THE TOXICITY, HEMOLYTIC ACTIVITY, AND PROTEIN COMPONENTS OF THE VENOM FROM CERTAIN SPIDERS OF THE FAMILY LYCOSIDAE (ARANEAE)

By

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(ARANEAE)

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PREFACE

Interest in spiders, their life histories, venom toxicity, and potential as biological control agents has resulted in several recent studies at Oklahoma State University. Dr. D. E. Howell, Professor and Head, Department of Entomology, and Dr. G. V. Odell, Associate Professor of Biochemistry, Oklahoma State University, were involved in spider venom research upon my arrival and suggested several areas of research I might pursue. An interest in spiders and their venoms had been created by earlier conversations with Dr. Roger H. Grothaus, a personal friend and a fellow Naval medical entomologist. So it seemed natural to follow a research problem so important to the public health and of such interest to researchers at Oklahoma State University.

Indebtedness is expressed to: The United States Navy for making the study possible; Dr. D. E. Howell and Dr. G. V. Odell for their leadership and guidance throughout the study; Dr. W. A. Drew, Professor of Entomology, and Dr. R. D. Eikenbary, Associate Professor of Entomology, for their suggestions and criticisms of the research project and for reviewing the manuscript; Dr. H. K. Wallace, Professor and Head, Department of Zoology, University of Florida, Gainesville, Florida, for tentative identification of the spiders used in the toxicity studies; Terry Biery, Curtis Bush, Neil McCullum, and Mr. Karl L. (Pete) Masters for aiding in the collection of spiders; Joyce Sternberg and Judy Hall for electrophoresis operations; and to Iris Anita Peterson, my wife, for aid in preparing the manuscript.

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INTRODUCTION

Up until the last twenty years almost all cases of spider bite, reported in the United States, resulting in serious injury or death were assigned to the infamous black widow spider. Arachnidism was synonymous with "Lactrodectism." However, recent reports indicate that many different species of spiders could be responsible for spider bites in human beings, known as arachnidism. There is sufficient evidence to indicate that the bite of some of the wolf spiders, family Lycosidae, would be capable of causing a toxic response.

To evaluate the public health importance of a spider several factors must be considered. It is necessary to know if the venom presents a toxic hazard to human beings. This is usually accomplished by evaluating its biological activity on lower animals. The spider must be present in locations frequented by man to allow for contact between the two. Finally, the spider must occur in these locations in sufficient numbers to make the number of possible contacts which could occur significant. The primary objective of this study was to determine the species of lycosids, occurring in this area, that represent a potential toxic hazard to man. A second objective was to characterize the protein components of the venom, gut contents, and hemolymph of a representative member of the family Lycosidae.

REVIEW OF LITERATURE

Early History of Arachnidism

Thorp and Woodson (1945) relate that the first recorded spider bite in the Colonies occurred on September 3, 1726, in what is now Needham, Massachusetts. The first reference to any specific spider was made in 1812 in Peter Smith's "Indian Doctors Dispensatory" which made reference to the toxicity of black widow spiders. They further indicate that most of the early reports of spider bites traceable to black widows occurred in southern states. A bibliography of the literature on all aspects of venom and venomous animals was presented by Harmon and Pollard (1948). Most of the information pertaining to human cases of arachnidism has come from clinical situations where the patient has usually described and in some cases produced the spider. One must have serious doubts as to the validity of species named under these circumstances.

Spiders of Public Health Importance

There are many instances, recorded in the literature, where an individual has been bitten by a spider and the spider was recovered and identified. Some of those which have been reported as occurring in the United States are listed below. Schmaus (1929) recorded the effects of *Loxosceles rufescens* Dufour bite (Loxoscelidae). Blair (1934) allowed himself to be bitten by *Latrodectus mactans* (Fabricius) (Theridiidae).

Tinkham (1946) reported the effects of Peucetia viridans Hentz venom (Oxyopidae), which had been squirted into the patient's eye from a distance of about ten inches. Results of the bite of Loxosceles reclusa Gertsh and Muliak was outlined by Atkins, Wingo and Sodeman (1957). Furman and Reeves (1957) described the symptoms resulting from the bite of Chiracanthium inclusum Hentz (Clubionidae). Waldron and Russell (1967) disclosed that Loxosceles unicolor Keyserling had been responsible for a case of "necrotic arachnidism." Waldron (1968) reports on a case of spider bite involving Thiodina sylvana Hentz (Salticidae). There are also references in the literature to cases of spider bite where the offending spider was not recovered but inferences were made to the probable species involved.

Many authors differ on the toxic species of spiders present in the United States and the potential danger they represent for human beings. Minten (1959) stated that there is no indigenous species of "tarantula" known to be very dangerous. Those spiders he reported as dangerous were: the large Lycosa, Latrodectus sp., and P. viridens. Baerg (1958) reported that the bite of the "tarantula," Dugesiella hentzi Girard, resulted in death for two of the six rats bitten. However, he did not consider this species dangerous to man. Baerg (1959) listed those spiders which were considered poisonous to man, in descending order of severity, as Latrodectus mactans, Loxosceles reclusa, Latrodectus bishopi Kaston, Latrodectus geometricus Koch, Chiracanthuim diversum Koch and C. inclusum. Horen (1963) separated the toxic species of spiders into those that are moderately or severely toxic and those that are slightly toxic. Those listed in the former category were Latrodectus bishopi, L. geometricus and the L. mactans complex. Scott

(1963) considered Latrodectus mactans, Loxosceles reclusa and C. diversum to be the more dangerous species in the United States.

Reports of injury or death resulting from spider bites in foreign countries are rather sparsely scattered in the literature. Macchiavello (1947) reported "gangrenous spot" of Chile was due to the bite of the spider Loxosceles laeta (Nicolet). Mackinnon (1948) described the same conditions resulting from the bite of L. laeta in Uruguay. Gertsh (1949) observed that the genera Phoneutria and Ctenus (Ctenzidae) have neurotoxic venom. McKeown (1952) listed Latrodectus hasseltii Thorell and Atrax robustus Cambridge as the two toxic species in Australia. Finlayson (1956) stated that L. geometricus and Latrodectus indistinctus Pickard-Cambridge are the most important poisonous spiders in South Africa. A death due to the bite of the trapdoor spider Euctimena tibialis Rainbow is recorded by Savory (1964) as having occurred in Australia. These few references serve to illustrate the fact that there are many different species of toxic spiders which have been identified and leads one to be aware of the possibility of related toxic species in the United States.

Impact of Spiders on Public Health

Horen (1966) in reviewing the recorded spider bites in the United States found that only 615 cases had been reported up to the early 1940's, with 38 deaths. The period 1950-1959 showed that 65 of 460 fatalities caused by venomous animals were a result of poisonous spider bites. Parrish (1963) stated that of these 65 deaths, two were attributable to Loxosceles reclusa and the rest were caused by Latrodectus spiders. Earlier Parrish (1959) pointed out that most of the

fatalities (59%) occur between May and October. In Oklahoma from 1950 through 1954 only three deaths were reported as resulting from venomous animals. All of these were the result of spider bites (Parrish, 1959). Scott (1963) stated that injury from arthropod venoms was a common public health hazard in the United States, with millions of people being bitten or stung each year. From those envenomizations he estimates that about 25,000 will result in severe injury and about 26 in death. According to him spiders are rated second in the total number of human fatalities from envenomizations, with an average of eight people dying every year from spider bites.

Lycosids as Toxic Spiders

Thorp and Woodson (1945) and Gertsh (1949) related the story of how in the 17th century the peasants in Italy were being stricken by a strange disease called tarantism. Its symptoms were weeping, grotesque postures, and madness. The only cure was purported to be wild, unrestrained dancing. The condition was said to be brought on by the bite of a spider, *Lycosa tarantula*. A search of available literature failed to reveal the author of *L. tarantula*. Savory (1928) described Fabrès work with *Lycosa narbonnensis* Walckenaer. He stated that after allowing the spider to bite a sparrow on the leg, the bird immediately lost the use of the leg and died within two days. In a related experiment a mole was bitten on the nose and died in three days. Thorp and Woodson (1945) stated that *Lycosa raptoria* Walckenaer, a Brazilian wolf spider, may cause marked symptoms on human beings. However, they reported those species of *Lycosa* which were encountered around the Los Angeles area bit vigorously without coaxing but the wound was only

annoying and not painful. Gertsh (1949) asserted that *L. raptoria* is reported to cause "progressive necrosis" in human beings. *Lycosa murina* Nicolet was reported by Donoso-Barros (1948) as causing "cutaneous arachnidism" in Chile. Minten (1959) listed the large *Lycosa* as being dangerous spiders in the United States. *Lycosa carolinensis* Walckenaer and *Lycosa punctulata* Hentz were classified by Horen (1963) as being slightly toxic to man. Grothaus and Teller (1968) summarized the clinical report resulting from the bite of *Lycosa miami* Wallace. They stated that the swelling and pain subsided on the second day. Snetsinger (1969) records having been bitten by both *L. carolinensis* and *Lycosa helluo* Walckenaer with the resulting swelling and pain subsiding in a day or two.

The wolf spider kills its prey by three methods, according to McKeowan (1952). The spider may either crush the prey with its chelicerae, inject it with venom or both. He further observed that the prey is squeezed dry since only fluids are capable of passing through the spider's small oral cavity. Kaston (1953) stated that spiders are capable of controlling the quantity of venom injected. The morphology of a spider's venom apparatus was outlined by Comstock (1948). He said there were two venom glands, each discharging venom through a long duct which opened near the tip of the apical segment of the chelicera. Dugés (1836) observed that the venom glands of lycosids occupy a large segment of the cephalic region. He further stated that by his observations, hunting lycosids were not afraid to take on larger prey.

Ecology of the Lycosids

Gertsh (1949) characterized the family Lycosidae as occupying

almost every terrestrial habitat and being dominant predators in all. He stated they were most numerous in prairie regions, with many species being burrowing spiders. Of the problem spiders found in midwestern and northeastern homes, the most common family reported by Snetsinger (1969) was Lycosidae. From this family *L. helluo* and *L. carolinensis* were the two most frequently found species. He further observed that fall was the time of highest spider infestation rates in the home.

In comparing *Lycosa rabida* (Walckenaer) with two other *Lycosa* Kuenzler (1958) found that dense vegetation was a requirement for *L. rabida*. He found the species to be easily excited causing it to scramble onto high grass or into thicker grass near the ground. However, the spider would attack an enamel marking brush when finally cornered. He observed it not to be a burrower but to be most frequently found above the ground surface on grasses, as did Barnes (1953). Gertsh (1949) stated he had observed *L. rabida* in grassy areas along roadsides over most of the United States. Eason and Whitcomb (1965) reported that *L. rabida* was abundant in old pastures and grasslands in Arkansas. They stated that this species matures during early summer and midsummer, with only the immature progeny overwintering. The spider was more frequently collected by them during August, when over 50% of the total collected for the year was taken. However, they were able to collect it from July through November.

Branson (1966) updated the spider fauna collected in Oklahoma and listed a total of 33 species of lycosids. Bailey, Grothaus and Drew (1968) added five species to that list. Thus there are a total of 38 species of lycosids which have been reported for the state.

Venom Evaluation Variables

The evaluation of a venom, and the variables which are present in making such an evaluation, has received considerable attention from many authors. Carmichael (1940) in studying the effects of ambient temperature on the toxicity of rattlesnake venom found that rats held at 48 to 60 F required a minimum fatal dose two to three times that of rats held at 90 to 98 F. In evaluating the venom of Latrodectus hasseltii on white mice, Wiener (1956) found that at 37 and 0 C the toxicity of the venom was 100 times greater than at 18 to 24 C. Stahnke (1965) observed a similar phenomenon when he subjected rats to high and low temperature stress prior to inoculation of scorpion or rattlesnake venom. At both temperature extremes an increase in the toxic effect of the venom was observed. Benton, Heckman and Morse (1966) found that the optimum ambient conditions for survival of white mice and guinea pigs, subject to either cobra or honey bee venom, was 78 F and 45% relative humidity. These authors reported that a temperature increase or decrease would increase mortality. However, changes in humidity would not always result in an increase in mortality.

In reporting on Vellard's work, Maretic and Stanić (1954) related that the pH of spider venom becomes alkaline and increases in toxicity above 25 C and acidic and less toxic at lower temperatures. Bücherl (1956) reported that for Phoneutria fera Perty the venom has a neutral pH for adults but is acidic in immatures.

Seasonal variation has been examined as a possible explanation for variation in venom toxicity. Schöttler (1954) noted both qualitative and quantitative variation in venom obtained from captured scorpions, leading him to suspect possible seasonal variation. Keegan, Hedeen and

Whittemore, Jr. (1960) showed that black widow spiders held at 35 F and room temperature during the same time of year exhibited no significant difference in venom toxicity. The authors expressed their LD_{50}'s as fraction of venom gland per test animal. They found the LD_{50} for white mice to be from 0.33 to 0.23 in November and from 3.29 to 2.45 in April and May.

