

BIOLOGICAL AND CHEMICAL CHARACTERIZATION OF
EXTRACT OF BROWN RECLUSE (LOXOSCELES
RECLUSA) SPIDER VENOM APPARATUS

By

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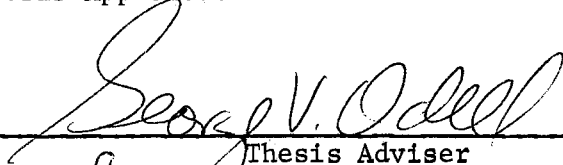
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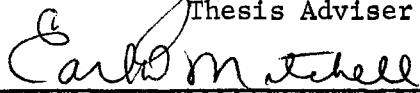
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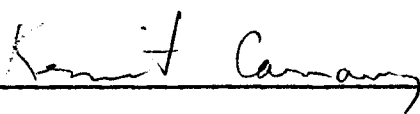
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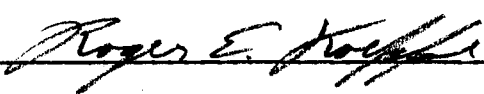
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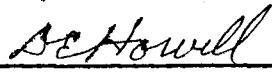
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Dedicated to my wife,
Lois Marie

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NOMENCLATURE

A ₂₈₀	absorbance at 280 nm
A ₂₆₀	absorbance at 260 nm
anti	antiserum against
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
Chymo A	chymotrypsinogen A
Cyt C	cytochrome C
disc	discontinuous
GTP	guanosine 5'-triphosphate
HMW	high molecular weight fraction
hr	hour(s)
i.d.	intradermal
i.m.	intramuscular
i.p.	intraperitoneal
i.v.	intravenous
iso	isoelectrofocused fractions
LD ₅₀	lethal dose for 50 percent of the treated animals
LMW	low molecular weight fraction
mA	milliampere
mM	millimolar
Myo	myoglobin
NAD	nicotinamide adenine dinucleotide
nm	nanometer

NOMENCLATURE (Continued)

Oval	ovalbumin
OXT	extract of poison apparatus
PMN	polymorphonuclear
pI	isoelectric point
Pi	inorganic phosphate
RBC	red blood cells
R _f	mobility relative to the solvent front
SDS	sodium dodecyl sulfate
s.c.	subcutaneous
TLG	thin-layer gel
Tris	tris-(hydroxymethyl)-amino methane
UV	ultraviolet
μg	microgram
μl	microliter

CHAPTER I

INTRODUCTION

The brown recluse spider, Loxosceles reclusa Gertsch and Mulaik, has been found throughout the south central United States (1). Atkins et al. (2,3) first described the effect of the venom of L. reclusa on rabbits and hypothesized that this spider was the cause of necrotic spider bites which were known to occur in the south central United States. Further studies with laboratory animals by Smith and Micks (4) and Morgan (5) firmly established the lethal and necrotic effects of the brown recluse spider venom.

Necrotic lesions in humans attributed to brown recluse envenomation have been described by Atkins et al. (3), Dillaha et al. (6,8), and Lessenden and Zimmer (7). At least three deaths, two children (7,9) and one adult (9,10) have been attributed to the brown recluse spider. Dillaha et al. (6) described the following symptoms of L. reclusa bites. Local reactions can include pain, bleb formation, erythema, ecchymosis, necrosis ending in eschar and ulcer formation. The systematic reactions may include fever, nausea, malaise, hemolysis, thrombocytopenia and in rare cases, death.

Steroid therapy has been proposed as treatment for brown recluse envenomation by Dillaha et al. (6,8). In a series of 10 rabbits challenged with equal amounts of pooled venom, intravenous methyl prednisolone was seen to protect all three rabbits receiving this steroid six hr

following venom injection.

Five of six animals receiving the steroid 24 to 48 hr after the challenge died within three days as did the single untreated control (6). Ten human victims of brown recluse bites were treated with steroids. Five did not develop necrotic lesions. The failure of the treatment with the other five was attributed to an excessive length of time between bite and treatment (8).

Recently Berger (11) described two cases of brown recluse envenomation where the spiders were positively identified. In neither case did necrosis develop. In fact the effects of the bites were compared to those of a flea. Neither of the victims had been previously bitten by spiders. Two months after envenomation, both showed positive lymphocyte transformation to brown recluse venom. When tested at the time of envenomation, neither was positive. The assay for transformation was based on the fact that only sensitized lymphocytes will transform into blast cells and undergo mitosis. Mitotic rate was assayed by the incorporation of tritiated thymidine into daughter cells. Berger felt these data indicated that humans could be immunized against L. reclusa. He also felt that steroids were not an effective treatment. The "successes" of steroids have been with people who would have shown no necrosis. Experiments in animals with brown recluse spider venom have not supported Dillaha's (6) claim of the effectiveness of steroids (12,13).

Morgan (5) conducted toxicity studies of L. reclusa in several animals. The venom was collected by electrostimulation and pooled in saline. Protein was quantitated by the spectrophotometric method of Waddell (14). Approximately 70 μ g of venom protein was obtained from each spider. Twenty five or more μ g of venom protein was found to be lethal

to rabbits whether injected intravenously (i.v.) or intradermally (i.d.). Skin lesions were produced by as little as 0.1 μ g venom i.d. The lethal i.d. dose in guinea pigs was found to be approximately 20 μ g. Sublethal doses did not generate as large a lesion as was produced in rabbits. The intraperitoneal (i.p.) lethal dose in mice was found to be approximately 15 μ g while the venom was found to be nontoxic to rats. Morgan (5) and Atkins (3) found both male and female venoms toxic, but Morgan (5) reported that males gave only about half the volume of venom as did the females.

Norment and Smith (15) found that brown recluse venom (collected by electrostimulation) caused hemolysis in vivo of hemocytes of the common house cricket, Acheta domesticus. Norment and Vinson (16) tested the effect of the venom on the tobacco budworm larvae, Heliothis virescens. All of the fat body was destroyed as was the striated muscle tissue of the body wall and the digestive tract. Some changes in hemolymph protein of treated specimens were found by acrylamide gel electrophoresis.

Grothaus (17) reported that the brown recluse spider often "regurgitated" when biting. Nazhat (18) found this regurgitated material to be a definite problem when trying to identify the protein components of L. reclusa venom collected by electrostimulation. Polyacrylamide disc gel electrophoresis patterns of the material collected from different spiders were highly variable. Grothaus tested the regurgitated material, as well as other body fluids, and found them to be nontoxic to mice.

Buffkin et al. (19) reported that an extract of eggs of Loxosceles was nontoxic while that of the eggs of the black widow, Latrodectus hesperus, contained the toxic components of the adult.

Enzymes such as proteases, hyaluronidase, and phosphodiesterase

have been reported as common components of spider venoms (20). The enzymatic activities present in brown recluse venom have been investigated quite thoroughly. Nazhat (18) reported that L. reclusa venom (collected by electrostimulation) contained no protease or phospholipase C or D activity. Some lipase activity was observed. Hall (21) reported that L. reclusa venom (collected by electrostimulation) contained no phospholipase A activity, although some indication of hyaluronidase activity was found. Wright et al. (22) reported protease, esterase and hyaluronidase activities were present in brown recluse venom (collected by microdissection). Alkaline phosphatase, collagenase and phospholipase A were absent. The hyaluronidase activity was separated from the esterase activity by polyacrylamide gel electrophoresis. Kinetic properties of the hyaluronidase as related to enzyme and substrate concentrations were investigated and the pH optimum was determined to be 6.2. A rabbit antiserum to the venom was developed and was shown to inhibit the activity of the hyaluronidase in vitro and in vivo.

Denny et al. (23) reported hemolysis of red blood cells (RBC) in vivo and in vitro. In the first series of test animals, the venom preparation injected (i.v.) was a saline homogenate of dissected venom glands. Nine dogs were challenged with the homogenate of approximately 1.5 glands per dog. All dogs showed depressed platelet counts, loss of reticulocytes and a slight rise in plasma hemoglobin. The hematocrit dropped slightly in spite of the fact that all dogs exhibited severe dehydration. Four dogs were injected (i.v.) with venom obtained by electrostimulation in a second experiment. Each dog received 37 µg of venom protein as measured by the method of Waddell (14). Two of the four dogs died. The remaining pair exhibited mild hemolysis. The effect of venom

collected by electrostimulation was tested in vitro with human RBC. After 48 hr two ml of human blood showed 26 percent hemolysis with 3.7 µg of venom protein while controls showed only two percent. No description of the treatment of the blood before hemolysis tests were begun was included in the paper. Heating of the serum to inactivate complement prior to starting the hemolysis tests resulted in no loss of activity. On the basis of the above data Denny et al. have proposed that brown recluse spider venom has a direct hemolytic action on red blood cells.

Smith and Micks (4) investigated the hemolytic properties of three species of *Loxoxceles*, L. laeta, L. reclusa and L. refescens. The venom was collected by electrostimulation. An abdominal extract of all three was also prepared. Extracts of all three species had a direct hemolytic effect on human RBC while venom had none.

Kniker et al. (24) have reported that L. reclusa venom contains a potent inhibitor of hemolytic complement activity in vitro. This activity was observed only at very low venom concentrations as direct hemolysis by the venom became a factor at higher concentrations. The assay consisted of sensitized sheep erythrocytes with serum added for complement. The complement inhibitor was found to be nondialyzable, excluded from Sephadex G-75, and stable over a wide range of pH, heat, and storage conditions.

Smith and Micks (25) reported that brown recluse lesions are characterized by early and pronounced infiltration of polymorphonuclear (PMN) leukocytes. PMN leukocytes in test rabbits were depleted by injecting nitrogen mustard. These treated rabbits were then challenged with L. reclusa venom. The venom was collected by electrostimulation and used immediately after collection. Depletion of PMN leukocytes inhibited

lesion formation in treated animals while control animals developed lesions. The effect of complement depletion prior to venom injection was tested in guinea pigs and was found to also inhibit lesion formation. On the basis of these experiments Smith and Micks proposed that lesion formation was due to an "Arthus-like" reaction. Puffer et al. (26) have reported that the venom of L. reclusa evoked an "Arthus-like" reaction in the microcirculation of mouse gastrolial mesentery.

Atkins et al. (3) reported that a single large female rabbit, which had been bitten or injected seven times with L. reclusa venom, tolerated the injection into the ear vein of 2.5 glands macerated in saline. A control rabbit died. Both Smith and Micks (4) and Denny et al. (23) reported that multiple injections of L. reclusa venom led to the production of serum antibodies. Antibodies were detected by immunodiffusion studies. Smith and Micks (4) obtained a Loxosceles antiserum from the Instituto Butantan, São Paulo, Brazil. This antiserum was not produced with L. reclusa venom. The venoms of L. reclusa, L. refescens, and L. laeta were tested with the antiserum by the immunodiffusion technique. All three venoms produced three apparently similar precipitation lines. Immuno-electrophoresis studies revealed four antigenic components in all three species, but the patterns were distinctly different. Attempts to protect rabbits with three ml of antiserum injected after venom challenge resulted in only a very negligible reduction in lesion size.

Some of the components of L. reclusa venom were successfully radio-labeled by Elgert et al. (27). Spiders were fed ^{14}C -leucine in water. Radioactive bands were noted in disc gel electrophoretic patterns of venom collected by microdissection. No correlation of biological activity to radioactive bands was made.

Pinkston (28) has described the morphology of the L. reclusa poison apparatus. Based on the observation of secretory packets and cell fragments in the gland lumens, a holocrine secretory process was proposed.

Several spider venoms have been fractionated by a variety of procedures. McCrone and Hatla (29) reported the separation of Latrodectus mactans venom into seven components by vertical acrylamide gel electrophoresis. The venom of Atrax robustus has been separated into five components by a series of ultrafiltration membranes (30). Schanbacher et al. (31) described the fractionation of Dugesiella hentzi venom using Sephadex G-100 column chromatography. Suarez et al. (32) have fractionated a Loxosceles laeta venom gland extract into two major components using a small (2.2 X 30 cm) G-100 Sephadex column. The slower eluting component contained the necrotizing materials (as tested with rabbits) and showed only one distinct and two faint bands on polyacrylamide gel electrophoresis. In addition the whole extract was reported to contain no phospholipase C, collagenase, protease, or phosphodiesterase activity. No data were given to support the above statements.

The objectives of this work were to describe the biological activities of the toxic components of L. reclusa venom, isolate these components, and determine their chemical characteristics.

The abundance of the brown recluse spider and the toxicity of its venom make this study not only desirable, but necessary.

CHAPTER II

EXPERIMENTAL PROCEDURES

A. Specimen Collection

The specimens of L. reclusa were collected in central and north-eastern Oklahoma and southeastern Kansas. The spiders were captured at night in individual plastic cups (Premium Plastics, Inc.). Large numbers of specimens could only be obtained during the summer months. If the spiders were not used immediately, they were frozen in the cup with dry ice, then stored at -15°C until needed.

B. Dissection of Poison Apparatus

The entire poison apparatus including chelicerae was dissected by a slight modification of the method of Pinkston and Howell*. The modification consisted of freezing the spider with dry ice, then allowing it to thaw before beginning the dissection process. The original procedure was dissection of a live spider. Hemolymph was collected in capillary pipets during the dissection process and pooled.

C. Preparation of Extract of Poison Apparatus (OXT)

The extract of the poison apparatus (OXT) was prepared by lightly

* Personal communication.

crushing the apparatuses of 100 spiders, either all female or all male, in two ml of 0.02 M NH_4HCO_3 , pH 8.0, with a flattened glass stirring rod. The insoluble material was removed by centrifugation at about 7000 X g on a Sorvall Model RC-2 at 0°C for 15 min. The supernatant was stored at -15°C if not immediately used. All OXT's used for toxicity studies were routinely frozen and thawed twice before use.

D. Determination of Protein

The protein concentration of the OXT and the various fractions obtained from the OXT were assayed according to the method of Lowry et al. (33), using bovine serum albumin as the reference standard. In the case of the highly purified toxins where little material was available for assay, the volumes of the various reagents for the assay were reduced to increase sensitivity. Absorbance readings were obtained on a Perkin-Elmer Coleman 101 Spectrophotometer.

E. Toxicity Studies

Toxicity studies of the OXT were conducted with adult American cockroaches, Charles River CD-1 Strain white mice, 20 to 25 g, and New Zealand white rabbits. The cockroaches were anesthetized with CO_2 and injected intra-abdominally by the method described by Grothaus (17). The mice were anesthetized with diethyl ether and injected intraperitoneally. The rabbits were restrained and injected intraperitoneally. All tests included animals injected with solvent blanks. The Reed-Meunch method (34) was used for obtaining LD_{50} values.

The lesion producing ability of the OXT was approximately quantitated using the rabbit skin test of Kondo et al. (35). To test the

effectiveness of the OXT in generating a lesion in mice, nine mice were injected with doses ranging from 24 to 192 μg protein. All injections were made subcutaneously in the upper hind leg.

A separate group of six rabbits was used to test the effect of different sites of venom injection. Routes tested were intravenous (ear), intraperitoneal, subcutaneous (back) and intramuscular (hind leg). One rabbit was injected by each method with the exception of the i.v. route where three animals were tested. In all tests 360 μg of OXT protein was injected.

Toxicity of the hemolymph of brown spiders in mice by the i.p. route was also tested.

F. Freeze-Thaw Stability of OXT

The stability of the OXT to freezing and thawing was tested by injecting a series of mice with OXT which had been subjected to freeze-thaw varying numbers of times. The route of injection in each case was i.p., and the amount of OXT protein injected in each case was 200 μg diluted to 500 μl with 0.02 M NH_4HCO_3 .

G. Temperature Stability of OXT

The temperature stability of the OXT was tested by incubating the OXT for 30 min at various temperatures before injecting into mice. Again 200 μg of OXT protein was used and the route was i.p.

H. Effect of Buffer pH and Type on OXT Toxicity

To test the effect of different pH's and buffers the following scheme was used: 100 μg of the OXT (preparation for which LD_{50} was de-

terminated) was incubated with 300 μ l of the buffer to be tested for two hr at 0°C, then it was injected intraperitoneally into mice.

I. Effect of Proteases on Toxicity of OXT

To test the effect of proteases on the toxicity of the OXT the following experimental procedure was used. The proteases utilized were Pronase and trypsin (Calbiochem grade B) and the buffer was Tris, 0.046 M, pH 8.1, containing 0.0115 M CaCl_2 . In the initial experiment 350 μ g of OXT was utilized for each test animal, and this amount was preincubated for 30 min at room temperature with 20 μ g of protease. In a second experiment only 180 μ g of OXT protein was used and preincubation time was one hr. Injections were made intraperitoneally into 20 to 25 g white mice.

J. Protease Assays

To insure that the proteases used in the above experiment were active and to test the protease activity of the OXT the enzyme assay method of Hummel (36) was used. The highest amount of OXT protein assayed by this method was 100 μ g. In addition, the protease assay of Kunitz (37) was used to test the activity of the OXT. Two different substrates were utilized: one percent bovine serum albumin and one percent casein. The buffer in both cases was 0.1 M sodium phosphate, pH 7.6. Incubation temperature was 37°C and aliquots were taken for acid-soluble protein determination at 30 min and four hr. The maximum amount of the OXT assayed for protease activity was 120 μ g protein per ml of assay mixture. For comparison trypsin was tested for protease activity in the same system.

K. Phosphohydrolase Assays

Phosphohydrolase activity of the OXT was determined by two methods. The first method consisted of measuring the free inorganic phosphate (Pi) released from nucleotides by the OXT. The method was that of Marsh (38). All reagent volumes were one-fourth those described by Marsh. The buffer was 100 mM Tris-HCl, five mM in $MgCl_2$, two mM in KCl and pH 6.8. The total volume was one ml in each assay. All absorbance readings were taken on a Beckman DU Spectrophotometer. Both ATP and GTP (10^{-4} M) were utilized as substrates. The second method of phosphohydrolase determination was that of Garen and Levinthal (39). The amount of p-nitrophenyl phosphate was only half that described. The amount of OXT protein assayed for activity was 50 μ g. The pH and temperature optima of the OXT were established by the first assay procedure. In both cases 36 μ g of OXT was used; total sample volume was 0.5 ml and 9×10^{-9} M ATP was used as the substrate. Blanks contained all components including 36 μ g of OXT protein which had been denatured by heating at $100^{\circ}C$ for five min. In the temperature optimum assays, blanks were included at each temperature and enzyme reaction time was 90 min. In the pH optimum experiments reaction time was 45 min.

L. Hyaluronidase Assays

Hyaluronidase assays were conducted with both OXT and hemolymph by the method of Tolksdorf, et al. (40).

M. Hemolysis Tests

The hemolytic properties of the OXT were investigated by a number of procedures. In all cases the red blood cells (RBC) were washed with

normal saline until no hemoglobin was apparent in the supernatant then the cells were diluted 1:10 with normal saline. The original test solutions contained 0.1 ml diluted RBC, 1.0 ml normal saline and varying amounts of OXT. Separate samples were incubated at 24 and 37°C. Aliquots of supernatant were taken for hemoglobin determination at 45 min, 3 hr and 12 hr. The modified benzidine method of Crosby and Furth (41) was used to determine hemoglobin release. The same tests were repeated with 0.1 ml human serum added for complement. A third series was with serum and 0.07 M CaCl_2 . The second series of tests previously described was repeated using rabbit RBC and rabbit serum. In all cases, 0, 50, 100 and 150 μg of OXT were used with duplicates of each concentration.

N. Thin-Layer Gel Chromatography

Initially small amounts of OXT were fractionated by the process of G-100 thin-layer gel filtration (TLG). The method is that of Radola (42). The elutant was 0.02 M NH_4HCO_3 , pH 8.0. The reference proteins were obtained from Pharmacia with the exception of bovine crystalline albumin which was obtained from Mann Research Laboratories. The standards used to establish the molecular weight curve were cytochrome C (cyt C), myoglobin (myo), chymotrypsinogen A (chymo A), ovalbumin (oval) and bovine serum albumin (BSA). The standard curve was of inverse migration distance (relative to cytochrome C) versus log molecular weight.

O. Fractionation of OXT

0.1. Column Chromatography

The final fractionation procedure of the OXT consisted of Sephadex G-25 and G-100 column chromatography, preparative disc gel electrophore-

sis, and finally Sephadex G-50 column chromatography. The low molecular weight fraction of the OXT was further separated by Bio-Gel P-2 column chromatography with the final purification of some components being accomplished with Dowex ion exchange resin. A flow diagram of the procedure is included in the Results Chapter. All fractions were concentrated by lyophilization. The G-25 column was 0.8 X 80 cm and had a flow rate of 5.5 ml/hr. The G-100 column was 1.2 X 60 cm and had a flow rate of 8 ml/hr. The G-50 column was 2 X 20 cm and had a flow rate of 30 ml/hr. In all the above columns the elutant was 0.02 M NH_4HCO_3 and the volume collected per fraction was 1.1 ml. The ion exchange resin used was Dowex 1 X 8 (200-400 mesh) in the formate form. Column size was 1 X 25 cm and the flow rate was 1 ml/min. Fraction size was two ml. The gradient was of the two reservoir constant mixing type and is described below.

