A STUDY OF PROTEIN AND PEPTIDE COMPONENTS OF VENOMS OF LOXOSCELES RECLUSA GERTSCH AND MULAIK AND DUGESIELLA HENTZI (GIRARD)

By

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A STUDY OF PROTEIN AND PEPTIDE COMPONENTS OF VENOMS OF LOXOSCELES RECLUSA GERTSCH AND MULAIK AND DUGESIELLA HENTZI (GIRARD)

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CHAPTER I

INTRODUCTION

Reptiles, arthropods, arachnids, and mammals have representative members with venom. Birds are the only non venomous higher organisms. These venoms are used to secure prey and to defend against their predators. Among the many arachnids found in nature relatively few are harmful to man; however, spiders are ever present in man's environment. Due to this close proximity many cases of arachnidism are reported each year in the United States. Most cases of arachnidism are usually considered and reported to be from the genus Latrodectus; however, bites of the members of the genus Loxosceles can also cause severe reactions in man. Spider venoms are usually grouped into the two general classes, which are neurotoxic and cytotoxic. This grouping is based on the victims reaction to the bite. The black widow and other members of the genus Lactrodectus are neurotoxic, while the members of the genus Loxosceles are usually cytotoxic; however, both venoms can have mixed effects. An antivenom is available for the genus Lactrodectus, but little is known about the treatment of a bite from a spider belonging to the genus Loxosceles. Since the bite by a member of the genus Loxosceles seems to be a growing problem in this region (1), the venom of this spider should be chemically and biologically characterized.

One member of the genus Loxosceles, the Loxosceles reclusa, was found by Gertsch and Muliak (2) to inhabit southeastern and central

United States. The Loxosceles reclusa commonly known as the brown recluse, fiddleback, or brown spider is potentially more dangerous than the black widow due to its insignificant appearance and its preferred indoor habitat. The combined factors of a highly necrotic venom and a close association with man would strengthen this proposal. This makes the spread of this species to other states very easy as suggested by Waldron and Russel (3). The spider is not aggressive, does not attack, and bites only when molested. Many victims are bitten in bed during the night or while dressing. The venom of the L. reclusa can not only cause necrotic lesions, but also hemolytic anemia and death in man (4). Very little information is in the literature on the biological activity and the chemical composition of the venom.

Arthropod venoms have been studied and found to contain quinones, acetylcholine, histamine, histidine, formic acid, and proteins. Enzymes such as hyaluronidase and small peptides have been found in wasp and bee venoms. Snake venoms also contain proteins with enzymatic activity such as: proteinases, cholinesterase, hyaluronidase, phosphatase, and phospholipases A and B; in addition to these large proteins small peptides have also been found (5).

This thesis will report the results of studies on the protein components of L. reclusa venom, the biological activity, and the hemolytic activity of the whole venom and the individual protein components. Certain enzyme assays will also be reported for the whole venom. The venom of the tarantula, Dugesiella hentzi, has also been studied. Tarantula, D. hentzi venom was found by Grothaus to cause death in mice (6). According to Horen (7) the tarantula, a member of the mylomorph family, is considered to be innocuous to man in the United States.

CHAPTER II

REVIEW OF THE LITERATURE

Most literature on venoms and venom fractionation is concerned with reptiles. These fractionations were conducted with large amounts of venom which is comparatively easy to obtain. This review of the literature will be limited to spider and arthropod venoms where only small amounts of venom were obtained, with particular consideration for the Loxosceles reclusa venom.

Horen published data on all reported or potentially dangerous spiders in the United States (8). Gertsch and Muliak reported that the adult females of the L. reclusa are from seven millimeters to twelve millimeters in length with the average being nine millimeters. The males average about eight millimeters with the same size range. They describe the color of the spider as yellowish to dark orange or reddish brown marked with a darker pattern in a shape similar to that of a violin (9). According to Gertsch and Horen (8,9) these spiders can be found under rocks and tree bark but most are found in homes and buildings where man lives.

Most arachnidism in the United States is attributed to the genus Lactrodectus; however, due to increasing reports of bites from a brown spider the genus Loxosceles was suggested as a source of necrotic arachnidism. Nicholson and Nicholson reported a case of hemolytic anemia in a three year old girl following the bite of a large brown spider (10).

James, et. al. described reactions of two children to the bite of an unidentifiable "bug". Both children developed large lesions and both passed "black" urine. One child required skin grafts to heal the lesion, while the other child was left with a large scar. These lesions were described as "viserocutaneous arachnidism". This type of lesion also resulted from a bite of L. laeta, a member of the genus Loxosceles, which is found in South America (11). "Black urine" is caused by a large amount of hemoglobin metabolites in the urine resulting from the destruction of red blood cells (12). Blattner also described the reactions of several people to bites from spiders other than the black widow. He noted the resemblance of the bite to that of L. laeta (13). Atkins, et. al. compared the bite of L. laeta of South America to L. reclusa in North America, and concluded that L. reclusa was the cause of necrotic bites found in this region. Experiments performed by Wingo in which experimental animals were bitten by L. reclusa spiders showed that there could be little doubt as to the relationship between the brown spider and the cutaneous or systemic loxoscelism (14). Dillaha, et. al. summarized data obtained from sixteen patients bitten by the brown spider (15). Horen reported that both cutaneous and systemic reactions can occur and that little is known about treatment (7). Dillaha, et. al. described the bite of the L. reclusa as a brief stinging sensation at the time of the bite or it may not be felt at all. Little pain occurs until two to eight hours later at which time a noticeable erythema around the bite is seen. A blister forms rapidly and after several days the blister becomes a dark hard mass of dead tissue. This area becomes depressed and the margins separate. The hard area usually referred to as a scab is lost leaving an ulcer behind. This

ulcer may heal naturally but in some cases skin grafts are required (16). Other descriptions of the bite are reported by Atkins, et. al. (17). It was also noted by Atkins that there was a variation in biting. Some spiders bite and are detached very quickly, others remain with fangs sunk into the skin for five to ten seconds and some extreme cases held the skin until forcibly detached. The longer the fangs remain attached the more venom was injected and the greater the reaction for that animal. The rabbit and the guinea pig were found to be sensitive to the bite of the brown spider. It was noted that while rabbits and guinea pigs were very susceptible, rats were extremely resistant (18). Denny, et. al. used dogs for injection of the spider venom. The injections were intravenous. The venom caused death, visceral hemorrhages and other effects. The appearance of antibodies was demonstrated by an immunodiffusion technique. Antibodies were found in dogs and rabbits after two injections and antiserum prepared by hyperimmunization of a rabbit with multiple subcutaneous injections of the venom showed an ability to inhibit the in vitro hemolytic action (4).

According to Weiner, et. al. the spider venom is most toxic to those arthropods and insects on which the spider normally feeds (19). For this reason many insects are used to test the toxicity of the venom. Norment, et. al. used the hemocytes of the common house cricket Acheta domesticus (Linaeus) to test toxicity of the L. reclusa venom (20).

Morgan found that male brown spiders gave less than half the venom that was obtained from the females. However, it was just as toxic. Spiders milked during abnormally high or low temperatures gave less venom than those spiders milked at normal temperatures.

Grothaus used white mice to test the biological activity of the

L. reclusa venom. The venom was injected in .13, .25, .26, .3, and .48 mg. amounts. The mouse injected with .13 mg. gave no response, while all others began to behave abnormally in six to twelve hours. The mice injected with .26 and .48 mg. died within twelve to forty-eight hours. All other animals developed lesions at the point of injection. White rabbits were also injected and lesions were observed (6).

Nazhat separated L. reclusa venom into protein components by disc electrophoresis using polyacryamide gels containing persulfate. Seven bands were observed, three of which were biologically active when tested by injecting cockroaches. Six of these bands gave hemolysis with red blood cells (21).

The genus Lactrodectus which has toxic venom has been studied for many years. The venom is neurotoxic and effects are usually nausea, muscle stiffness and other pains (22). Keegan, et. al. found seasonal variation in the toxicity of the black widow spider venom, the venom being most toxic in November and least toxic in May. These tests were not performed using whole venom; instead the cephalothoracic contents which contains two venom glands were used. Thus the actual amount of venom present was uncertain (23). L. mactans venom was separated into its components by acrylamide gel electrophoresis by McCrone and Hatala (24). Seven bands were found. The "B" fraction was found to be lethal to mice. Strong ultraviolet absorption suggested non-protein components were present. Three non-protein components were found in the venom. The molecular weight of the lethal "B" fraction was estimated by using light scattering. McCrone compared the lethality of several Lactrodectus venoms. Dissected venom glands were used to obtain the venom. He found that all species have potent venoms, but L. geometricus, the brown widow,

has the most potent venom of the genus Lactrodectus (25). McCrone also compared the Lactrodectus spiders of North America by immunological and electrophoretic techniques. L. mactans mactans, L. variolus, and L. bishopi gave eight major fractions while L. geometricus gave six bands. They all contained several common antigens and therefore an antivenom preparation against one will neutralize the effects of several of them (26). Frontali, et. al. worked with L. tredecimuguttatus venom which electrophoretically separated into five components when a cellulose powder column was used. Sulfhydryl and disulfide bonds were necessary for activity in two isolated components from L. tredecimguttatus venom (27).

The Aphonopelma tarantula of Arizona produces little if any apparent physiological effect when it bites man. However, injections of 10 mg. of venom into the groin of a rat produced, within 20 minutes, severe convulsions and death in one hour. The rats were examined and the reactions were found to be of the same type of syndrome as that produced by venom of the scorpion, Centruroides sculpturatus. Stahnke and Johnson compared these two venoms by using disc electrophoresis. The tarantula had ten protein fractions while the scorpion had sixteen protein fractions. A heterologus test showed the anti-sera obtained from rabbits contained at least one large band which could also be interpreted as three bands (28). Grothaus observed that the tarantula, Dugesiella hentzi, venom was lethal to a mouse when 3.11 mg. of venom was administered subcutaneously. A cockroach was injected with 0.35 mg. of venom and died within 30 minutes (6). Nazhat used disc electrophoresis to separate the protein components of the tarantula, Dugesiella hentzi, and found no bands present, which would suggest low molecular

weight proteins.

