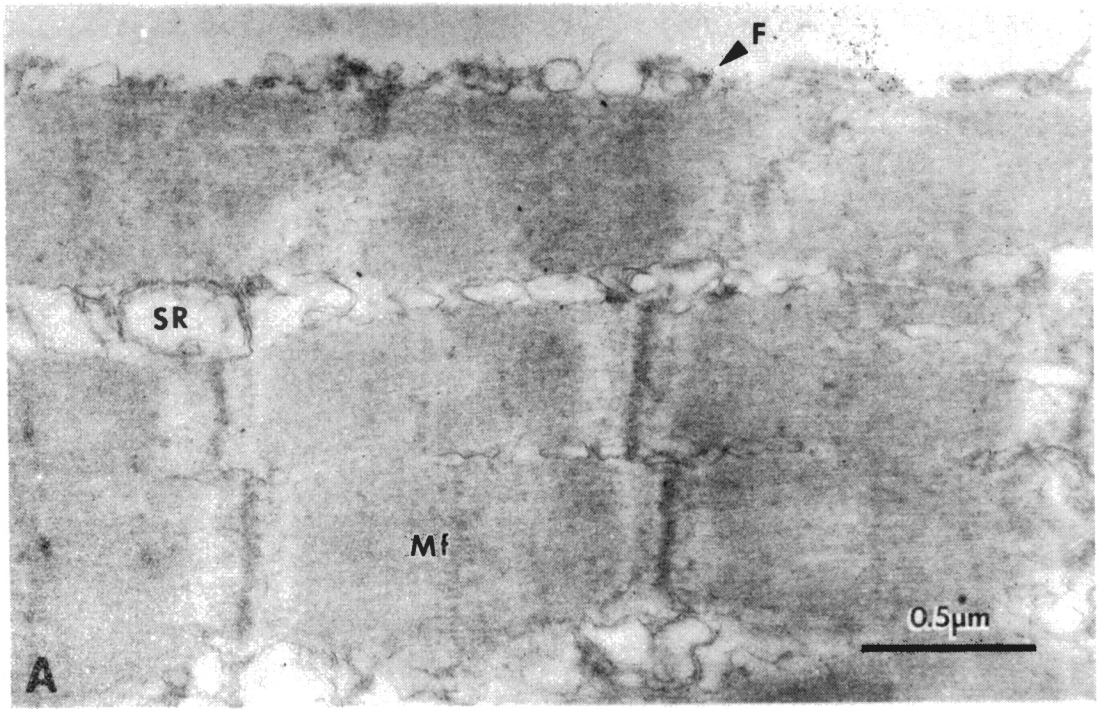
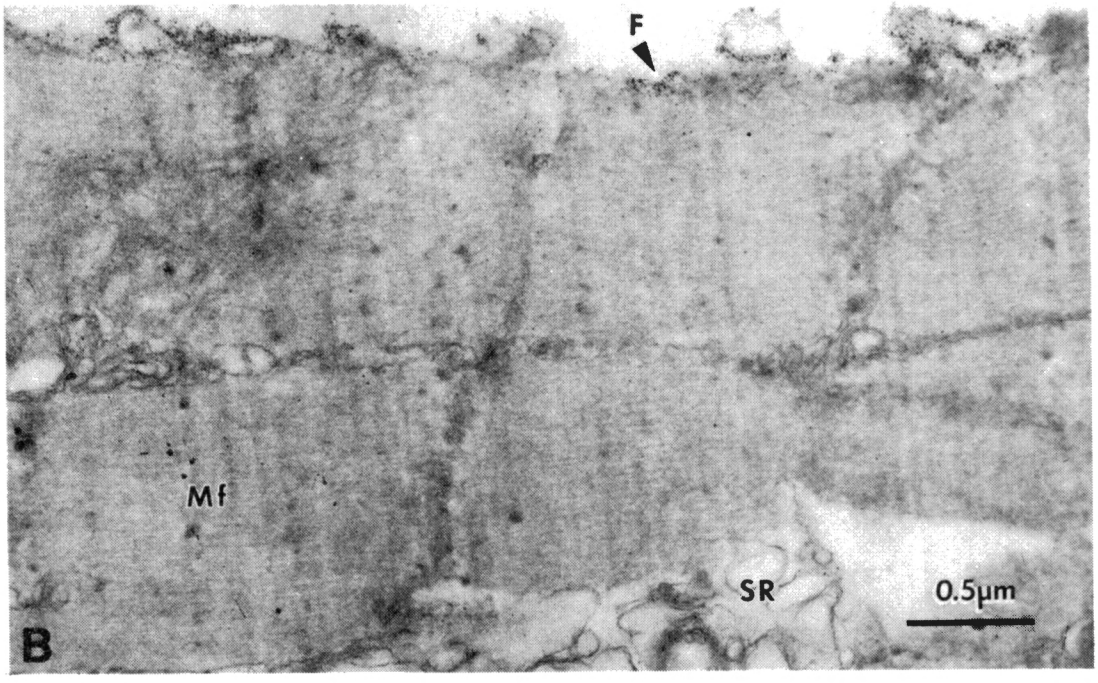