Elution Scheme

50 ml H_2O in lower reservoir
 50 ml H_2O
 50 ml 4 N formic acid
 50 ml 4 N formic acid + 0.3 N ammonium formate
 50 ml 4 N formic acid + 0.6 N ammonium formate
 50 ml 4 N formic acid + 0.9 N ammonium formate
 50 ml 4 N formic acid + 1.0 N ammonium formate

0.2. Assay of Fractions

The toxicity of the various fractions was determined by bioassay with mice, roaches and rabbits. Combinations of certain fractions were also tested for toxicity. The protein components of the fractions were assayed by disc gel electrophoresis.

0.3. Disc Gel Electrophoresis

Both the analytical and the preparative disc gel electrophoresis were by the same method, primarily that of Ornstein (43). The gels, pH 9.5, were seven percent acrylamide and five mm in diameter. The stacking gels measured 1.5 cm while the separating gels were 10 cm in length. The samples were buffered at pH 8.3 and contained 10 percent sucrose and tracking dye. Electrophoresis of samples was at four mA/gel with cold water circulating through the cooling jacket of the apparatus. The water was in a closed system containing a pump and an aluminum coil submerged in an ice and water mixture. In the case of the analytical gels, electrophoresis was continued until the tracking dye was one cm from the bottom of the gel and protein bands were visualized with coomassie blue. Certain gels were stained with Schiff's reagent by the method of Zacharias et al. (44). The validity of the method was tested by staining a gel containing transferrin (Sigma). In the preparative disc gel method, electrophoresis was continued for one hr after the tracking dye had eluted from the end of the gel to yield better resolution. Normal run times were 3 to 4 hr. Multiple samples were separated with only one being stained to give the location of bands. Major bands were cut from the gels, the acrylamide sections crushed with a glass stirring rod and extracted into 0.02 M NH_4HCO_3 by stirring in a 4°C cold room for 12 hr. The validity of the extraction was tested by electrophoresis of the individual isolated fractions. In some cases the homogeneity of the isolated bands was tested by electrophoresis at different pH's and gel concentrations. Additional gel types used were pH 4.3, seven percent acrylamide, pH 4.3, 15 percent acrylamide and pH 9.5, 15 percent acrylamide. Hemolymph samples of both male and female spiders were separated on

analytical disc gels and compared to the patterns of toxic purified fractions of the OXT.

P. Quantitation of Protein Using Disc Gel Scans

A very crude estimation of the amount of protein present in the major bands of analytical gels was accomplished by the following technique. The gels were scanned at 550 nm on a Beckman DU Spectrophotometer with a Gilford Model 410 Gel Scanning Attachment. The scan pattern was cut from the chart paper and weighed, then the major peaks were cut from the scan and weighed. From these measurements the percent per band of the total could be approximated. Lowry protein determinations on material extracted from these same bands on preparative gels were also made.

Q. Determination of Molecular Weights

Q.1. Column Chromatography

The G-100 column described previously was standardized for molecular weight determination. Protein standards used were horse heart cytochrome C (California Corporation for Biochemical Research), calf intestine alkaline phosphatase (Calbiochem grade B), chymotrypsinogen A (Sigma) and ovalbumin (Sigma). The retention times of chymotrypsinogen A and ovalbumin were determined by their absorbance at 280 nm. Cytochrome C was determined by its absorbance at 550 nm while alkaline phosphatase was determined by its enzymatic activity on p-nitrophenyl phosphate as determined by the method of Garen and Levinthal (39).

Q.2. Sodium Dodecyl Sulfate (SDS) Gel

Electrophoresis

Molecular weights were also determined by SDS polyacrylamide gel electrophoresis. The method is that described by Weber and Osborn (45). The gels were five mm X 10 cm and were 10 percent acrylamide. Electrophoresis was at eight mA/gel and the molecular weight standards were the same as described for TLG. Gels were pre-run for four hr and electrophoresis of samples was for four hr.

R. Isoelectric Focusing

Isoelectric focusing was accomplished by the method described by Haglund (46). The pH range of ampholine utilized was 5 to 8. An Ampholine Column LKB 8101 was used and the material was isoelectrofocussed for 72 hr and 1.1 ml fractions were collected. The pH of the fractions was determined using a Fisher Model 320 pH Meter with a microelectrode.

S. Amino Acid Analysis

Hydrolysis of protein and determination of amino acid composition was by the procedure of Moore and Stein (47). Hydrolysis was accomplished with 6 N HCl at 110°C for 22 hr. Actual analysis was accomplished on a Beckman Model 120C Amino Acid Analyzer. One tenth μ M of norleucine was added to each sample prior to hydrolysis.

T. Determination of Spectrophotometric and

Chromatographic Characteristics

of Nucleotides

Ultraviolet (UV) spectra were obtained on a Cary 14 recording

spectrophotometer unless otherwise indicated. Buffers used for nucleotide spectra and solvent systems used in paper chromatography were as described in P.L biochemicals' Ultraviolet Spectra of 5'-Ribonucleotides (48). All four "Pabst" systems were used in the chromatography and nucleotides were visualized by UV quenching. The chromatograms were also sprayed with ninhydrin and phosphate sprays (49). All sprays were tested with known nucleotides chromatographed in the Pabst systems. In some cases the nucleotides were hydrolyzed for seven min with 1.0 N HCl at 100°C to produce mononucleotides (50). All reference nucleotides were obtained from Sigma.

U. Luciferase Assay

The firefly luciferase assay of Strehler and Totter (51) was used to assay the ATP content of the low molecular weight fraction of the OXT. The validity of the assay was tested with ATP standards. The firefly extract was purchased from Worthington.

V. Preparation of Antisera

Antisera were prepared for the OXT and two purified toxins. Animals used for antisera production were adult New Zealand white rabbits. For OXT antiserum two rabbits were used. Injections were once a week for four weeks. The first two injections contained 7.5 µg OXT protein and were placed intramuscularly in alternate hind legs. The third injection was 15 µg placed intramuscularly in the hind leg. The last injection contained 15 µg and was by the i.p. route. For the purified toxin antisera, toxins were obtained from 64 spiders. Again four weekly spaced injections were used. Only one rabbit was used for each toxin antiserum.

The first injection for each was 1/7 of the total available isolated toxin injected in the hind leg. The second, third and fourth injections were 2/7 of the total with the second and third injections being placed intramuscularly while the last was by the intraperitoneal route. All rabbits were bled via the ear vein 10 days after the last injection. The blood was allowed to stand overnight at 4°C then the sera were separated by centrifugation.

W. Detection of Antibodies:

in vitro and in vivo

The presence of antibodies was detected by three techniques: Ouchterlony, immunoelectrophoresis and a RBC agglutination method. The Ouchterlony and immunoelectrophoresis techniques were as described by Clausen (52). The support medium for both tests was Ionagar No. 2S (Colab Laboratories). The buffer was 0.05 M veronal and electrophoresis apparatus and gel punch were purchased from Gelman. Electrophoresis was for two hr at 80 volts. Visualization of antigen-antibody reactions was accomplished with amido black. The agglutination test consisted of coating washed human RBC (diluted 1:10 with saline) with antiserum, then allowing the coated RBC to react with OXT. Each test solution contained 0.5 ml saline, 0.05 ml diluted RBC, 0.05 ml antiserum and 25 µg OXT. Duplicates were run on each antiserum and test were made at 24 and 37°C. A second series of tests was with antisera and OXT preincubated together for 30 min before adding the RBC. To check for the extent of agglutination, a third series of tests with OXT protein varying from 2 to 20 µg was conducted. All agglutination tests included controls containing normal rabbit serum.

Prior to the generation of antibodies, human, mouse and rabbit sera were tested by the Ouchterlony technique for the possible existence of natural antibodies. In addition a mouse skin extract was tested. The extract was prepared by homogenizing 1.5 g of mouse skin in 10 ml of 0.05 M veronal buffer. The insoluble residue was removed by centrifugation. In all experiments 100 μ g of OXT was used as the antigen and a maximum of 10 μ l of sera or extract was used as a possible source of antibodies.

The ability of the antisera to neutralize the biological effects of the OXT was tested with mice and rabbits. With mice three series of tests were conducted. In all three 25 to 30 g white mice were injected by the i.p. route. In the first two tests OXT was preincubated with antisera before being injected. In the third test antiserum was injected 30 min after OXT. With rabbits OXT preincubated with antisera was injected subcutaneously.

X. Effect of Heparin on OXT Toxicity

An experiment to test the effect of heparin on the OXT in mice was devised. Eight mice were injected intraperitoneally with 4.25 units heparin/mouse prior to being challenged with 300 μ g of OXT protein injected i.p. The second injections were 30 min after the heparin treatment. Controls included mice injected with heparin alone and venom alone.

Y. Effect of OXT, OXT Fractions and Purified

Toxins on Plasma Coagulation Time

To test the effect of OXT and various OXT components on the coagula-

tion time of plasma, the following experiment was conducted. Plasma was separated from out-dated human blood by centrifugation at 1000 X g for 10 min at room temperature. Since the blood contained citrate to chelate Ca^{++} , an excess of CaCl_2 was added. Each assay for coagulation time contained 0.5 ml plasma and 0.2 ml one percent CaCl_2 W/V. Coagulation time was taken as the difference between the time of addition of CaCl_2 and time of full loss of liquid plasma. All assay mixtures and controls were preincubated at 37°C for 10 min prior to the addition of CaCl_2 to initiate coagulation.

The first series of tests compared the coagulation time of plasma + CaCl_2 to that of the same mixture containing 50 μg of OXT protein. Coagulation times were also determined for a second set of controls containing volumes of 0.02 M NH_4HCO_3 and 0.10 M NH_4HCO_3 equal to that of the OXT. A second series of tests compared controls to assays containing various amounts of OXT. Other tests were conducted to compare the coagulating ability of various fractions and toxins.

Z. Pathological Examinations

The rabbits used in the LD_{50} determination were also used for pathological examinations. Immediately after death the heart, kidneys, and sections of liver, large and small intestines were removed and placed in 10 percent formalin. The samples (13 rabbits) were given to Dr. Billy Ward of the Department of Veterinary Pathology, OSU, for microscopic histological examination. In addition several series of mice were given to Dr. Ward after being injected with different amounts of OXT by different routes. Skin lesions of rabbits were also made available for examination.

CHAPTER III

RESULTS AND DISCUSSION

A. Specimen Collection, Dissection and Preparation of OXT

Over 5000 spiders have been used in this study. The ease of obtaining specimens indicates the extent of infestation. L. reclusa must be considered an important health problem because of the known toxicity of its venom. In view of the low egg production and long development time between egg and adult of the brown recluse (53), no attempt was made to rear sufficient spiders for venom fractionation and characterization.

Several lines of evidence indicated that a whole gland extraction process was the best method of obtaining brown recluse venom. This evidence included: Grothaus' (17) observation of spiders "regurgitating" stomach contents when biting; Nazhat's (18) findings of the variability of L. reclusa venom obtained by electrostimulation as assayed by disc gel electrophoresis; and Pinkston's (20) observation of secretory packets and cell fragments in the lumen of L. reclusa glands and postulation of a holocrine secretory process. The possibility of dissecting out the glands and squeezing them to collect the venom as described by Wright (54) was considered, but was discarded due to the viscosity of the gland contents and the necessity of obtaining the maximum amount of venom. It should be noted that the glands were not extensively homogenized in the preparation of the OXT. The obvious disadvantage of an extract is that

many components of the cells in addition to the venom are also obtained. Biological activities and chemical components of the extract are significant only if they can be directly related to the known toxic properties of the venom. The work of Grothaus (17) and Nazhat (18) also raises another important question; the validity of identification of enzymatic and other biological activities in brown recluse venom obtained by electrostimulation. The "regurgitated" material may contribute to the venom's overall toxicity, but toxic characteristics of the spider bite are present in extracts of the poison apparatus only.

By freezing and thawing the spiders before beginning dissection, the muscles of the cephalothorax are fractured from the exoskeleton, thus allowing the carapace to be removed easily. This simple procedure allowed the glands to be removed much more quickly than by the original method of removing the apparatus from a live spider. Based on extracts from 500 spiders, OXT's had an average protein concentration of 5.0 mg/ml, variation being from 3.6 to 5.7 mg/ml. These data indicated that about 100 μ g of OXT protein was obtained from the average spider.

B. Toxicity Tests

In the determination of LD_{50} (48 hr) in mice, 44 animals were used with 21 surviving. Figure 1 shows the data obtained treated by the Reed-Meunch method (34). Where the two curves cross is the approximate LD_{50} . The value obtained was 4.6 μ g OXT/g body weight. Fourteen rabbits were used in the LD_{50} (48 hr) determination with three surviving. The value obtained was 6.0 μ g OXT/Kg body weight. In the roach determination of LD_{50} (24 hr) 34 roaches were used with 11 surviving. The value was 30 μ g/roach. Doses of up to 600 μ g OXT protein were injected i.p. into rats

with no apparent ill effects. The OXT used in rats was tested in mice and found to be toxic. Obviously the OXT does not affect all species alike. Variations of response within a species have also been reported. As Berger (11) has proposed, the severe necrotic lesion in humans may represent a very extreme response of a few individuals rather than the average response.

Figure 2 shows the relationship between lesion size in rabbits and amount of OXT protein injected as tested by the method of Kondo et al. (35). Lesion size per μg OXT protein was approximately 0.66 mm. In the mouse lesion tests, the five mice with the higher doses died within 48 hr. The four surviving mice were sacrificed after five days and their skin was removed and examined. No sign of a lesion was found in either dead or surviving mice. Figure 3 shows this lack of skin response in the mouse which had the highest dose and survived.

The method of presenting venom challenge to rabbits has a marked effect on OXT toxicity. Of the routes tested, i.p. and s.c. were lethal while i.m. and i.v. were not. The i.m. route produced a lesion while the i.v. route produced the effect shown in Figure 4. This picture was taken 24 hr after injection. No necrosis was ever apparent and even the pictured effect was gone within a week. Preliminary experiments with mice performed with Dr. Billy Ward indicated that the OXT was equally lethal whether injected i.p., s.c., i.v., i.d., or i.m.

The hemolymph of the spider was found to be essentially nontoxic. Only one mouse died, and it had been injected with one mg of hemolymph protein. Another mouse injected with the same dose survived. Eight mice injected with lesser amounts of hemolymph protein also survived.

Figure 1. LD₅₀ (48 hr) of the OXT as Determined in Mice

The mice were 20-25 g Charles River CD-1 Strain injected (i.p.) with OXT diluted to 0.5 ml with 0.02 M NH₄HCO₃. The method of interpreting the data is that of Reed and Meunch (34). The LD₅₀ is taken as the point of intersection of the two curves. Figure 1 shows 4.6 µg OXT protein/g body weight as the LD₅₀ (48 hr) in mice.

Figure 2. Dependence of Lesion Size in Rabbits on the Amount of OXT Protein

The rabbits used were New Zealand Whites and the OXT was diluted to 0.2 ml total volume with 0.02 M NH₄HCO₃ prior to injection. The method utilized was that of Kondo et al. (35). The rabbit was sacrificed 24 hr after injection and the skin was removed. Lesion size was estimated using averages of several measurements.

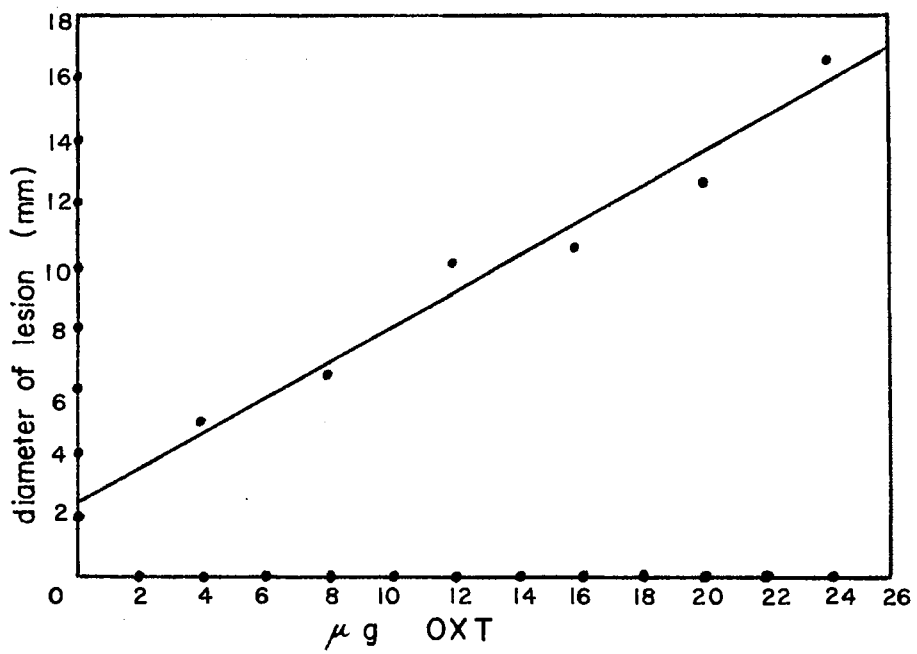
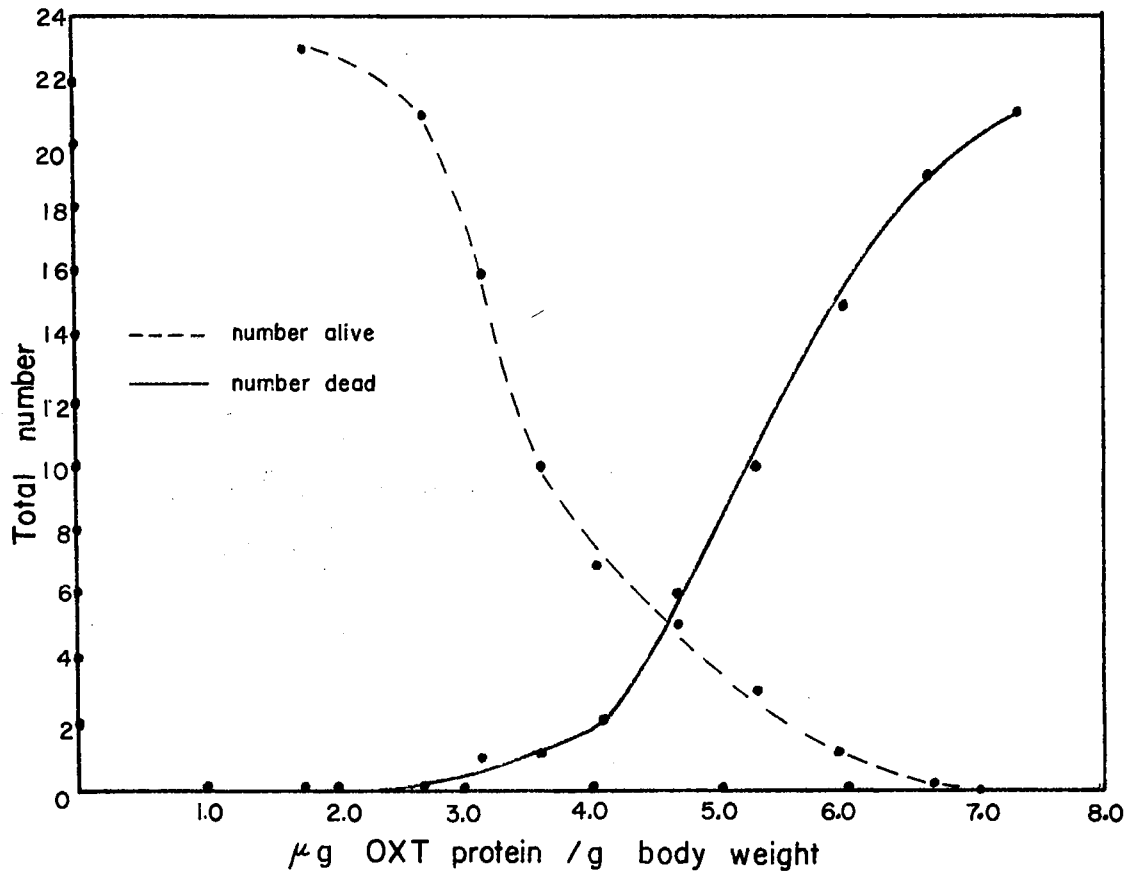


Figure 3. Mouse Subcutaneous Tissue at Injection Site of OXT

The picture was taken five days after injection. Even though the mouse was injected (s.q.) with 96 μ g of OXT protein, no lesion was apparent.

Figure 4. Rabbit Response to OXT Injected i.v.