It has been shown by LeBez, et. al. that the venom of Lactrodectus tredecimguttatus can be labeled with phosphorous 32 by feeding the spider ^{32}P in water. The spider is then allowed to bite a guinea pig. The venom was found to spread quickly in the body of the sacrificed animal. This labeling procedure could then be used to follow the action of the venom in the animal (29).

Welsh and Batty have found 5-hydroxytryptamine in the venom and in the venom containing parts of some arthropods, such as scorpions, social wasps, and bee venoms. This compound doesn't contribute to the toxicity of the venom, but when present it produces severe pain. It may also increase the uptake of the toxic components by increasing the permeability of the cells and increasing the rate of blood flow at the site of the puncture and venom injection (30).

McIntosh and Watt studied venom from the scorpion, Centruroides sculpturatus, finding that this venom was stable over a wide pH range and resistant to enzymatic digestion. In immunoelectrophoresis ten precipitin bands were observed. Toxicity was found to be dependent on the presence of sulfhydryl groups (31). In another paper the neurotoxic principle of the C. sculpturatus was investigated (32). Watt concluded that one or two components were concerned with toxicity of the venom. The toxin is believed to be a peptide of low molecular weight on the basis of dialyzability. Due to the low ultraviolet absorption in the 260-265 nm range it is assumed that only small amounts of aromatic amino acids are present in the toxic fraction (33). Israeli scorpions were investigated by Nilzan and Shulov to find protein antigens for immunologic studies, and to determine if morphologically distinct species were

different in their venoms also. The proteins that were found were of poor antigenicity and therefore poor antisera would be produced. The venoms were found to be different when checked by electrophoresis (34). Russel, et. al. studied the venom of the scorpion, Vejonis spinigerus. The chemical composition was determined by disc electrophoresis in which thirteen bands were found. On Sephadex G-50 four major peaks were observed (35). Alpha hemolysis was found in the scorpion, Nebo herichonticus by Rosin (36). Ibrahim found phospholipase activity in scorpion venoms (37).

Bee venom contains histamine releasing components according to Fredholm and Haegermark (38). O'Connor, et. al. found in the venom of the honey bee, Apis mellifera, a complex mixture of lipids, free amino acids, carbohydrates, peptides, proteins, and enzymes (39). Nelson and O'Connor studied the non-protein components of the honey bee and found six lipid-like compounds, several free amino acids, and several peptides (40).

Ants venom have also been studied and Lewis, et. al. found phospholipase activity present in the Australian bulldog ant, Myrmecia pyriformis (41). Stinging ants normally use their venom offensively to kill prey for food but if the nest is attacked the ant may use its venom to repel the intruder (42).

CHAPTER III

EXPERIMENTAL PROCEDURE

Collection of Spiders

L. reclusa spiders were collected in various buildings at the OSU campus and surrounding area. These spiders were also collected at Phillips University in Enid. The tarantulas and black widows were collected in the areas surrounding Stillwater. The spiders were fed flies and cockroaches. The tarantulas were given flies, cockroaches and water.

Venom Collection

Venom was collected on small pieces of cigarette paper according to the technique of Grothaus and Howell (43). The cigarette paper was used to collect the venom of the black widow and fiddleback, while glass capillary tubes or tubes of polyethylene were used to collect the tarantula venom. The fiddleback and black widow spiders were placed in a multiple cell holder designed by Nazhat (21). This apparatus was further improved by designing a circular multiple holding cell which allows one person to "milk" spiders while another removes and replaces spiders. The biting stimulus source was a Heathkit audio generator model IG-72 which has a frequency range of 1 cycle/second to 1000 cycles/sec. and an output of 0 to 10 volts. By touching the fiddleback or black widow with electrodes using a stimulus of 7 volts and 20 cycles/

sec., the spiders bite the cigarette paper and release their venom. In order to observe the spiders while collecting venom a dissecting microscope was used. The microscope is essential since one must be able to visibly detect venom release. This is necessary because not all spiders release venom when they bite the cigarette paper. D. hentzi was stimulated with a higher voltage of 30 volts at 20 cycles per second. Glass capillary tubing was first used to collect venom; however, these tubes were easily broken by the tarantula, while sometimes damaging his fangs. Polyethylene tubes were then used to collect the venom since they bend with the pressure that is applied with the tarantulas bite.

Venom Recovery

Venom collected on paper was placed directly on the top of polyacrylamide gels in a twenty per cent sucrose solution for electrophoresis. To apply this type of sample to a Bio-gel column the venom was eluted from the paper with a 0.05 M phosphate buffer pH 7 into 50 microliter or less. This sample was then placed on the Bio-gel column.

Tarantula venom was removed from the tubing by a microliter syringe and added to an amount of 20% sucrose that gave a final concentration not less than 10% in sucrose. The sample was then layered on to the polyacrylamide gels. To place the tarantula venom on the Bio-gel columns the venom was removed from the tubing by a microliter syringe and placed directly on the column.

Electrophoresis

Approximately 10 µg of protein is obtained with each bite of the L. reclusa. This amount varies so that one must have at least 10 spider

bites per small square of cigarette paper and usually more in order to obtain band patterns of the protein components. The polyacrylamide gels used were 7% gels, pH 8.9. However, they ran at pH 9.5. The buffer was a glycine tris buffer pH 8.5. The 7% separating gel was made mixing two solutions A and C of the following composition:

A. 1 N HCl 43 ml, Tris 36.3 gm N,N,N',N' tetramethylethylene diamine 0.23 ml, H_2O to 100 ml (pH 8.8 - 9.0)

C. Acrylamide 28 gm, Bis-acrylamide 0.735 gm, H_2O to 100 ml

Once A and C were mixed, an equal volume of catalyst was added (.14% ammonium persulfate) and the solution was added to glass columns which had been treated with column coat for 10 min. The gels were polymerized in the dark for thirty minutes and then pre-electrophoresed at 5 milliamps per sample to remove the ammonium persulfate. The samples were then layered on in 10 - 20% sucrose solutions and electrophoresed at 5 milliamps per sample for one hour.

Location of Protein Components

After samples have been electrophoresed on polyacrylamide gels the protein components can be located either by staining or scanning. By running all samples in duplicate one gel can be frozen while the other gel is stained with a dye such as amido black and destained to locate the various protein components. The gels are then placed side by side and the unstained gel can be sectioned. This technique allows one to obtain a visible record of the number and the location of the protein components. When unstained gels are scanned, the adsorption at 280 nm was used to locate the protein components. The gel is transported at a

constant speed and the resulting graph can be used to locate the protein components.

Chromatography on Bio-gel Columns

All columns were 90 cm in height and 6 mm in internal diameter. The columns were treated with dimethyldichlorosilane to prevent wall effects. A glass filter paper was placed at the bottom of the column and the Bio-gel columns were then poured. The column flow rate was kept at 20 µl/min by using a peristaltic pump (LKB Recychrom 4912A) which was then connected to a drop counter fraction collector (LKB Ultrorac 7000). The buffer used was 0.05 M phosphate buffer pH 7.0 and 0.5 ml samples were collected. Protein peaks were determined by 280 nm absorption.

Concentration of Samples

A Biomed microconcentrator which concentrates by ultrafiltration was used to concentrate all samples.

Biological Activity

The venom of the L. reclusa and D. hentzi venom components were injected into cockroaches. The cockroaches were checked at 6, 24, and 48 hours.

Guinea pigs were injected subcutaneously with venom components of the L. reclusa and D. hentzi. The guinea pigs were checked every twelve hours for the development of a wheal.

Enzyme Assays

Hyaluronidase

Venom was added to 0.5 ml of hyaluronic acid in a concentration of 1.2 mg/ml in 0.1 M acetate buffer pH 3.5 and 0.15 M in sodium chloride. The mixture was incubated overnight at 37°C. The reaction was stopped by the addition of 0.01 ml of 4 N sodium hydroxide, which adjusts the mixture to approximate neutrality. The mixture was then tested for N-acetylhexosamines. This was done by adding 0.1 ml of potassium tetraborate to the above sample. The sample was then heated in a boiling water bath for three minutes and then cooled in tap water. Three milliliters of dimethylaminobenzaldehyde was added and the samples were heated at 36 - 38°C for 20 minutes. Samples were then read at 544 mμ.

Phosphatidase A

Venom was incubated with 0.5 ml of solution of labeled lecithin (1-acyl-2-(^{14}C-oleoyl)-GPC. The solution was incubated at 36°C overnight, and then diluted to 5 ml with saline. Sodium hydroxide (4N) was added to obtain an alkaline pH of 9.0. The solution was then extracted with chloroform to remove the unreacted lecithin and lysolecithin. After extraction at alkaline pH the solution was acidified with 6 N HCl to a pH of 2.0. Chloroform was then used to extract the free acid. The chloroform extracts were then placed in counting vials and the chloroform was evaporated. After evaporation Brays scintillation fluid was added to the vials and the samples were counted. Phosphatidase activity is then determined by the location of the radioactivity. If no phosphatidase A activity is present then the majority of the radioactivity

would be found in the unreacted lecithin which is found in the basic extraction. If phosphatidase A activity is present then the majority of the radioactivity would be found in the free acid or the acidic extraction. Therefore, the phosphatidase A activity is indicated by the location of the radioactivity in either the acidic or basic extraction.

CHAPTER IV

RESULTS AND DISCUSSION

Disc polyacrylamide gel electrophoresis is a useful technique for the separation of mixtures of proteins and large peptides. These gels also permit the use of small samples and yield good resolution between individual proteins. The gels are polymerized by using the chemical catalyst ammonium persulfate, which is a strong oxidizing agent. Due to the presence of the ammonium persulfate chemical alteration of the proteins separated in the gel does occur.