Horen (1963) was concerned about the depth of injections on laboratory animals, suggesting that deep injections may be the reason for the lack of local reactions in some cases. He suggested that the venoms would have the greatest effect when injected into tissue of low vascularity (dermis), and hence would not be diluted.

Several authors have explored the variations in venom toxicity between a male and female spider of the same species. Wiener (1957) reported that the male *Atrax robustus* was more toxic to mice and guinea pigs than the female by both direct bite and venom injection techniques. However, Kaire (1963) working with the same species declared the venom of both sexes to be equal in toxicity. Morgan (1969) found the venom of both sexes of *Loxosceles reclusa* to be equal in relative toxicity, although the male released less venom.

There has been a great deal of variation in the selection of laboratory animals for use in biological assay of spider venoms. Thorp and Woodson (1945), in reporting on previous research on black widow spiders, stated that cats were used as assay animals in early experiments, but they were superseded by rats which were later replaced by the more susceptible guinea pigs. They reported dogs as being resistant to black widow venom. Herms, Bailey and McIvor (1935) found guinea pigs to be much more susceptible than rats to black widow spider

venom. Shulov (1952) and Grothaus (1967) reported that mice were susceptible to black widow spider venom. Maretic and Stanić (1954) listed the laboratory animals more susceptible to Latrodectus venom in descending order: mice, guinea pigs, rats, and kittens. They reported rabbits and dogs as being resistant. Finlayson (1956) used mice and guinea pigs while Wiener and Drummond (1956) utilized only mice in reporting positive sensitivity tests to various species of Latrodectus.

Rabbits have been successfully employed in venom evaluation of several species of the genus Loxosceles: MacKinnon and Witkind (1953), Atkins et al. (1958), Micks and Smith (1963), Grothaus (1967), and Keh (1968). Shulov, Ickowicz, and Pener-Solomon (1962) as well as Grothaus (1967) were unable to produce typical necrotic lesions on mice with the venom of Loxosceles spiders, even though they were able to achieve mortality. Atkins et al. (1958) achieved both the formation of typical necrotic lesions and in many cases mortality by using guinea pigs as assay animals. Denny, Dillaha, and Morgan (1964) reported that dogs injected with venom of the brown spider, Loxosceles reclusa, exhibited toxic reactions to it but did not form typical necrotic lesions.

Wallace and Sticka (1956) reported that mice, rats, guinea pigs, and rabbits were all resistant to the venom of Atrax robustus. They felt that man possessed a peculiar sensitivity to the spider not shared by other animals. Baerg (1958) was successful in obtaining mortality in white rats using venom from the "tarantula," Dugesiella hentzi (Gerard) (Theraphosidae). However, he felt that results on test animals should not be overstated in predicting the effect of the venom on human beings. Grothaus (1967) stated that if sufficient venom was

used a "wet" lesion could be obtained on white mice using venom from certain members of the family Lycosidae.

Symptomology and Acquired Immunity

According to Herms, Bailey and McIvor (1935) the bite of a black widow spider on guinea pigs produced marked excitation, sneezing and coughing, respiratory spasms, abdominal distension, purplish color at bite site, labored breathing, nasal exudate, diaphragm paralysis, respiratory paralysis, and a convulsive death.

Atkins et al. (1958) reported that following the bite of a female *L. reclusa* a rabbit developed a wheal at the bite site in about 7 hours. This increased in size during the next day with lethargy, stiffness in the leg, and copious yellow feces developing on the second day. On the third day a weight loss of over 7 oz was noted and the edges of the wheal had thickened resulting in the formation of a pocket in the center. On the fifth day the rabbit had returned to normal with the exception of the bite site which was a purplish blue with thick red edges. Sloughing of the center portion started on the 10th day and lasted until the 15th. On the 15th day healing started and was completed by the 35th day. They observed guinea pigs to exhibit similar symptoms. Grothaus (1967) observed a similar sequence of events for *L. reclusa* on a rabbit.

Shulov, Ickowicz, and Pener-Solomon (1962) showed the bite of *Loxosceles rufescens* exhibited delayed symptoms on white mice resulting in paralysis and death, but did not cause any pathological symptoms at the bite site. However, their studies with several species of *Latrodectus* showed immediate onset of slowly ascending paralysis,

terminating in death.

Keh (1968) found that direct bites of female *Loxosceles unicolor* on rabbits resulted in responses ranging from the formation of a wheal with erythema and localized swelling, to more severe cases with lesions formed 24 hours after the bite. This was followed by formation of an eschar which later ruptured and drained. After three weeks the primary eschar fell off revealing the secondary eschar which had formed under it.

MacKinnon and Witkind (1953) noted that rabbits built up an immunity to *Loxosceles laeta* bites following several exposures. Atkins et al. (1958) found that rabbits could be immunized after four injections of *L. reclusa* venom.

Methods of Venom Recovery

There are several methods of venom recovery that have been used in past studies conducted on spiders. The direct and most natural method is to simply allow or encourage the spider to bite the assay animal. This method was employed by Herms, Bailey and McIvor (1935), Shulov (1952) and Keh (1968), as well as many others. Shulov, Ickowicz and Pener-Solomon (1962) recognized that the reaction to the venom could not be equated to the amount of venom injected by using this method.

Keegan, Hedeen, and Whittemore, Jr. (1960) removed the cephalothorax, macerated it in saline, and then injected the mixture into the test animal. D'Amour, Becker and Riper (1936) removed the venom glands and injected the macerated glands, in a saline diluent, into the test animal. Lebez (1954) showed that venom obtained by allowing the spider to bite a cotton pad was more toxic than either dried venom or

macerated venom glands.

Baerg (1958) "milked" "tarantulas" by placing electrodes at the basal segment of the chelicerae. Denny, Dillaha, and Morgan (1964) obtained "pure" venom by electrical stimulation of the spider. They then collected the venom from the fangs with capillary tubes. Maretic (1967) collected the venom of orthognath spiders by mechanical pressure or by a 40 volt (d-c) electrical shock. Grothaus and Howell (1967) recovered spider venom by touching the leg of the spider with a pair of electrodes from a sine wave generator. They achieved the greatest venom yield with the least mortality with approximately 6-7 volts at 15-20 cps. The venom was collected on cigarette paper, held between the spider's fangs during the "milking" procedure. Smith and Micks (1968) shocked the spiders they were working with with 200 volts for 1/10 second and collected the released venom from the fang with capillary tubes.

Venom Induced Hemolysis

For some time workers have been interested in the hemolytic activity of spider venoms as a key to their mammalian toxicity. Kyes (1910) using red blood cells, washed with saline, found that ox, sheep and goat cells were not hemolyzed by spider venom. However, red blood cells of man, as well as many other animals, were readily hemolyzed. Kellogg (1915) described some early work on spider venom hemolysis in which macerated poison glands produced no hemolysis to red blood cells, but maceration of the entire body of the spider resulted in total hemolysis. Ray (1945) also noted that snake venom would not lyse the red blood cells of sheep, goats or oxen but was effective on guinea

pigs and dogs.

Kelen, Rosenfeld and Nudel (1960-1961-1962) reported that *Lycosa* venom would strongly lyse ox and sheep red blood cells and would weakly lyse human red blood cells.

Most of the more recent work on red blood cell hemolysis by spiders has been conducted with venom from necrosis inducing spiders. Kelen, Rosenfeld and Nudel (1960-1961-1962) reported that certain *Loxosceles* venoms will markedly hemolyze human red blood cells. Denny, Dillaha and Morgan (1964) found there was a direct hemolytic action of brown spider venom on human red blood cells with maximum hemolysis reaching a mean of 26.2%. Nazhat (1968) obtained 36% hemolysis using brown spider venom on human red blood cells. However, Smith and Micks (1968) found no hemolytic activity on human red blood cells for either brown spider venom or the two other species of *Loxosceles* tested. They were able to produce hemolysis for all three species by using abdominal extracts.

Venom Characterization by Electrophoresis

Various methods have been used by researchers to characterize toxic venoms. One of the earlier methods was through the use of paper electrophoresis. Muic, Stannic and Meniga (1956) found six protein constituents, using this method, for the venom of *Latrodectus tredecimguttatus* Rossi. Fischer and Bohn (1957) reported four protein components for both *Lycosa erythrognathus* Lucas and *Phoneutria fera*. Wiener (1963) identified eight separate components from *Atrax robustus* venom. Maretic (1967) used paper electrophoresis and noted 5 to 7 protein components from orthognath spider venom. Lebez et al. (1968)

used Se^{75} labeled venom to obtain the electrophoretic pattern of Hogna (Lycosa) tarentula venom on filter paper.

The use of disc electrophoresis has recently become an important tool in the characterization of venoms. McCrone and Netzloff (1965) used a Conalco Model 12 disc electrophoresis apparatus to fractionate the venom components of certain Latrodectus spiders. They showed that antivenin prepared against one of the species would neutralize the lethal effects of all four species being studied. They further proved, by gel electrophoresis, that although the venoms appeared to have the same basic nature they showed species-specific qualitative differences. McCrone and Hatala (1967) used the supernate of homogenated black widow spider venom glands in the characterization of seven protein bands by gel electrophoresis. Nazhat (1968) utilized the Conalco polyacrylamide gel disc electrophoresis apparatus to obtain 5 to 7 protein bands from brown spider venom and seven bands from black widow spider venom. She claimed "tarantula" venom had no protein components since no bands had been obtained by electrophoresis. However, later work has shown that "tarantula" venom does contain protein (G. V. Odell, personal communication).

Frontali and Grasso (1964) used an extensive process to separate three different protein components from Latrodectus tredecimguttus venom. They obtained five protein fractions by using column electrophoresis on cellulose powder. These workers reported gel filtration on Sephadex G-200 showed three protein fractions with two showing biological activity. Following this they purified the venom by filtration on Sephadex G-100, recovered the extract, and by chromatography on a column of DEAE-Sephadex A-50 were able to completely separate the three

toxic bands. Smith and Micks (1968) utilized an immuno-electrophoresis technique to show that the venoms of three species of Loxosceles were different.

Quantitative Estimates of Venom Contents

Fischer and Bohn (1957) said that, on a dry weight basis, the protein concentration of Phoneutria fera venom was 54% of the total weight of the whole venom. Maretic (1967), working with an orthognath spider, did not estimate the protein content of the venom but stated that 76.7% of fresh venom was water. Stahnke and Johnson (1967) used the Itzhaki and Gills method for estimating protein amounts. They found the venom of the "tarantula" (Aphonopelma) to be 5% protein while that of a Centuroides scorpion was 62% protein. McCrone and Hatala (1967) measured the relative concentrations of protein fractions, eluted from black widow spider venom, as absorbance at 240 mu using a Beckman DU Spectrophotometer. The protein concentration was found to be 53.7% of the total volume of the venom. They estimated the molecular weight of the single lethal protein to be 5,000 ± 1,000 by means of a light scattering technique. Morgan (1969) determined, by using Waddell's method, that 18% of brown spider venom was protein.

MATERIALS AND METHODS

Test Organisms

Two types of test animals were used to study the effects of lycosid venoms. Initially, the venom of a collected spider was injected into guinea pigs. If a lesion was obtained on guinea pigs, the spider's venom was then further challenged on domestic swine (Yorkshires).

The guinea pigs used in the tests were obtained, through a local supplier, from a guinea pig farm near Wichita, Kansas. Only white, virgin females were used to reduce possible variations in susceptibility due to sex. The animals weighed 300-500 g when used for testing purposes. They were maintained in self-cleaning wire cages and were fed Purina Guinea Pig Chow each day. Water was available at all times. The room temperature was maintained at 85 F ± 5.

The domestic swine were kept two to a stall in a nearby barn. Ambient temperature in the barn was approximately that being recorded outside. Food and water were available at all times. The animals weighed between 40-115 lb. during the testing period and were all males.

Laboratory Materials

Field Collection of Spiders. Two methods were used to capture lycosid spiders. Field collections were made both during the day and at night, by placing a baby food jar over the spider, allowing it to

crawl into the jar, and then covering the jar with a perforated screw top. A canvas fishing creel was used to carry the baby food jars. A hand trowel was carried in the creel to aid in the extraction of burrowing species. Pencils and paper were also carried in order to date and label all collected specimens. Field collections were made at night with the aid of a battery operated head lamp. Twenty pit traps were constructed in locations which appeared to be suitable habitats for lycosids. Construction consisted of excavating a hole of sufficient size to accommodate a quart jar. It was necessary that the inner surface of the jar be clean or the spider could gain enough traction to climb out. Best results were obtained by having the jar mouth flush with the ground and free of overhanging grass. The jar was shaded by a conical sheet metal canopy with a single pointed leg. The leg was pressed into the ground far enough to hold the canopy about 3 inches above ground. These traps were examined at regular intervals, with the captured spiders being removed into individual containers and labeled. After capture the spiders were taken to the laboratory, placed in individual cartons, and fed.