Three hundred sixty μ g of OXT protein was injected via the ear vein. The picture was taken 24 hr after injection. The only evidence of response was the areas of discoloration which also disappeared within a week.



C. Freeze-Thaw Stability of the OXT

The OXT is stable to freezing and thawing as is shown by Table I. This table also indicates that the OXT may be activated by freezing and thawing. This OXT was lower in toxicity than usual as 200 µg should have been fatal to all mice. This could represent variation of response within a species and it certainly points out the shortcomings of bioassays.

D. Temperature Stability of Toxicity of the OXT

As Table II shows, the toxic components of the OXT as assayed with mice are inactivated at the higher temperatures. Some general observations should be included here. The OXT loses toxicity when stored at 4°C. Half-life appears to be approximately one week. Lyophilized OXT has been successfully stored at -15°C for several months.

E. Effect of pH and Buffer Type on OXT Toxicity

The choice of 0.02 M NH_4HCO_3 as the buffer for all column separations, etc., was an obvious one in view of the data in Table III. It does offer some protection against pH change and it is easily removed by lyophilization. The toxic ability of the OXT seems fairly stable to pH, but Tris and phosphate buffers appear to inactivate it. An early attempt to use a phosphate buffer in a column purification procedure resulted in the loss of toxicity in the OXT of 600 spiders.

F. Effect of Proteases on OXT Toxicity

Tables IV and V show that Pronase and trypsin both decrease the toxicity of the OXT in mice. In the initial experiment a large amount

TABLE I
FREEZE-THAW STABILITY OF OXT* AS DETERMINED
BY TOXICITY IN MICE

No. of freeze-thaws	No. of mice injected (i.p.)	No. dead at 48 hr
0	4	1
2	4	3
4	4	3
6	4	3
8	2	2

*Each mouse was injected with 200 μ g of OXT protein.

TABLE II
TEMPERATURE STABILITY OF TOXICITY OF OXT TO MICE*

Temperature $^{\circ}$ C**	No. of mice injected (i.p.)	No. dead at 48 hr
0	4	4
24	4	3
37	4	4
55	4	0
70	4	0

*Each mouse was injected with 200 μ g of OXT protein.

**Incubation time was 30 min.

TABLE III
EFFECT OF VARIOUS BUFFERS* ON THE TOXICITY OF OXT IN MICE

buffer	pH	No. of mice injected (i.p.)	No. dead at 48 hr
0.1 M sodium acetate	4	6	6
0.1 M sodium acetate	5	6	4
0.1 M sodium phosphate	6	6	2
0.1 M sodium phosphate	7	6	3
0.1 M tris-HCl	8	6	1
0.1 M tris-HCl	9	6	2
0.02 M NH_4HCO_3	8	6	6
H_2O	-	6	6

*One hundred μg of OXT was incubated with each buffer for two hr at 0°C prior to being injected i.p. into mice.

TABLE IV

EFFECT OF PROTEASES ON OXT TOXICITY IN MICE: I*

No. of mice injected	Material injected (i.p.)	No. dead at 48 hr
4	trypsin + OXT	1
4	pronase + OXT	4
2	trypsin	0
2	pronase	0

*Conditions were as follows: 350 μ g OXT, 20 μ g proteases, and 30 min incubation at room temperature.

TABLE V

EFFECT OF PROTEASES ON OXT TOXICITY IN MICE: II*

No. of mice injected	Material injected (i.p.)	No. dead at 48 hr
2	OXT	2
4	trypsin + OXT	0
4	pronase + OXT	1

*Conditions were as follows: 180 μ g OXT, 20 μ g proteases, and one hr incubation at room temperature.

of OXT was used because incubation with enzymes was in a Tris buffer. The second experiment with a lower amount of OXT was conducted to show that both Pronase and trypsin destroy toxicity.

G. Protease Assays

No protease activity was detected in the OXT. Figure 5 shows this lack of activity and compares the activities of the indicated amounts of pronase and trypsin in Hummel's assay (36). Figure 6 shows similar results with the method of Kunitz (37). Wright (22,54) has reported the presence of a protease in brown recluse venom by an assay procedure similar to that of Kunitz (37). Activity was found only with heat denatured casein as the substrate. Over 25 μ g of venom was required for activity and two hr incubations were used. Possible explanations for lack of detection of protease in this study are that the enzyme is denatured by freezing and thawing or that assay incubation times were insufficient. Also, the activity of the enzyme reported by Wright was very low. It is possible that the OXT contains a highly specific protease for which the correct assay conditions have not been determined.

H. Phosphohydrolase Assays and Preliminary Characterization of the Enzymatic Activity

Table VI shows the activity of the OXT with ATP and GTP while Figure 7 shows its activity with p-nitrophenyl phosphate. Table VI indicates that ATP is a better substrate than GTP and the reaction rate is still fairly linear after 90 min of incubation. Figure 7 shows a very slow hydrolysis of p-nitrophenyl phosphate. Figures 8 and 9 indicate crude estimations of the pH and temperature optima of the phosphohydrolase

Figure 5. Protease Assay by the Method of Hummel (36)

Figure 6. Protease Activity (With One ml of One Percent Substrate) of the OXT Compared to That of Trypsin

The assay was conducted by the method of Kunitz (37).

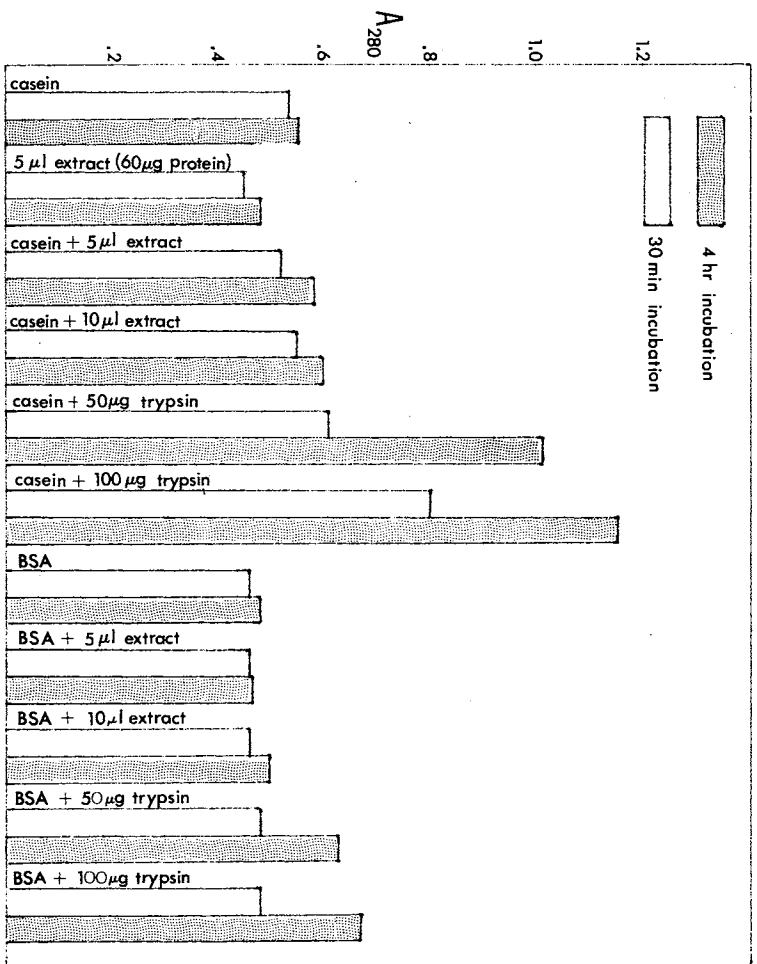
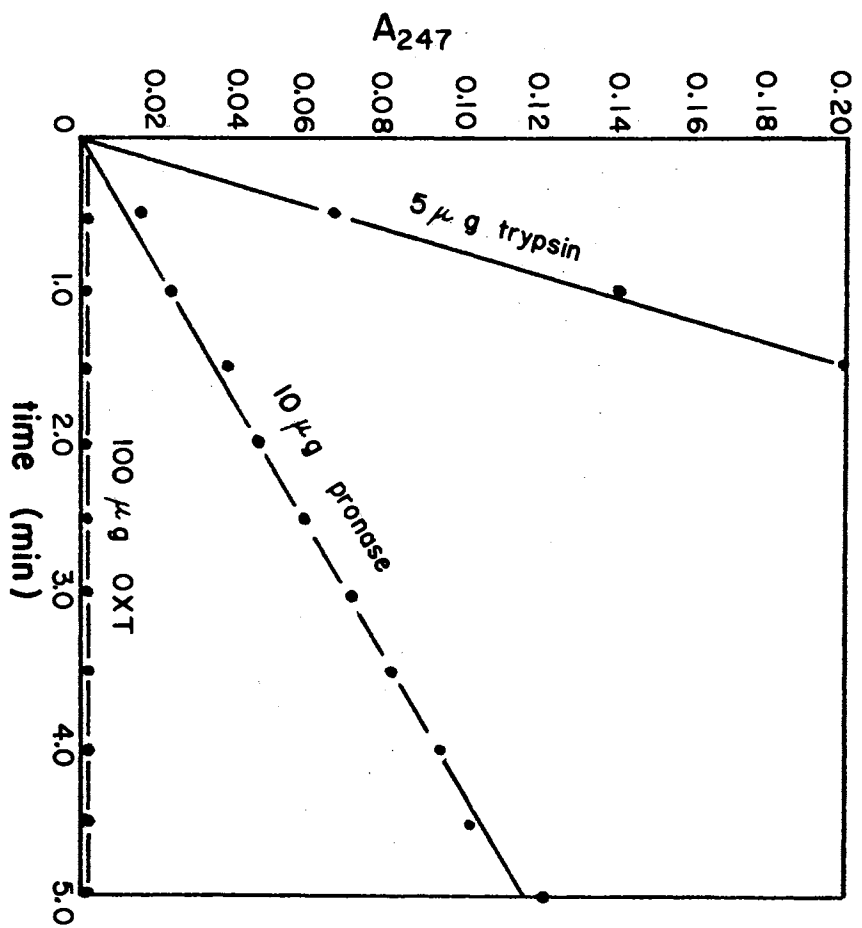


TABLE VI
PHOSPHOHYDROLASE ACTIVITY OF OXT WITH ATP AND GTP*

Substrate (10^{-4} M)	30 min incubation	90 min incubation
	Moles Pi released/mg OXT/min	
GTP	2.68×10^{-8}	2.03×10^{-8}
ATP	0.97×10^{-8}	0.98×10^{-8}

*Activity was determined by the release of free Pi by the method described by Marsh (38). Each value represents the difference between the active mixture and a control with the same components except the OXT had been heat denatured.

Figure 7. Phosphohydrolase Activity of OXT With
p-Nitrophenyl Phosphate

The assay is that of Garen and Levinthal (39). The
assay mixture contained 50 μ g of OXT protein.

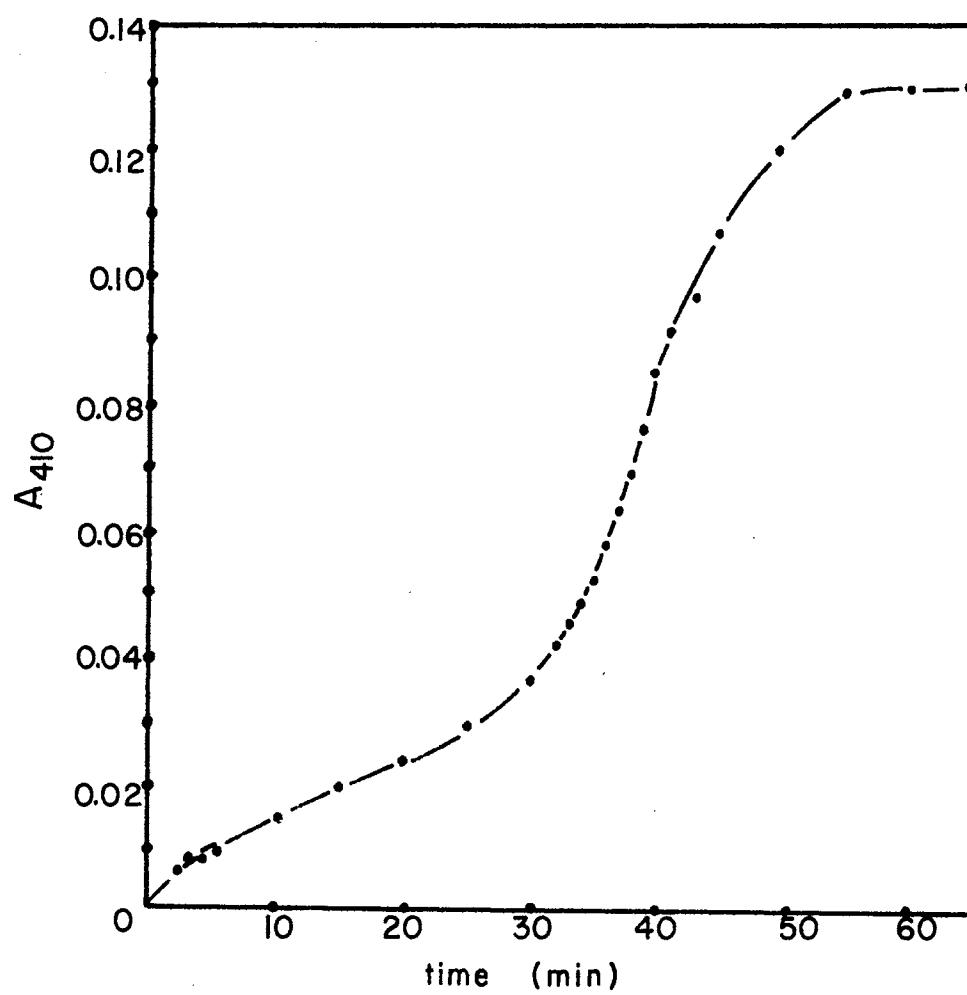
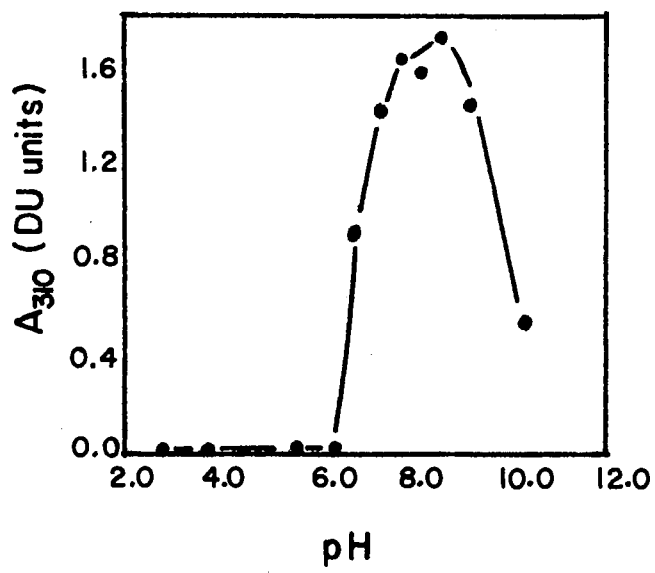
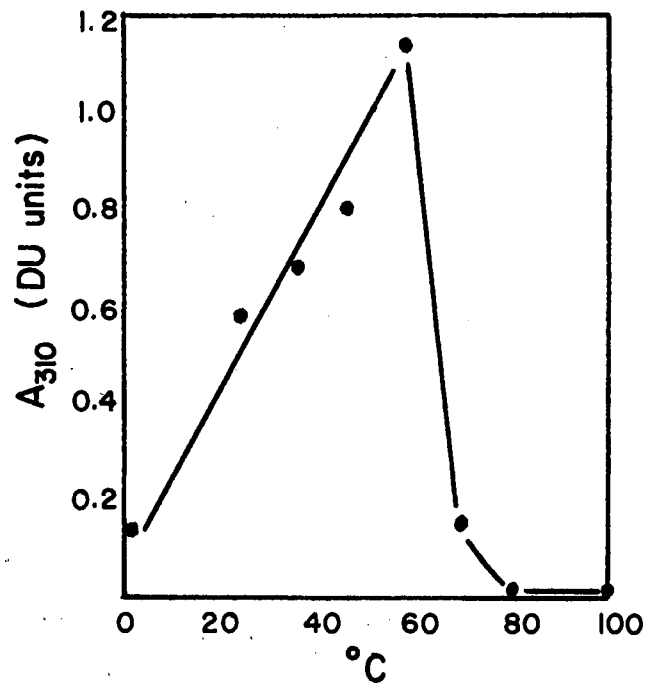


Figure 8. Temperature Optimum of the Phosphohydrolase Activity of the OXT

Assay conditions are described in the procedures chapter, page 12.

Figure 9. pH Optimum of the OXT Phosphohydrolase Activity

Assay conditions are described in the procedures chapter, pages 12-13.



activity. These values were determined to optimize conditions for assays on fractions obtained from the OXT. The sigmoid shape of the phosphohydrolase activity curve in Figure 7 may indicate product activation but this aspect was not investigated further. It is interesting to note that Wright (22) detected no phosphatase activity in venom obtained by microdissection.

I. Hyaluronidase Assays

Hyaluronidase activity was present in the OXT. Wright (22) has characterized this activity extensively and no further work was attempted other than to correlate the location of hyaluronidase activity with one of the fractions isolated from the OXT. Ninety μg of hemolymph protein displayed no hyaluronidase activity indicating that the enzyme was present in the OXT only. Shanbacher et al. (31) have suggested that hyaluronidase is a spreading factor for other toxic components present in venoms. Wright (22) thinks that hyaluronidase has a similar role in the brown recluse spider.

J. Hemolysis Tests

No hemoglobin release was obtained with any of the 24° hemolysis tests. A slight amount of hemoglobin was released in all the 37° assays, but the amount was fairly constant from assay to assay and had no relation to the amount of OXT present. The lack of hemolytic activity is in direct conflict with the findings of Denny et al. (23) but is in agreement with Smith and Micks (4). It is important to note that Denny et al. used venom obtained by electrostimulation.

K. Thin-Layer Gel Chromatography

TLG chromatography experiments revealed four major coomassie blue positive components in the OXT. The approximate average molecular weights of these components were 72,000, 53,000, 35,000, and 24,000. Resolution of components was not complete, but this partial separation indicated that Sephadex column chromatography would be effective in fractionating larger quantities of OXT.

L. Fractionation of OXT and Biological

Properties of Resulting Fractions

Figure 10 illustrates the separation achieved with the G-25 column described earlier. In this particular case the OXT separated was obtained from 100 female spiders. The separation pattern with male OXT is the same. Lethality to mice and rabbits and lesion causing ability are in the high molecular weight fraction (HMW). HMW is also toxic to roaches. The low molecular weight fraction (LMW) had an A_{280}/A_{260} ratio of 0.61 indicating the possible presence of nucleotides. The HMW had an A_{280}/A_{260} value of 1.40.

Figure 11 shows the separation of the HMW of 200 female spiders by the G-100 column. Fraction III is toxic to mammals (both lesion causing and lethal) while IV is toxic to roaches. The data for localization of roach toxin(s) is given in Table VII. The amounts of fractions in the 10 μ l volumes (Table VII) were approximately equal to the amount of each obtained from one spider. Twice the quantity of OXT injected approximated the amount obtained from one spider. If the amounts of protein of all fractions are summed, a value of 91 μ g is obtained. This compares well with 104 μ g protein obtained from one spider in the OXT used, es-

Figure 10. Separation of OXT on G-25 Sephadex

The column was 0.8 X 80 cm and had a flow rate of 5.5 ml/hr. The buffer was 0.02 M NH_4HCO_3 and the OXT was obtained from 100 female spiders. A_{280} values were obtained on a Perkin-Elmer Coleman 101 Spectrophotometer.