Venom of the Loxosceles reclusa, Lactrodectus mactans, and Dugesiella hentzi were electrophoresed on polyacrylamide gels containing ammonium persulfate. Nazhat reported that in gels containing ammonium persulfate the L. reclusa venom consisted of seven protein components, and the venom of L. mactans also contained seven protein components. The D. hentzi showed no bands on polyacrylamide gels containing ammonium persulfate. These results are shown in Figure 1. Nazhat further reported venom hemolytic activity for the whole venom and fractionated venom components obtained from polyacrylamide gels. The whole venom gave a relative hemolytic activity of 36%. All protein components gave some hemolytic activity with the exception of the third component. The majority of the activity was found in the first, and the fifth through the seventh components. The first and seventh protein components gave 100% hemolysis. Fractionated venom was injected into cockroaches. The

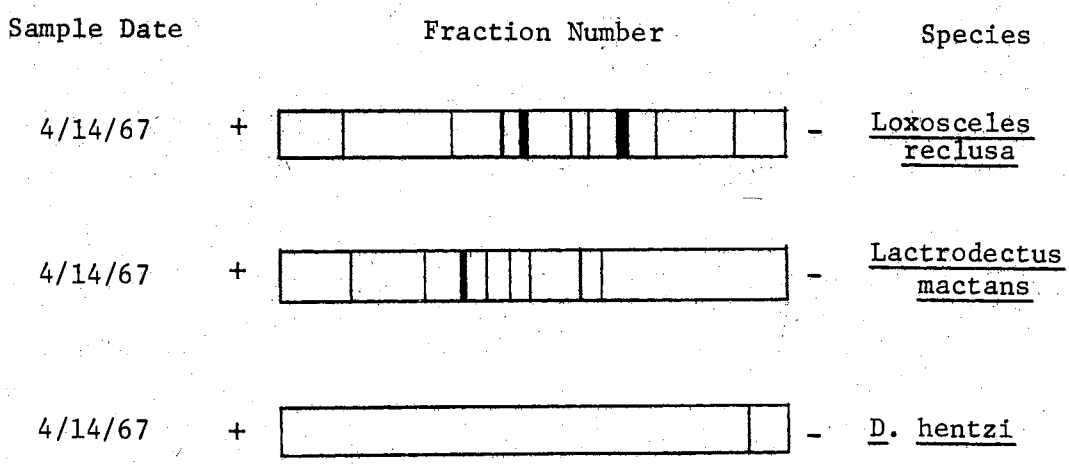

Figure 1. Protein Components of the Venoms of Loxosceles reclusa, Lactrodectus mactans, and D. hentzi on 7% Polyacrylamide Gels pH 8.9 Containing Ammonium Persulfate

third and fourth components were found to be lethal to the cockroaches.

Due to the possible effect of the ammonium persulfate on the proteins, steps were taken to remove the ammonium persulfate from the gel. This was accomplished by pre-electrophoresing the gels for thirty minutes. The samples were layered directly onto the gels using a twenty percent sucrose solution. The samples were then electrophoresed for one hour. When venom was separated into its components using gels without ammonium persulfate, only four protein components were found in the Loxosceles reclusa and Lactrodectus mactan venoms. At the same time six and seven protein components were found respectively in the male and female venom of D. hentzi. Figure 2 shows the results of the various venoms separated on gels containing no ammonium persulfate. The reduction of the number of protein components in the Loxosceles reclusa and Lactrodectus mactans venom indicates that the ammonium persulfate was acting on some of the proteins in the venom in such a way that two bands were seen instead of one. The appearance of protein components in the D. hentzi venom would also indicate that some alteration of the proteins present by the ammonium persulfate had occurred.

It was suggested by Brewer (44) that the ammonium persulfate did not alter the proteins present in the gels since the ammonium persulfate migrated ahead of the tracking dye, and therefore it could not come in direct contact with the protein. Instead Brewer attributes the modification of the protein to the oxygen rich environment in the gel, which was formed by the action of the ammonium persulfate on the reducing agents in the gel. To remove the oxygen rich environment Brewer suggested layering an anionic reducing agent ahead of the sample or by using a continuous input of a sulfhydryl-rich environment. When this technique

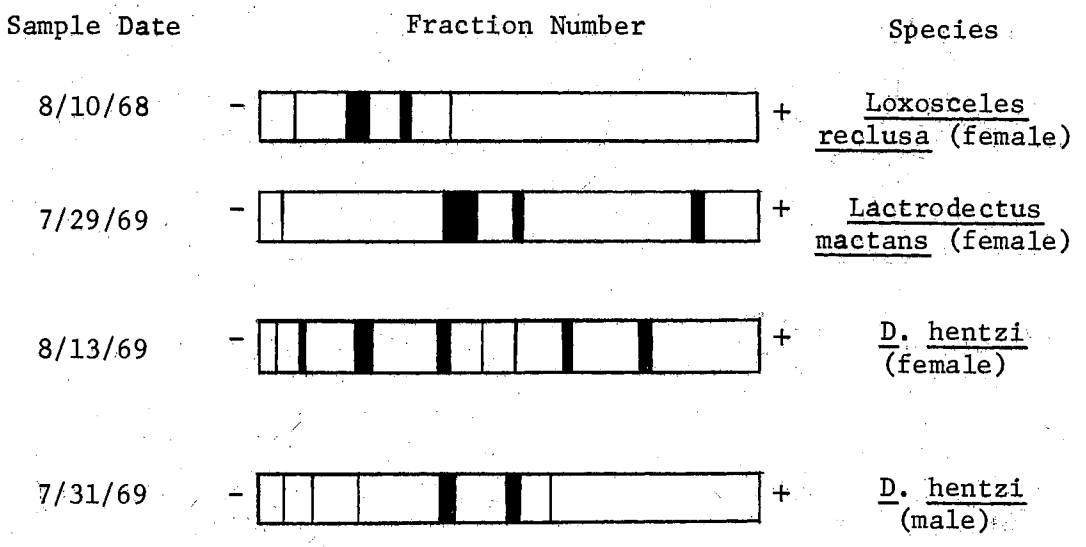

Figure 2. Protein Components of the Loxosceles reclusa, Lactrodectus mactans, and D. hentzi Venoms Observed with 7% Polyacrylamide Gels pH 8.9 Containing no Ammonium Persulfate

was tried using L. reclusa venom the number of protein components found were the same in number and position as those obtained with gels containing ammonium persulfate. Since the sulfhydryl-rich environment produced the same results as the gels containing ammonium persulfate, gels that are pre-electrophoresed to remove the ammonium persulfate were used to separate the venom into its respective components. Figure 3 shows the results obtained on these types of gels.

Protein bands were located in unstained gels by using 280 nm absorption of each band in a scanner described under methods. This technique was also used with stained gels. The resulting graph of migration distance vs absorption cannot be used to quantitate the amount of protein present. Certain problems were encountered when using the unstained gel. If the gel is left in the glass sample tube one obtains absorption peaks due to imperfections in the glass tube. The other alternative is to remove the gel and scan; however, if any nicks or gel column imperfections are formed in the gel during removal these points will also produce peaks. For these reasons scanning was used only with stained gels. Even with stained gels the scan is subject to limitations. Figure 4 shows a scan of L. reclusa venom. In this sample the bands were separated, and the scan shows each peak distinctly (the only exception being that) although the baseline did not return to zero between the peaks. In D. hentzi venom six and seven bands are seen, and they are relatively close together. When these gels were scanned (Figures 5 and 6) some bands, depending on their intensity are either seen as one large distinct peak or are seen as a large peak with a shoulder. By using a lower scanning speed more of the bands are resolved as seen in Figure 7. Due to the limitations of the scanning methodology gel

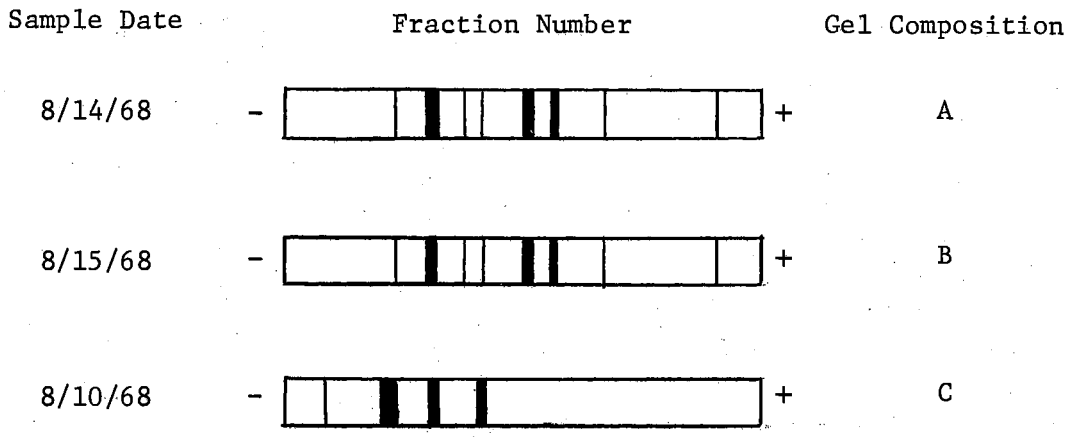

Figure 3. Protein Components in L. reclusa Venom with Variation in the Gel Composition. (A) with persulfate, (B) with persulfate and tris-thioglycolate, (C) without persulfate