CT
I
S
Mi
SR
Mf
1.0μm
B

Figure 10. Electron Micrographs of *In Vivo* Immunoferritin Localization

a. Longitudinal section of part of a muscle cell of mouse muscle injected with myotoxin and incubated first with rabbit anti-myotoxin *a* IgG and second with goat anti-rabbit IgG conjugated with ferritin. Note presence of ferritin (F) localized along the sarcolemma. Also, note dilatated sarcoplasmic reticulum (SR) and myofibril (Mf).

b. Longitudinal section of part of a muscle cell of mouse muscle injected with myotoxin and incubated first with rabbit pre-immune IgG and second with goat anti-rabbit IgG conjugated with ferritin. Note the increased amount of ferritin (F) along the sarcolemma, as compared with Figure 10a, indicating nonspecific binding. Also, note dilatated sarcoplasmic reticulum (SR) and myofibril (Mf).


F
SR
Mf
0.5μm
A


F
Mf
SR
0.5μm
B

nonspecific binding and making localization of myotoxin impossible. The amount of ferritin on the sarcolemma varied from cell to cell (Figures 10a and 10b) within a given section of experimental or control muscle.

CHAPTER IV

DISCUSSION

The fractionation of C. v. viridis venom resulted in the isolation of two myotoxic components. Fraction 3 from the carboxymethyl Sephadex column produced myonecrosis in mice identical to that reported by Ownby et al. (1976) and by Cameron and Tu (1977) as induced by myotoxin a. Only myotoxin a was used in this study and is subsequently referred to as myotoxin.

The injection of myotoxin into rabbits elicited the formation of specific antibodies. The formation of one precipitin line between myotoxin and antiserum indicated that at least one antibody exists to the myotoxin, and since there was never any precipitin lines formed between preimmune serum and myotoxin, the possibility of the precipitin line being due to non-immune sources was very remote. Also the line between myotoxin and antimyotoxin serum reacted with complete identity with the line between crude C. v. viridis venom and antimyotoxin, confirming the presence of the purified myotoxin in its homologous crude venom.

The gel-diffusion results indicated no reaction between commercial antivenin and myotoxin. There were no precipitin lines that formed between crude C. v. viridis venom and commercial antivenin that identified with the precipitin line that formed between antimyotoxin serum and C. v. viridis venom (Figure 5d). The absence of antibodies in antivenin to myotoxin components could explain its relative ineffectiveness in

preventing myonecrosis induced by rattlesnake (Crotalidae) envenomation. At least two factors could account for the poor immunogenicity of myotoxic components: (1) their small molecular weight (myotoxin a, 4100), and (2) their low concentration, due to dilution, resulting from mixing four crude venoms in the preparation of the immunizing antigen for production of antivenin.

In localization of myotoxin, utilizing fluorescein, it was necessary to use a variety of controls in order to demonstrate the fluorescence due only to myotoxin. Controls that were used for direct localization studies included: (1) sections of PSS-injected muscle incubated in myotoxin, followed by incubation by either (a) antimyotoxin IgG conjugated with FITC absorbed with myotoxin, (b) preimmune IgG conjugated with FITC; (2) sections of PSS-injected muscle not incubated in myotoxin incubated in either (a) antimyotoxin IGG conjugated with FITC, (b) antimyotoxin IgG conjugated with FITC absorbed with myotoxin, or (c) preimmune IgG conjugated with FITC. Results from the in vitro myotoxin treatment of adjacent sections followed by incubation with antimyotoxin conjugated with FITC showed bright fluorescing of the sarcolemma and structures within the cell (Figures 6a and 7a). The adjacent section treated with antimyotoxin that had been absorbed with myotoxin showed removal of nearly all the fluorescence along the sarcolemma and within the muscle cell (Figures 6b and 7b). The amount of fluorescence removed by absorbing out the antimyotoxin IgG represents the myotoxin that was bound to the muscle cells. By using adjacent sections identical muscle fibers can be compared, decreasing interpretation errors due to nonspecific fluorescein binding. Problems with resolution and nonspecific binding of fluorescein-labeled antibodies preclude more precise localization. Nonspecific