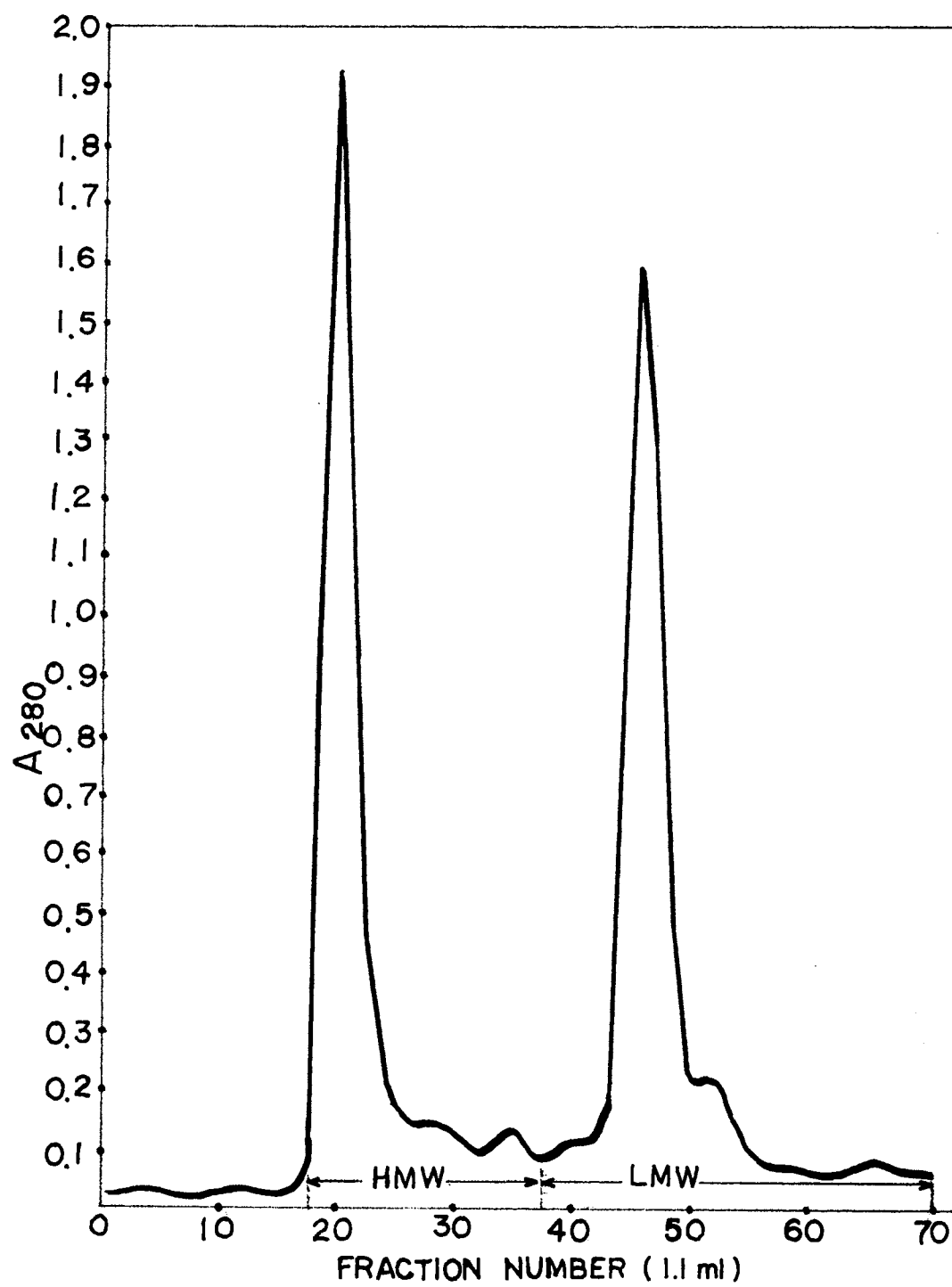


Figure 11. Separation of G-25 HMW on G-100 Sephadex

The column was 1.2 X 60 cm and had a flow rate of .8 ml/hr. The buffer was 0.02 M NH_4HCO_3 . Absorbance values were obtained on a Perkin-Elmer Coleman 101 Spectrophotometer. The G-25 HMW was obtained from 200 female spiders.

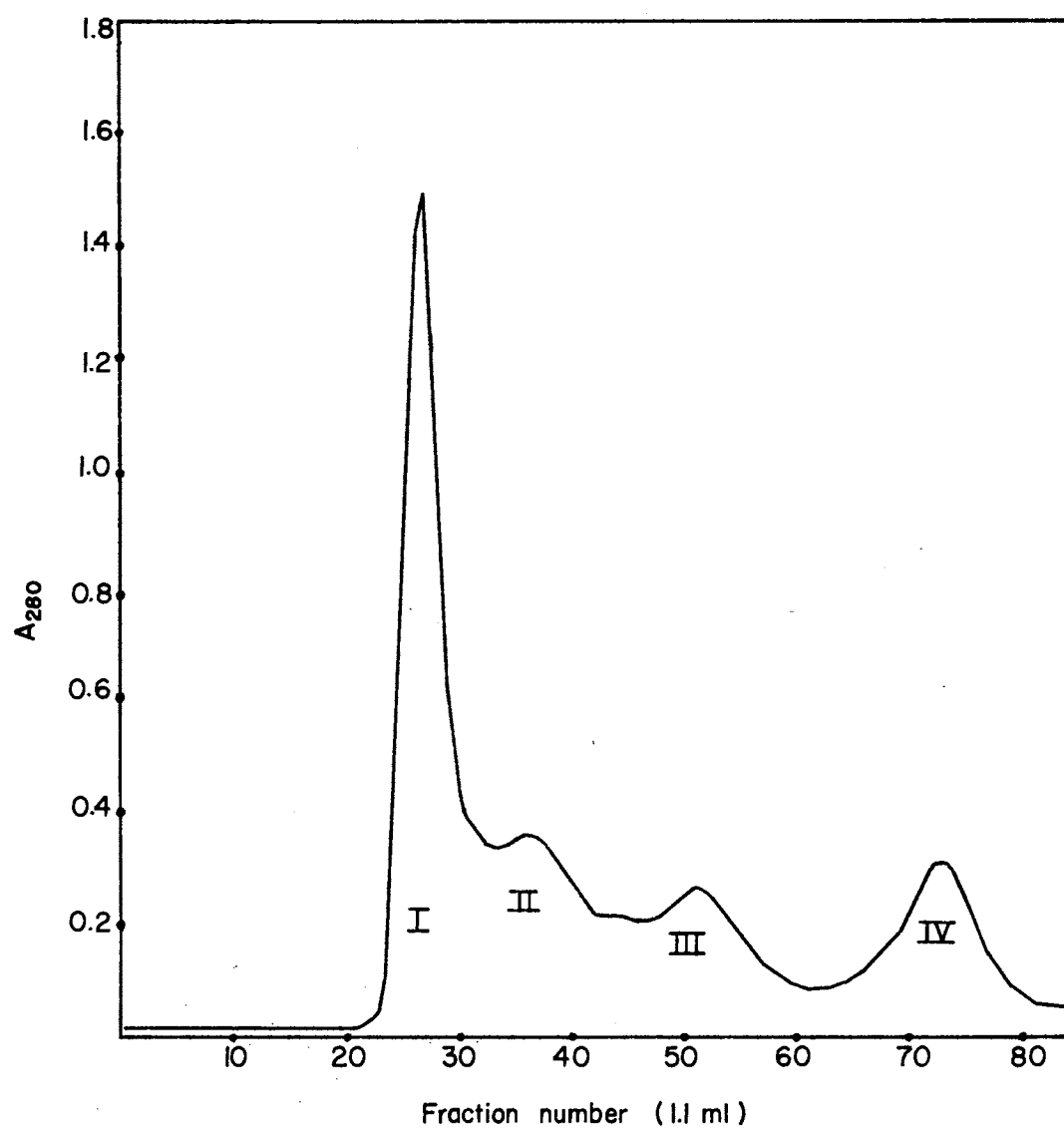


TABLE VII
TOXICITY OF OXT FRACTIONS TO ROACHES

No. of roaches	Material injected*	Volume injected μg	Amount protein μg	No. dead at 24 hr
5	G-25 LMW	10	28	0
5	G-100 I	10	20	2
3	G-100 I	15	30	1
5	G-100 II	10	17	0
5	G-100 III	10	16	0
5	G-100 IV	10	9	2
4	G-100 IV	15	14	4
3	OXT	10	52	3
3	0.02 M NH_4HCO_3	15	--	0

*All samples were dissolved in 0.02 M NH_4HCO_3 and injected intra-abdominally into adult American Cockroaches.

pecially in view of the fact that these fractions were obtained from a different OXT. As shown in Table VII, fraction IV contains toxicity to roaches while III is nontoxic. Fraction II contains some toxicity, but it does not increase with an increase in protein. It should be stated that the effect of both OXT and fraction IV is immediate paralysis of the roaches even with sublethal amounts of protein. Fraction II does not show this property.

Figure 12 shows analytical disc gels (coomassie blue stain) of G-25 and G-100 fractions compared to that of OXT. Fraction G-100 III showed no bands when stained by the Schiff method (47) while 50 μ g of transferrin was very visible by the same technique. This is weak evidence for the absence of glycoproteins as major components of G-100 III. A scan of the coomassie blue stained analytical gel of fraction III is shown in Figure 13. The bands were further separated by the preparative technique. The fast running G-50 column was used to remove acrylamide from the protein obtained from the preparative gels. Figure 14 shows such a separation. A_{220} was read rather than A_{280} due to the small amount of protein present. The acrylamide must be removed because it is toxic and also it interferes with Lowry protein determinations.

Toxicity to rabbits was found to be located in the bands labeled toxins 1 and 2 on Figure 13. The remainder of the bands were nontoxic in quantities up to the amount of these components obtained from 100 spiders. Figure 15 shows the reproducibility of preparative gels of different G-100 fraction III's. The gel to the extreme right is of male G-100 fraction III. A single faint band is apparent between toxins 1 and 2 in the male fraction III only. This band (obtained from 64 spiders) was nontoxic to mice. Figure 16 shows disc gel patterns of (from left

Figure 12. Disc Acrylamide Gel Electrophoresis Patterns of G-25 and G-100 Fractions Compared to OXT

The gels contained (from left to right) G-100 I, 60 μ g, G-100 II, 50 μ g, G-100 III, 50 μ g, G-100 IV, 30 μ g, G-25 HMW, 60 μ g, G-25 LMW, 90 μ g, and OXT, 50 μ g. The gels were pH 9.5, seven percent acrylamide and electrophoresis was at four mA/gel for 1.25 hr. The gels were stained with coomassie blue.

Figure 13. Scan of Analytical Disc Gel Electrophoresis Pattern of G-100 Fraction III

The gel was scanned at 550 nm on a Beckman DU Spectrophotometer with a Gilford Model 410 Gel Scanning Attachment. The gel was pH 9.5, seven percent acrylamide and electrophoresis was at four mA for 1.25 hr. The stain was coomassie blue.

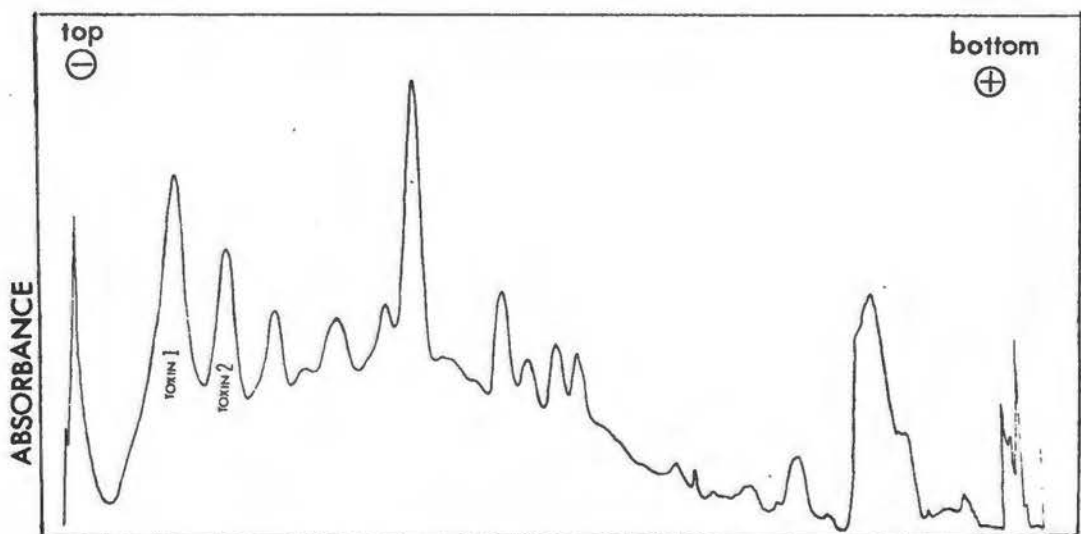
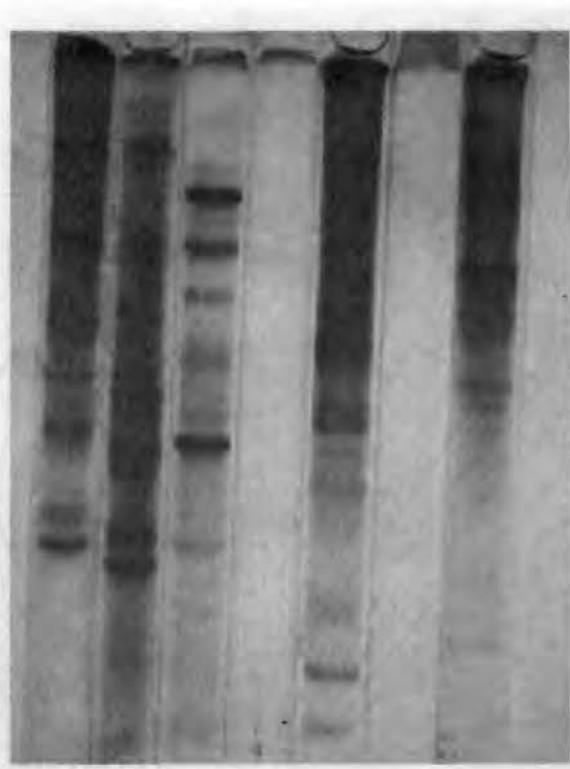


Figure 14. Separation of Acrylamide From a Protein Band
Cut From a Preparative Disc Gel

The column was 2 X 20 cm G-50 Sephadex and had a flow rate of 30 ml/hr. Elutant was 0.02 M NH_4HCO_3 and the toxin was obtained from 64 spiders. A_{220} values were obtained on a Perkin-Elmer Coleman 101 Spectrophotometer.

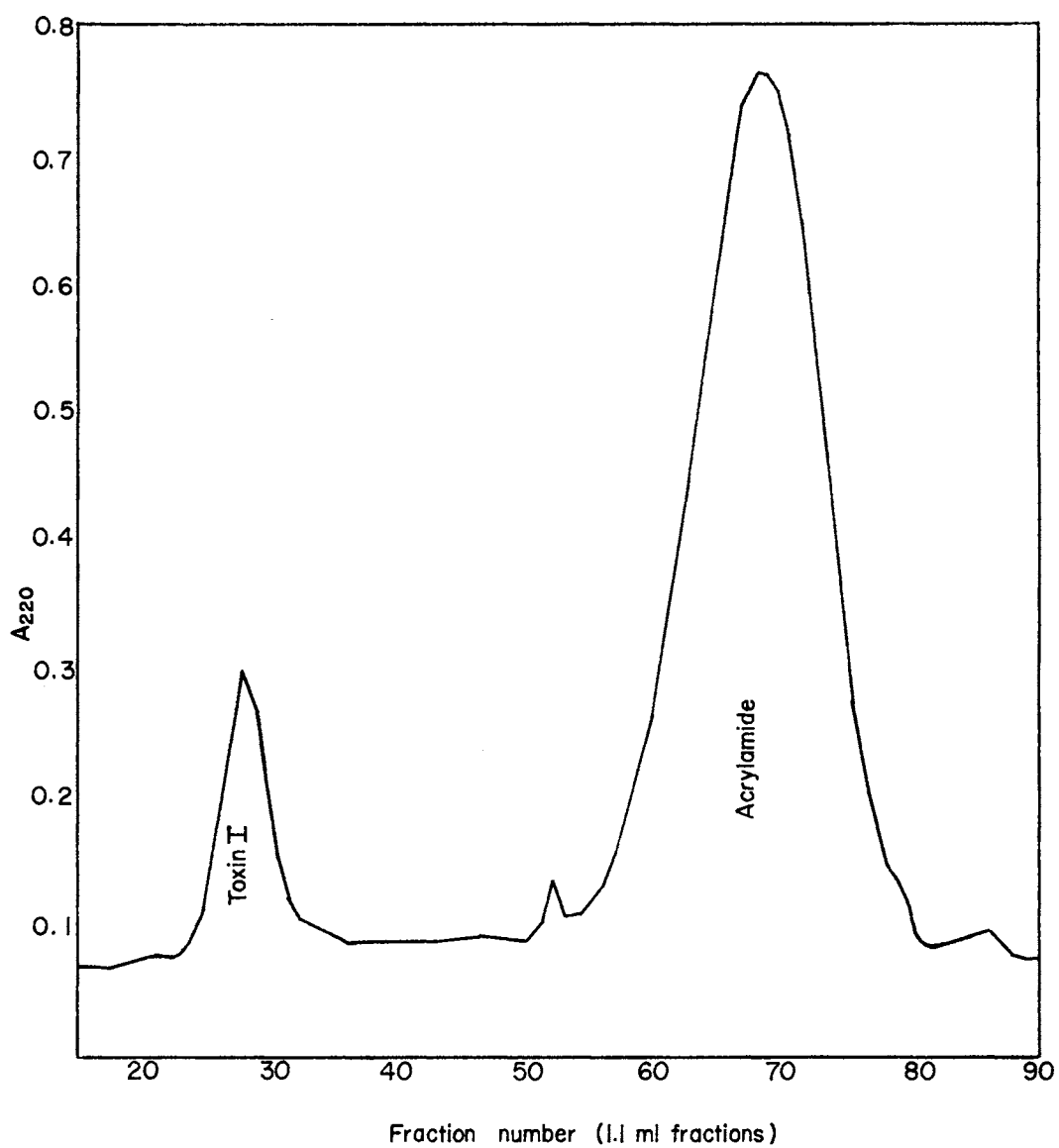
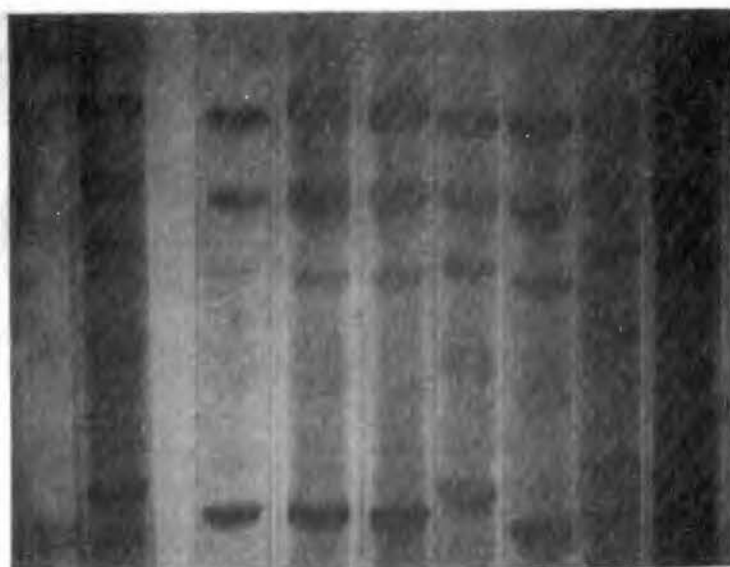


Figure 15. Coomassie Blue Stained Gels From Different G-100 Fraction III Preparative Electrophoretic Separations

The gels were pH 9.5, seven percent acrylamide and electrophoresis was at four mA/gel for three hr. Most gels contained approximately 180 μ g of Fraction III protein.

Figure 16. Comparison of Hemolymph With Toxic G-100 Fraction III by Disc Gel Electrophoresis

From left to right the gels contained 0.5 μ l female hemolymph, 0.5 μ l male hemolymph, and 180 μ g female G-100 fraction III. The gels were pH 9.5, seven percent acrylamide and electrophoresis was at four mA/gel for 1.25 hr. The gels were stained with coomassie blue.



to right) 0.5 μ l male hemolymph, 0.5 μ l female hemolymph, and 180 μ g of female G-100 fraction III. Obviously the toxic bands are absent in the hemolymph.

Toxin 1 appears to be the lesion causing agent in rabbits when small amounts are injected s.c. Larger amounts of toxin 1 are lethal to mice (i.p.) and rabbits (i.p. or s.c.). Table VIII is a purification table of the OXT including toxicity tests (i.p.) in mice. As Table VIII shows, the G-100 fraction III represents 17 percent of the OXT protein and retains 41 percent of the original toxicity to mice. The second method of quantitating the amount of toxins 1 and 2 using analytical disc gel scans yields 138 μ g toxin 2 and 250 μ g toxin 1 from the OXT of 100 spiders. This method is of limited value. Fishbein (55) states that coomassie blue follows Beer's Law only over a very narrow range and the binding of the stain varies with the type of protein. Fishbein also presents data that show that less coomassie blue is absorbed by the protein the further it migrates in the gel. Regardless, it appears that incomplete extraction has been achieved from the preparative gels due to the very great difference between Lowry determinations on the extracted toxins and scan determinations on the gels. Longer extraction times yielded no increase in extracted protein. The possibility also exists that the protein of toxins 1 and 2 can not be accurately quantitated by the Lowry technique. The data on determination of the LD_{50} of the OXT have been presented earlier. Forty mice were used for the G-25 HMW determination with 26 surviving. For the G-100 fraction III, 28 were used of which 18 survived. For the purified toxins 1 and 2 only five 25 g mice were used for each. The data are summarized in Table IX. These data indicate that only toxin 1 is lethal to mice. This was not expected

TABLE VIII
PURIFICATION TABLE OF SPIDER TOXINS*

Fraction	Total protein (mg)	LD ₅₀ in mice μg/g body weight	lethal units**	Toxicity retained %
OXT	10.0	4.6	2.2×10^3	100
G-25 HMW	6.2	4.3	1.4×10^3	64
G-100 III	1.7	2.0	0.9×10^3	41
Toxin 1	0.041	~1.0	~41	0.02
Toxin 2	0.045	---	-----	----

*The protein concentrations represent an average of 5 different preparations of 100 spiders each.