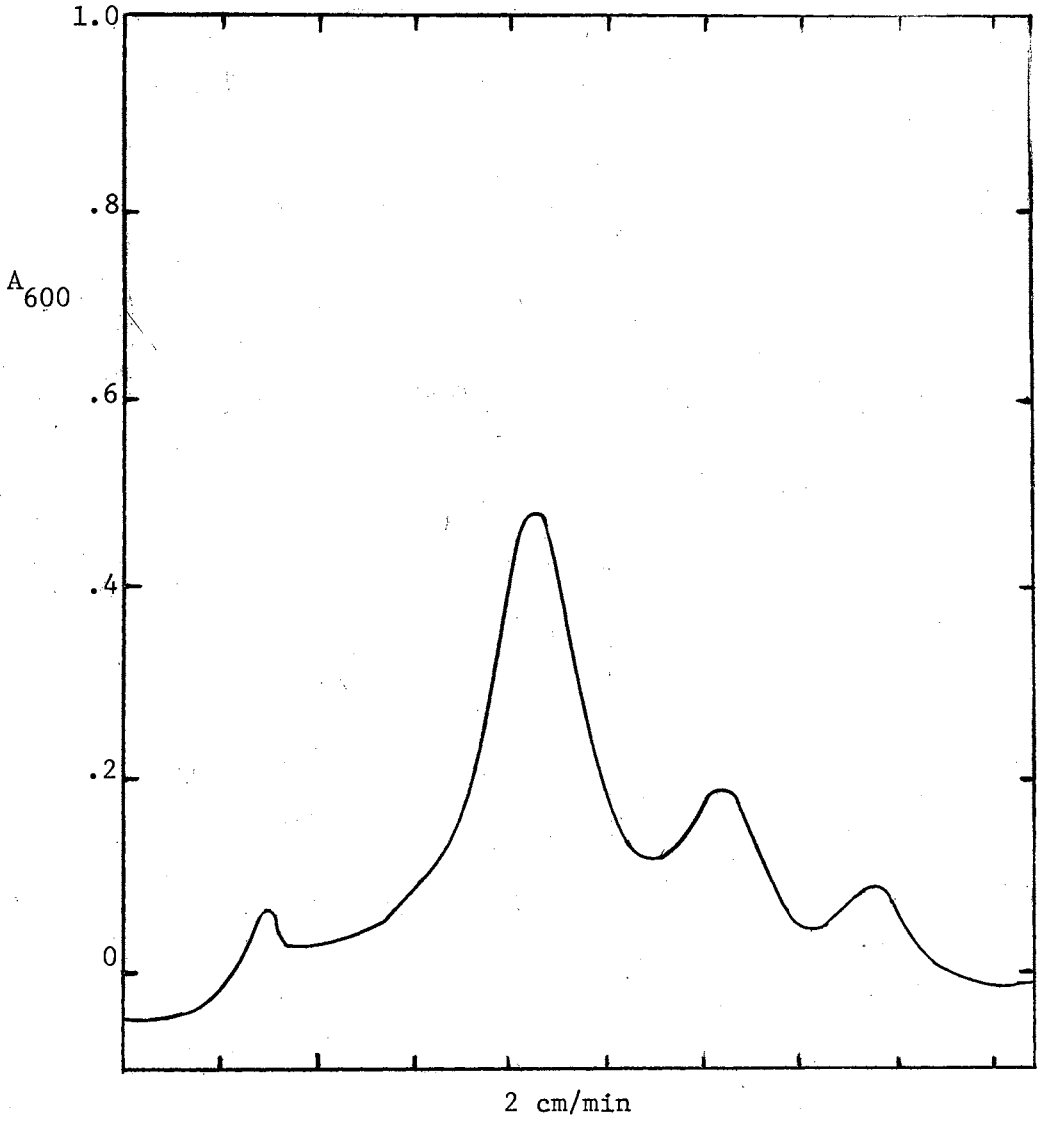

Figure 4. L. reclusa Venom Components in a Stained Gel Without Persulfate. Scanned at 600 nm, slit 0.2 mm, and at a scan speed of 2 cm/min in a Gilford Linear Transport Model 2410

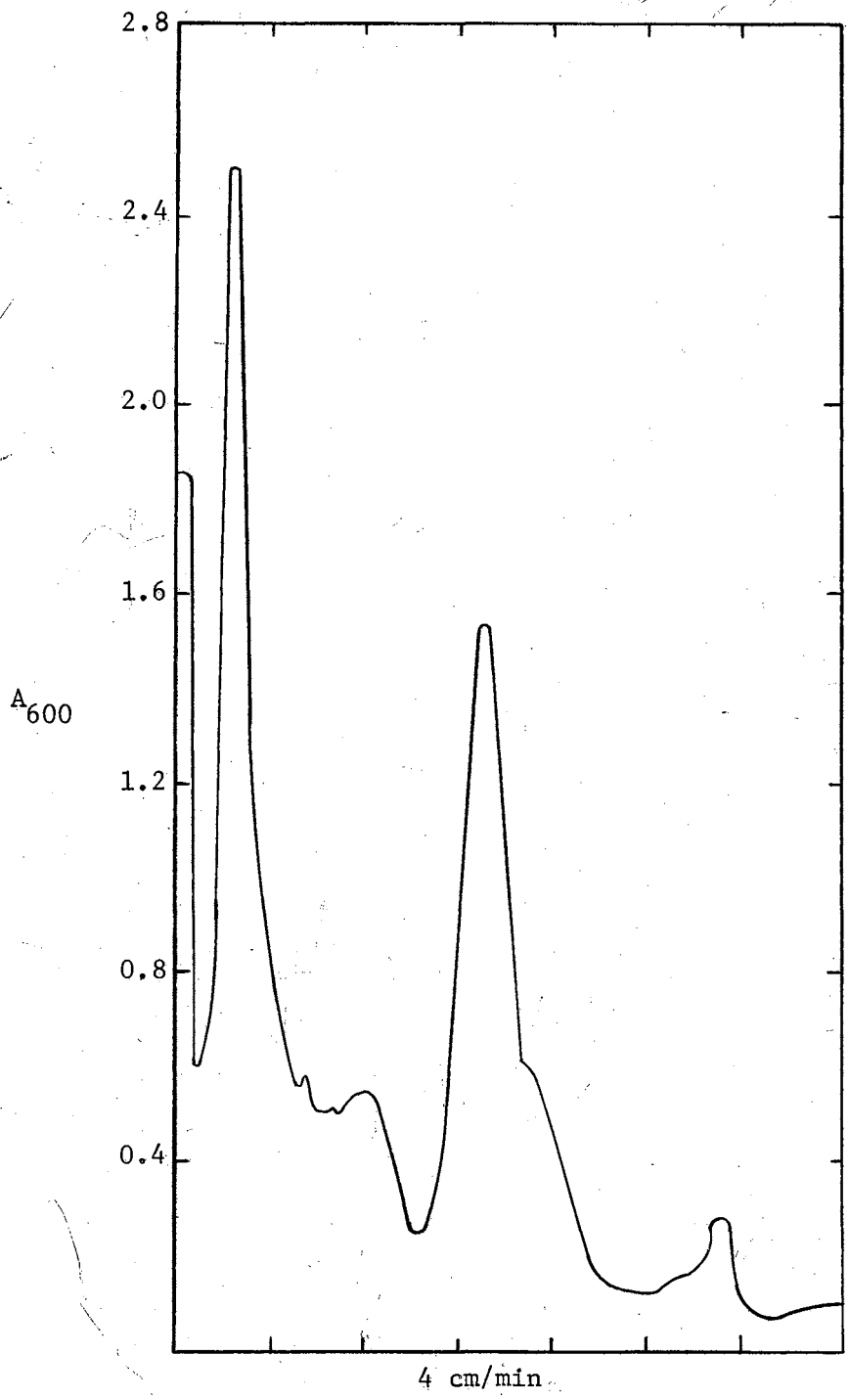

Figure 5. D. hentzi Female Venom Components in a Stained Gel Without Persulfate. Scanned at 600 nm, slit 0.2 mm and at a scan speed of 4 cm/min in a Gilford Linear Transport Model 2410