Laboratory Requirements of Lycosids. The cannibalistic habits of lycosids made it necessary to provide each spider with its own container. The containers were either half-pint or pint ice cream cartons. The lid was removed from the carton and replaced with a clear plastic petri dish lid. The plastic lid allowed easy viewing of the spider and simplified its removal from the carton. It was found that most lycosids require an available source of moisture. To facilitate the watering of the spider, a cork borer was used to puncture a hole in the side of the carton of sufficient size to permit the snug entry

of a 4-dram vial. The vial was filled with tap water, plugged with a cotton plug and placed in the prepared hole. The spiders were fed 5 to 10 house flies once every 2 weeks. The flies were reared in the laboratory using standard rearing techniques. The spiders were not studied for at least 4 days after feeding. This delay was to reduce venom variability which may result from recent depletion of the venom supply. Room temperature was maintained at 85 F ± 5.

Spider Restrainers. Spiders used in the animal studies were restrained in a single expanded plastic cell described by Grothaus and Howell (1967). When the venom was to be recovered from a series of spiders of the same species, a multiple spider restrainer was constructed. A 1 x 1 inch strip of expanded plastic was glued to a ½ x 1 inch board, approximately 18 inches long. The plastic was split, about 3/4 of the way through, down its entire length. Pockets were formed at regular intervals within the split by burning out an area with a hot metal rod. The spiders were then placed in the pockets with the anterior end of the cephalothorax protruding from the slit. The spiders were held in place by the firm pressure of the plastic against the body maintained by wrapping dental floss around the restrainer and over any exposed legs.

Venom Recovery Equipment. The spiders were stimulated to release venom by electrical shock. This shock was provided by a sine wave generator (Heathkit model IG-72) with positive and negative electrodes. The venom was collected from the fangs of restrained spiders by means of glass capillary tubes. These tubes had an internal diameter of 0.9 to 1.1 mm. The tubes were heated and pulled out to further reduce the internal diameter. A dissecting microscope was used to observe the

spider. A Mettlar Analytical Balance (model H 16) was utilized to weigh the venom.

Electrophoresis Equipment. Spider venom and body fluids were examined for protein fractions by using a Hoefer Electrophoresis Unit (model DE-102). A Hoefer Destainer (model DE-105) with a petroleum grade charcoal filter was used to destain the gels.

Testing Procedure

Venom Recovery. The spider to be "milked" was anesthetized with CO_2 by the introduction of the gas through a tube inserted in the hole on the side of the carton. The spider was then placed in a plastic cell or in the multiple spider restrainer and positioned under the microscope. The lycosids, as a whole, would bite readily and repeatedly at objects held within reach of their fangs, but were sometimes reluctant to release venom. If the electrodes were applied immediately to the base of the chelicerae the usual result was that the spider would not bite but would simply fold its fangs back into the fang furrow. The expulsion of gut contents or venom mixed with gut contents was the typical result of this action. To compensate for this it was necessary to antagonize the spider by stroking one of its palpi with the electrodes before stimulating it to bite. The spider was then permitted to strike a capillary tube or electrode and the expelled venom was collected in preweighed capillary tubes. The optimum setting on the generator for venom recovery was 6 volts at 20 cps. The tubes containing the collected venom were then reweighed. The difference between the "premilking" and "postmilking" weights represented the amount of venom collected. Rubber gloves were always worn while conducting weighing and "milking" operations to eliminate the possibility of

increasing the weight of the capillary tubes by the addition of body oils.

Venom Injection. Prior to inoculation, the guinea pig's hip was trimmed free of hair with electric clippers and then, in most cases, shaved with an electric razor. All syringes and needles were sterilized in an autoclave prior to injection of venom. A 0.25 cc syringe fitted with either a 26 or 27 gauge needle was used to administer the injections. Prior to the addition of venom, 0.1 cc of saline (0.9%) was drawn into the syringe. The needle was inserted in the large end of the capillary tube and the venom extracted. Only the venom from one spider was used to inject a guinea pig.

The guinea pig was encouraged to climb into a glass quart jar, which was lying on a table. This prevented the animal from turning around and limited its movements. The shaved leg was then extended. Intradermal inoculations were made by slipping the well-sharpened needle proximally, with the aperture up, under the first few layers of skin. Insertion was no more than 1/8 to 1/4 of an inch. If the needle opening was not visible beneath the surface of the skin a new site was selected and the needle reinserted. The injection was made and the needle removed quickly. Following injection the guinea pigs were returned to individual cages for observation. The maximum elapsed time from "milking" to guinea pig inoculation was 10 minutes.

The outer surface of the hip was selected as the injection site since it was a large fleshy surface and easily visible. It had an added advantage in that any localized response to the venom might manifest itself by affecting the walking behavior of the animal.

The venom for injection of swine was collected in the same manner

as described above. The venom was then transported to the barn where the swine were held. There was no preparation of the swine prior to inoculation. The area immediately behind the ear was selected as the injection site. This location was selected since the skin in this area is soft and can readily be penetrated by a needle. Other advantages were the scarcity of hair, protection from physical damage offered by the ear, relative cleanliness of the area, and the light character of the skin at that location. A syringe containing 0.1 cc of 0.9% saline was again used to draw the venom from the tubes, as previously described. One or two men served as swine holders. They restrained the swine and pulled the ear forward to expose the injection site for intradermal injections. The technique used for swine inoculation was similar to that used on guinea pigs. However, needle insertion was usually 1/4 to 1/2 inch for swine. The maximum elapsed time from "milking" to swine inoculation was 20 minutes.

Venom Evaluation. After a guinea pig had been inoculated it was held in an individual cage and observed at intervals of 1, 2, 5, 12, 24, 48, and 72 hours. If no lesion or other pathological conditions were noted the animal was returned to a holding cage with two other guinea pigs. Those guinea pigs exhibiting visible symptoms were retained for further observation. Each guinea pig was injected with venom only three times with at least a 2-week delay between injections. The hip used was alternated for each injection.

The swine were kept two to a stall throughout the testing period. Ear notches were used to differentiate the swine. Observations were again made at 1, 2, 5, 12, 24, 48, and 72 hours. Each swine was injected only three times and never received the venom of the same

species of spider twice. There was an average of 5 days' delay between injections. The ear used was alternated for each injection.

Notes on observable pathological conditions and behavioral changes were kept for each observation period. All lesions were measured by length and width, with notes taken on scab formation and retention times.

When venom testing was complete, the spider was preserved in 70% ethyl alcohol and held for shipment to a spider taxonomist. An attempt was made to collect a series of males and females of each species. This was done to reduce the possibility of inconclusive results due to an individual spider's reluctance to release venom or the difference that may exist between male and female venom.

Red Blood Cell Hemolysis. Two different methods were used to evaluate the hemolytic action of Lycosa rabida venom. One type involved the use of washed human red blood cells. Each of eight plastic centrifuge test tubes received 1 cc of fresh whole blood and 3 cc of 0.9% saline. These tubes were centrifuged at 10,000 rpm at 0 C for 15 minutes. The supernate was then carefully poured off and 3 cc of saline was again added to each tube and the cells gently resuspended in the saline. This washing process was repeated four times.

Two separate tests were conducted using washed red cells. One was conducted using cells suspended in 2 cc of saline and the other utilized packed red blood cells with no saline added. In either case a known amount of venom was introduced into each of six tubes by flushing it out of capillary tubes with a pipette filled with saline. Three to four drops of saline were used to insure total removal of the venom.

Two tubes were held as controls. The tubes were allowed a 30-minute contact time while packed in crushed ice. They were then centrifuged for 10 minutes at 10,000 rpm and 0 C. The extent of the hemolysis was recorded as none, slight, moderate, or complete. If no hemolysis was noted the tubes were placed in the cold room overnight at a temperature of 4 C. The following day the cells were again spun down at 10,000 rpm for 10 minutes in a refrigerated centrifuge. The extent of the hemolysis was recorded as before.

The second method involved placing a drop of whole fresh human blood on a microscope slide. A drop of venom was then either placed on a cover slip before laying it on the blood, or added alongside the cover slip after it had been placed on the blood slide. In either case the results were viewed under a compound microscope. Notes were made on red cell responses to the venom and hemolysis was evaluated as being either present or absent.

Electrophoresis. Five-inch columnar tubes were acid cleaned and precoated with a silicone base column coater, to facilitate gel removal. One end of each tube was plugged with a rubber stopper. The tubes were placed, stopper down, in a filling rack. Equal amounts of stock solutions A and C were mixed in a flask. An equal volume of catalyst, ammonium persulfate, was then added to the flask and the mixture stirred. The tubes were filled from this prepared solution. A small amount of water was layered on the top of each tube to prevent formation of a meniscus by the polymerizing gel. The tubes were allowed to stand for 30 minutes, in total darkness, to fully polymerize the 7% polyacrylamide gels. The tubes were then removed, the water drawn off, and buffer 10x added to each tube. Buffer 10x contains 3.0 g of Tris

and 14.4 g of glycine to which water is added to bring the total volume to one liter. The columns were placed in vertical slots in the electrophoresis unit. The lower section of the unit was filled, to one inch from the top, with buffer 10x at a pH of 8.33. The upper section was filled with 400 ml of buffer 10x at a pH of 8.34. Electrophoresis was conducted at 5 mA per sample tube for 30 minutes to eliminate any residual catalyst. Room temperature during electrophoresis was 25 C. The venom or body fluid to be electrophoresed was removed from the capillary tubes by inserting the needle of a 10 μl syringe into the large end of the tube. In some cases it was necessary to blow the material out of the capillary tubes onto a piece of parafilm. There it could be picked up by the syringe. The sample was layered on top of the gel. Electrophoresis was then continued at the same rate for one hour. The gels were removed from the unit and stained with 0.5% aniline black in 7% acetic acid. After 3 hours the dye was drained off and the gels placed in the destainer, filled with 7% acetic acid and allowed to remain overnight. Finally the gels were removed from the destainer and placed in stoppered test tubes filled with 7% acetic acid.

Since proteins of the same molecular weight but slightly different charge tend to appear as separate bands on disc electrophoresis, Hedrick and Smith (1968), an attempt was made to counteract this fractionating action. Sodium dodecylsulfate has the ability to "hold" proteins of similar molecular weight together thus allowing them to migrate to a similar point in the gel and appear as a single band. In certain instances, prior to the addition of the sample and final electrophoresis, the following reagents were added to each gel column: 15 μl of 1% sodium dodecylsulfate (SDS) (0.01 g SDS in 1 ml 20%

sucrose) and 15 μl of 1% mercaptoethanol (ME), (10 μl ME in 1 ml 20% sucrose).

RESULTS AND DISCUSSION

Survey Data

Since the purpose of the study was to evaluate the mammalian toxicity of the venom and to estimate the potential public health hazard of representative members of the family Lycosidae, it was decided to concentrate on collecting in areas frequented by human beings. Most of the collecting was conducted around houses, outbuildings, barns, sheds, etc. Only large specimens, which were apparently in the adult stage, were collected for "milking." This was done on the assumption that adult spiders represent a greater hazard than immatures if for no other reason than their increased capacity for venom storage. The majority of the *Lycosa antelucana* Montgomery were collected in and around homes with the remainder being found around buildings with cement or other type of solid foundations. The *Shizocosa avida* (Walckenaer) were taken either around buildings with solid foundations or in pit traps. *Lycosa rabida* (Walckenaer) was by far the more common species collected in these situations with about half of these being collected around homes and the other half around buildings with solid foundations. This species was nearly always taken from tall, thickly matted Bermuda grass. It was more frequently found at about ground level near the base of the foundation. The most successful collecting technique was to kneel by the building, then while holding the baby food jar in the right hand and the lid in the left, pull the grass away

from the building. If the spiders were present a gentle shaking of the grass would cause them to run. They would usually run either along the ground next to the foundation or up the foundation, rarely out into the grass toward the collector. The collected members of *Lycosa helluo* Walckenaer were found around buildings with solid foundations and in one case in a burrow near a house. The majority of the *Lycosa carolinensis* Walckenaer collected were found in burrows. They were usually around homes or buildings with solid foundations. However, one was collected from a burrow in an open field. No males of this species were collected during the study. One *Lycosa punctulata* Hentz was collected near a building with a solid foundation. Almost all of the *Shizocosa ocreata* Hentz collected were collected from pit traps located near a shed. The *Lycosa acompa* Chamberlain collected were found near homes and structures with solid foundations. One *Lycosa gulosa* Walckenaer and one *Lycosa apothetica* Wallace was found near a building with a solid foundation.

Collections were made from March through August of 1968. In addition, *L. rabida* was collected from June through September in 1969 as well as in 1968. *L. antelucana* was collected in every month from March through August. *S. avida* was collected in March and June. *L. helluo* was taken during May and June. *L. carolinensis* was found during June and August. A single *L. punctulata* was collected in March. The reason for the low incidence of this species is probably due to the fact that the first mature spiders of this species are not found until September and were most frequently collected in October and March by Eason and Whitcomb (1965). The males of *S. ocreata* were collected in about equal numbers with females, in pit traps, during May and June.