fluorescence of the muscle in the direct labeling techniques could be due to either nonspecific binding of the Fc portion of the IgG molecule or to molecular fluorescein/protein (mol. F/P) ratios being too high. To eliminate binding of the Fc portion of IgG, pepsin digestion could be used to separate the Fab'_2 fragments (Wylie et al., 1979). It was determined by The and Feltkamp (1970) that nonspecific fluorescence was present in all cases in which the mol. F/P ratio was higher than 5, and nonspecific fluorescence was not present, if the mol. F/P ratio was below 3. Since the FITC conjugate used in this study had a mol. F/P ratio of 1.8, this was probably not the source of nonspecific fluorescence. However, the mol. F/P ratio obtained for the fluorescein conjugated antibody was an average value, and the IgG solution would contain a percentage of mol. F/P ratios above and below this value. In order to be sure that no mol. F/P ratios above 5 were present in the antibody conjugate, it could be fractionated on a gel-filtration column, and mol. F/P ratios calculated for each fraction. The mol. F/P ratios, that were above 5, could then be discarded.

These problems could explain the failure of the indirect labeling technique to produce consistent and valid results. To avoid these problems, the first antibody (antimyotoxin IgG) could be separated into only Fab'_2 fragments of IgG, thereby reducing nonspecific Fc fragment binding. The second antibody could be Fab'_2 fragments conjugated with FITC made against Fab'_2 fragments of rabbit IgG. In the indirect fluorescein labeling, the mol. F/P ratio of the second antibody could have been checked.

In vitro localization of myotoxin showed definite binding to the sarcolemma and inside muscle cells, but this was in an *in vitro* situation and may not represent what actually occurs in an *in vivo* situation.

However, the muscle damage produced by myotoxin *in vitro* is similar to that induced by *in vivo* treatment with myotoxin, at least at the time interval studied. Results obtained from *in vivo* direct localization studies were difficult to interpret due to poor fluorescence which was probably due to a low concentration of myotoxin within the muscle. Low amounts of myotoxin could result from there being only limited amounts of myotoxin available for binding to muscle cells or to myotoxin being rapidly metabolized by muscle cells.

Incubation of muscle in myotoxin resulted in localization of the toxin along the sarcolemma as well as inside the cells. This means that myotoxin could be binding to both sarcolemma and sarcoplasmic reticulum, and to other cellular organelles including myofilaments. Thus localization at this level (light microscopy) does not allow us to pinpoint the actual site of myotoxin.

The primary action of myotoxin as proposed by Ownby et al. (1976) is dilatation of the sarcoplasmic reticulum, which could result from several types of initial action. This could result from inhibition of ion transport at the level of the sarcolemma. Sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase), an enzyme necessary for the active transport of Na^+ out of the cell and K^+ into the cell, is inhibited by ouabain, a cardiac glycoside. In skeletal muscle treated with ouabain the initial effect is dilatation of the sarcoplasmic reticulum (Ginn et al., 1968; Knight, 1978). This dilatation of sarcoplasmic reticulum is the same initial effect observed when skeletal muscle was treated with myotoxin (Ownby et al., 1976; Knight, 1978). The Na^+ - K^+ pump is closely associated with the plasma membrane of cells and any alteration in the cell membrane, such as blocking the Na^+ - K^+ pump with ouabain, would

result in a change in its permeability characteristics. It was suggested by Ginn et al. (1968) that blocking the pump resulted in the influx of sodium, chloride, and water, and the efflux of potassium. The water accululated in the endoplasmic reticulum causing enlargement, and eventual expansion of all cellular compartments, and distortion of cell membranes. However, it was shown by Knight (1978) that myotoxin does not demonstrate the same physiological responses as ouabain. Knight proposed that myotoxin did not affect the $Na^+ - K^+$ ATPase system, but instead might affect the calcium transport mechanism. This would have the same ultrastructural changes as ouabain but could explain the physiologic response of initial spasms and decline in contractile response reported by Knight (1978). Myotoxin a could be altering the sarcolemma, causing it to become more permeable to calcium ions and, subsequently, to water. Calcium in the sarcoplasm could then trigger contraction and also additional Ca^{++} release from the sarcoplasmic reticulum (Endo, 1977; Crevey et al., 1978) which could eventually lead to myofilament degradation (Duncan and Smith, 1978) as observed by Ownby et al. (1976) using myotoxin a.

REFERENCES

Andres, G. A., Hsu, K. C., and Seegal, B. C.: Immunological techniques for the identification of antigens or antibodies by electron microscopy. In: Handbook of Experimental Immunology, Vol. 2, pp. 34.1-34.45 (Weir, D. M., Ed.), Oxford, England, Blackwell Scientific Publications, 1973.