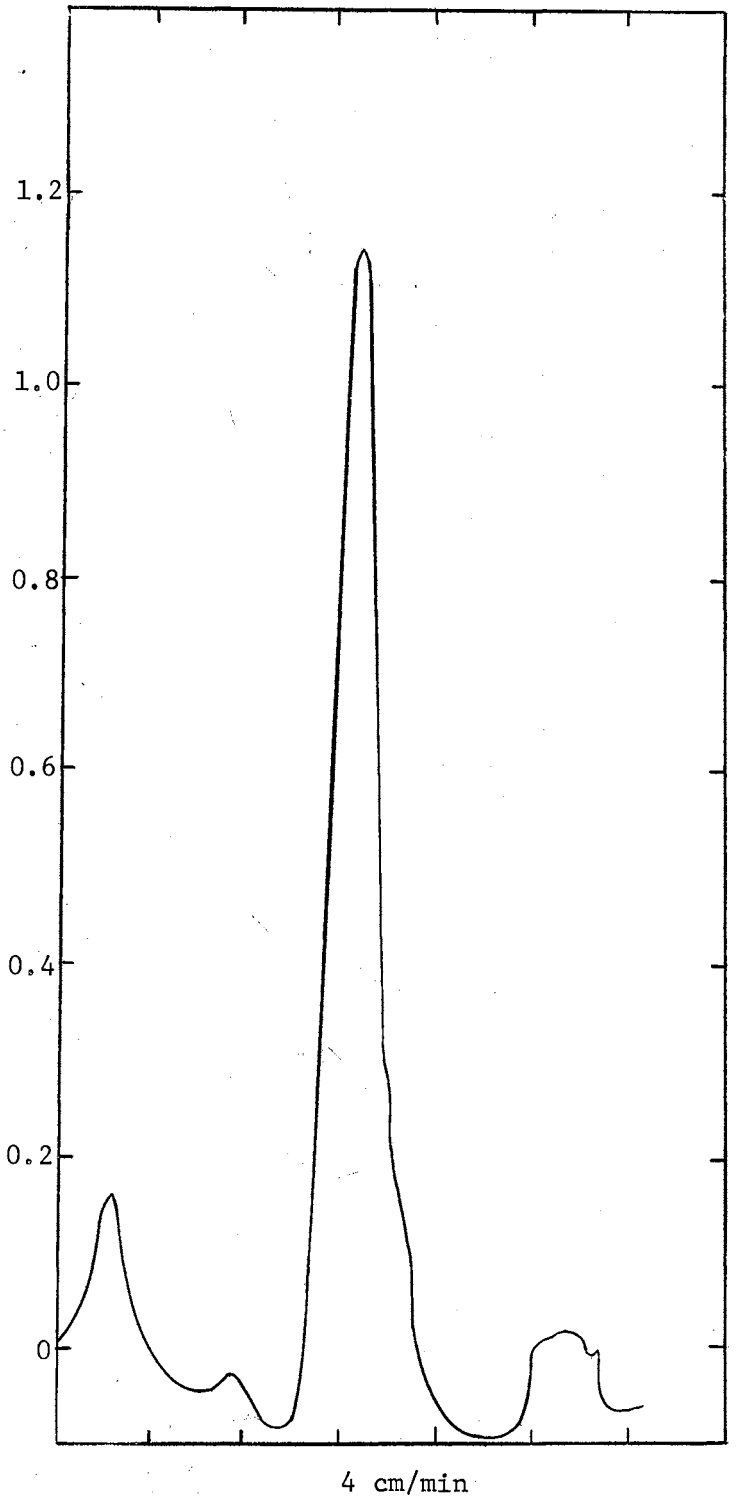

Figure 6. D. hentzi Venom Components in Stained Gels Without Persulfate. Scanned at 600 nm, slit 0.2 mm, and at a scan speed of 4 cm/min in a Gilford Linear Transport Model 2410

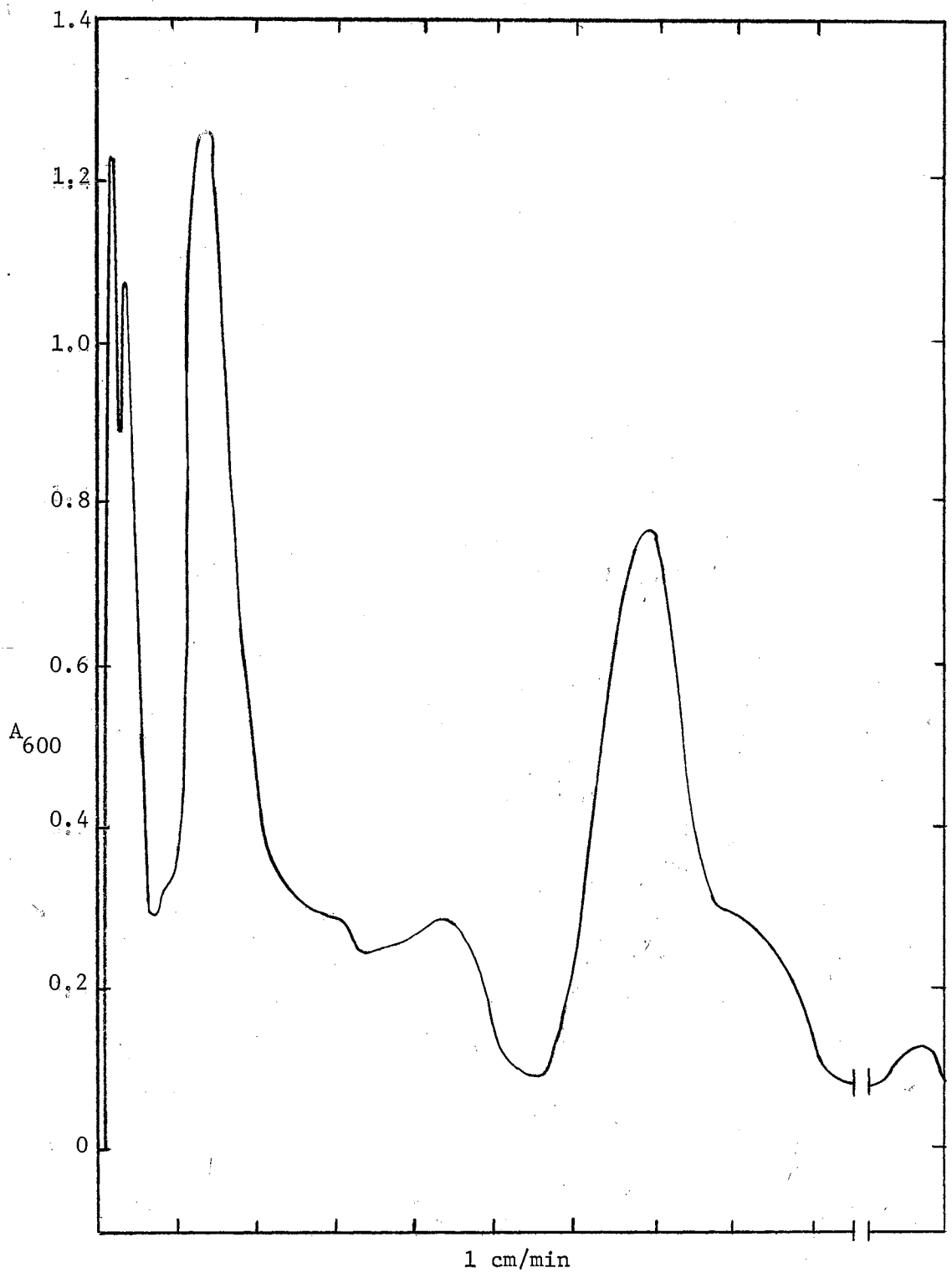

Figure 7. Female Venom of D. hentzi in Stained 7% Polyacrylamide Gel pH 8.9 Containing no Ammonium Persulfate Scanned using a Gilford Linear Transport Model 2410 at 600 nm, slit 0.2 mm, and at a scan speed of 1 cm/min

staining was necessary and by scanning at low speeds good graphs of stained gels were obtained. Kruski states that when using a densitometer, samples of higher molecular cause an increase in gel concentration resulting in a lower peak area, while spreading results in a larger peak area (45). For this reason peak area cannot be relied on for exact quantitative estimation of the concentration of the proteins present. The established fact of a variance in dye binding capacity by different proteins also presents an error.

By using standard proteins of known molecular weight one can obtain an estimation of the molecular weight of an unknown substances. Problems are encountered when electrophoresing standard compounds since many are not present as a monomeric unit. The native charge of the proteins also affects the migration distances. Hendric and Smith (46) noted that it is possible to separate charge and size isomers in disc polyacrylamide electrophoresis. They also noted that variation of the "bis" concentration affected the sieving properties of the gel. Since charge differences of proteins can cause migration of the protein not as a function of molecular weight, the charge effect should be minimized in the molecular weight estimation. Shapiro, et al. (47) used sodium dodecyl sulfate (SDS), an anionic detergent, and were able to minimize the native charge differences for proteins with molecular weights ranging from 13,700 to 160,000. When electrophoresing proteins in the presence of SDS and 2-mercaptoethanol some aggregation occurs. This aggregation can be eliminated by carboxymethylation. Standard proteins electrophoresed in the presence of SDS and 2-mercaptoethanol gave a linear plot when the distance migrated was plotted vs the log of the molecular weight of the compound. This would indicate that all the proteins migrated as anions

due to complex formation with the SDS, the migration distances apparently depending primarily upon molecular weight instead of charges.

To obtain another estimation of molecular weight of venom components Bio-gel columns were used. These columns were 90 cm in height and had an internal diameter of 6 millimeters. In such tall narrow columns wall effects become a major concern. By coating the column with dimethyldichlorosilane before pouring of the gel one can reduce this effect. Blue dextran was first passed through the Bio-gel P-60 column until the band remained compact and uniform through the entire gel column. Once this was accomplished with blue dextran a sample of bovine serum albumin was passed through the column to saturate any sites that could bind protein. Next standard proteins were passed through the column in order to obtain a standard curve for molecular weight estimation as shown in Figure 8. Once these standard curves were found to be reproducible a sample of L. reclusa venom was applied to the gel and the elution pattern was obtained by reading the absorption of the fractions at 280 mμ. This is shown in Figure 9. As can be seen from this graph the elution pattern is uneven and the peaks are not distinct. This is due to the lack of constant flow. When protein samples are placed on these tall narrow columns the flow rate is reduced. Due to this reduction in flow rate or even stoppage of the flow the proteins tend to diffuse; and therefore, when they are eluted from the column, they are spread over many tubes. This causes broad flattened peaks which overlap.