After June, however, no specimens of either sex were collected by the traps. All the specimens of *L. acompa* were collected in June. *L. gulosa* and *L. apothetica* were both taken in March.

In 1968 *L. rabida* was collected in June, July and August with more females being collected in June and more males in August. In 1969 they were collected from June through September. Once again more females were collected in June and more males in August. However, in September only males and immatures were collected. It was also noted, for September, that a great deal of searching was required to recover one or more spiders. The month of June yielded more than 60% of the total number of spiders collected for 1969.

A summary of collection data for 1968 is contained in Table I. The code for the collection site abbreviations is: (F) buildings with solid foundations (other than homes); (H) homes; (P) pit traps located near buildings, sheds, or other permanent structures. These data are not intended as a reflection of the total habitat or behavioral patterns of any of the spiders listed. This is only information gained from the months collections were made, under the restriction of limited types of collection locations.

Laboratory Observations

The necessity of providing a source of moisture has already been mentioned. If moisture was not made available most of the lycosids would die within a very short period of time. Most of the adult *Lycosa rabida* kept in the laboratory during 1968 died during September and October of that year. The spiders were placed on shelves in the laboratory and were therefore exposed to the ambient temperature and

TABLE I

COLLECTION DATA ON SPECIMENS OF THE FAMILY LYCOSIDAE FROM PAYNE COUNTY, OKLAHOMA[a]

Species	Collection Site	Month(s) Collected	Month More Frequently Collected[b]	% of Total
Lycosa antelucana Mont.	F & H	Mar. - Aug.	All	15
Shizocosa avida (Walck.)	F & P	Mar. & June	---	7.5
Lycosa helluo Walck.	F & H	May & June	---	7.5
Lycosa carolinensis Walck.	F & H[c]	June & Aug.	June	13
Lycosa punctulata Htz.	F	Mar.	---	2.5
Shizocosa ocreata Htz.	P	May & June	June	13
Lycosa acompa Ch.	F & H	June	---	7.5
Lycosa gulosa Walck.	F	Mar.	---	2.5
Lycosa apothetica Wallace	F	Mar.	---	2.5
Lycosa rabida (Walck.)	F & H	June - Aug.	Aug.	29

[a]Collected around houses, outbuildings, and other permanent structures during March through August 1968 and "milked" in the laboratory.

[b]Listed only if more than five specimens were collected.

[c]Majority of specimens collected in burrows.

humidity conditions of the room. Those collected in 1969 were maintained in a Precision Scientific Co. low temperature incubator (model 808) at 27 C ± 1. As of November, 1969, the majority of the adults were still alive. Most of the previous mortality occurred from manipulation during "milking" operations.

Guinea Pig Injections

Guinea pigs were selected as laboratory animals due to their reported sensitivity to venoms.

Subcutaneous Injections. Four spiders were "milked" and the venom injected subcutaneously. This meant the needle was inserted on a plane almost parallel to the leg with the venom being introduced just under the dermal layers. Using this technique, the venom from *Lycosa carolinensis* resulted in an erythematous swelling, at the inoculation site, 2 hours after injection. The area gradually receded in size and lightened in color during the next 48 hours, when it appeared to have returned to normal. The venom of three other species of lycosids had no effect when injected in this manner.

TABLE II

VENOM YIELD AND RESULTS OF SUBCUTANEOUS INJECTIONS

Species	Venom Obtained & Injected mg	Guinea Pig Response
Schizocosa ocreata Htz. ♂	5.0	Negative
Shizocosa avida (Walck.) ♀	0.5	Negative
Lycosa helluo Walck. ♀	3.1	Negative
Lycosa carolinensis Walck. ♀	4.8	Red & Swollen

Intracutaneous Injections.

(1) Lycosa rabida. This species was "milked" a total of 16 times during July and August. Discounting the two "milkings" containing gut contents, an average venom yield of 6.5 mg for females and 4.4 mg for males was obtained.

The most severe reaction shown by the guinea pig to this species was to 8.8 mg of female venom, Figure 1. One hour after injection a black ring, 10 x 8 mm in diameter, encompassed a 7 x 6 mm white central pocket. The tissue surrounding the ring was erythematic and swollen. The animal was lethargic. After 5 hours the central area began to fill with lymph and tissue fluids. At 12 hours the lesion was about 10 mm in diameter with the 7 x 6 mm central area covered by an eschar. By the next day the eschar had sunk into the central pocket making the surrounding tissue appear to be welling up around the lesion. After 96 hours the eschar was beginning to contract and the neighboring tissue was light red and only slightly swollen. By the 8th day the scab had begun to pull away from the lesion. After 10 days the primary scab had been lost and a second scab was revealed underneath. Two weeks following injection the secondary scab was gone and the area completely healed with scar tissue. A 2 mm subcutaneous bluish spot remained in the center of the denuded area.

Three of four guinea pigs showed similar responses to female L. rabida venom. The single exception resulted only in localized erythema for 48 hours. Minor variations occurred in the length of time which elapsed between each event and in the coloration of the central pocket. In those cases where a lesion was formed several separate events always occurred. First, the injection site always developed a dark subdermal

area, visible within at least one hour after inoculation. This area was usually the same size or somewhat smaller than the lesion. Second, a central eschar was formed 5 to 12 hours following inoculation. The third event was the loss of this primary scab 8 to 11 days after injection. The final event was the loss of the secondary scab from 11 to 16 days following inoculation.

Venom of the male L. rabida produced similar results to that of the female in only one of four inoculations. The typical response was seen after injection of 8.9 mg of venom, Figure 2. Small primary eschars were formed at the 3.3 and 3.4 mg level. However, these were gone after 72 hours revealing no secondary scabs. One injection containing venom and gut contents failed to form a primary eschar.

(2) Lycosa antelucana. This species was "milked" a total of 12 times during June, July and August with an average venom yield of 5.4 mg for females and 3.7 mg for males.

A female carrying an egg case released 14.6 mg of venom, which after inoculation followed a similar sequence of events as those outlined for the female L. rabida. However, in this case the guinea pig was quite adversely affected by the venom. This was best expressed by its listlessness and lethargic response to stimuli, for 12 hours after injection. The surrounding tissue remained erythematic for 48 hours. The resulting lesion, Figure 3, was 15 x 11 mm. In a separate test, the venom from two female spiders was pooled. The resulting lesion behaved in a typical manner, except there was an hour's delay in the start of observable symptoms. A third injection of 0.6 mg of venom resulted in only local swelling and light erythema which dissipated in 2 days.

Two guinea pigs were inoculated with venom from two male _L. antelucana_. Both developed lesions with small scabs, 1 mm in diameter, at the injection site. An apparent return to normal occurred after 72 hours.

(3) _Lycosa carolinensis_. In only one or two cases was the "milking" of this spider accomplished without the regurgitation of gut contents. This species was particularly discontent about being restrained and harassed. Consequently, it struck any object within range with concurrent release of venom and copious gut contents. These combined fluids and venom were recovered from each "milking" and ranged in weight from 4.8 to 30.3 mg. Another peculiarity about this species was that the only sex collected was female.

In one instance, where the author is reasonably confident that only venom was collected during "milking," a 13 x 10 mm lesion developed from the injection of 17.3 mg of venom, Figure 4. The animal displayed an extreme response to this venom remaining lethargic for about 24 hours. The surrounding tissue became erythematic and swollen and remained so for almost 5 days. Healing was somewhat delayed with the secondary scab remaining 16 days following inoculation. A second test with 30.4 mg of venom plus gut contents resulted in the formation of a typical lesion. Once again, the animal remained lethargic for about 12 hours following injection. Healing was completed in 14 days.

(4) _Lycosa helluo_. The first test with this species resulted in a 12 x 10 mm lesion, following the injection of 5.3 mg of venom from a female spider, Figure 5. This animal expired while the lesion was being photographed. The hot lights, recent envenomization, and fear resulting from handling probably contributed to its death.

An injection of 10.8 mg of venom plus gut contents from a female spider resulted in the formation of a typical lesion. Although the area surrounding the lesion was affected for 72 hours following injection, as indicated by the erythema, the animal never appeared lethargic or reluctant to move. Healing progressed normally with the loss of the secondary scab occurring on the 15th day.

An erythematic area was formed one hour following injection of 4.8 mg of venom. However, no scab was formed and the area returned to normal in 5 days.

(5) Other Lycosids. Two female *Lycosa acompa* were "milked" and the resulting venom inoculated into two separate guinea pigs. Injection of 6.4 mg of venom plus gut contents resulted in the formation of a 13 x 8 mm lesion, Figure 6. The primary eschar was formed about 2 hours following injection. The remainder of the healing process proceeded at the expected rate with the primary scab falling off by the 8th day, Figure 7, revealing the preformed secondary eschar. The secondary scab became detached by the 16th day, Figure 8. The other inoculation resulted only in a slight redness at the injection site.

A female *Shizocosa ocreata* yielded 1.9 mg of venom which when injected caused the formation of a 5 x 3 mm lesion. The resulting primary eschar was less than 1 mm in diameter. The entire healing process was completed in 7 days with no secondary eschar being formed. The venom of the other species tested resulted only in swelling or redness or both.

Table III lists the individual spiders whose venom was tested on guinea pigs. The abbreviations and symbols which will be used in the

ensuing tables are as follows:

Neg. - - no observable response to venom

Sw. - - - swelling at injection site

Er. - - - erythema at injection site

L. - - - reluctance of the animal to move

Sc. - - - formation of an eschar

Sp. No. - specimen number

♂ - - - adult male

♀ - - - adult female

* - - - regurgitated material also collected

TABLE III

VENOM YIELD OF SPECIMENS AND INJECTION RESULTS ON GUINEA PIGS

Sp. No.	Species	Venom Obtained & Injected mg	Lesion Size mm	Animal Response	Healing Time Days
J-13	_Lycosa acompa_ Ch. ♀	6.4*	13 x 8	Er-Sw-Sc	14
J-14	_Lycosa acompa_ Ch. ♀	0.6		Er.	3
A-12	_Lycosa antelucana_ Mont. ♀	0.6		Sw-Er	2
A-13) A-14)	" " ♀ " " ♀	3.0	10 x 6	Sw-Er-Sc	8
A-15	" " ♀	14.6	15 x 11	Sw-Er-L Sc	14
A-51	" " ♂	7.7	1 x 1	Er-Sc	3
A-52	" " ♂	3.0	1 x 1	Er-Sc	3

TABLE III (CONTINUED)

Sp. No.	Species	Venom Obtained & Injected mg	Lesion Size mm	Animal Response	Healing Time Days
J-12	_Lycosa apothetica_ Wallace ♀	0.6		Er	3
E-11	_Lycosa carolinensis_ Walck. ♀	30.4*	15 x 14	Er-Sw-Sc L	14
E-14	" " ♀	17.3	13 x 10	Sw-Er-Sc L	16
J-11	_Lycosa gulosa_ Walck. ♀	5.2		Er	3
B-12	_Lycosa helluo_ Walck. ♀	5.3	11 x 8	Sw-Er-L	Died[a]
D-11	" " ♀	10.8	15 x 12	Sw-Er-Sc	12
F-11	" " ♀	4.8		Er	4
G-11	_Lycosa punctulata_ Htz. ♀	3.4		Er	4
C-11	_Lycosa rabida_ (Walck.) ♀	11.2[b]		Er	3
C-12	" " ♀	8.8	10 x 10	Sw-Er-Sc L	14
C-13	" " ♀	7.0	7 x 6	Er-Sw-Sc	11
C-14	" " ♀	6.2*	5 x 3	Er-Sw-Sc	16
C-51	" " ♂	8.9	13 x 11	Er-Sw-Sc L	18
C-52	" " ♂	3.3	1 x 1	Er-Sw-Sc	3
C-53	" " ♂	8.6		Er-Sw	2
C-54	" " ♂	3.4	3 x 2	Er-Sw-Sc	4
B-51	_Shizocosa avida_ (Walck.) ♂	1.7		Er-Sw	2

TABLE III (CONTINUED)

Sp. No.	Species		Venom Obtained & Injected mg	Lesion Size mm	Animal Response	Healing Time Days
H-11	Shizocosa ocreata Htz. ♀		0.9		Er	2
H-12	" "	♀	3.1		Er	2
H-13	" "	♀	1.9*	1 x 1	Er-Sw-Sc	7

[a]Died while lesion was being photographed.

[b]Venom milky in appearance.

Four control guinea pigs were each inoculated in the hip with 0.1 cc of saline (0.9%). The injection site had been prepared by shaving the hair. No changes in tissue color or condition were noted at the inoculation site at any time during the 48-hour observation period.

Domestic Swine Injections

Those species which had produced relatively severe lesions on guinea pigs were selected for further observations on the effects of their venom on domestic swine.