**A lethal unit is defined as the μg/g LD₅₀ of that fraction and is obtained by dividing total protein by LD₅₀ (μg/g).

TABLE IX
EFFECTS OF TOXINS 1 AND 2 IN MICE

Toxin	protein injected (i.p.) μg	result (48 hr)
1	5	survived
1	10	survived
1	20	survived
1	30	died
1	40	died
2	5	survived
2	10	survived
2	20	survived
2	30	survived
2	40	survived

as mice do not develop skin lesions. Mice injected with toxin 2 seemed hyperactive for the first hour following injection, but no permanent effect was seen even though animals were observed for two weeks post-injection.

The effect of toxin 1 in rabbits is summarized by Table X. Figures 17 and 18 show the lesion generated by 28 μ g of toxin 1 at 24 hr and 34 days after injection. The effect of toxin 2 in rabbits is shown in Table XI. The s.c. route was included to reduce toxicity and allow lesion formation. The animal injected with the lowest dose of toxin 2 exhibited all the symptoms shown by earlier rabbits with lethal doses other than convulsions and death. These symptoms were also the same as shown by rabbits in the OXT LD₅₀ experiment. The symptoms include a comatose state starting 4 to 6 hr after injection and lasting approximately six hr. During this time the rabbits exhibit severe diarrhea. The rabbits usually die after 12 to 20 hr although some have survived almost 48 hr. Death is preceded by violent convulsions. Figure 19 shows the site of the 12 μ g injection of toxin 2 immediately after death. Obviously there is no severe reaction of the skin to toxin 2 indicating that it is not the lesion causing agent.

Fractions of OXT were also approximately quantitated as to lesion causing activity by the method of Kondo et al. (35). The data is shown in Table XII. Four rabbits were used to obtain these data. The OXT value is based on the average of six induced lesions, while the G-25 HMW is on four. Both the G-100 III and toxin 1 values are based on the average of two lesions each. Figure 20 is a photograph showing two of the lesions generated for Table XII. The light source was behind the skin. The small lesion was due to 18 μ g of G-25 HMW while the very large

TABLE X
EFFECT OF TOXIN 1 IN RABBITS

Toxin 1 μg (Lowry)*	Route of injection	Result
45	i.p.	dead (24 hr)
20	i.p.	dead (7 days)
28	s.c.	lesion
10	s.c.	small lesion

*Each amount of purified toxin was injected into a single rabbit.

TABLE XI
EFFECT OF TOXIN 2 IN RABBITS

Toxin 2 μg (Lowry)*	Route of injection	Result (48 hr)
25	i.p.	dead
16	i.p.	dead
12	s.c.	dead
6	s.c.	survived

*Each amount of purified toxin was injected into a single rabbit.

TABLE XII
LESION CAUSING ABILITY OF OXT AND ISOLATED
FRACTIONS IN RABBITS

Fraction	Diameter of lesion/ μ g protein mm
OXT ¹	0.7
G-25 HMW ²	1.3
G-100 III ³	2.1
Toxin 1 ⁴	1.8

¹The value represents the average of six lesions induced in a single rabbit.

²The G-25 HMW value represents the average of 4 lesions induced in two rabbits.

³The G-100 III value represents the average of 2 lesions induced in two rabbits.

⁴The toxin 1 value represents the average of 2 lesions induced in two rabbits.

Figure 17. Effect of Toxin 1 24 hr After Injection

The rabbit was injected (s.c.) with 28 μ g of purified toxin 1.

Figure 18. Effect of Toxin 1 34 Days After Injection

This is the same rabbit shown in Figure 17.

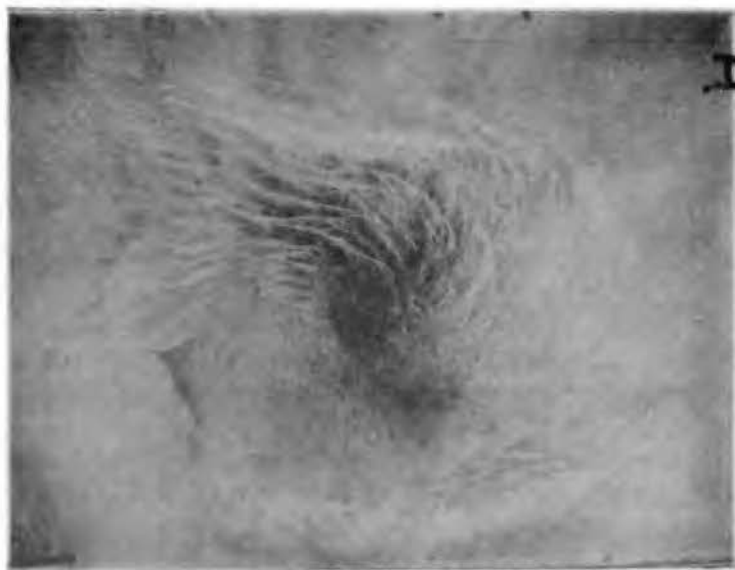
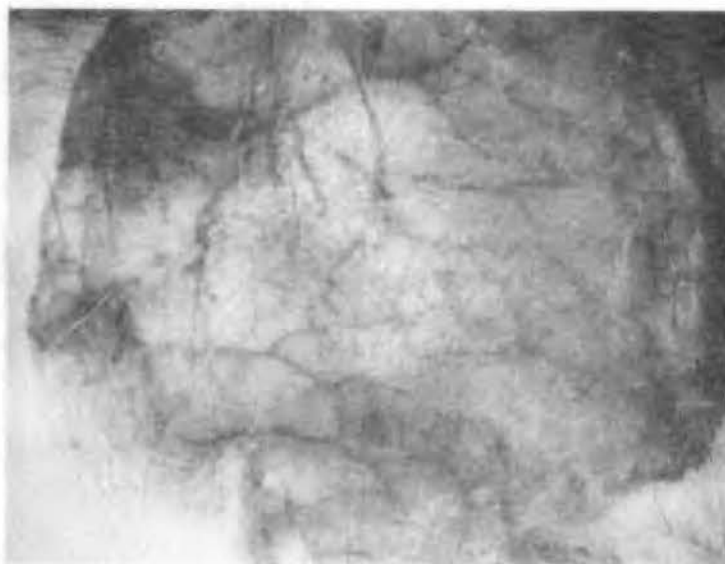


Figure 19. Lack of Skin Reaction in Rabbit With
Lethal Dose of Toxin 2

The rabbit was injected (s.c.) with 12 μ g of purified toxin 2 and died 12 hr after injection. The skin was removed immediately after the rabbit died.

Figure 20. Rabbit Skin Lesions Generated by G-25
HMW and G-100 Fraction III

The lesion pictured on the left was generated by 18 μ g of HMW protein while the lesion on the right was by 16 μ g of G-100 fraction III protein.



lesion was due to 16 μ g of G-100 fraction III. The values obtained in all rabbit skin lesion tests should be considered as approximations. The method has many variables including depth and location of injection, intraspecies variation in response to venom, and determination of exact size of lesion.

Figures 21 and 22 show purified toxin 2 electrophoresed in different gel systems. In all toxin 2 appears to be a single band. Attempts to prove purity of toxin 1 by the same method were not as conclusive. Figure 23 shows toxin 1 electrophoresed in the same gel system in which preparative electrophoresis was accomplished. Toxin 2 was included for comparison. Toxin 1 is very diffuse. In the other gel systems toxin 1 gave a very diffuse band or none at all. The lack of a sharp band on disc gel electrophoresis of toxin 1 could be due to its instability under the described experimental conditions. This instability could account for the drastic decrease, during purification, of lethality in mice and lesion causing ability in rabbits. A second possible cause of this decrease in toxicity is that other components separated from the toxin have a synergistic effect on its biological activity. A third possibility, at least with regard to the toxicity of toxin 1 in mice, is that one of the other components of OXT G-100 fraction III is toxic, but is denatured during the process of preparative electrophoresis.

Numerous problems were encountered during the development of the isolation procedure for toxins 1 and 2. All column steps must be maintained at 4°C as toxicity is lost when separation is attempted at room temperature. The G-25 column step appears unnecessary. If the OXT were placed directly on the G-100 column, the LMW fraction should come out in the salt peak. This was tried twice (200 spider's OXT each time). Sepa-

Figure 21. Electrophoresis of Purified Toxin 2
in Different Gel Systems

Gel systems utilized included (from left to right)
pH 4.3, seven percent acrylamide, pH 4.3, 15
percent acrylamide, and pH 9.5, seven percent
acrylamide. The amount of toxin applied to each
gel was 1/5 the amount obtained from 64 spiders.
Electrophoresis was at four mA/gel for 1.75 hr.

Figure 22. Electrophoresis of Purified Toxin 2

The gel was pH 9.5, 15 percent acrylamide and con-
tained 1/5 the purified toxin 2 obtained from
64 spiders. Electrophoresis was at four mA for
1.75 hr.

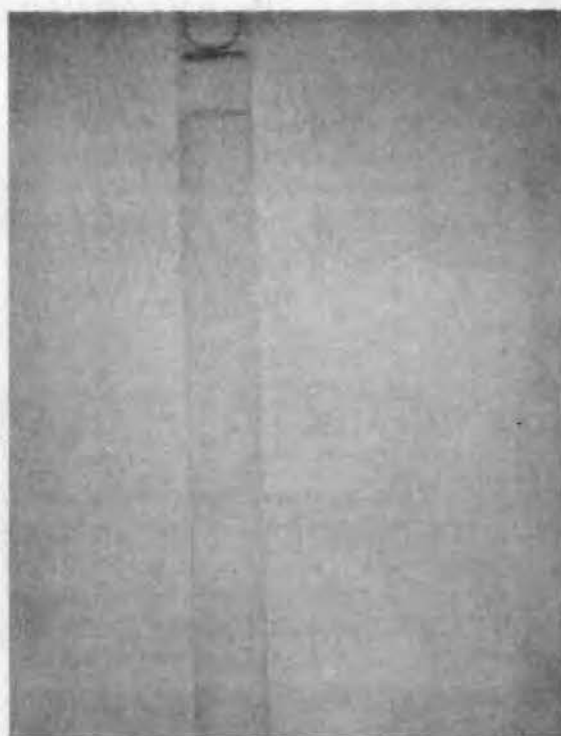
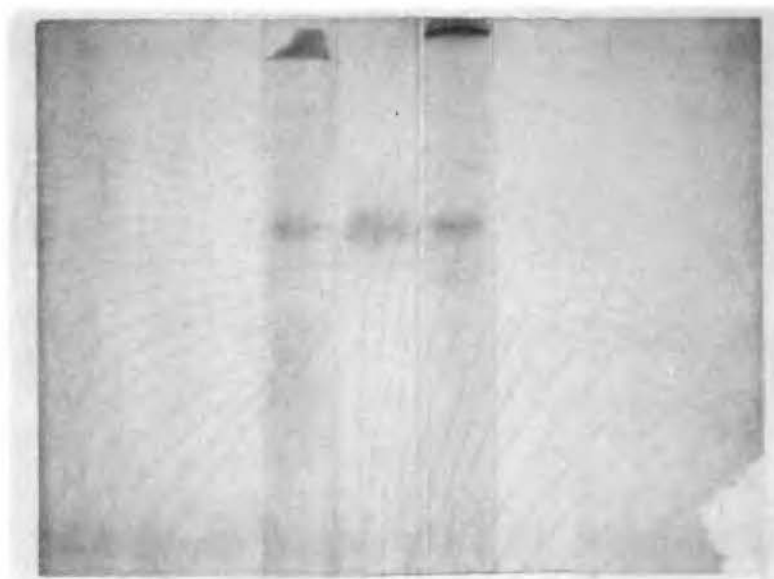
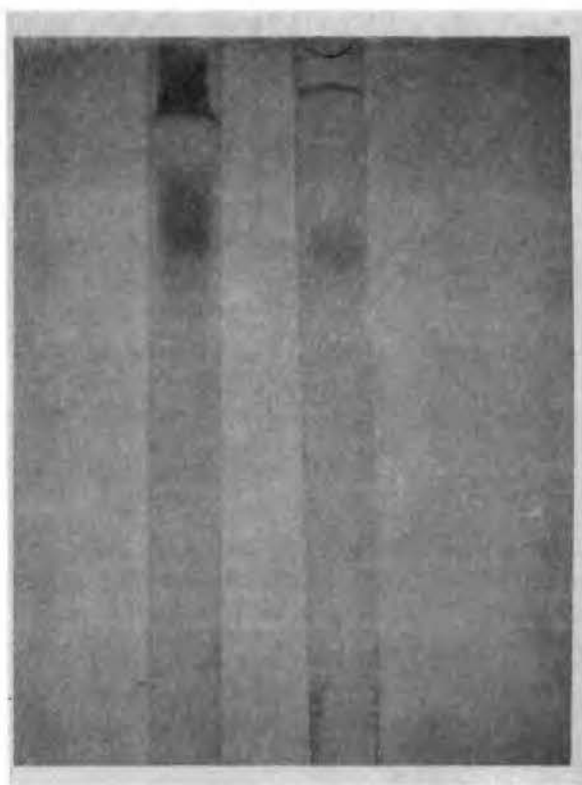


Figure 23. Electrophoresis of Purified Toxins 1
and 2

The gels were pH 9.5, seven percent acrylamide and electrophoresis was at four mA/gel for 1.75 hr. The amount of toxin applied to each gel was approximately five μ g.



ration was similar to Figure 11 with an added salt peak although resolution was not obtained. Analytical disc gel electrophoresis of peaks from this separation revealed no toxins 1 or 2. Toxicity (as tested in mice) was absent from all fractions. Evidently some detoxifying component is removed during the G-25 separation or insolubilized by the subsequent lyophilization. Solubilization of the G-25 HMW prior to G-100 chromatography does result in a slightly opaque solution indicating some insoluble component(s). As mentioned earlier, type of buffer is also important. If the electrophoresis apparatus was not cooled during the preparative electrophoresis step, toxic components were denatured.

M. Isoelectric Focusing

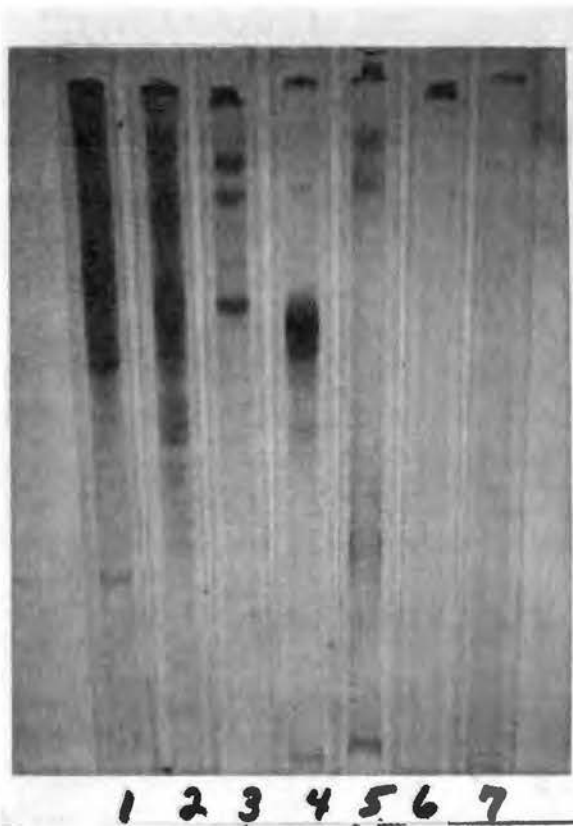
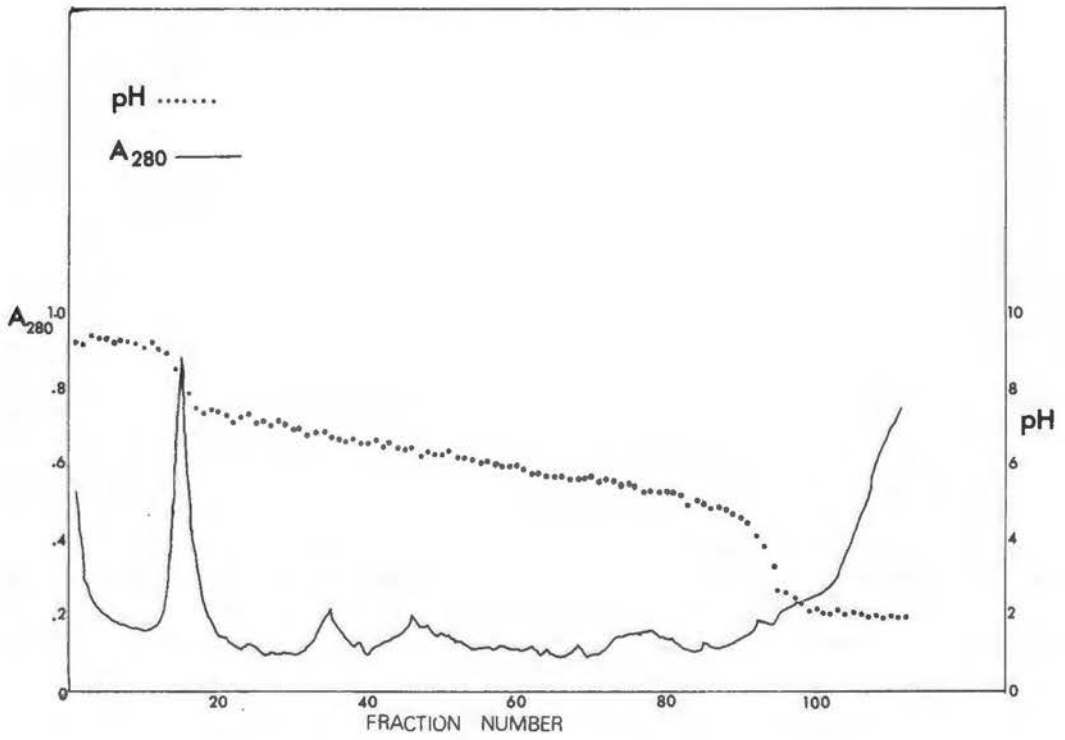
The G-100 fraction III of 400 female spiders was separated on an isoelectric focusing column. Figure 24 shows the results. Figure 25 shows analytical disc gel patterns of the following: gel 1 - 30 μ g OXT, gel 2 - 30 μ g G-25 HMW, gel 3 - 30 μ g G-100 III, gel 4 - a portion of isoelectric run fractions (iso) 71-84, gel 5 - iso 12-23, gel 6 - iso 30-40 and gel 7 - iso 41-54. The iso 12-23 contains the toxic activity. Attempts to separate the ampholine from the protein by dialysis were successful with iso 71-84, but dialysis of iso 12-23 resulted in the loss of biological activity. Disc gel electrophoresis of the dialyzed material revealed no bands indicating that the toxins either bound to or passed through the dialysis membrane. Figure 24 indicates that the isoelectric pH of both toxins 1 and 2 is approximately 8.3 since both toxins 1 and 2 are found in iso 12-23. A higher pH range of ampholine might achieve separation of toxin 1 from toxin 2, but the amount of G-100 fraction III required and the length of time involved were prohibitive,

Figure 24. Isoelectric Focusing of G-100 Fraction III

The G-100 fraction III was obtained from 400 female spiders. The isoelectric focusing procedure was that of Haglund (49). Fraction size was 1.1 ml. A_{280} values were obtained on an Hitachi Perkin-Elmer Coleman 124 Spectrophotometer. The ampholine was pH 5-8. The pH of the fractions was determined using a Fisher Model 320 pH Meter with a microelectrode.

Figure 25. Isoelectrofocused Fractions Compared to OXT, HMW, and G-100 Fraction III by Disc Gel Electrophoresis

The gels contained the following samples: gel 1 - 30 μ g OXT, gel 2 - 30 μ g G-25 HMW, gel 3 - 30 μ g G-100 III, gel 4 - iso 71-84, gel 5 - iso 12-23, gel 6 - iso 30-40, and gel 7 - iso 41-54. The gels were pH 9.5, seven percent acrylamide and electrophoresis was at four mA/gel for 1.75 hr.



especially in view of the difficulty in removing the ampholine.