Axelsen, N. H., Kroll, J., and Weeke, B. (Eds.): A Manual of Immunoelectrophoresis Methods and Applications. Ch. 23, p. 162, Universitetsforlaget, Oslo, 1973.

Cameron, D., and Tu, A.: Characterization of myotoxin a from the venom of prairie rattlesnake (Crotalus viridis viridis). Biochemistry 16: 2546-2553, 1977.

Clausen, J.: Immunochemical techniques for the identification and estimation of macromolecules. In: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 2, part III (Work, T. S. and Work, E., Eds), Amsterdam: North Holland, 1969.

Crevey, B. J., Langer, G. A., and Frank, J. S.: Role of Ca^{2+} in maintenance of rabbit myocardial cell membrane structural and functional integrity. Journal of Molecular and Cellular Cardiology 10: 1081-1100, 1978.

Duncan, C. J., and Smith, J. L.: The action of caffeine in promoting ultrastructural damage in frog skeletal muscle fibres. Archives of Pharmacology 305: 159-166, 1978.

Endo, Makoto: Calcium release from the sarcoplasmic reticulum. Physiological Reviews 57 (1): 71-108, 1977.

Ginn, F. L, Shelburne, V. D., and Trump, B. F.: Disorders of cell volume regulation. American Journal of Pathology 53: 1041-1059, 1968.

Huang, T. T., Blackwell, S. J., and Lewis, S. R.: Hand deformities in patients with snakebite. Plastic and Reconstructive Surgery 2 (1): 32-36, 1978.

Knight, J. P.: A comparative study of the effects of ouabain, rattlesnake (Crotalus viridis viridis) venom, and myotoxin a on the contractile response and ultrastructure of rat skeletal muscle. Unpublished M.S. thesis, Oklahoma State University, July, 1978.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193: 265-275, 1951.

Ownby, C. L.: Pathogenesis and chemical treatment of hemorrhage induced by rattlesnake venom. Ph.D. thesis, Colorado State University, August, 1975.

Ownby, C. L., Cameron, D., and Tu, A. T.: Isolation of myotoxic component from rattlesnake (Crotalus viridis viridis) venom. Electron microscopic analysis of muscle damage. American Journal of Pathology 85: 145-166, 1976.

Ownby, C. L., and Tu, A. T.: Chemical neutralization of snake venoms. In: Venoms: Chemistry and Molecular Biology, p. 435. New York: Wiley, 1977.

Reisfeld, R. A., Lewis, U. J., and Williams, D. E.: Disc electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281-283, London, 1962.

Richardson, K. C., Jarrett, L., and Finke, E. H.: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Technology 35: 313-323, 1960.

Stringer, J. M., Kainer, R. A., and Tu, A. T.: Myonecrosis induced by rattlesnake venom. An electron microscopic study. American Journal of Pathology 67: 127-140, 1972.

The, T. H., and Feltkamp, T. E. W. Conjugation of fluorescein isothiocyanate to antibodies. I. Experiments on the conditions of conjugation. Immunology 18: 865-881, 1970.

Vick, J. A.: Symptomatology of experimental and clinical crotalid envenomation. In: Neuropoisons, Vol. 1, pp. 71-86 (Simpson, L. L., Ed.), New York, Plenum Press, 1971.

Wylie, D. E., Damsky, C. H., and Buck, C. A.: Studies on the function of cell surface glycoproteins. I. Use of antisera to surface membranes in the identification of membrane components relevant to cell-substrate adhesion. Journal of Cell Biology 80: 385-402, 1979.

VITA[2]

William Michael Woods

Candidate for the degree of

Master of Science

Thesis: PRODUCTION OF ANTISERUM TO MYOTOXIN A FROM PRAIRIE RATTLESNAKE (CROTALUS VIRIDIS VIRIDIS) VENOM AND LOCALIZATION OF MYOTOXIN A IN MOUSE SKELETAL MUSCLE

Major Field: Physiological Sciences

Biographical:

Personal Data: Born in Chickasha, Oklahoma, March 29, 1955, the son of William H. Woods and Carol J. Woods.

Education: Graduated from Chickasha High School, Chickasha, Oklahoma, in May, 1973; received the Bachelor of Science degree from Oklahoma State University in May, 1977, with a major in Zoology; completed requirements for the Master of Science degree at Oklahoma State University in July, 1979.

Professional Experience: Teaching assistant, January, 1978, to May, 1978; laboratory assistant, July, 1978, to August, 1978; teaching assistant, September, 1978, to December, 1978; research assistant, January, 1979, to June, 1979 (all professional experience at Oklahoma State University).