Lycosa rabida. The reaction of swine to L. rabida venom varied from a slight erythema to the formation of a dark bluish stripe. Swelling was common following most inoculations. The dark blue stripe, when present, changed to a reddish color about 12 to 24 hours following injection. Four days after injection no tissue involvement was visible

and the area appeared to be normal.

Lycosa antelucana. The most severe response to this species occurred following the injection of 13.9 mg of venom from a female spider. One hour following injection a red stripe 13 x 3 mm with a 10 x 2 mm central, raised, whitish area appeared at the inoculation site. After 5 hours the stripe was still present but the cental portion was now 8 x 6 mm and reddish-blue in color. Very little swelling or other involvement of surrounding tissue was noted. After 24 hours the central area was white again, 10 x 2 mm in size, and surrounded by blue-colored tissue. There was marked swelling at the site. After 48 hours the swelling was gone and the site was erythematic. The area appeared normal on the 3rd day following inoculation.

Swelling and erythema were common effects from this spider's venom. In all cases the affected area returned to normal in 2 to 3 days.

Lycosa carolinensis. A female of this species yielded 23 mg of venom and regurgitated material. The injected material caused the formation of 12 x 8 mm dark stripe. After 2 hours a 6 x 3 mm bluish central area was formed and some local swelling was observed, Figure 9. This condition persisted for 24 hours at which time the stripe became light red and the central area was whitish. Some swelling still remained. At 48 hours the swelling and the central area were gone although the light red stripe was still present. Three days following injection the area was only slightly erythematic and by the 4th day appeared to be completely normal.

Other Lycosids. Lycosa helluo venom produced a 10 x 8 mm dark stripe with a 6 x 5 mm central whitish area. Swelling accompanied the

tissue discoloration. However, the central area dissipated in 24 hours and the pig appeared to be normal after 48 hours. On another occasion L. helluo venom caused the formation of a 12 x 4 mm dark stripe with accompanying swelling at the inoculation site. After 3 days the area appeared to have returned to normal.

Lycosa acompa and Shizocosa avida venom caused only minor swelling and erythema. In both cases all visible symptoms were gone after 48 hours.

Loxosceles reclusa. In an effort to determine its effect on domestic swine, two pigs were injected with L. reclusa venom in the same manner used for lycosids. The venom from two females was pooled to obtain 0.3 mg. One hour after injection a 15 x 2 mm dark red area was visible at the injection site. At the 2-hour observation period the stripe was 15 x 4 mm with a 4 x 2 mm black central area. Some swelling had developed by this time. After 5 hours the dark stripe had enlarged to 25 x 10 mm and the central area was blackish-blue and 10 x 6 mm in size. The animal was observed frequently scratching and flopping his ears at this time. The area was markedly swollen. These conditions persisted until the 24-hour observation. At that time an 8 x 4 mm dark scab was present in an area that was slightly erythematic. There was little swelling at that time. The swelling and erythema were gone after 48 hours. However, the scab persisted for about a week.

A second inoculation with 2.0 mg of brown spider venom plus regurgitated material resulted in only slight swelling and erythema. Both conditions were no longer visible after 24 hours.

TABLE IV

VENOM YIELD OF SPECIMENS AND INJECTION RESULTS ON DOMESTIC SWINE

Sp. No.	Species	Venom Obtained & Injected mg	Animal Response
J-13	Lycosa acompa Ch. ♀	0.6	Sw-Er
A-12	Lycosa antelucana Mont. ♀	2.9	Sw-Er
A-13	" " " ♀	6.1	Sw-Er
A-14	" " " ♀	2.1	Sw-Er
A-15	" " " ♀	14.0	Cen[a]-Sw Er
A-51	" " " ♂	1.2	Er
A-52	" " " ♂	3.0	Neg.
E-14	Lycosa carolinensis Walck. ♀	23.0*	Cen-Sw Er
E-15	" " " ♀	12.9	Sw-Er
B-12	Lycosa helluo Walck. ♀	12.1	Cen-Sw Er
D-11	" " " ♀	12.0	Sw-Er
C-12	Lycosa rabida (Walck.) ♀	4.6	Sw-Er
C-13	" " " ♀	0.8	Er
C-14	" " " ♀	11.3	Sw-Er
C-15	" " " ♀	33.7*	Sw-Er
C-52	" " " ♂	1.3	Er
C-53	" " " ♂	2.6	Sw-Er
C-55	" " " ♂	3.1	Er
C-56	" " " ♂	12.2	Sw-Er
B-52	Shizocosa avida (Walck.) ♂	1.9	Er

TABLE IV (CONTINUED)

Sp. No.	Species	Venom Obtained & Injected mg	Animal Response
L-11) L-12)	*Loxosceles reclusa* Gertsh & Mulaik ♀ " " ♀	0.3	Cen-Sw Er-Sc
L-13	" " " ♀	2.0*	Sw-Er

[a]Indicates more severe tissue involvement within area of erythema.

Two control swine were each inoculated behind the ear with 0.1 cc of saline (0.9%). No changes in tissue color or condition were noted at the inoculation site at any time during the 48-hour observation period.

Species Selection

In order to study the protein components and hemolytic activity of lycosid venom it was necessary to select one species representative of the family Lycosidae. *Lycosa rabida* (Figures 10, 11) was selected for several reasons. First, it was found to be the more common lycosid in the areas surveyed. Large amounts of pooled venom would be needed for some of the venom component studies, making it necessary to "milk" several spiders of the same sex at one time. Also, both male and female venom produced typical lesions when inoculated into guinea pigs. None of the spiders tested which produced lesions in guinea pigs appeared to be significantly more or less toxic than *L. rabida*.

Hemolysis

In order to evaluate the possible human response to *L. rabida* venom, a series of tests was conducted to determine the venom's ability to lyse red blood cells.

Washed red cells were prepared as previously described. In two experiments 2 ml of 0.9% saline was added to the test tubes prior to adding the venom. These tubes were held under refrigeration for one test and allowed to remain at room temperature on the other occasion. Those tubes held at 4 C overnight showed no hemolysis the following day while those including controls, held at 85 F ± 5 showed total hemolysis after 24 hours.

Another test involving washed red cells was conducted without adding saline after the supernate had been poured off on the final washing. The venom was added directly to centrifuge tubes containing the packed red cells. No hemolysis was observed after 30 minutes in any of the tubes. However, after 17 hours, at 4 C, the tubes were spun down and hemolysis was noted in all of the tubes containing female venom. Slight hemolysis was also noted in one of the control tubes which could have resulted from cell destruction during mixing.

The concentration of cells, by not adding saline, allowed the venom to contact a larger surface area of a greater number of red blood cells. This increased contact area appeared on gross examination of the tubes to aid in red cell lysis, resulting in a reddish coloration of the supernate. Under these conditions "complete" hemolysis occurred only once, as shown in Table V, indicating a low level of hemolytic activity.

TABLE V

RESULTS OF HEMOLYSIS STUDIES ON WASHED HUMAN RED BLOOD CELLS

Tube No.	Sex of Spider	Venom Added[a] mg	Saline (0.9%) Added ml	Results	
				30 min	17-24 hr
1	♀	Unknown	2	Neg.	Neg.
2	♀	Unknown	2	Neg.	Neg.
3	♂	Unknown	2	Neg.	Neg.
4	♂	Unknown	2	Neg.	Neg.
Control	--	--	2	Neg.	Neg.
Control	--	--	2	Neg.	Neg.
1[b]	♂	2.1	2	Complete	Complete
2[b]	♂	0.9	2	Neg.	Complete
3[b]	♂	2.9*	2	Complete	Complete
Control[b]	--	--	2	Neg.	Complete
1	♀	2.8*	--	Neg.	Complete
2	♀	1.2	--	Neg.	Slight
3	♀	1.7	--	Neg.	Slight
4	♂	1.1	--	Neg.	Neg.
5	♂	3.0	--	Neg.	Neg.
6	♂	2.5	--	Neg.	Neg.
Control	--	--	--	Neg.	Slight
Control	--	--	--	Neg.	Neg.

[a]Venom from _L. rabida_ spiders.

[b]Held at room temperature.

To see if red cell lysis could be observed microscopically fresh human blood was placed on a glass microscope slide, Figure 12, and venom from a female L. rabida was added to the cover slip prior to placing it on the blood slide. Lysis of the red cells occurred almost immediately following placement of the cover slip, Figure 13. Red blood cell "ghosts" could be seen. It was noted that about 80% of the red cells under the cover slip had been lysed. A blood slide was prepared for use as a control. No morphological changes were noted in the red cells during a 2-hour observation period. When a drop of venom was placed alongside of the cover slip on a slide containing fresh human blood, the red cells could be seen rushing out from under the cover slip and into the venom drop. As they migrated out to the perimeter of the drop they became a progressively lighter red until at the perimeter they were completely colorless, Figure 14. This migration occurred in a matter of seconds. Closer examination of the venom drop showed the cells had become misshapen. The degree of distortion increased as they moved toward the perimeter. Finally a point was reached where the integrity of the cell was completely disrupted and an interface was formed between misshapen cells and by-products of cell destruction, Figure 15.

It appeared from these observations that L. rabida venom was capable of red blood cell lysis. Since hemolysis did not extend to all cells under the cover slip and seemed to be short-lived or self-limiting in its action, the venom's efficacy as a hemolytic agent is probably low.

Disc Electrophoresis

Reference Proteins. Reference proteins of known molecular weight

were obtained and their mobility through 7% polyacrylamide gels was determined. The 7% gels were used throughout the study to reduce the variability in migration limits that result from variation in gel concentrations, Kruski and Narayan (1968). The proteins used and the purchasing source are as follows: apoferritin (Mann Research Laboratory), ovalbumin (Mann Research Laboratory), cytochrome "C" (Calbiochem), insulin (Calbiochem), gamma globulin (Mann Research Laboratory), ribonuclease (Calbiochem), bovine serum albumin (Mann Research Laboratory), chymotrypsinogen "A" (Sigma Chemical Company).

The gels were prepared for electrophoresis in the manner previously described. Each column received 15 μl of 1% sodium dodecylsulfate and 15 μl of 1% mercaptoethanol. The reference protein was then layered on the top of the gel, with each column receiving 75 μg, and electrophoresed. The resulting gels were stained with aniline black and then destained. The proteins migrated from the negative to the positive end of the gel. The migration of the protein, as indicated by the band, was measured from the top or negative end of the gel to the proximal and distal face of the band. The mobility limits of the band were then recorded as cm of migration, Table VI.

This information is presented graphically, in Figure 16, as a plot of the log of the molecular weight with the mobility of the protein in the gel. A best fit line was then drawn (by eye) through these points.

Lycosa rabida Venom. A total of 65 adult _L. rabida_ were "milked" to provide the venom for electrophoretic analysis. Usually, the venom of 12 to 15 spiders of the same sex was pooled for each separation. The venom was collected in glass capillary tubes using techniques previously described. These capillary tubes were then placed in test

TABLE VI

ELECTROPHORETIC MOBILITY OF REFERENCE PROTEINS IN 7% POLYACRYLAMIDE GELS[a]

Tube No.	Protein	Mole Weight	Log Mole Weight	Mobility Limits cm
1	Insulin	5,700	3.7559	7.0-7.5
2	Ribonuclease	14,000	4.1461	6.0-6.3
3	Ovalbumin	40,000	4.6021	2.3-2.5 2.8-3.9
4	Cytochrome "C"	13,000	4.1139	6.3-6.9
5	Bovine Serum Albumin	69,000	4.8388	1.8-2.1 2.6-3.3
6	Gamma Globulin	153,100	5.1847	0.1-0.3
7	Apoferritin	450,000	5.6532	0.2-0.3, 0.6-0.7, 1.3-1.4, 6.4-6.7
8	Chymotrypsinogen "A"	23,650	4.3738	6.3-6.5 6.7-7.0

[a]15 μl of 1% sodium dodecylsulfate and 15 μl of 1% mercaptoethanol added prior to addition of 75 μg of reference protein.

tubes and transported to the laboratory in an ice bucket. The usual length of time between "milking" and the start of electrophoresis was about 20 minutes.

Early work on L. rabida venom, using a Gilford UV Scanner at 280 λ, showed from 2 to 3 peaks on four different separations using polyacrylamide gels. Although the experimental results were quite variable due to concentration of venom involved, indications are that very few proteins were present.

Later work, using gels without sodium dodecylsulfate or mercapto-ethanol added, showed three distinct bands in both male and female venom. Exact amounts of venom used in these experiments were unknown.

A 1% solution of sodium dodecylsulfate and mercaptoethanol in 20% sucrose solution was prepared and 40 μl were added to each prepared column. Then venom amounts of 1 or 2 μl were layered on top of each gel prior to electrophoresis. The stained gels showed two minor and one major band for females and males, Figure 17. It can be stated that at least one protein is present in L. rabida venom. The migration of the major protein band was found to be 2.3 to 2.4 cm for males and 2.4 to 2.5 cm for females. Using the mobility limits of the reference proteins as a guide the log molecular weights for the venom were found to be 4.87 for males and 4.90 for females, with estimated molecular weights of 74,100 and 79,400, respectively.