N. Molecular Weight Determinations

The G-100 column was calibrated and the molecular weights of G-100 fractions III and IV were determined. Figure 26 shows the plot obtained. Fraction III had a molecular weight of 32,400 while that of IV was 6,800. Since IV shows only one distinct and one faint band on polyacrylamide gel electrophoresis, Figure 12, the value of 6,800 probably represents the major component.

SDS polyacrylamide gel electrophoretic determinations of the molecular weights of purified toxins 1 and 2 revealed molecular weights of 34,000 for both. This is an average value, the composite of three separate experiments. Toxin 1 is slightly heavier than toxin 2, 33,800 to 34,200 but it is doubtful if the method is this accurate. Figure 27 is a typical example of one of the SDS experiments. This particular determination yielded results of 33,000 for both toxins. Incidentally, both toxins migrated as a single band under the conditions of SDS electrophoresis.

O. Amino Acid Composition of Toxins 1 and 2

Table XIII shows the amino acid composition of toxins 1 and 2. Calculations were based on an average molecular weight of 34,000 for both toxins. The identification of methionine sulfoxide is tentative. The toxins used for analyses were isolated from 400 spiders. Analyses of toxins from 100 spiders were also accomplished but only qualitative information on the identity of amino acids could be obtained as peaks were too small for accurate integration. The recovery of norleucine from

Figure 26. Molecular Weight Determination of
G-100 III and IV by Sephadex
G-100 Column Chromatography

The column was 1.2 X 60 cm and had a flow rate of eight ml/hr. The buffer was 0.02 M NH_4HCO_3 and the protein standards were detected by the procedures described on page 16.

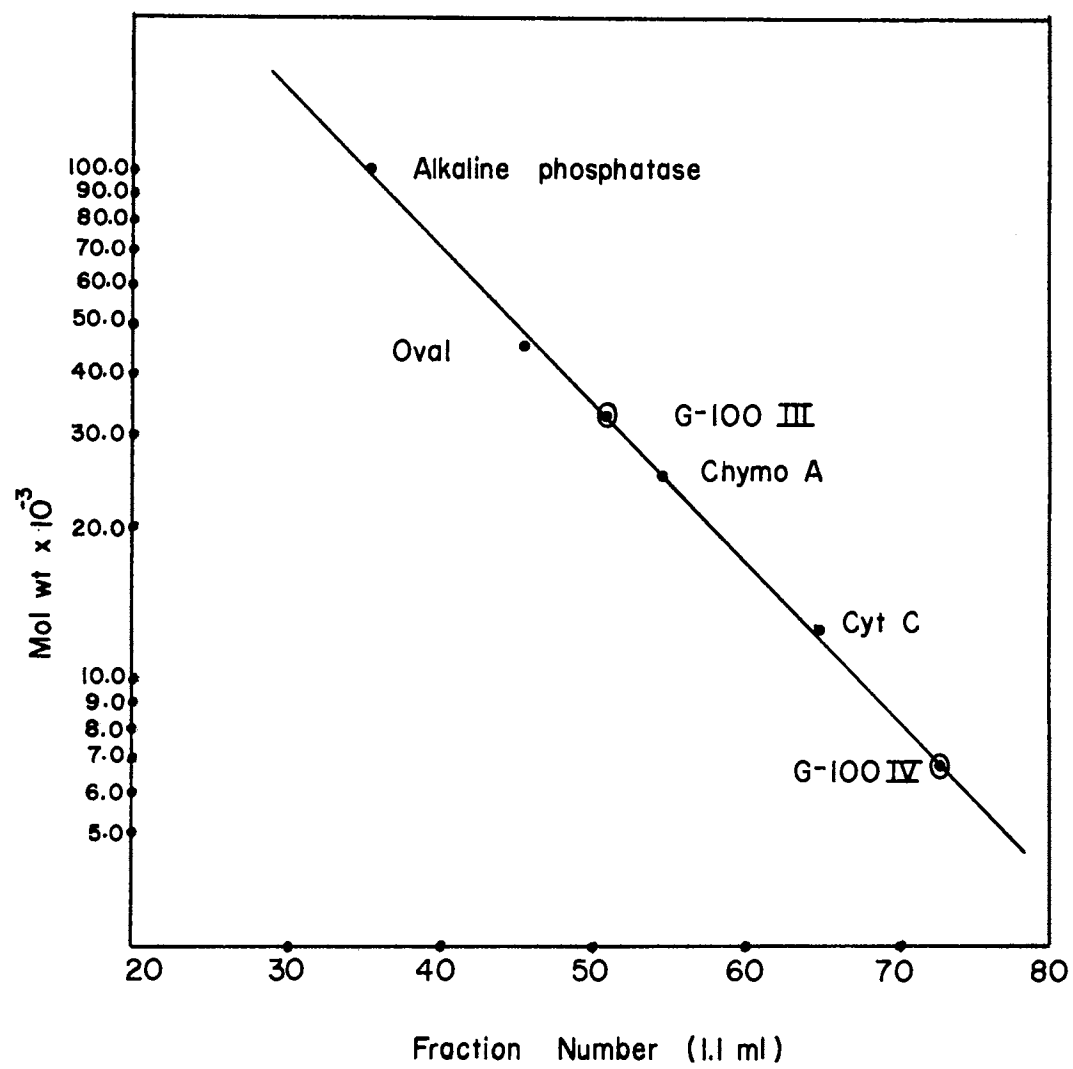


Figure 27. Molecular Weight Determination of
Toxins 1 and 2 by SDS Polyacryl-
amide Gel Electrophoresis

The method utilized was that of Weber and Osborn (45). The gels were 5 mm X 10 cm and were 10 percent acrylamide. Electrophoresis was at 8 mA/gel. Gels were pre-run for 4 hr and electrophoresis of samples was for 4 hr. Bands were visualized with coomassie blue.

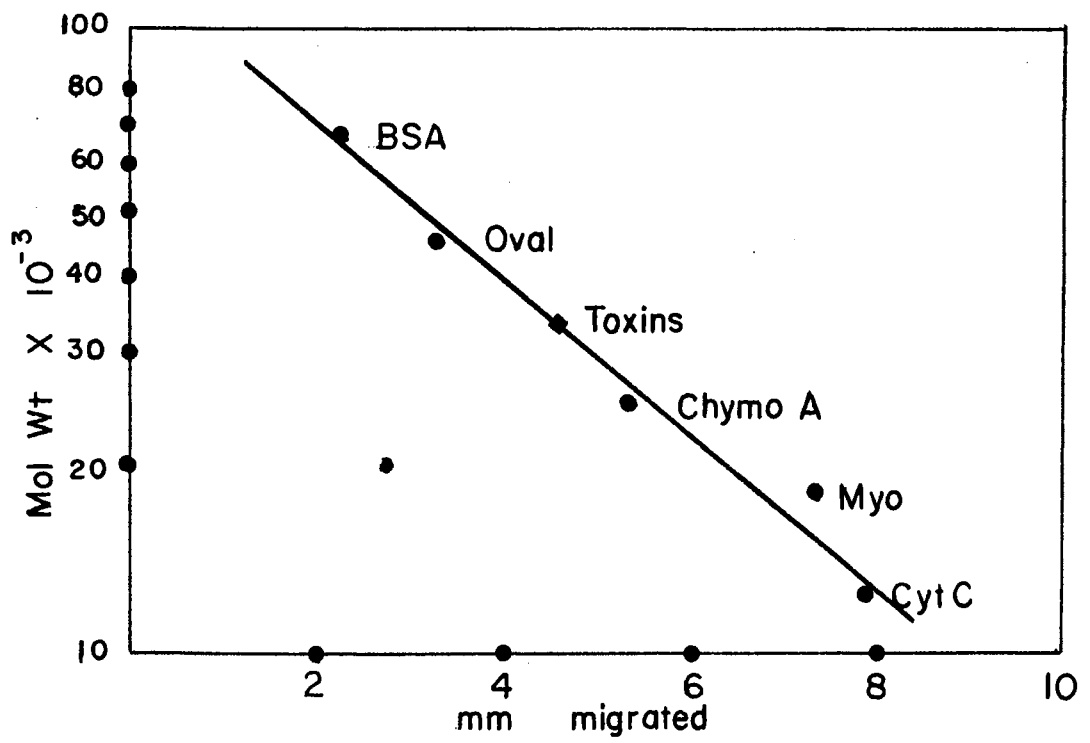


TABLE XIII
AMINO ACID COMPOSITION¹ OF TOXINS 1 AND 2²

Amino Acids	Toxin 1	Toxin 2
	No. of residues ³	
Lys	9	10
Arg	17	18
Asx	54	49
Thr	22	21
Ser	17	20
Glx	26	31
Pro	10	13
Gly	45	36
Ala	30	31
$\frac{1}{2}$ Cys	8	9
Val	24	25
Met	2	--
Ile	15	14
Leu	23	20
Tyr	7	7
Phe	15	15
Methionine Sulfoxide ⁴	trace	trace
Total residues	324	319

¹Due to the small amount of purified toxins obtained, no analysis for tryptophan was attempted.

²Hydrolysis of protein and determination of amino acids were by the procedures of Moore and Stein (47). Actual analysis was accomplished on a Beckman Model 120C Amino Acid Analyzer.

³Calculations were based on molecular weights of 34,000 for both toxins.

⁴This is a tentative identification based on retention time.

all analyses was approximately 50 percent. All analyses revealed large ammonia peaks probably indicating that not all of the acrylamide was removed by the G-50 Sephadex column.

P. Location of Hyaluronidase and Phosphohydrolase Activities in OXT Fractions

Figure 28 represents the determination of location of the phosphohydrolase activity. The activity of G-100 IV was not tested. The phosphohydrolase activity was present in the greatest amount in G-100 I. Figure 29 shows a similar determination of the location of hyaluronidase activity. The assay is of the turbidimetric type and a loss of absorbance at 540 nm represents hyaluronidase activity. The majority of the activity was present in G-100 II while almost no activity was present in I or III. As Tu (56) points out, early researchers working with animal venoms postulated that the combination of enzymes (proteases, phosphatases, hyaluronidase, etc.) was responsible for the toxicity of venoms; but, as purification procedures improved, enzymatic activity could be separated from the toxic components. This appears to be true with the brown recluse spider venom at least with respect to hyaluronidase and phosphohydrolase activities.

Q. Fractionation and Partial Characterization of LMW

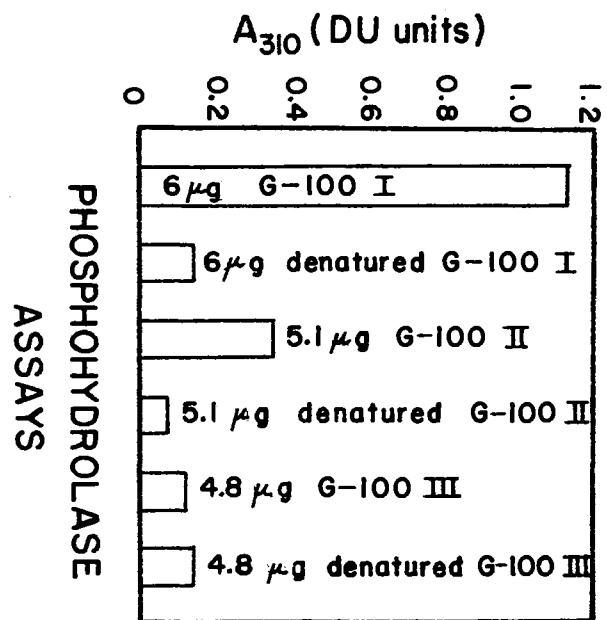
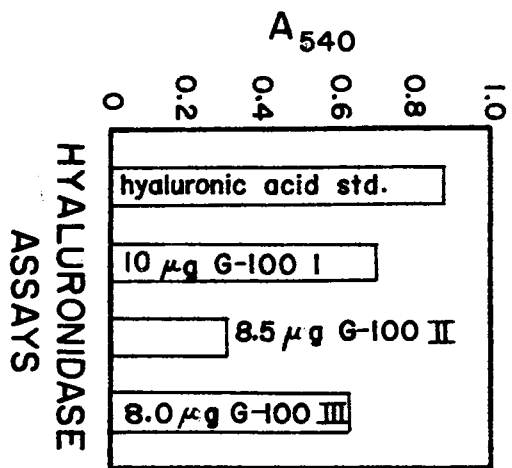
The G-25 LMW was further investigated for two reasons. First, as Table VIII shows, when LMW is removed, total toxicity decreases, and second, if LMW is not removed, toxicity is lost. These phenomena do not necessarily have to be directly related to the LMW as the loss of toxic-

Figure 28. Phosphohydrolase Activity of G-100
Fractions of OXT

Phosphohydrolase activity was determined by measuring the release of Pi from ATP. The method is described in the procedures chapter, page 12.

Figure 29. Hyaluronidase Activity of G-100 Fractions of OXT

A decrease in turbidity indicates hyaluronidase activity. The method utilized was that of Tolksdorf, et al. (40).



ity during the G-25 separation could be due to slow denaturation of the toxins. A possible cause of the loss of toxicity if the G-25 step is omitted has already been discussed. A third reason for study of the LMW was its high UV absorbance at 260 nm. Recently, Chan (57) has found that adenine nucleotides are major constituents of tarantula, Dugesiella hentzi, venom. This investigator was curious as to whether nucleotides were present in the LMW.

Q.1. Luciferase Assay on G-25 LMW

The luciferase assay (51) revealed no ATP present in the LMW fraction of 100 spiders. Using the absorbance values obtained at 280 and 260 nm the total amount of nucleotides tested was estimated to be 375 μ g by the method and nomograph of Adams (58). Figure 30 shows the sensitivity of detection of standard ATP by the luciferase method. The absence of ATP in LMW was not surprising since the OXT does contain phosphohydrolase activity.

Q.2. Toxicity of Recombined HMW and LMW

An attempt was made to regain the toxicity lost during the G-25 fractionation by recombining the HMW and LMW and testing the toxicity of the combination in mice. Table XIV summarizes the experiment and results. No synergistic effect of LMW on HMW was apparent. In fact the combination seems to be slightly less toxic than HMW alone.

Q.3. Isolation and Tentative Identification of LMW Components

Since the OXT is an extract, the hemolymph of 800 female spiders

Figure 30. Luciferase Assay ATP Standard Curve

The firefly luciferase assay of Strehler and
Totter (51) was used.

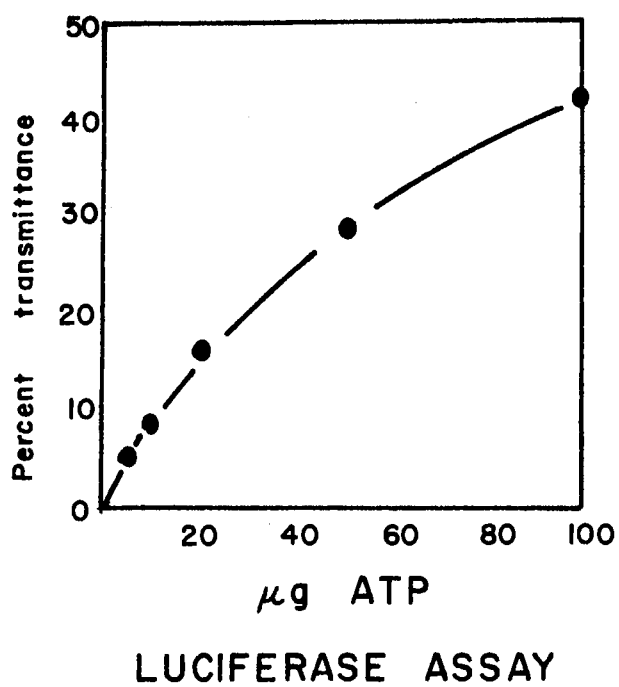


TABLE XIV
TOXICITY OF HMW, LMW, AND RECOMBINED HMW AND LMW IN MICE

No. of mice	Material injected (i.p.)	HMW protein/g body weight μg	No. dead at 48 hr
4	HMW + LMW*	3.2	0
4	HMW + LMW	3.6	0
4	HMW + LMW	4.1	1
2	HMW + LMW	4.5	1
2	HMW	2.8	0
2	HMW	3.1	1
2	HMW	3.6	1
2	HMW	3.8	2
2	LMW from 5 spiders	---	0
2	LMW from 20 spiders	---	0

* OXT from 100 spiders was separated into HMW and LMW by the G-25 column described in the Procedures Chapter. Each fraction was dissolved in 5 ml of 0.02 M NH_4HCO_3 . For the recombined fractions test, equal volumes of LMW and HMW were combined prior to injection. The protein concentration of the HMW solution was estimated by the procedure of Lowry *et al.* (33).

was collected for comparison. A hemolymph LMW was separated by use of the G-25 column. Both hemolymph and OXT LMW's were further fractionated by P-2 column chromatography. These separations are compared in Figure 31. Both separations show four major components (labeled 1,2,3 and 4 on each plot) but only peak 1 is different. The other three peaks were eluted from the P-2 column in identical locations. The OXT LMW was isolated from 340 female spiders so there is an obvious concentration of major components in the OXT. In view of their high concentration in the OXT, it was deemed worthwhile to attempt to identify all four major components of OXT LMW.

Figures 32, 33, 34 and 35 show ultraviolet spectra of OXT LMW P-2 peaks 1, 2, 3 and 4 respectively. Table XV lists the spectral data obtained from the UV spectra of the various peaks plus data on inosine and GTP obtained on the same instrument. The location of absorbance maxima and minima and the shape of the curves for peaks 1 and 2 indicated the probable presence of guanine nucleotides. The calculated data do not agree particularly well with GTP, especially at pH 11. Addition of one M KCN to peaks 3 and 4 caused no change in their spectra. The same treatment of NAD and deamino-NAD standards produced new absorbance maxima around 325 nm. This procedure eliminated the possibility of peaks 3 and 4 containing NAD or NAD analogs. The spectral data of peak 3 agreed well with that of inosine thus indicating its possible presence.

Initially thin layer chromatography was tried as a method of further fractionating and identifying the four OXT P-2 peaks but was found to require too much material. The paper chromatography systems described by Pabst Laboratories (now called P. L. biochemicals, inc.) (48) were superior. In Pabst system III peak 1 had an R_f of 0.33 which is close

Figure 31. P-2 Column Chromatography of OXT and Hemolymph LMW's

The column was 1.5 X 80 cm and had a flow rate of six ml/hr. The buffer was 0.02 M NH_4HCO_3 . The hemolymph LMW was from 800 female spiders while the OXT LMW was obtained from 340 female spiders.

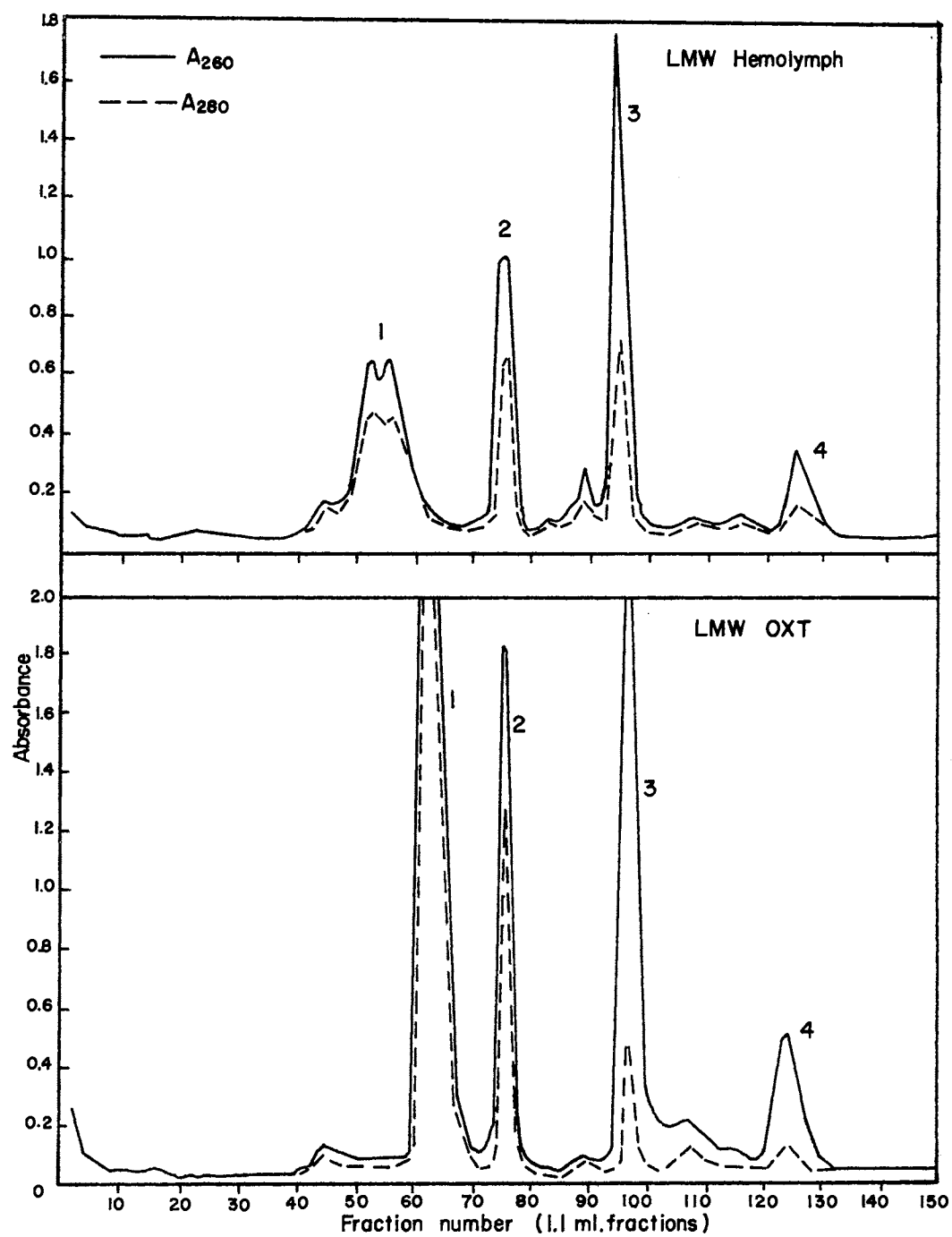


Figure 32. Ultraviolet Spectra of OXT LMW Peak 1

All spectra were recorded with a Cary 14. The sample size was one ml and the buffers were 0.1 N HCl, pH 1, 0.02 M sodium phosphate, pH 7, and 0.002 N NaOH, pH 11. The amount of material used for each spectra was that obtained from nine spiders.