A Bio-gel P-60 column was used to separate the D. hentzi venom into its components while giving an estimation of molecular weight. D. hentzi venom was collected and concentrated by lyophilization. When this sample

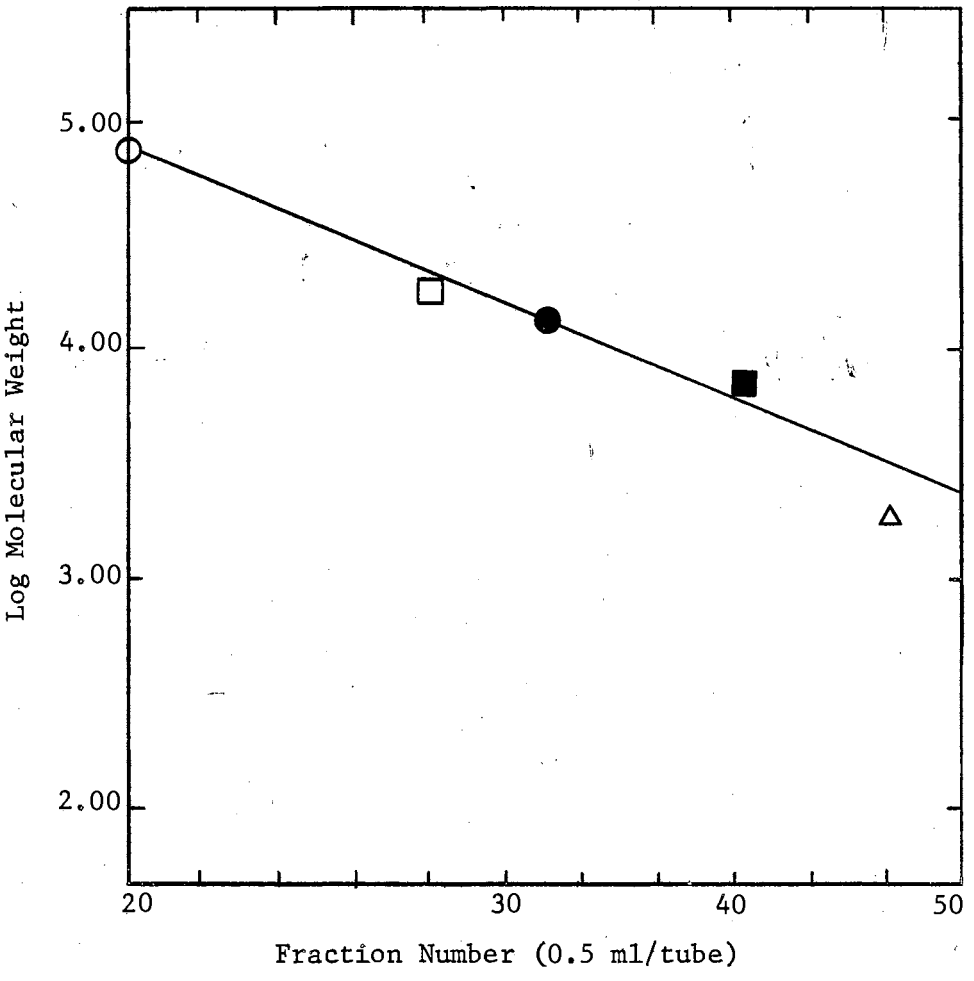

Figure 8. Calibration of Bio-gel P-60 Column (90 cm x 6 mm ID) using Reference Proteins and Eluting with 0.05 M Phosphate Buffer pH 7.0

- O Bovine serum albumin - 66,500 mw
- □ Insulin trimer - 17,400 mw
- O Cytochrome - 13,000 mw
- ■ Insulin - 5,800 mw
- Δ Bacitracin - 1450 mw

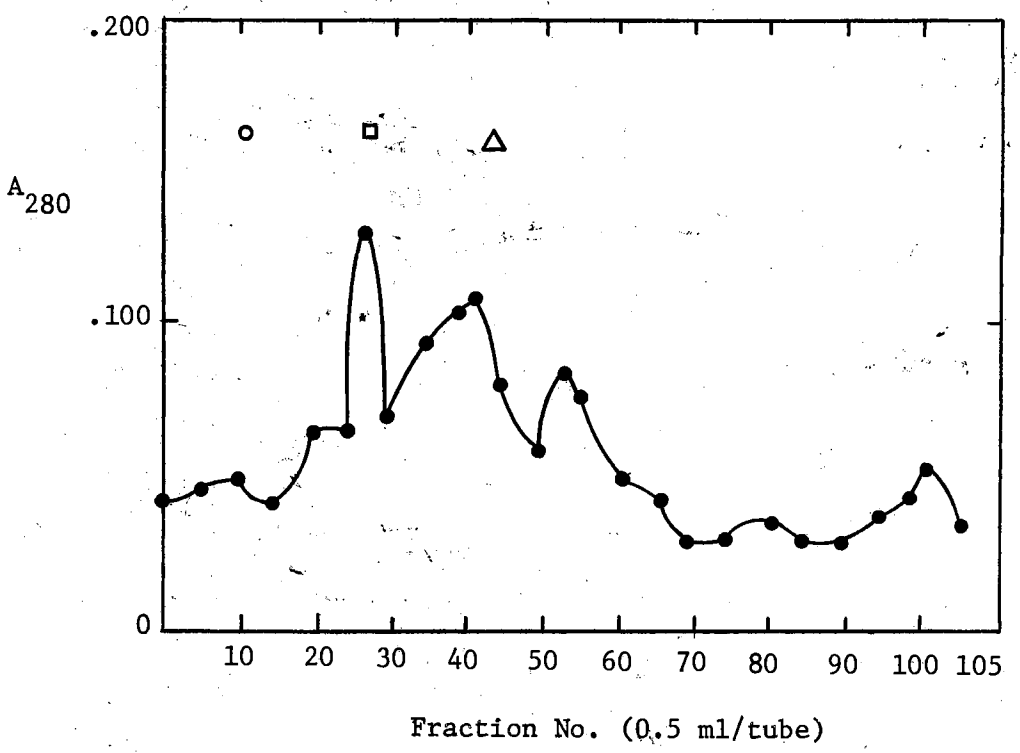

Figure 9. L. reclusa Venom Elution Pattern of Proteins from a Bio-gel P-60 Column, 90 x 6 mm ID

O Bovine serum albumin - 66,500 mw

□ Insulin trimer - 17,400 mw

Δ Bacitracin - 1450 mw

was placed on the column two peaks were observed as seen in Figure 10. One peak eluted after the salt peak, probably due to absorption to the gel. A repeat experiment was conducted and only one peak was observed as seen in Figure 11. The two single tube peaks were due to improperly cleaned tubes. Since lyophilization can be harmful to proteins a Biomed microconcentrator was used to concentrate the venom. This concentrator utilizes ultrafiltration to concentrate samples. When the D. hentzi venom was collected in capillary tubing, the sample was removed by a microliter syringe and placed directly on the column. By placing samples directly on the column two peaks were observed as shown graphically in Figure 12. These peaks elute in a region in which accurate molecular weight information is not attainable due to non linearity with respect to low molecular weights. Since Bio-gel P-60 is not valid in this region less than 5,800, a gel which is capable of resolving low molecular weight substances was poured. A Bio-gel P-2 column was poured and standardized as described for the P-60 column. The only difficulty encountered was the lack of known molecular weight marker compounds in this region. Small peptides were found to adsorb to the column material and give rise to false information. It was also noted that proteins with molecular weights that differed by only a few hundred grams per mole were not separated. This would mean that the peaks obtained from the Bio-gel columns may not be homogeneous. When D. hentzi venom was placed on Bio-gel P-2 columns two major peaks were observed with two minor peaks which show as shoulders. See Figure 13. The two major peak tubes containing the highest amount, determined by optical density, of the compound were concentrated using the Biomed microconcentrator. These two samples were then injected into cockroaches and the results of these

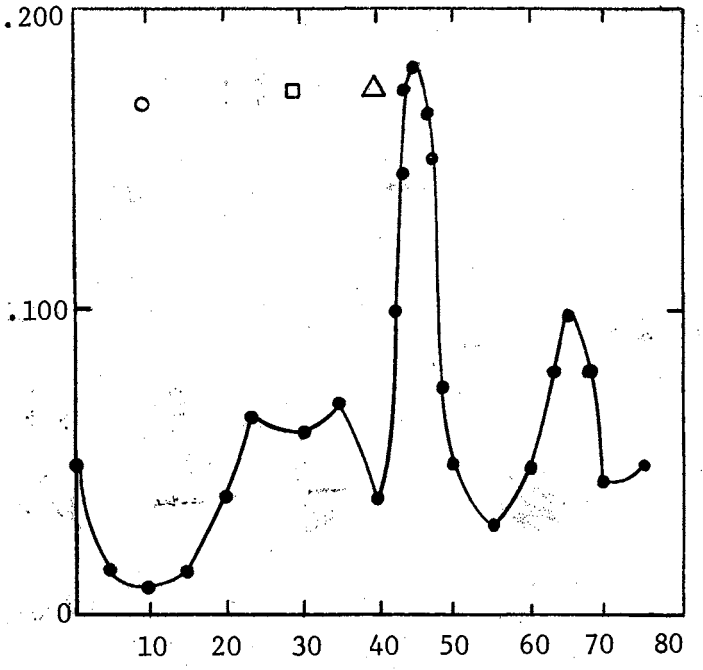

Figure 10. Elution Pattern of Protein in D. hentzi Venom from a Bio-gel P-60 Column 90 cm x 6 mm ID using 0.05 M Phosphate Buffer pH 7.0

O Bovine serum albumin - 66,500 mw

□ Insulin trimer - 17,500 mw

Δ Bacitracin - 1450 mw

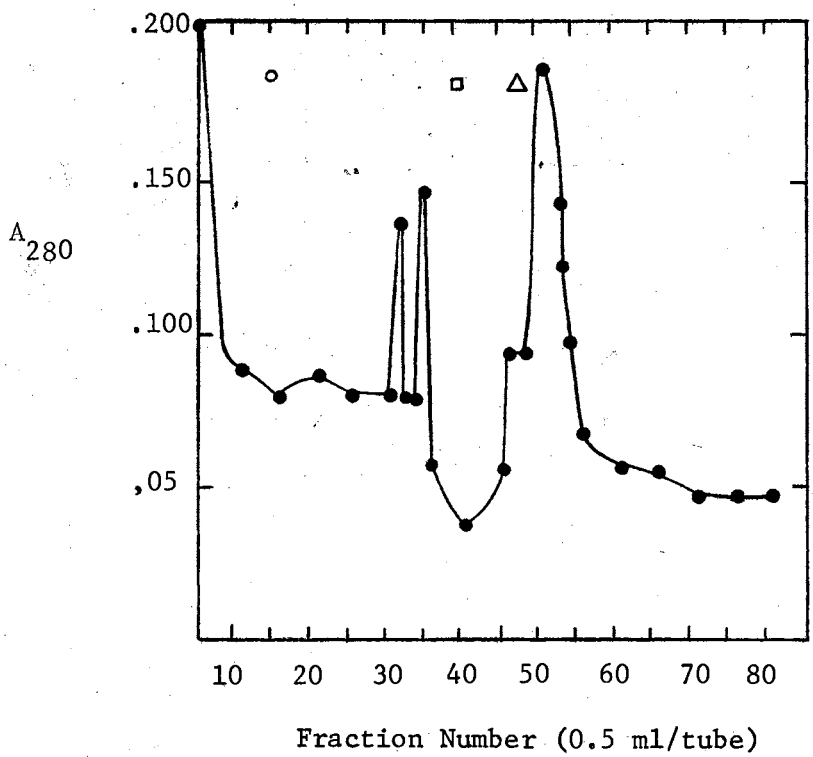

Figure 11. Proteins in D. hentzi Venom Eluted from a Bio-gel P-60 Column 90 cm x 6 mm ID using 0.05 M Phosphate Buffer pH 7.0