Lycosa rabida Gut Contents. Gut contents were collected from the spider by placing the electrodes from the generator at the base of the palpi and shocking the spider with 6 volts at 20 cps. If the spider was not harassed prior to being shocked the usual result was the expulsion of fluid through the buccal cavity. This material was

collected in capillary tubes and transported to the laboratory in test tubes set in an ice bucket.

Earlier work, with varying amounts of material, showed 2 to 4 peaks when scanned with the Gilford scanner.

The material was later electrophoresed using 2 µl per column. Two tubes each contained 40 µl of a 1% solution of sodium dodecylsulfate and mercaptoethanol; the other two did not. The results are presented in Table VII. Three bands were observed in all cases, although the migration limits of the proteins and the size of the bands differed.

TABLE VII

ELECTROPHORETIC MOBILITY OF SPIDER[a] GUT CONTENTS IN 7% POLYACRYLAMIDE GELS

Tube No.	Sample Size	Gel Additions	Mobility cm
1	2 µl	---	0.3, 0.6-0.7, 0.8-0.9
2	2 µl	---	0.3, 0.5-0.6, 0.8-0.9
3	2 µl	SDS & ME[b]	0.1, 0.3, 0.8-1.0
4	2 µl	SDS & ME	0.1, 0.3, 0.9-1.0

[a]Lycosa rabida (Walck.)

[b]Sodium dodecylsufate and mercaptoethanol

Based on these measurements the log molecular weight of the protein components was found using the graph of reference protein mobility, Figure 16. The tubes containing sodium dodecylsulfate and

mercaptoethanol were the only ones measured. The bands at 0.1 and 0.3 cm did not migrate far enough for an accurate estimate of molecular weight but both are over 150,000. The band measuring 0.8 to 1.0 cm had a log molecular weight of 5.15 with an estimated molecular weight of 141,000.

Lycosa rabida Hemolymph. Hemolymph was obtained by breaking off a leg near the trochanter. Capillary tubes were used to draw off the oozing material. The capillary tubes were transported to the laboratory in test tubes placed in an ice bucket.

Scanning of electrophoresed gels showed the presence of 3 to 4 peaks. The amount of the material used for electrophoresis was not known.

Electrophoresis of hemolymph at various concentrations gave four bands when run either with or without sodium dodecylsulfate and mercaptoethanol, with only migration limits and the size of the bands differing, Table VIII. There was a single exception; a tube with 1 μl of hemolymph, and containing sodium dodecylsulfate and mercaptoethanol, formed only two bands.

The estimated molecular weights were computed, as before, using the log molecular weight obtained from Figure 16. The molecular weight of the band migrating 0.3 cm was estimated only as greater than 150,000. The band migrating 0.8 to 0.9 cm had a log molecular weight of 5.15 and an estimated molecular weight of 141,000. The band found in the 0.9 to 1.1 cm range had a log molecular weight of 5.12 and an estimated molecular weight of 132,000. The last band had a range of 2.0 to 2.1 cm and log molecular weight of 4.90; its estimated molecular weight was 79,400.

TABLE VIII

ELECTROPHORETIC MOBILITY OF SPIDER[a] HEMOLYMPH IN 7% POLYACRYLAMIDE GELS

Tube No.	Sample Size	Gel Additions	Mobility cm
1	1 μl	---	0.3, 0.7, 0.9, 1.0
2	2 μl	---	0.3-0.4, 0.7, 0.8-0.9, 0.9-1.0
3	1 μl	SDS & ME[b]	0.4, 0.9
4	2 μl	SDS & ME	0.3, 0.8-0.9, 0.9-1.0, 2.0-2.1
5	3 μl	SDS & ME	0.3, 0.8-0.9, 1.0-1.1, 2.0-2.1

[a] *Lycosa rabida* (Walck.)

[b] Sodium dodecylsulfate and mercaptoethanol

The estimated molecular weights of the proteins are not intended as accurate measurements of actual molecular weights but serve only to relate the size of the unknown proteins to that of the reference proteins. More work on lycosids is indicated before the total venom components and the toxic properties of each can be fully determined.

SUMMARY AND CONCLUSIONS

A total of 10 species from the family Lycosidae were found to cause a toxic response when injected into guinea pigs. All 10 species were collected in Payne County, Oklahoma, near buildings and other structures frequented by man. These species are listed below in descending order of frequency collected: Lycosa rabida; Lycosa antelucana; Shizocosa ocreata and Lycosa carolinensis; Lycosa acompa, Lycosa helluo and Shizocosa avida; Lycosa apothetica, Lycosa gulosa and Lycosa punctulata. These specimens are available in the Department of Entomology Museum, Oklahoma State University, or through the Department of Zoology, University of Florida.

Lycosa rabida was by far the more frequently collected species. It was found in tall Bermuda grass, above the surface of the ground, near homes and other buildings with solid foundations. Its presence in these locations in large numbers increases the likelihood of contact with man during mowing, trimming or gardening activities.

Subcutaneous injections of venom resulted in a toxic response by guinea pigs in only one instance, by Lycosa carolinensis. All the species so tested were later found to cause a toxic response in guinea pigs when inoculated intradermally.

Intradermal inoculations caused the formation of primary eschars on 16 out of 28 inoculations on guinea pigs. Those species which caused lesions requiring 12 days or longer to heal were Lycosa acompa, Lycosa antelucana, Lycosa carolinensis, Lycosa helluo and Lycosa rabida.

All other species tested produced symptoms which disappeared in 2 to 7 days. In no case did injections of less than 3.0 mg of venom produce a lesion. When a lesion was formed the course of events, related to healing, was similar regardless of the species of lycosid involved. This seemed to indicate a similarity of venoms between those species which produced lesions.

None of the lycosids tested on domestic swine produced lesions. *Lycosa antelucana*, *Lycosa carolinensis*, and *Lycosa helluo* caused the most severe localized response of those tested. In none of the tests were visible symptoms present after 4 days. Two domestic swine inoculated with *Loxosceles reclusa* venom failed to show typical "necrotic" lesions. However, one did form an eschar as a result of an injection, but healing was completed in about a week. The response of domestic swine to all spiders tested indicates a fairly high degree of resistance to the toxic effects of the venoms injected.

From experiments conducted using washed human red blood cells and fresh whole human blood it was shown that *Lycosa rabida* venom does have a hemolytic effect. However, the level of hemolysis is relatively low.

Using disc gel electrophoresis, *Lycosa rabida* venom was shown to contain one major protein band; gut contents, three bands; and hemolymph, four bands. Molecular weights of the protein bands were estimated by comparing gel migration limits to those of reference proteins. The estimated molecular weight of the major venom protein was 74,100 for males and 79,400 for females. Two of the three proteins found in gut contents had estimated molecular weights of over 150,000. The third was estimated at 141,000. The molecular weights of the protein bands in hemolymph were estimated as: over 150,000; 141,000; 132,000;

and 79,400.

Estimation of the public health importance of those species of lycosids studied is a difficult task. Based on the results obtained from laboratory animals, *Lycosa acompa*, *Lycosa antelucana*, *Lycosa carolinensis*, *Lycosa helluo*, and *Lycosa rabida* would seem to have the capabilities to cause a toxic response in human beings. The two species more likely to be encountered in this area would be *Lycosa antelucana* and *Lycosa rabida*, since *Lycosa carolinensis* is a burrowing spider and *Lycosa acompa* and *Lycosa helluo* were found less frequently. Since mortality from venom injection alone was not seen in guinea pigs it would be very unlikely to occur in man, except for hypersensitive individuals.

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APPENDIX

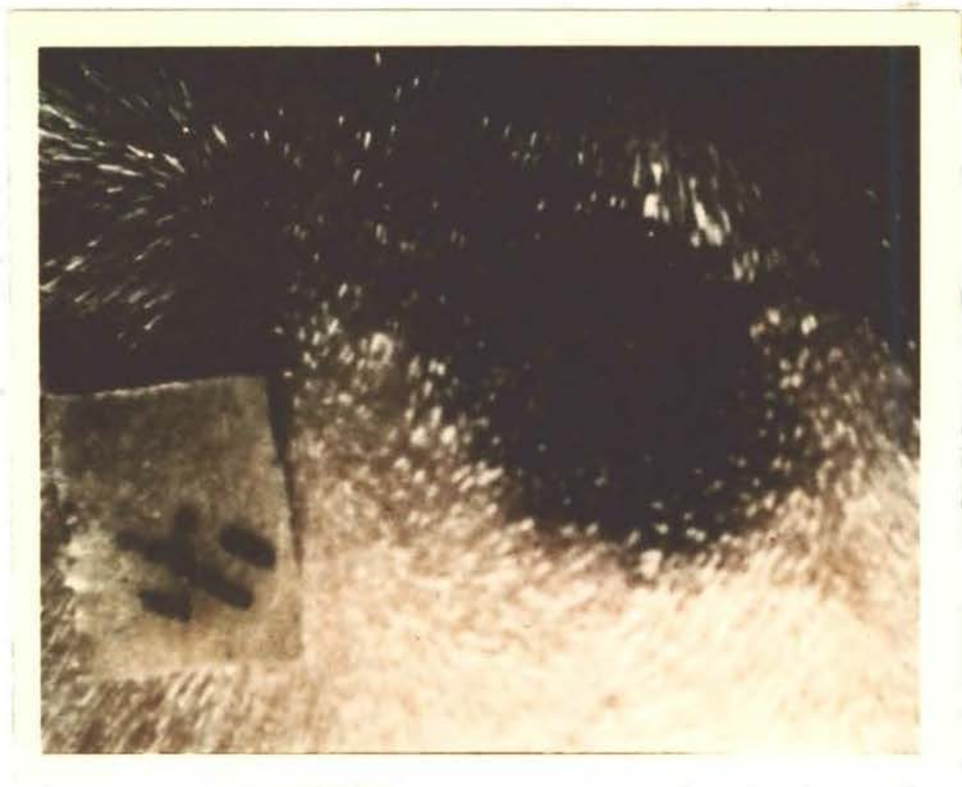

Figure 1. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa rabida ♀, 5 Hours After Injection.

Figure 2. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa rabida ♂, 4 hours After Injection.

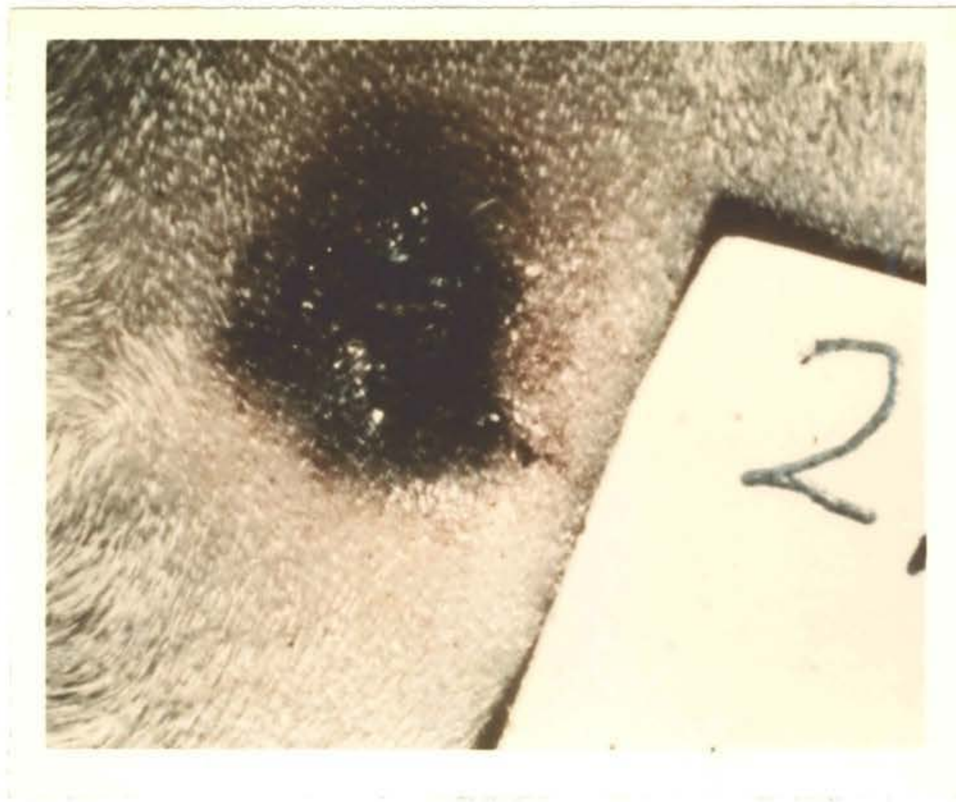

Figure 3. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa antelucana ♀, 3 Hours After Injection.