Figure 33. Ultraviolet Spectra of OXT LMW Peak 2

All spectra were recorded with a Cary 14. The sample size was one ml and the buffers were 0.1 N HCl, pH 1, 0.02 M sodium phosphate, pH 7, and 0.002 N NaOH, pH 11. The amount of material used for each spectra was that obtained from 43 spiders.

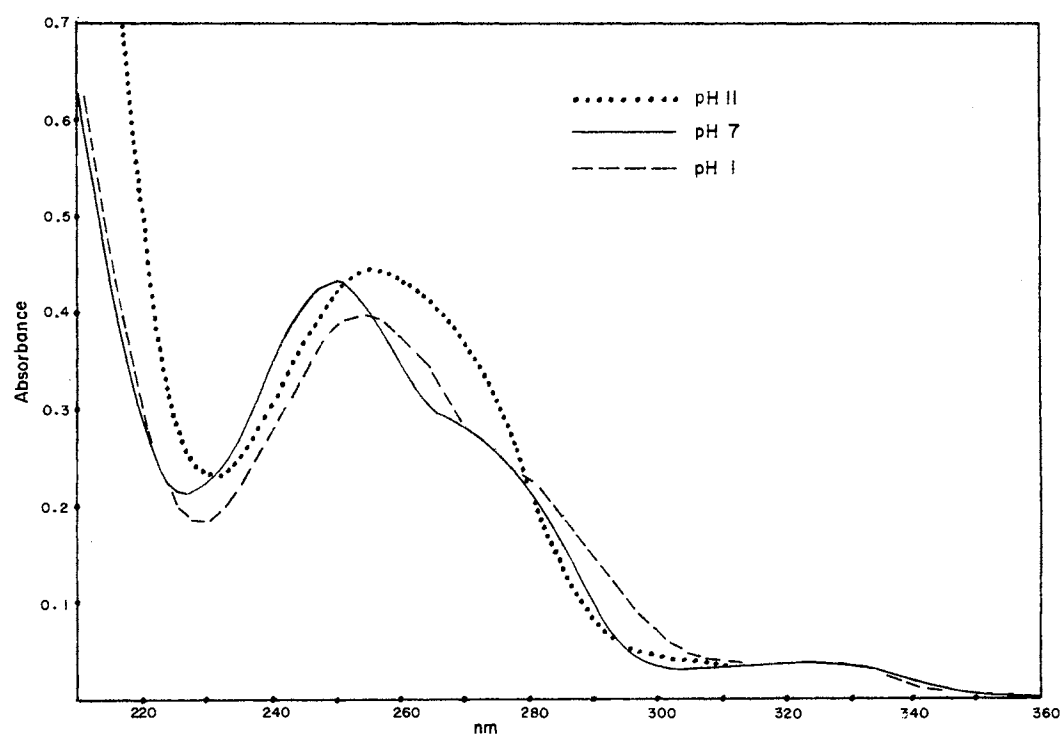
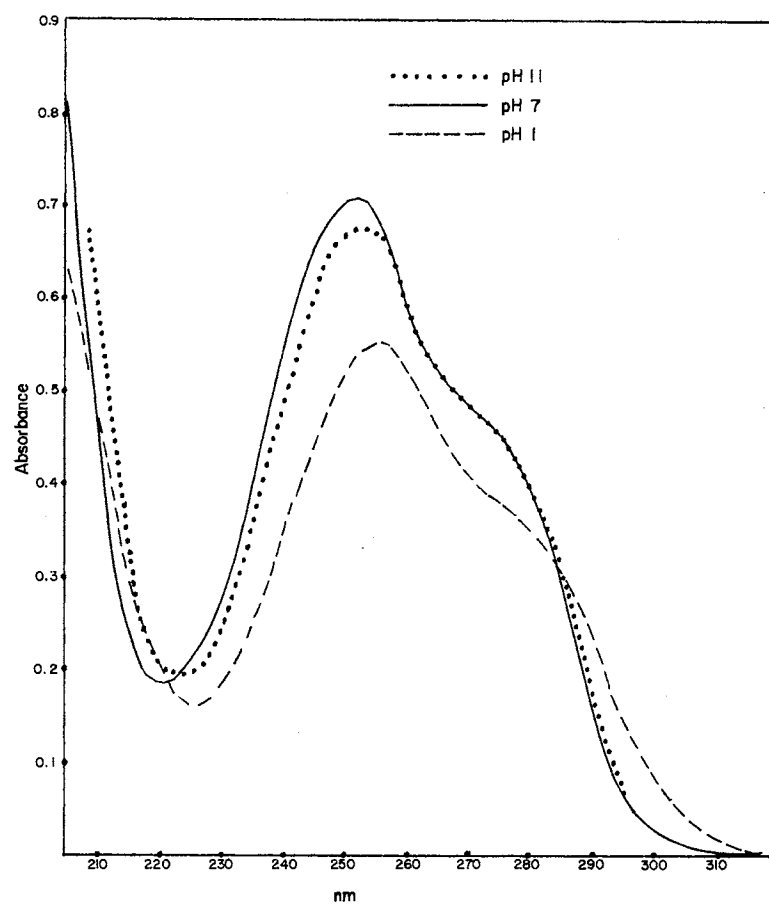


Figure 34. Ultraviolet Spectra of OXT LMW Peak 3

All spectra were recorded on a Cary 14. The sample size was one ml and contained the amount of material obtained from 32 spiders for each spectra. The buffers were 0.01 N HCl, pH 2, 0.02 M sodium phosphate, pH 7, and 0.002 N NaOH, pH 11.

Figure 35. Ultraviolet Spectra of OXT LMW Peak 4

All spectra were recorded on a Cary 14. The sample size was one ml and contained the amount of material obtained from 68 spiders. The buffers were 0.01 N HCl, pH 2 and 0.02 M sodium phosphate, pH 7.

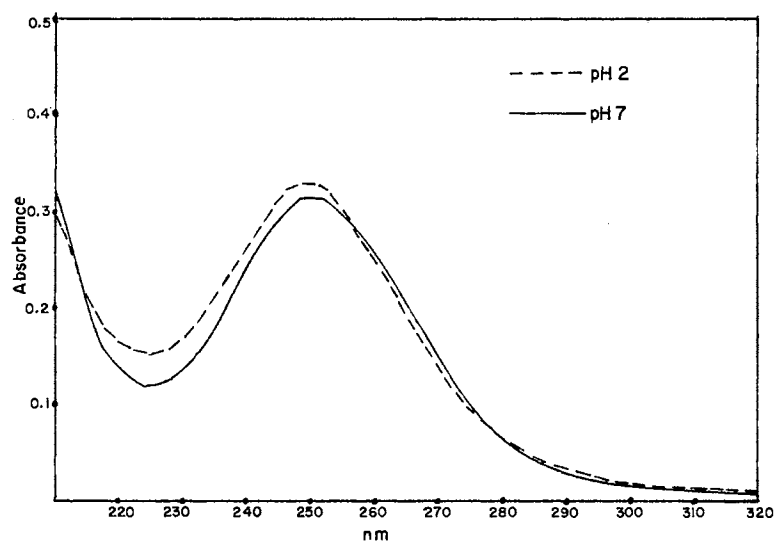
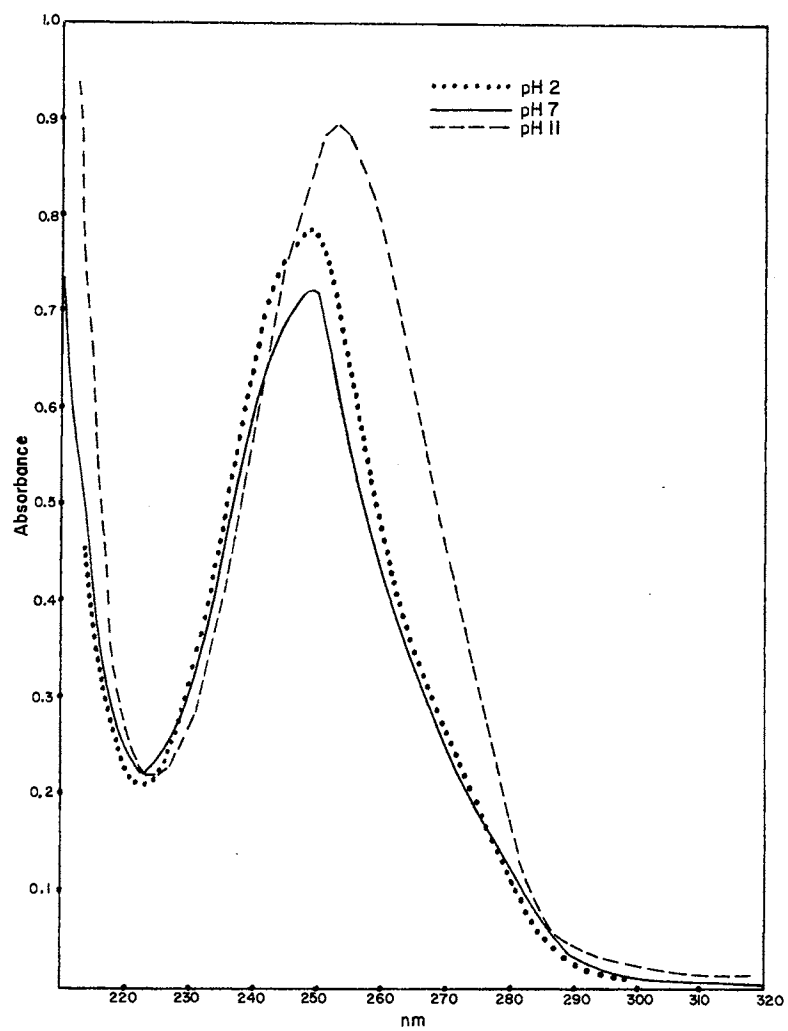


TABLE XV
UV SPECTRAL DATA* OF OXT LMW COMPONENTS

Sample	pH	λ_{max}	λ_{min}	$A_{280/260}$	$A_{250/260}$
OXT LMW peak 1	1	256	227	0.68	0.94
OXT LMW peak 1	7	252	222	0.67	1.14
OXT LMW peak 1	11	253	224	0.69	1.11
OXT LMW peak 2	1	254	229	0.61	1.03
OXT LMW peak 2	7	255	227	0.63	0.99
OXT LMW peak 2	11	255	231	0.53	0.97
OXT LMW peak 3	2	249	223	0.26	1.65
OXT LMW peak 3	7	249	223	0.28	1.68
OXT LMW peak 3	11	253	225	0.23	1.08
OXT LMW peak 4	2	250	225	0.23	1.25
OXT LMW peak 4	7	250	225	0.23	1.20
Inosine	2	249	223	0.24	1.50
Inosine	7	249	222	0.24	1.70
Inosine	11	253	225	0.18	1.05
GTP	1	256	228	0.67	0.95
GTP	7	252	223	0.64	1.18
GTP	11	258	230	0.60	0.91

* All data was obtained on a Cary 14 recording spectrophotometer.

to the literature value of 0.34 for guanosine (48). The only standards included with this separation were GTP (experimental R_f 0.62; literature value 0.61) and ATP (experimental R_f 0.41; literature value 0.48). Both GTP and peak 1 were eluted from the chromatogram with water and again their spectra were determined. The results are listed in Table XVI. The chromatogram contained no ninhydrin positive spots. Phosphate spray was not used because the chromatography solvent system contained a phosphate buffer. Hydrolysis of GTP and OXT peak 1 with 1.0 N HCl produced products which migrated in Pabst III with R_f 's of 0.18 for hydrolyzed GTP and 0.16 for hydrolyzed peak 1. Neither hydrolysis product would migrate in Pabst II, indicating that the product of hydrolysis was probably GMP. In Pabst IV the following values were obtained: GTP R_f = 0.23, peak 1 (not hydrolyzed) R_f = 0.22. Both gave a positive phosphate test while one ninhydrin positive spot was found with peak 1.

OXT peak 2 migrated with R_f of 0.35 in Pabst III which again is close to the literature value for guanosine. When peak 2 was eluted from the chromatogram, the spectral data listed in Table XVII were obtained. The hydrolysate of OXT peak 2 had two quenching components, one of which was immobile and one which had an R_f the same as guanosine as revealed by chromatography in Pabst II. Chromatography of unmodified peak 2 in Pabst IV gave two UV quenching and five ninhydrin positive components. The faster moving quenching component had an R_f similar to that of GMP (0.39 as compared to 0.37) but its spectrum had an irregular minimum at 233 nm (pH 1). The spectrum did show the characteristic guanine shoulder at 280 nm. This component did not give a positive phosphate test, but this may have been due to its low concentration. The slower migrating quenching component of OXT peak 2 had an R_f similar

TABLE XVI

UV SPECTRAL DATA* OF CHROMATOGRAPHED OXT P-2 PEAK 1 AND GTP

Sample	pH	λ max	λ min	280/260	250/260
peak 1	1	256	229	0.66	0.99
GTP	1	256	228	0.67	0.96
peak 1	7	252	223	0.67	1.18
GTP	7	253	226	0.65	1.11
peak 1	11	255	234	0.55	1.00
GTP	11	255	232	0.58	0.94

*All spectra were obtained on a Hitachi Perkin-Elmer Model 124 spectrophotometer.

TABLE XVII

UV SPECTRAL DATA* OF CHROMATOGRAPHED OXT P-2 PEAK 2 AND GTP

Sample	pH	λ max	λ min	280/260	250/260
peak 2	1	256	228	0.67	1.00
GTP	1	256	228	0.67	0.96
peak 2	7	252	223	0.67	1.18
GTP	7	253	226	0.65	1.11
peak 2	11	255	232	0.56	0.97
GTP	11	255	232	0.58	0.94

*All spectra were obtained on a Hitachi Perkin-Elmer Model 124 spectrophotometer.

to GTP (0.22 to 0.23). Its UV spectrum at pH 1 was very similar to those of guanine nucleotides.

As indicated earlier OXT LMW peak 3 had spectral values very similar to inosine. Chromatography of peak 3 in Pabst III revealed no ninhydrin or phosphate positive components. Peak 3 migrated with an R_f of 0.42 while inosine had an R_f of 0.43. Chromatography in Pabst I revealed an average R_f for both peak 3 and inosine of 0.46.

Very little of OXT peak 4 was obtained so chromatography was accomplished only in Pabst III. Two UV quenching and one ninhydrin positive components were present. No standards have been chromatographed which gave R_f 's similar to those obtained for the unknowns in OXT peak 4.

The Dowex column procedure worked quite well with nucleotide knowns as is shown by Figure 36. OXT LMW peak 1 and 2 would not elute from the column with the gradient described indicating that neither contained any of the common guanine moieties. Peak 1 required 2N ammonium formate + 4N formic acid for removal while peak 2 was removed by 1N NaOH after it was not eluted by 2N ammonium formate + 4N formic acid. Peak 3 eluted from the column as a single peak in the same location as inosine. Peak 4 eluted as a doublet in the H_2O portion of the gradient. Table XVIII summarizes the spectral properties of these two peaks at pH 7 while Figure 37 shows their spectra. The earliest eluting peak is titled LMW P-2 peak 4-1 while the second peak is LMW P-2 peak 4-2.

Spectra of the four peaks obtained from the hemolymph LMW by P-2 column chromatography were almost identical to those obtained from OXT LMW. No adenine moieties were apparent in either LMW.

Figure 36. Separation of Standard Nucleotides
With Dowex 1 X 8 Formate

The elution scheme is included in the Procedures chapter, pg. 14. The column was 1 X 25 cm and had a flow rate of 1 ml/min. Five hundred μ g of each nucleotide, with the exception of ITP, was used. Only 100 μ g of inosine was placed on the column.

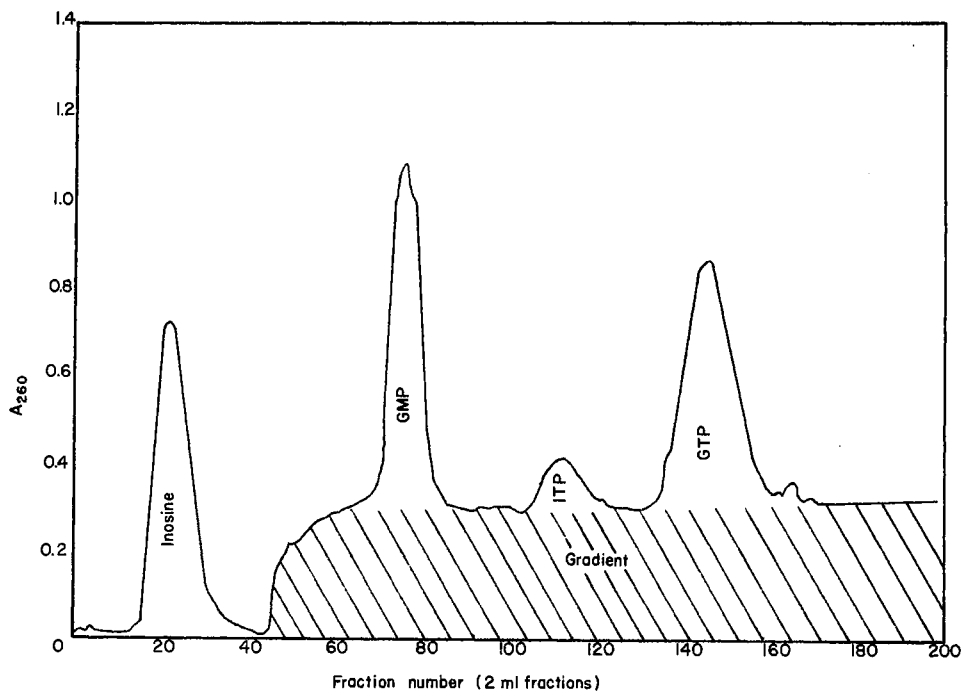


Figure 37. Ultraviolet Spectra of the Two Components of OXT P-2 Peak 4

The 2 components were separated by Dowex 1 X 8 formate and spectra were obtained on a Cary 14. The buffer was 0.02 M sodium phosphate, pH 7. Sample size was one ml and contained the amount of material isolated from 170 spiders.