O Bovine serum albumin - 66,500 mw

□ Insulin trimer - 17,500 mw

Δ Bacitracin - 1450 mw

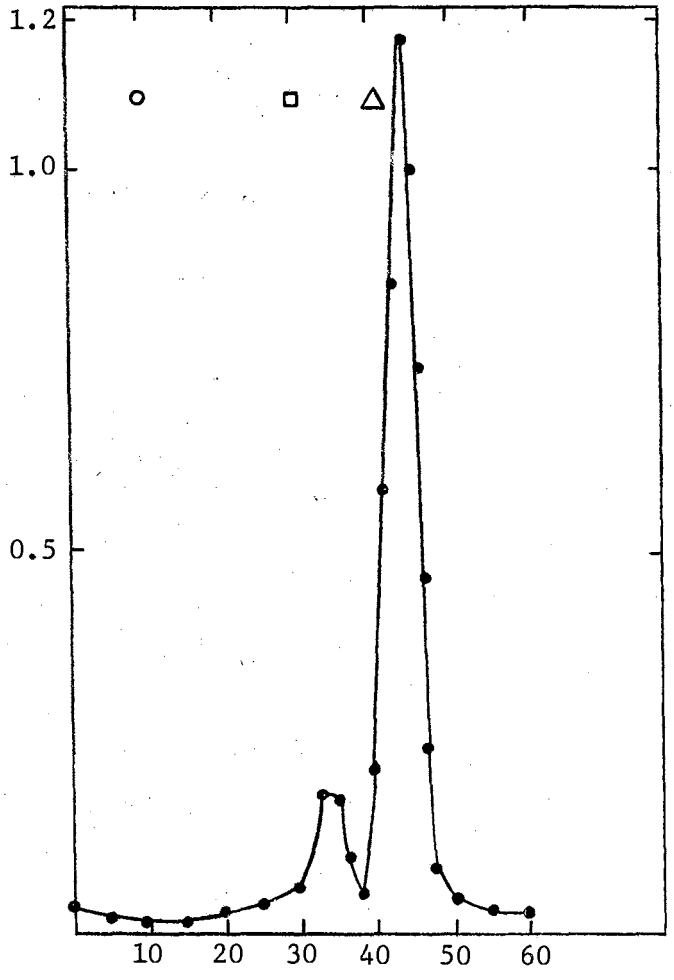

Figure 12. Elution Pattern of Proteins from D. hentzi Venom with a Bio-gel P-60 Column, 90 cm x 6 mm, using 0.05 M Phosphate Buffer pH 7.0 for Elution

O Bovine serum albumin - 66,500 mw

□ Insulin trimer - 17,500 mw

Δ Bacitracin - 1450 mw

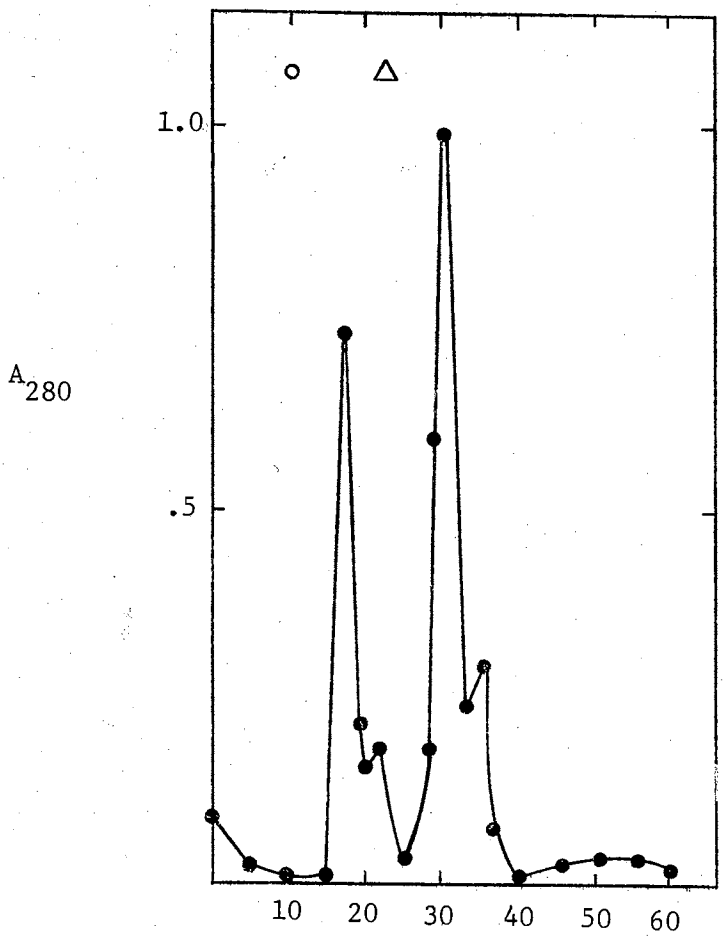

Figure 13. D. hentzi Venom Protein Elution Pattern from a Bio-gel P-2 Column, 90 cm x 6 mm, using 0.05 M Phosphate Buffer pH 7.0 for Elution

O Bovine serum albumin - 66,500 mw

Δ Bacitracin - 1450 mw

injections are seen in the first table. As can be seen from the table both fractions contained substances toxic to the cockroaches. In a second experiment using a Bio-gel P-2 column four peaks were found as seen in Figure 14. The tube containing the most of these four components, the tube with the highest optical density, was concentrated and injected into cockraoches. The results of these injections are shown in Table II. These peaks are not homogeneous as there is contamination with the other closely related peptide or protein components, for this reason only the peak tubes (tube with the highest optical density) were concentrated for biological tests. From this table one can see that the second and fourth components had no effect on the cockroaches. The first and third peaks contained lethal substances. The first peak was at the void volume and therefore could consist of any number of peptides or proteins above the molecular weight of 1450. Data from the Bio-gel P-60 column indicates that the molecular weight is lower than that of insulin which is 5800. From these columns it would appear that there are at least three proteins with molecular weights equal to or below 1450, and possibly other proteins between 1450 and 5800.

D. hentzi venom was also placed on the amino acid analyzer to determine if free amino acids were present. Five samples were analyzed; however, only three are reported here due to machine malfunction on one sample and contamination of one venom sample with stomach contents. This contamination was noted during venom collection and the large number of free amino acids present confirmed the suspected contamination. Table III shows the free amino acid composition of the three venom samples. Paper chromatography was also used for separation of low molecular weight compounds and a typical separation is shown in Figure 15. When

TABLE I

BIOLOGICAL ACTIVITY OF TWO MAJOR COMPONENTS OF D. HENTZI VENOM*

Observation Time	6 Hours		24 Hours		48 Hours	
	Cockroach		Cockroach		Cockroach	
	1	2	1	2	1	2
Peak from Bio-gel P-2 Column						
1	dead	on back	dead	on back	dead	dead
2	on back	normal	on back	normal	dead	normal
Control	normal	normal	normal	normal	normal	normal

*Injection of the two major components of D. hentzi venom isolated using a Bio-gel P-2 column 90 cm x 6 mm ID.

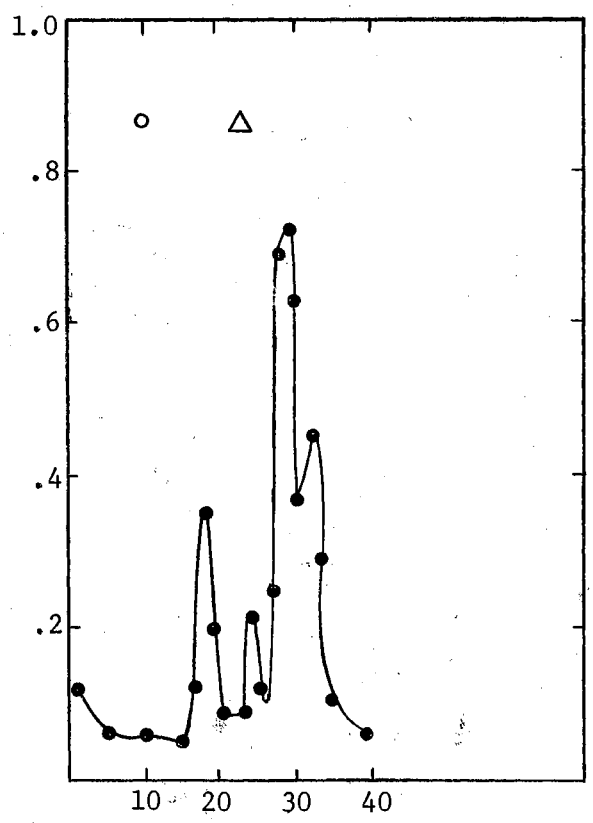

Figure 14. D. hentzi Venom Protein Elution Pattern from a Bio-gel P-2 Column, 90 cm x 6 mm ID, using 0.05 M, Phosphate Buffer pH 7.0 for Elution

O Bovine serum albumin - 66,500 mw

Δ Bacitracin - 1450 mw

TABLE II*

BIOLOGICAL ACTIVITY OF FOUR D. HENTZI VENOM COMPONENTS

Observation Time	6 Hours		24 Hours		48 Hours	
	Cockroach		Cockroach		Cockroach	
	1	2	1	2	1	2
Peak from Bio-gel P-2 Column						
1	dead	normal	dead	normal	dead	normal
2	normal	normal	normal	normal	normal	normal
3	normal	normal	dead	on back	dead	dead
4	normal	normal	normal	normal	normal	normal
Control	normal	normal	normal	normal	normal	normal

*Injection of the four major components of D. hentzi venom isolated using a Bio-gel P-2 column 90 cm x 6 mm ID.