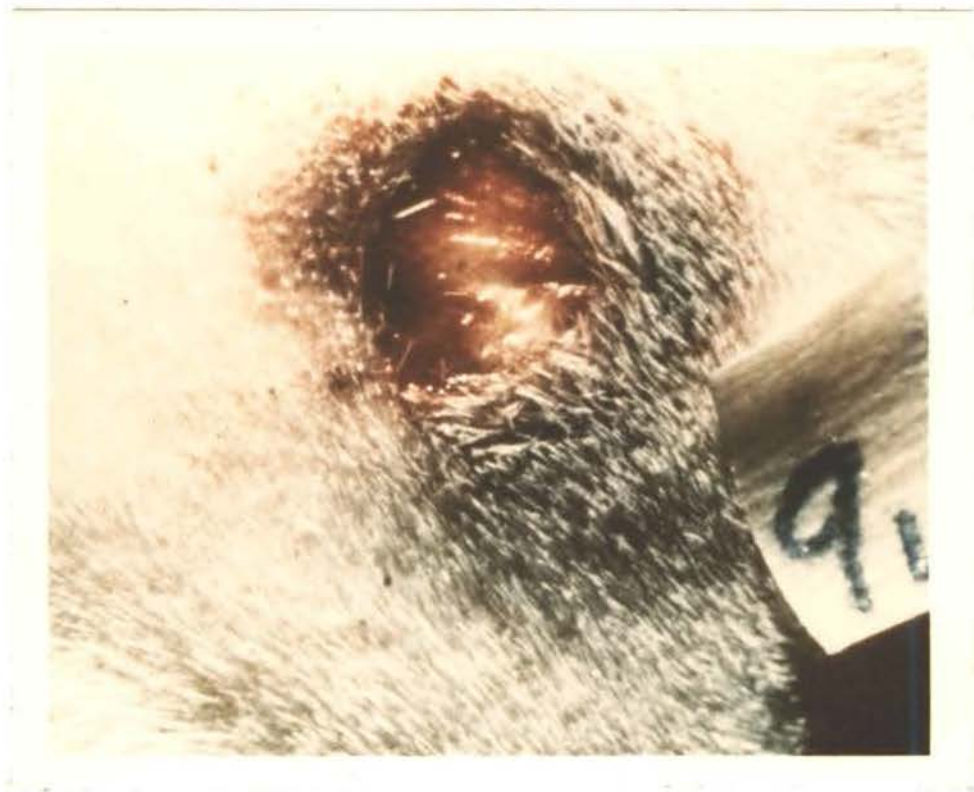

Figure 4. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa carolinensis ♀, 2 Hours After Injection.

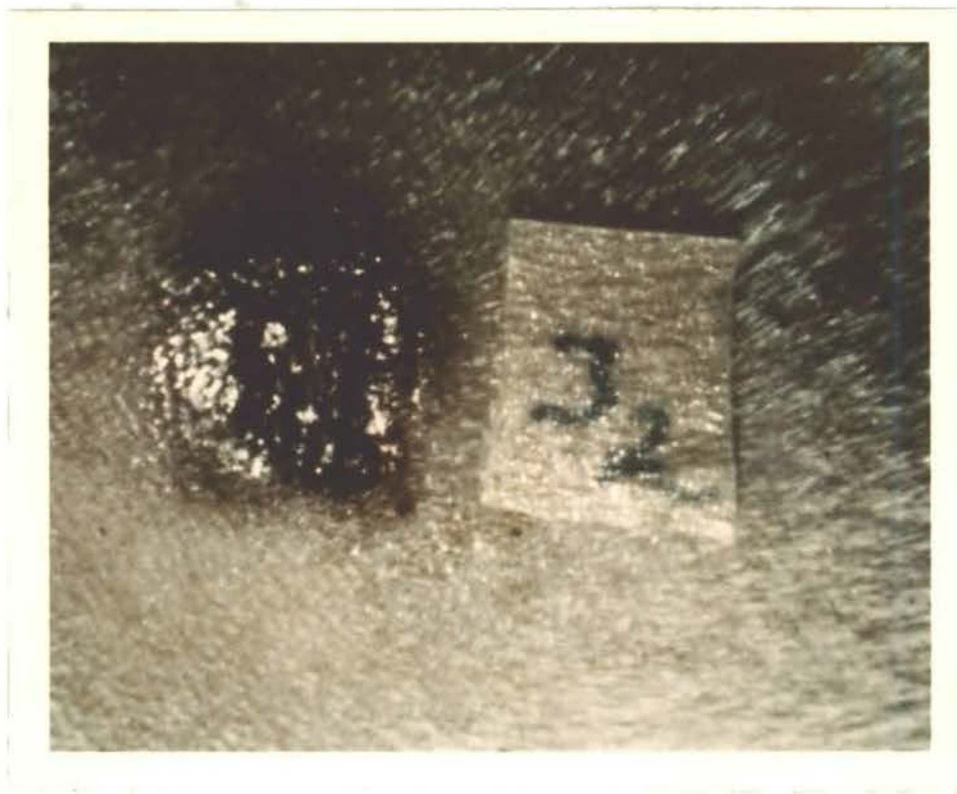

Figure 5. The Hip of a Guinea Pig Showing the Effect of Venom from *Lycosa helluo* ♀, 2 Hours After Injection.

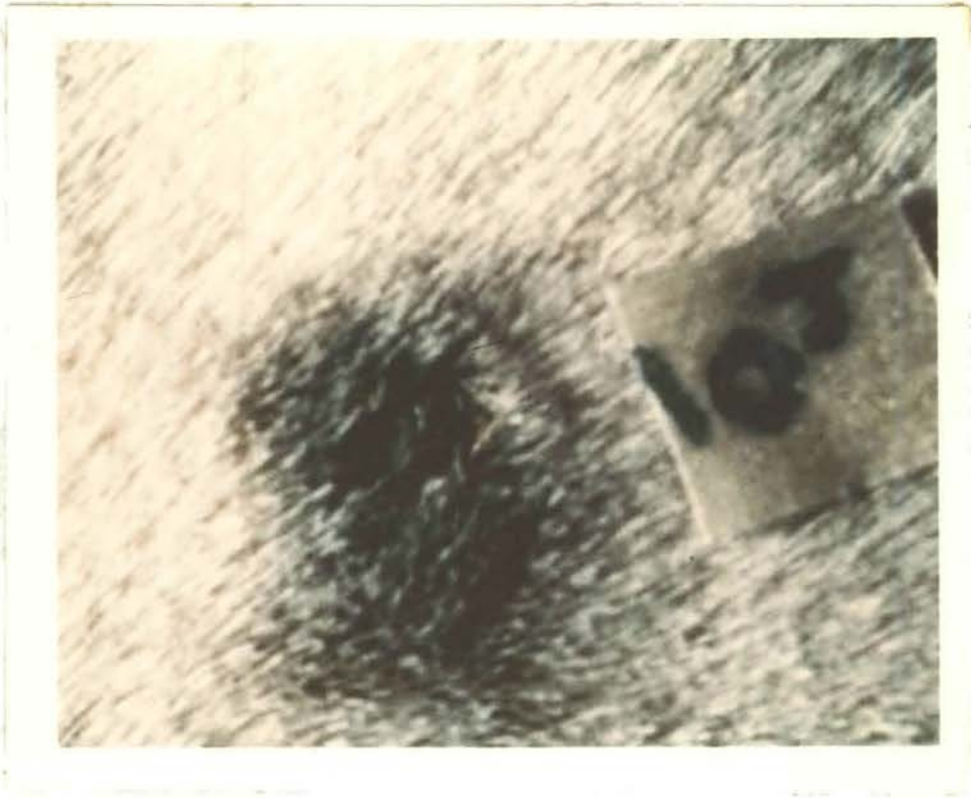

Figure 6. The Hip of a Guinea Pig Showing the Effect of Venom from *Lycosa acompa* ♀, 3 Hours After Injection.

Figure 7. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa acompa ♀, 8 Days After Injection.

Figure 8. The Hip of a Guinea Pig Showing the Effect of Venom from Lycosa acompa ♀, 16 Days After Injection.

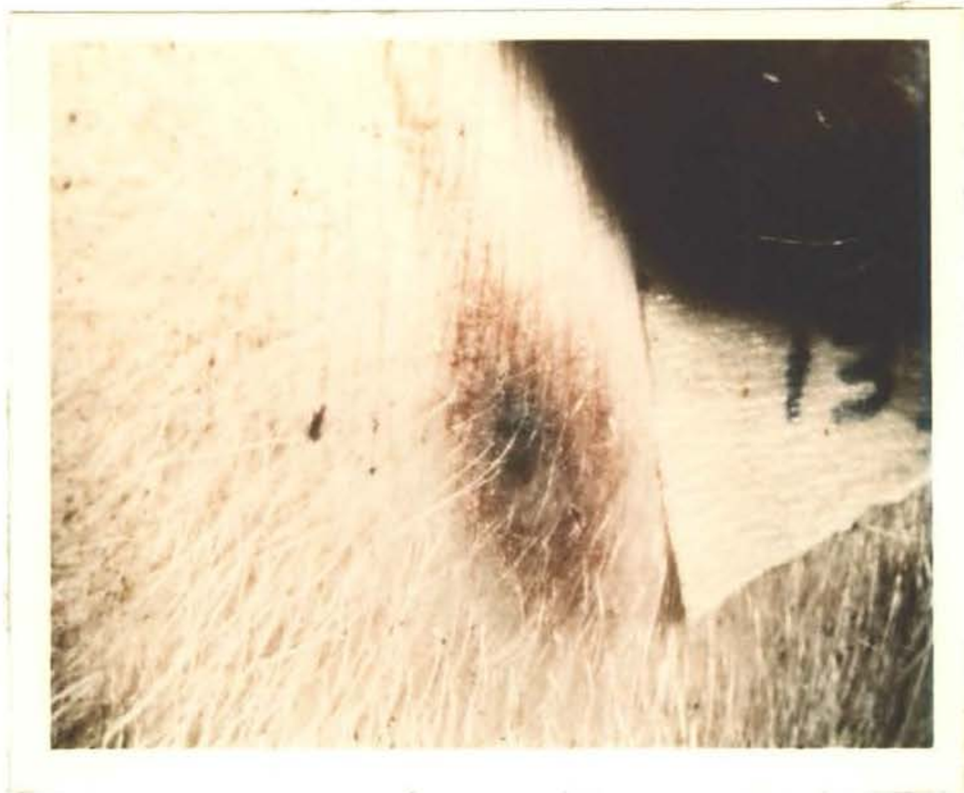

Figure 9. The Area Behind the Ear of a Domestic Swine Showing the Effect of Venom from Lycosa carolinensis ♀, 6 Hours After Injection.

Figure 10. Lycosa rabida (Walckenaer) ♂.

Figure 11. Lycosa rabida (Walckenaer) ♀.

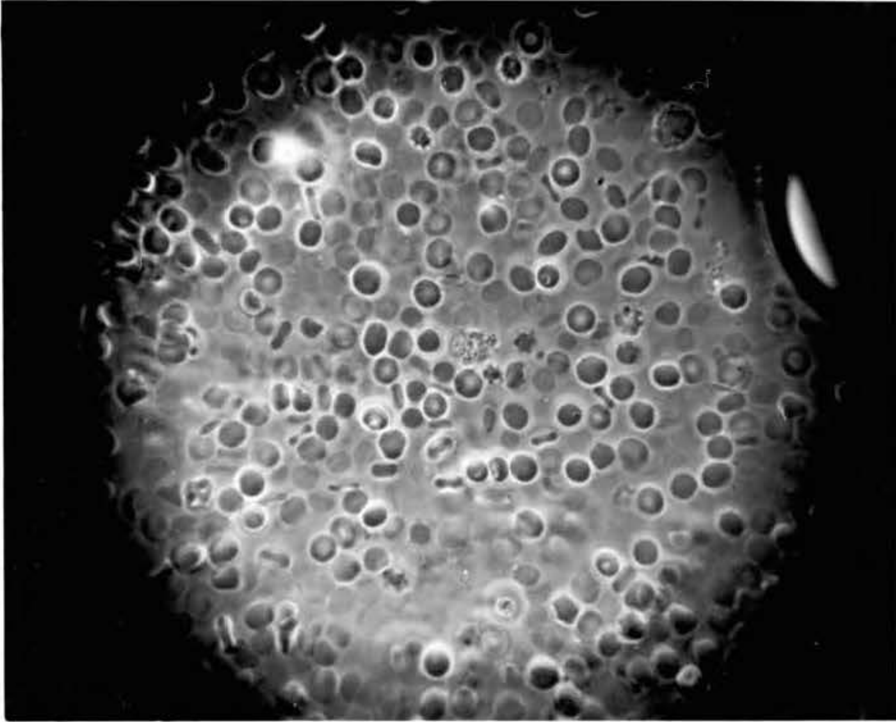

Figure 12. Normal Human Blood Cells (x450).