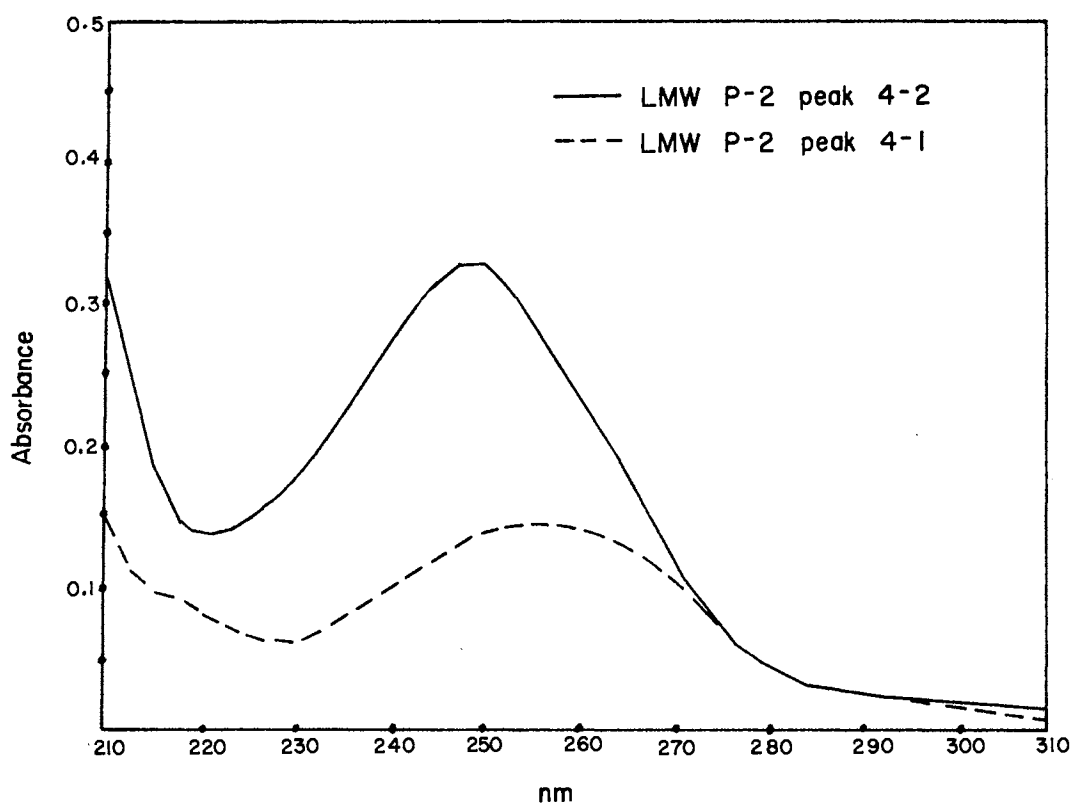


TABLE XVIII
SPECTRAL DATA OF COMPONENTS OF LMW P-2 PEAK 4 AT pH 7*

Sample	λ max	λ min	250/260	280/260
LMW P-2 peak 4-1	255	228	0.33	0.97
LMW P-2 peak 4-2	249	221	0.18	1.35

*Spectra were obtained on a Cary 14 recording spectrophotometer.

R. Results of Immunological Experiments

Antisera to toxin 1, toxin 2, and OXT were successfully produced. Figure 38 shows the double diffusion reactions of antiserum to toxin 1 (anti 1), antiserum to toxin 2 (anti 2), antiserum to OXT (anti OXT), and normal rabbit serum in patterns 1, 2, 3 and 4 respectively. In all four patterns 80 μ g of OXT was placed in the center well while the outer wells had 20, 40, 60 and 80 μ l of antisera starting in the upper left well and reading clockwise. Figure 39 shows essentially the same thing with the exception that pattern 4 contains anti OXT from the second rabbit. There is an obvious difference in the antisera obtained from the two rabbits.

Figure 40 shows an attempt to reveal whether the different antisera cross react. All outer wells contained 80 μ l sera while the center well contained 80 μ g OXT. Wells labeled 1 contained anti 1, 2 anti 2, and OXT anti OXT. The results were inconclusive even though the experiment was repeated. Examination of plates under a microscope revealed that there is an area of diffuse staining where the bands should touch.

Immunoelectrophoresis revealed five bands with the anti OXT while anti 1 and anti 2 showed only a single band. Figure 41 shows the stained slides.

The presence of antibodies in all antisera was detected by RBC agglutination. All test solutions incubated at 24°C gave agglutination within two hr while controls without OXT gave none. Incubation at 37°C gave agglutination with some hemolysis. Controls with normal serum and OXT gave no agglutination. When the OXT and antisera were preincubated for 30 min before adding the RBC, very little agglutination occurred. All lower amounts of OXT down to and including 2 μ g gave almost identical

Figure 38. Antisera Reactions to OXT

All center wells contained 80 μ g of OXT. Pattern 1 shows anti 1, pattern 2 anti 2, pattern 3 anti OXT, and pattern 4 normal rabbit serum. From the upper left reading clockwise outer wells contained 20, 40, 60, and 80 μ l sera. The support media was Ionagar No. 2S (Colab) and the buffer was 0.05 M veronal.

Figure 39. Comparison of the Two Anti OXT's by the Ouchterlony Technique

Conditions were the same as those in Figure 38 except pattern 4 contained anti OXT from the second rabbit.

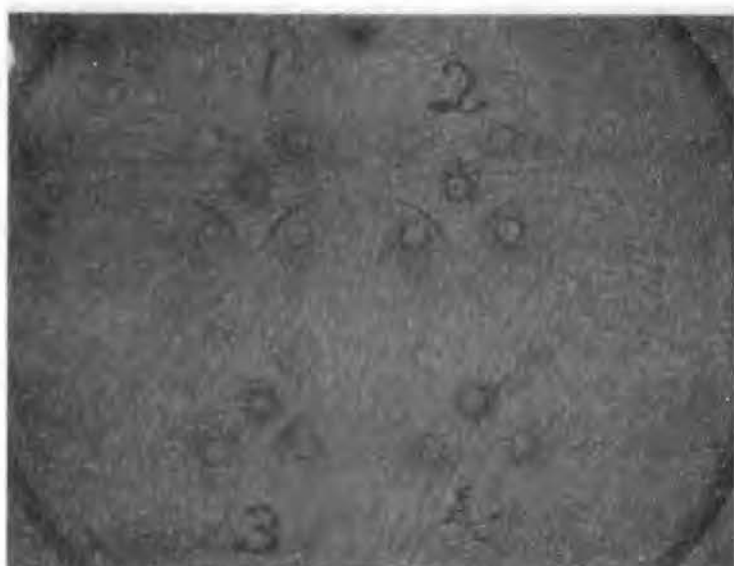


Figure 40. Cross Reaction Test Between Antisera

The center well contained 80 μ g OXT while the outer wells contained 80 μ l antisera. Wells labeled 1 contained anti 1; 2 anti 2; and OXT anti OXT.

Figure 41. Immuno-electrophoresis of Antisera

The outer wells were filled 8 times prior to electrophoresis while the slot was filled with OXT 4 times after electrophoresis. The slide labeled I contained anti 1 in the wells, 2 anti 2, and OXT anti OXT. Preparation of slides and electrophoresis procedure are described on page 21.



agglutination.

All of the above tests simply indicate that antibodies against the OXT and toxins 1 and 2 have been successfully produced.

Tables XIX and XX show the effect of OXT treated with antisera and injected into mice. Anti 1 appears to neutralize the toxic effects of OXT to mice. The survival of the mice injected with anti 1 at 30 min after 275 μ g OXT challenge should be noted in Table XX. Even though mice protected with anti 1 did survive through the original critical 48 hr, most died within two weeks of OXT injection. The antisera neutralization tests in rabbits were inconclusive. In the initial experiment 50 μ g of OXT was incubated with 200 μ l of antisera. All three antisera were tested. None of the rabbits showed any response. The second experiment utilized 300 μ g of OXT and 200 μ l of antisera. Only anti 1 and anti 2 were tested. A third rabbit was injected with 300 μ g OXT in 200 μ l saline as a control. All three rabbits exhibited similar skin lesions after 10 days. The failure of antisera in neutralizing lesion formation in rabbits could be due to low antibody concentration. The antibody fraction should be purified from the antisera to increase neutralization ability.

No natural antibodies against OXT were detected in mouse, human or rabbit sera or in the mouse skin extract. This seems to rule out an "Arthus-like" complex in formation of L. reclusa necrotic lesions.

The successful production of anti 1 and anti 2 does indicate that an antibody affinity column for one-step toxin purification might successfully be prepared.

TABLE IX

NEUTRALIZATION OF OXT WITH ANTISERA: TEST I*

No. mice	Material injected (i.p.)	No. dead at 48 hr	Comment
2	160 µg OXT	2	
2	50 µl anti 1	0	
2	50 µl anti 2	0	
4	50 µl anti 1 + 160 µg OXT	2	The 2 mice that died survived over 24 hr.
4	50 µl anti 2 + 160 µg OXT	4	All mice were dead within 12 hr.

*All samples were incubated for 30 min at room temperature, then diluted to 500 µl total volume with 0.02 M NH_4HCO_3 and injected into 25g white mice.

TABLE XX

NEUTRALIZATION OF OXT WITH ANTISERA: TEST II*

No. mice	Material injected (i.p.)	No. dead at 48 hr	Comment
2	500 µl normal serum + 275 µg OXT	2	
2	500 µl saline + 275 µg OXT	2	
2	500 µl anti 1 + 275 µg OXT	0	One mouse died after 5 days and the other after 15 days.
2	500 µl anti 2 + 275 µg OXT	1	Surviving mouse died after 4 days.
2	500 µl anti OXT + 275 µg OXT	1	Surviving mouse died after 8 days.
2	275 µg OXT; 500 µl anti 1 injected 30 min later	0	One mouse died after 12 days while the other was still alive at the end of a 20 day period.

*Conditions were the same as in test I except that 30 g mice were used.

S. Effect of OXT, OXT Fractions and Purified Toxins on Plasma Coagulation Time

Table XXI shows the effect of 50 μ g of OXT protein on the coagulation time of plasma. Assays containing NH_4HCO_3 coagulated at the same time as did the controls containing only plasma + CaCl_2 . Table XXII shows the effect of various amounts of OXT while Table XXIII compares the activities of the various toxic OXT fractions. Table XXV lists the activities of the two purified toxins while Table XXIV compares the four G-100 fractions. The ability to coagulate plasma is associated with the toxic fractions and specifically with toxin 1 although toxin 2 does have slight activity.

T. Effect of Heparin Treatment on OXT Toxicity in Mice

Table XXVI shows the effect of heparin treatment on OXT toxicity in mice. This was only a preliminary experiment, but it showed little advantage for the heparin treatment. Researchers at the University of Missouri have found that heparin treatment prevents lesions in rabbits injected with brown recluse venom (59). This led to the conclusion that the lesions were caused by a local blood clotting phenomena. The plasma coagulating activity associated with toxin 1 provides additional evidence that the cause of the lesion could be due to local blood clotting.

Figure 42 summarizes the fractionation of the OXT and indicates the location of the various biological activities.

U. Pathological Examinations

Dr. B. Ward found intestinal lesions in mice injected with lethal

TABLE XXI
EFFECT OF OXT ON PLASMA COAGULATION TIME

test	Control ¹ coagulation time ³ min ⁴	OXT ² coagulation time min
1	5.25	2.75
2	4.50	1.75
3	5.00	2.50
4	5.25	1.75
5	4.30	2.00

¹Controls consisted of 0.5 ml human plasma only.

²OXT assays contained 50 µg of OXT protein in addition to the plasma. Both OXT's and controls were preincubated for 10 min prior to the addition of CaCl₂ to initiate coagulation.

³Coagulation time was taken as the elapsed time between addition of the CaCl₂ and the loss of all liquid plasma.

⁴Time was estimated to the nearest 1/4 minute with the aid of a stop watch.

TABLE XXII
EFFECT OF OXT CONCENTRATION ON PLASMA COAGULATION TIME*

Sample	amount of Lowry protein μg	Coagulation time min
Test I		
Control	---	3.25
OXT	100	1.75
OXT	50	1.75
OXT	5	2.00
Test II		
Control	---	3.25
OXT	5.0	2.25
OXT	2.5	2.50
Test III		
Control	---	3.50
OXT	1.0	2.50
OXT	0.5	2.75
OXT	0.1	3.25

*Procedure was the same as in Table XXI.

TABLE XXIII
EFFECT OF OXT TOXIC FRACTIONS ON PLASMA COAGULATION TIME¹

Sample	amount of Lowry protein μg	Coagulation time min
Control	---	3.75
G-25 HMW ²	9	2.50
G-25 HMW	9	2.50
G-100 III ³	16	2.25
G-100 III	16	2.25

¹Procedure was the same as in Table XXI.

²G-25 HMW was the toxic HMW separated from the OXT by G-25 Sephadex.

³G-100 III was the toxic third peak separated from the G-25 HMW by G-100 Sephadex column chromatography.

TABLE XXIV
COMPARISON OF THE EFFECT OF G-100 FRACTIONS
ON PLASMA COAGULATION TIME*

Sample	amount of Lowry protein μg	Coagulation time min
Test I		
Control	---	3.00
G-100 I	2.0	3.00
G-100 II	1.7	3.00
G-100 III	0.8	1.75
G-100 IV	0.9	3.00
Test II		
Control	---	2.25
G-100 I	2.0	2.00
G-100 II	1.7	2.00
G-100 III	0.8	1.75
G-100 IV	0.9	2.25
Test III		
Control	---	2.25
G-100 I	2.0	2.25
G-100 II	1.7	2.00
G-100 III	0.8	1.50
G-100 IV	0.9	2.25

*Procedure was the same as in Table XXI.

TABLE XXV
EFFECT OF PURIFIED TOXINS 1 AND 2 ON PLASMA COAGULATION TIME¹

Sample	estimated protein ²	Coagulation time min
Test I		
Control	-----	4.00
Toxin 1	<1 µg	2.00
Toxin 2	<1 µg	3.25
Test II		
Control	-----	4.00
Toxin 1	<1 µg	2.50
Toxin 2	<1 µg	3.50

¹Procedure was the same as in Table XXI.

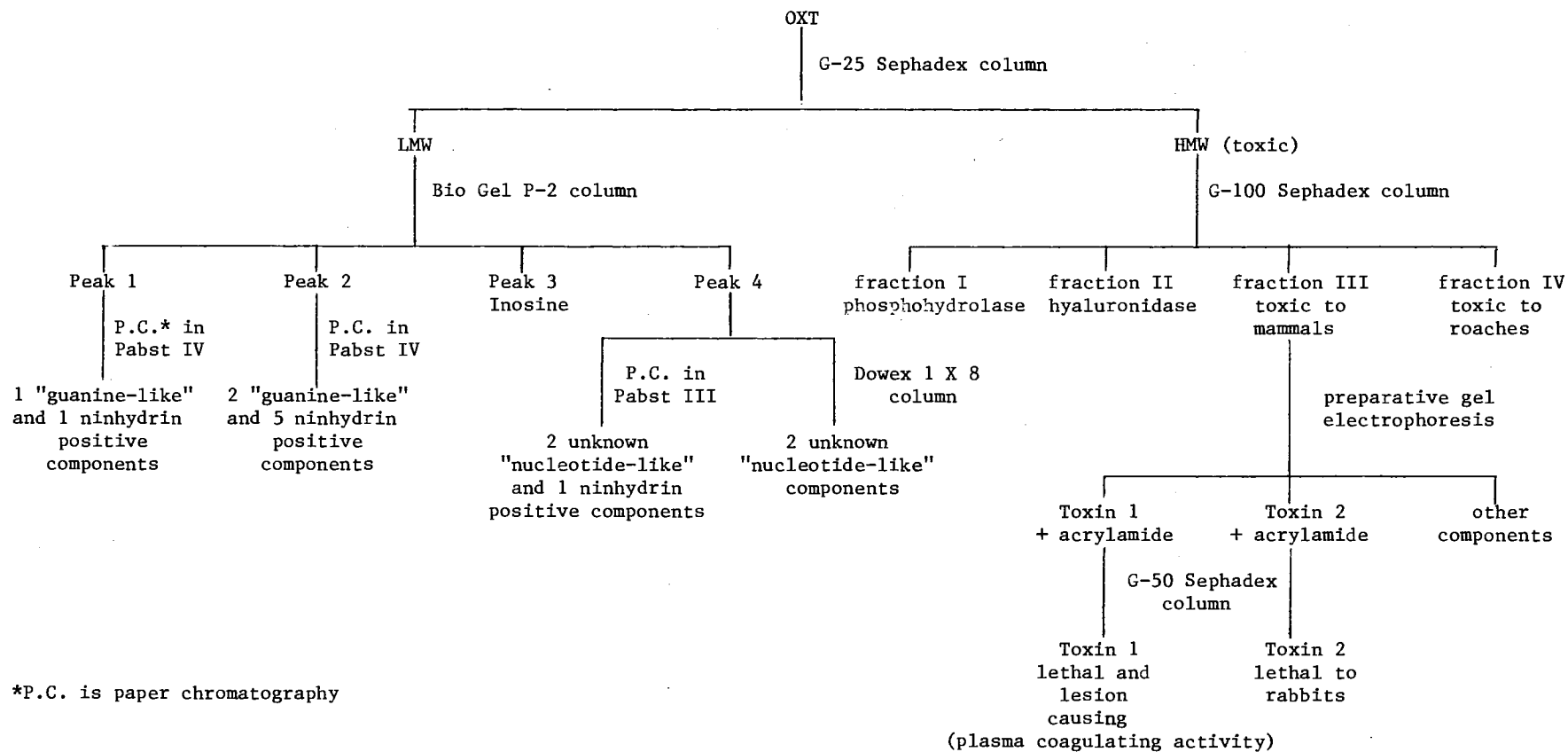
²Toxin 2 used in test I was 1/200 the amount obtained from 64 spiders while toxin 1 was 1/250 the amount obtained from 100 spiders. In Test II toxin 2 was as in test I but toxin 1 was obtained by washing the tube which contained the small amount used in test I with 25 µl of saline and using 10 µl of the wash.

TABLE XXVI

EFFECT OF HEPARIN TREATMENT ON OXT CHALLENGE IN MICE

No. of mice	material injected (i.p.)	No. dead at 24 hr	No. dead at 48 hr
8	300 μ g OXT	5	8
8	4.25 units heparin; 300 μ g OXT 30 min later	8	-
2	4.25 units heparin	0	0

Figure 42. Flow Diagram of OXT Fractionation With
Properties of Fractions



*P.C. is paper chromatography

doses of OXT. These were found whether the route was i.p. or s.c. No lesions were found in the intestines of treated rabbits. "The effect of the OXT in rabbits appears to be localized at the site of injection where it acts directly on the muscle, producing acute, calcified necrosis. The toxin, or its necrotic products, induce a major inflammatory reaction, characterized by neutrophilic exudation. Many of the vessels are congested and there is some evidence of thrombosis. No heart or kidney lesions were found in any of the specimens" (60). The intestinal lesions of mice found after OXT challenge by Dr. Ward were not sufficient damage to kill the mice in 48 hr. The means by which OXT produces death in test animals is not known.

CHAPTER IV

SUMMARY

An extract of the poison apparatus of the brown recluse spider, L. reclusa, has been prepared which retains the toxic biological activities of the venom. It is lethal to rabbits, mice and roaches. When injected i.d. or s.c. into rabbits it produces necrotic lesions. These toxic effects have been quantitated as to the amount of extract protein required. All LD₅₀'s are in the microgram range. The toxicity of the venom apparatus extract (OXT) is heat labile but stable to freezing and thawing. Toxicity to mice can be destroyed by the action of proteases. Toxicity of the OXT is diminished in the presence of phosphate or Tris buffers.

The OXT contains hyaluronidase and phosphohydrolase enzyme activities. The phosphohydrolase has been characterized as a rather non-specific, heat stable, alkaline phosphohydrolase. No protease activity was found. Also, the OXT has no hemolytic activity with human or rabbit erythrocytes, but it does decrease the coagulation time of human plasma.

The OXT has been fractionated by molecular exclusion chromatography, preparative polyacrylamide discontinuous electrophoresis, paper chromatography and ion exchange chromatography. Three toxic components have been identified. The component which is toxic to roaches has a molecular weight of 6,800 as determined by Sephadex column chromatography. The mammalian toxins, toxins 1 and 2, both have molecular weights of approximately 32 to 34,000 as determined by SDS polyacrylamide gel.

electrophoresis and Sephadex column chromatography. Both toxins appear to be electrophoretically homogeneous proteins. The isoelectric pH of both as determined by isoelectric focusing is 8.3. Amino acid analyses of toxins 1 and 2 have been obtained. Small amounts of toxin 1 cause lesions in rabbits. Larger amounts of toxin 1 are lethal to both mice and rabbits. Toxin 2 is lethal to rabbits but appears nontoxic to mice. Neither of the toxins has hyaluronidase or phosphohydrolase activity, but toxin 1 has the plasma coagulating activity. This suggests that the cause of the necrotic lesion is local blood clotting, as was earlier proposed by researchers at the University of Missouri. Future work will involve elucidation of the effect of toxin 1 on the blood clotting mechanism.

Antisera to toxin 1, toxin 2, and OXT have been prepared. The presence of antibodies was confirmed with immunodiffusion, immunoelectrophoresis and red blood cell agglutination techniques. None of the antisera were effective (as tested) in preventing toxic effects of the OXT in rabbits. Anti 1 appears to protect mice against the immediate lethal effects of the OXT.

The OXT contains a number of nontoxic "nucleotide-like" components which were partially characterized by paper and ion exchange chromatography and UV spectrophotometry. Only inosine was positively identified. No adenosine nucleotides were found.

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