TABLE III

FREE AMINO ACIDS IDENTIFIED IN *D. HENTZI* VENOM

Sample 1	Sample 2	Sample 3
unknown	unknown	unknown (aminobutyric acid)
	aspartic acid	aspartic acid
	threonine	threonine
serine	serine	serine
glutamic acid		glutamic acid
glycine	glycine	
isoleucine	isoleucine	
leucine	leucine	
	tyrosine	
phenylalanine	phenylalanine	
methionine		

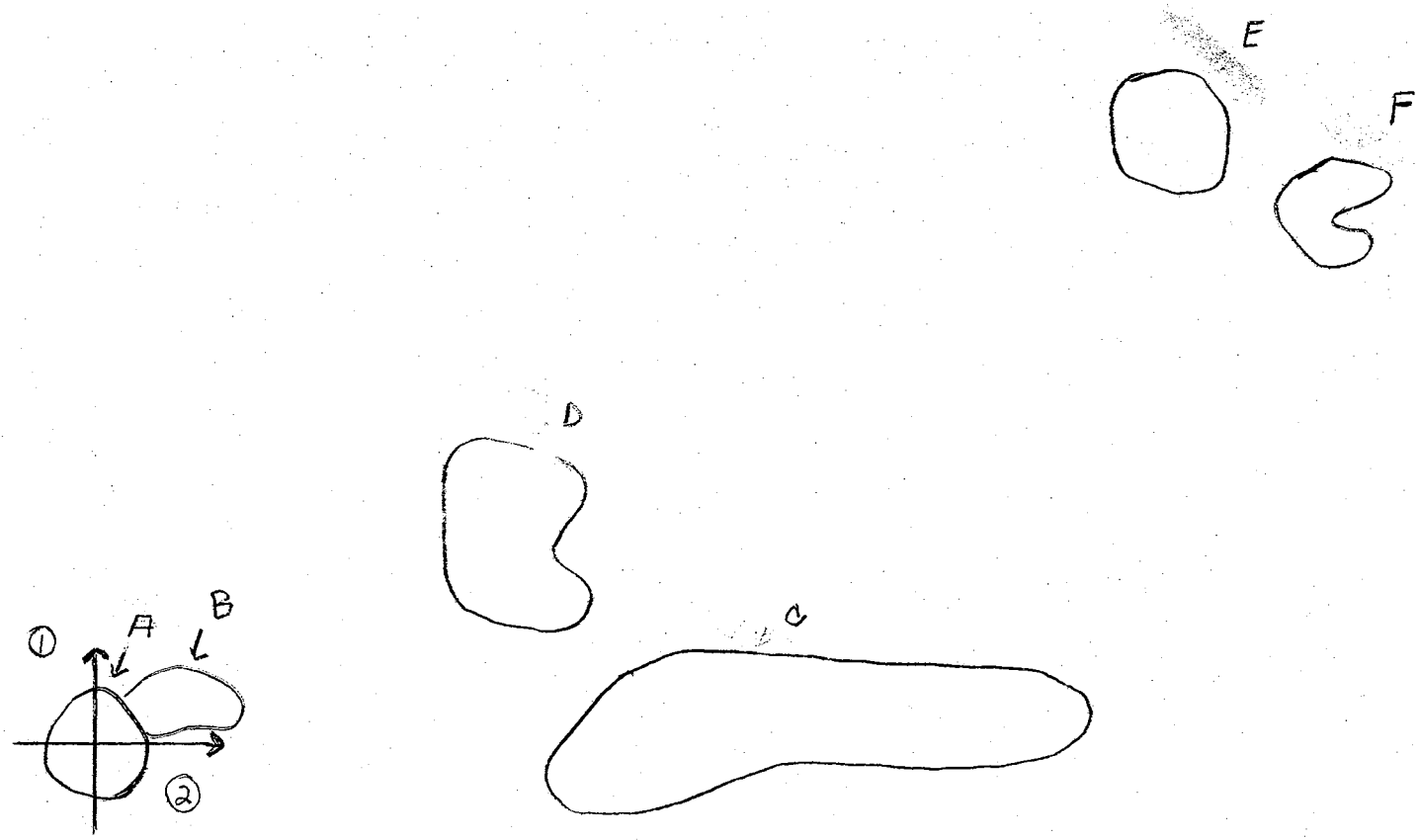

Figure 15. Paper Chromatograph of D. hentzi Venom. Solvents (1) N-Butanal:acetic acid: H_2O (4:1:1); (2) Phenol saturated with 6.3% sodium citrate and 3.7% sodium dihydrogen phosphate

compared to reference compounds only the spots E and D can be identified. Spot E could be tryptophan or methionine and D could be glutamic acid or serine.

The venom of the L. reclusa was tested for phosphatidase A activity by incubating whole venom with labeled lecithin 1 acyl-2-(^{14}C-oleoyl)-GPC. After incubation the sample was extracted by the procedure in Chapter III. For the results see Table IV. A positive control was obtained by using 500 µg of rattlesnake venom, and the negative control consisted of heat inactivated rattlesnake venom. No heat inactivated L. reclusa venom was used due to the difficulty in obtaining adequate venom. As seen from Table IV the basic extraction removes unreacted lecithin and lysolecithin in the chloroform layer. The control which consisted of lecithin and no venom shows that the extraction of the unreacted lecithin was not complete. When a control of heat inactivated venom was used the results were quite similar as mot of the radioactivity was in the chloroform phase of the basic extraction; however, due to poor extraction at the basic pH the acid extract contained considerable activity. As can be seen from Table IV the positive control, which consisted of 500 µg of rattlesnake venom and lecithin does show the presence of phosphatidase A. L. reclusa venom contained, according to this assay, no phosphatidase A activity. These results are inconclusive as a relatively small amount of L. reclusa venom was available for study. Ten spider bites will give about 100 µg of crude venom, and this can vary since not all spiders yield the same amounts of venom per bite. The L. reclusa spiders when kept in captivity also do not release venom after multiple venom extractions. Many spiders will not bite when shocked, but when placed in a container with a cockroach they will bite naturally,

TABLE IV

PHOSPHATIDASE A ACTIVITY OF TWO VENOMS

Treatment**	Control lecithin* without venom CPM*	L. reclusa venom CPM*	Rattlesnake venom CPM*	Control heat treated rattlesnake venom CPM*
Basic Extract, CHCl, (unreacted lecithin and lysolecithin)	9616	10820	422	8385
Acidic Extract, CHCl, (free labeled acid)	3901	4330	9157	3652

*Liquid Scintillation, Bray's solution no corrections.

**Obtained from Mr. Ed Hill, University of Michigan.

killing the cockroach.

Hyaluronidase activity was also assayed by the procedure described in Chapter III. The results are shown in Table V. The reaction mixtures were assayed for N-acetylhexosamines colorimetrically and N-acetylglucosamine 8 mM was used as a standard. In this assay 500 µg of rattlesnake venom produced 11 µg/ml of N-acetylhexosamines overnight at 37°C while L. reclusa venom produced 2.8 µg/ml of N-acetylhexosamines under the same conditions. This assay appears to be relatively insensitive for the accurate determination of hyaluronidase activity. A more sensitive assay is needed, however, this assay does indicate the presence of hyaluronidase activity in L. reclusa venom.

TABLE V

HYALURONIDASE ACTIVITY OF L. RECLUSA AND RATTLESNAKE VENOMS

Sample	L. reclusa venom	Rattlesnake venom	Blank Rattlesnake venom heat treated
A_{560}	0.004	0.077	0.0
	0.017	0.068	0.0

*Blank contained hyaluronic acid, and no venom.

CHAPTER V

RESULTS AND DISCUSSION

Summary

The venoms of Loxosceles reclusa, Lactrodectus mactans and D. hentzi were electrophoresed on polyacrylamide gels with and without ammonium persulfate. It was found that there was a decrease in the number of components in the Loxosceles reclusa and Lactrodectus mactans venom when separated on polyacrylamide gels without persulfate. There was an increase in the number of protein components from none to six or seven in the venom of D. hentzi. These results indicate that the ammonium persulfate had some effect on the protein components present in the venom. Due to these results the gels used to separate venom components were pre-electrophoresed to remove the ammonium persulfate. It was found that gel scanning was a useful tool for transcribing data from gel columns into linear graphs, but that these graphs cannot be used to quantitate with confidence the amount of protein present. It was also found that linear graphs can be plotted when samples separated on polyacrylamide gels are separated in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. By using this procedure described by Shapiro et al., migration in the gels is according to molecular weight and not charge since all proteins migrate as anions. The graph obtained is linear and can therefore be used to determine the molecular weight of unknowns.

A Bio-gel P-60 column was used to separate D. hentzi venom; however, the peaks were found to elute in a region where there is nonlinearity with respect to molecular weight. A Bio-gel P-2 column was then used to separate the D. hentzi venom. There were four peaks found, one of which eluted at the void volume. Two of these peaks were found to be lethal to cockroaches. From the data obtained from the Bio-gel columns there are at least three components of molecular weight 1450 or lower. The fourth component may consist of more than one component since it elutes at the void volume. Free amino acids were also found in D. hentzi venom by ion exchange chromatography.

L. reclusa venom was tested for phospholipase A activity and none was found to be present. An indication of hyaluronidase activity was found by incubating the venom with hyaluronic acid and testing for N-acetylhexosamines; however, due to the insensitivity of this assay several microassays should be tried before a conclusion is made.

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VITA

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