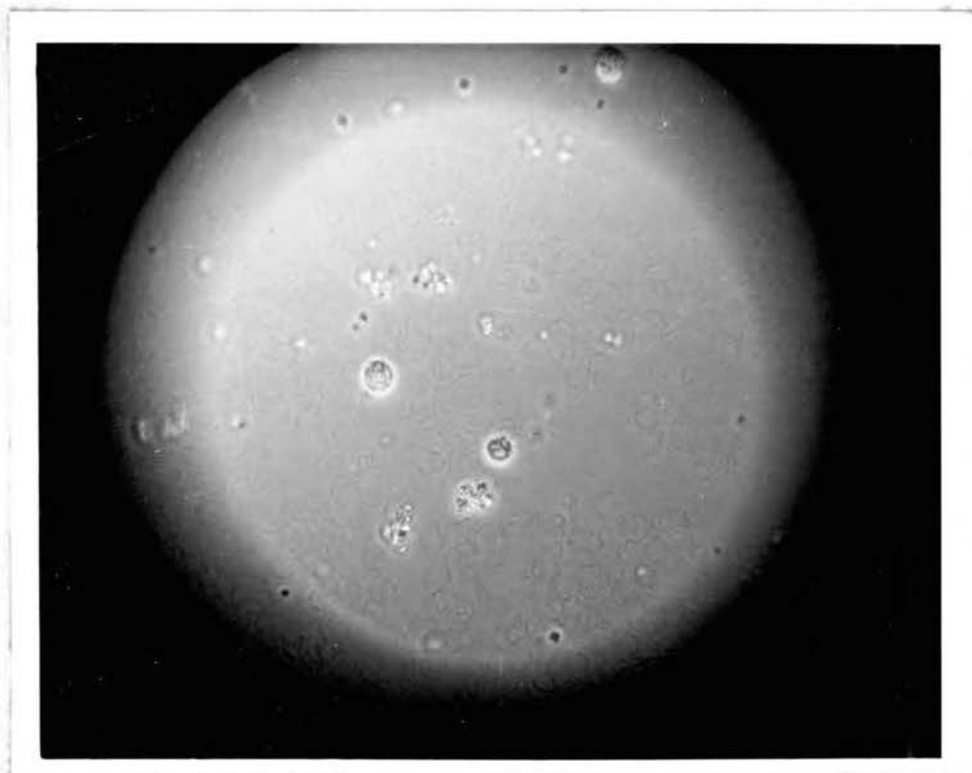

Figure 13. Effects of Lycosa rabida Venom on Human Blood, Note Red Cell "Ghosts" and Unlysed Lymphocytes (x450).

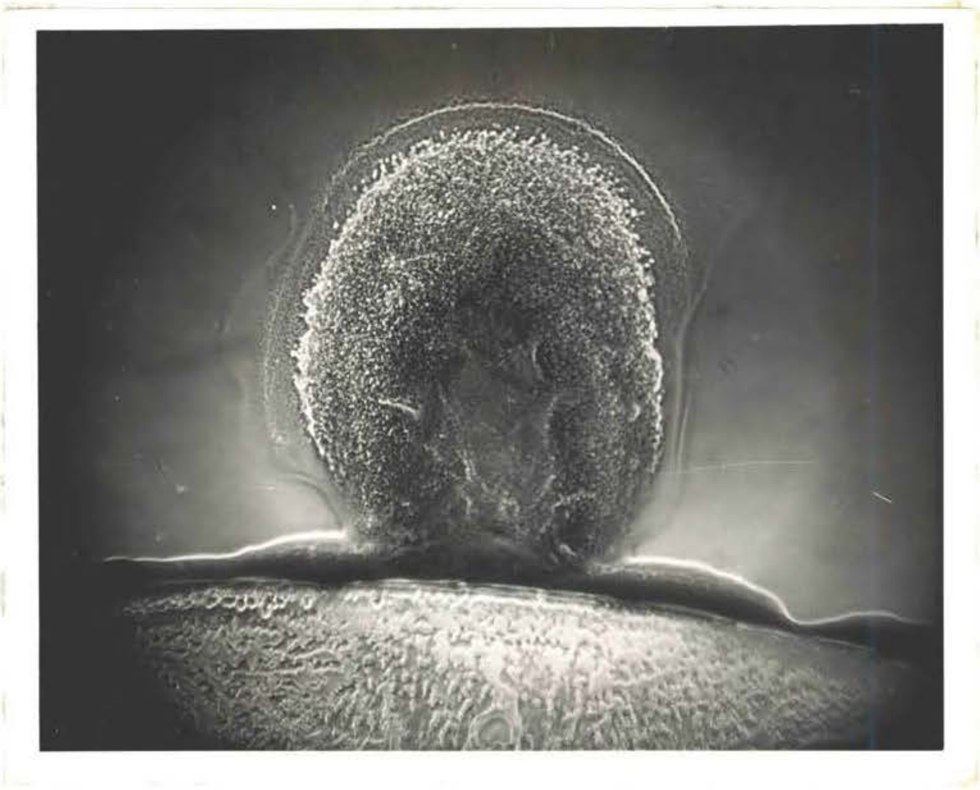

Figure 14. Human Blood Within a Drop of Lycosa rabida Venom Showing Cell Destruction (x200).

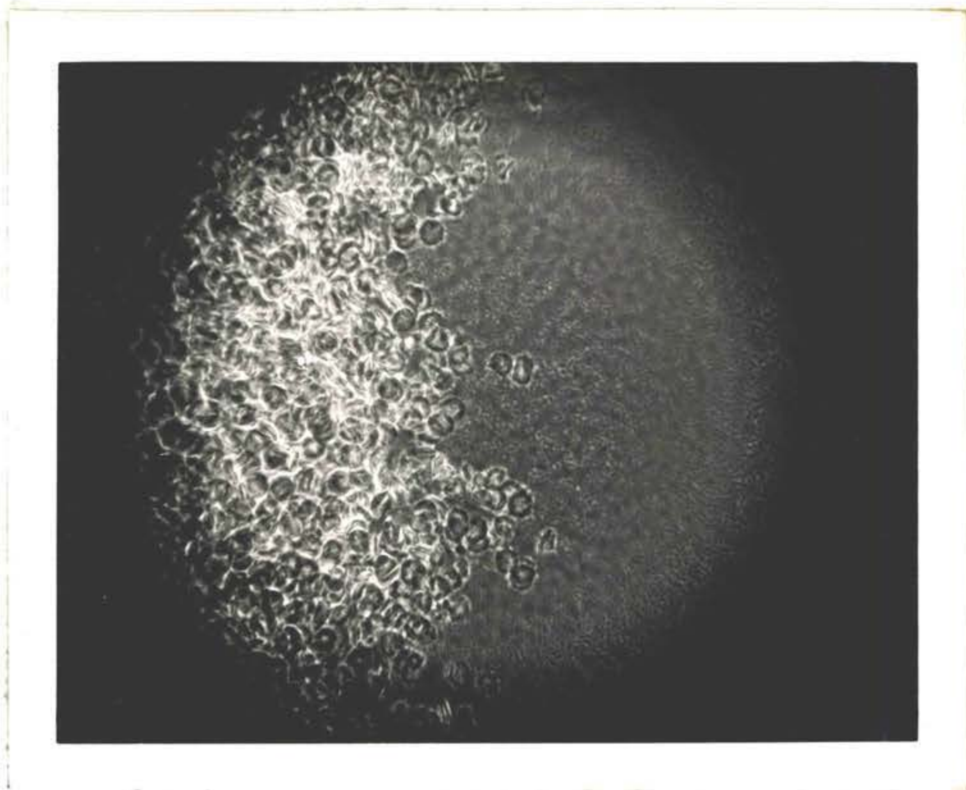

Figure 15. View of Venom Drop Showing Interface Resulting from Red Cell Destruction (x450).

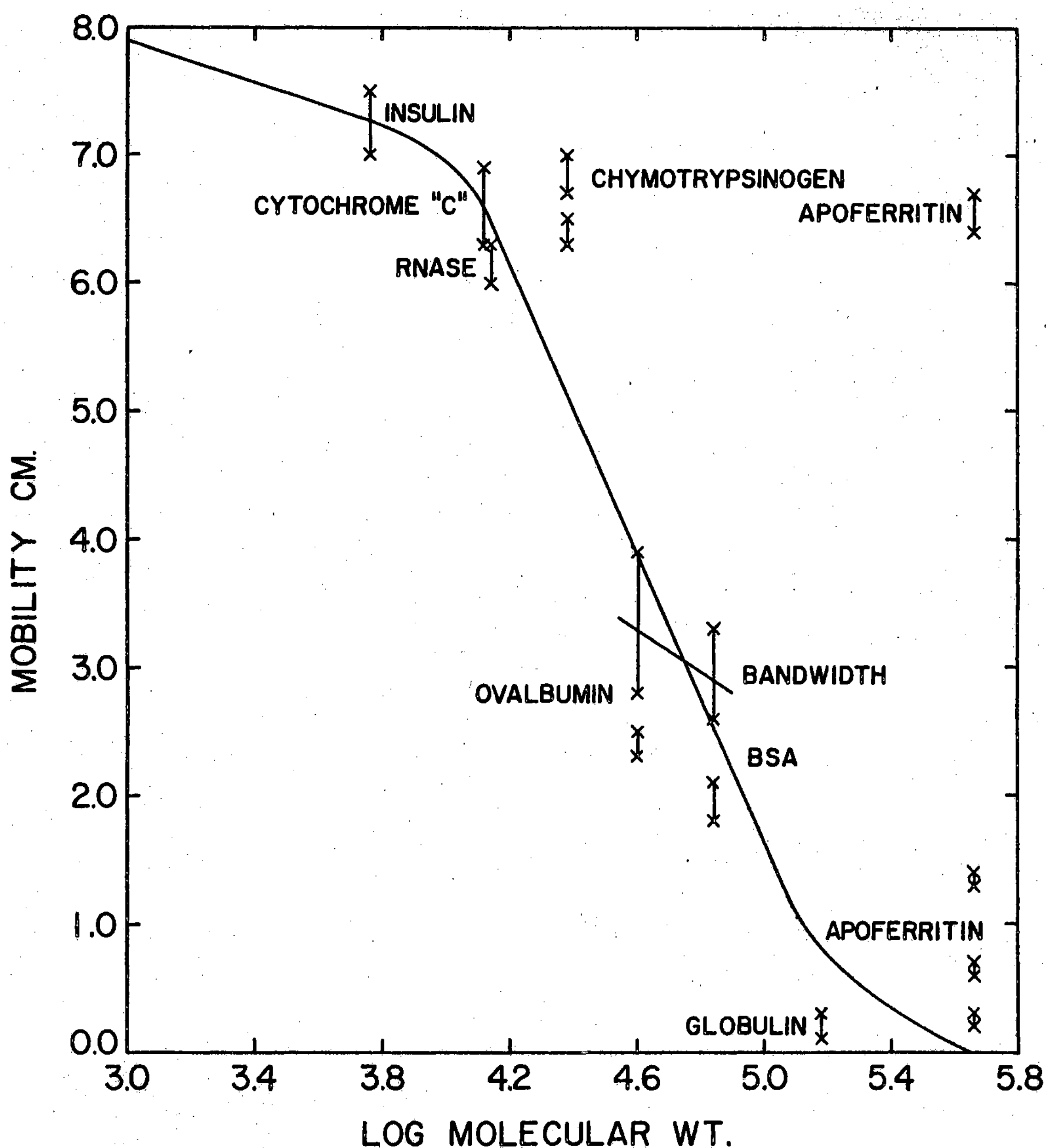

Figure 16. Graph of Reference Proteins Indicating Mobility in 7% Polyacrylamide Gels Containing Sodium Dodecylsulfate and Mercaptoethanol.

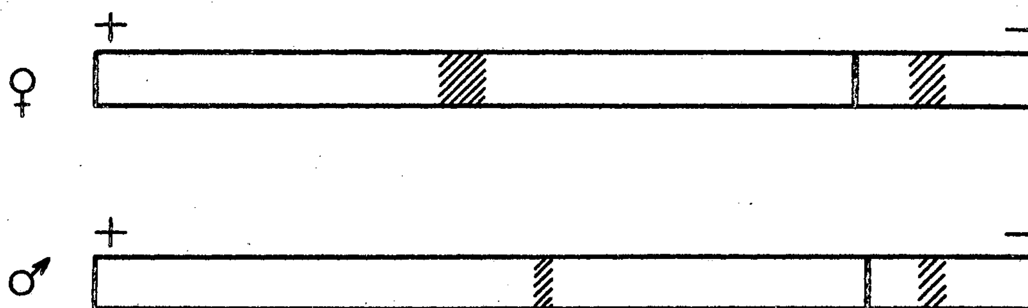

Figure 17. Migration of Lycosa rabida Venom in 7% Polyacrylamide Gels Containing Sodium Dodecylsulfate and Mercaptoethanol.

VITA

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Doctor of Philosophy

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